Exploration of Soil Resistome Through a Metagenomic Approach

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Abstract

Resistance toward antibiotics in microbes is considered to be one of the most significant challenges to modern medicine. The sum total of resistance genes against antibiotics in microorganism present in the soil is called the soil resistome and could serve as a source of resistance in microbes that can ultimately serve as a sink for drug discoveries. A deep knowledge of soil resistome and its multilateral interaction with advancement in drug development is essential for implementing suitable actions reducing spread of resistance in an efficient way. However, the soil resistome and its evolution are still in their infancy. The amalgamation of metagenomics with next-generation sequencing technology proved to be a robust methodological approach for exploring the soil microbiome, along with other related factors, especially the resistome. In this chapter, we have tried to incorporate the current knowledge on how the soil resistome is designed and discuss application of metagenomics to decipher hidden processes, particularly in respect to novel findings for medical diagnostics, controlling infections, and improving public health.

Keywords

Metagenomics · Antibiotic resistome · Rhizosphere · Microbial diversity

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1 Introduction

The soil is not only a natural reservoir of microbial diversity, but it also serves as a major source of the antibiotics used today in medical science. Most of the presently available antibiotics are synthesized as a bioactive compound of either bacterial or fungal origin. Microbial interaction within the soil presents a perfect model for understanding and developing novel drug discovery (Allen et al. 2015). Alternatively, the risk of emerging resistance in pathogenic strains has severely challenged this victory. Clinical environment is more prone to the occurrence of resistant pathogens due to introduction of new antibiotics (Lewis 2013). However, information related to the manifestation of "antibiotic resistance genes (ARGs)" in the soil are scarce. At present, culture-independent techniques—for example, metagenomics—have played an important role in revealing the diversity and existence of antibiotic resistance in soil. Deciphering the mechanisms related to antibiotic resistance may help in the identification of targets for the development of new drugs and effective antibiotics. Thus, gaining knowledge of ARGs will provide a platform to understand microbe-mediated diseases and their subsequent resistance. Earlier, Truman et al. (2014) illustrated underlying mechanisms of resistance for identification of novel antibiotics. They unravel the resistance mechanism against glycopeptide by *Streptomyces coelicolor* for the development of a novel screening procedure for antibiotic compounds. In the initial step of screening, the assay was performed to analyze cell wall stress using collection of bacterial extracts to analyze the inducers of sigE promoter, responsible for stress response in the cell wall. In the next step, mutant bioassay was performed to evaluate the extracts for activation of the sigE promoter, which in turn unveiled the presence of a glycopeptide antibiotic through the induction of resistance genes for glycopeptide. More recently, the culture-independent method has been used to identify a new antimicrobial by screening bacterial growth in diffusion chambers, in situ (Ling et al. 2015). This technique enabled the bacteria to access the required substrates from their natural environment. Using this approach, the author's revealed teixobactin which is a powerful antibiotic against clinically important Gram-positive bacteria. The authors suggested that although there was no resistance mechanism in *Mycobacterium tuberculosis* and *Staphylococcus aureus* strains against this antibiotic, they also recognized the role of horizontal gene transfer for acquiring resistant traits. For example, actinorhodin antibiotic cluster contains genes which encodes for export of proteins. Bacteria may develop resistance by acquiring mutations in the genes encoding antibiotic-related genes, e.g., DNA gyrase subunit A gene. However, pleiotropic effects occurring from these mutations may alter the metabolic pathways and consequently result in varying levels of resistance. Molecules having antibiotic activity at high concentration behave differently at low (but nontoxic) concentration, demonstrating main role in signaling and communication among bacteria, resulting in the induction of gene expression involved in virulence and further processes. An earlier study reported that some bacteria utilize antibiotics as an energy source increasing the possibilities of high antibiotic resistance in the natural environment

(Martinez 2008). Nevertheless, reports related to mechanisms of antibiotic resistance particularly in relation to soil are limited.

Soil contains particles of different size and composition, mineral, and organic compounds. Its heterogeneous structure creates a nexus of microenvironments allowing the existence of diverse microbial communities. Therefore, soil is considered to be a chief reservoir containing metabolic as well as genetic diversity and important source for identifying novel ARGs, increasing the area of knowledge about antibiotic resistance system. Over the decades, the metagenomics has revolutionized the area of soil microbiology by unearthing the hidden concepts. The alterations in emphasis from single isolate to diverse microbial communities, as well as from culturable to unculturable microorganisms, have reformed this field. Recently, Hug et al. (2016) established a new tree of life by aligning ribosomal protein sequences instead of 16S rRNA genes and was characterized to have an additional subdivision within the bacteria domain known as the candidate phyla radiation (CPR). Members included under the CPR group exhibit relatively small genomes with limited metabolic abilities, suggesting the existence of symbionts. It is interesting to observe that the lineages of CPR and other related groups without cultivable members contain maximum tree's diversity, signifying the role of culture-independent techniques for microbial diversity analysis. Certainly, these studies with the help of high-throughput next-generation sequencing (NGS) proved to unravel the soil ecological dynamics. Metagenomic technique involving both sequence-based and functional properties helped to demonstrate the functional and taxonomic diversity of microorganisms from natural environments. Sequence-based approach of metagenomic study comprises direct sequencing of total extracted DNA from an environmental sample. With the help of this approach, we can identify variation in the genes and diversity and abundance at taxonomic and functional levels along with microbial genome assembly. Advent of NGS technology has popularized the sequence-based metagenomics among scientific community and amplified the depth of sequencing by enabling full genome assembly. Moreover, combining different sequencing technologies resulting in short as well as long reads is considered to be more advantageous than using either technology alone (Sharon et al. 2015). Sharon et al. (2015) have successfully demonstrated the assembly of genomes having low abundance from complex terrestrial sediments. Short- and long-read sequencing has allowed researchers to demonstrate the abundance of microbial communities in a particular environment with numerous rare species. Alternatively, functional metagenomics is an important technique for identifying new genes of interest from diverse environmental samples. Typically, functional metagenomics encompasses creating metagenomic libraries; subsequently clones expressing the interested phenotype are being selected (e.g., antimicrobial or enzymatic activity, xenobiotic/antibiotic resistance, toxic compounds detoxifying, or environmental pollutant degrading). Since the phenotype-based screening provides important information about the gene function, therefore it could be employed for gene annotation. Recently, this approach was successfully used in the identification of amylolytic, cellulolytic, and lipolytic activities having potential industrial applications (Kim et al. 2015; Su et al. 2015; Alnoch et al. 2015). Functional

metagenomics is also useful for antimicrobial bioprospection (O'Mahony et al. 2015) and bioremediation of aromatic compounds present in soil system (Nagayama et al. 2015). Basically, ARG research involves functional metagenomics approach with the requirement of clone selection on agar plates added with antibiotics, followed by genetic content analysis. Soil microorganisms are important source of both antibiotic compounds and ARGs. Actually, more than 80% of all antibiotics being utilized in the clinic are derived from soil microorganisms, which are rich in antimicrobial resistance elements (Torres-Cortes et al. 2011). Therefore, exploring the resistome of soil will allow researchers to decipher the true diversity of resistance genes and will be helpful in identifying new resistance mechanisms, along with their ecological roles.

2 The Antibiotic Resistome

The antibiotic resistome is a framework to study resistance beyond the narrow point of view of the clinic, taking a wider outlook to incorporate resistance in all its forms, including within noninfectious environmental microbes (Perry and Wright 2014). The genetic and functional diversity in the resistome is vast and reflects the billions of years of evolution of microbes in close contact with toxic molecules of many origins (O'Brien and Wright 2011). These include inorganic metals and organic compounds made by microbes, plants, and animals (Li et al. 2016; Bérdy 2005). The molecules we term antibiotics are a subset of these toxic compounds (and here we must also include man-made compounds, as they are subject to the same evolutionary forces as natural substances).

In many cases, the molecular mechanisms that have evolved to evade nonspecific toxic molecules are identical and can be readily repurposed for antibiotic resistance. The sequencing of microbial genomes from diverse phyla and environments reveals that most (perhaps all) bacterial genomes harbor resistance elements, many in the form of intrinsic mechanisms (Cox and Wright 2013). These are the scars of the natural history of bacteria and the diversity of toxic molecules that they have encountered, including antibiotics.

Furthermore, many of these genomes display a variety of HGT signatures in the form of genes and pseudogenes encoding integrases, transposases, and gene sequences that confirm a long history of gene mobilization within and across microbial species and genera (Soucy et al. 2015). Such genomic resistance islands are found in many bacterial genera, frequently in the association of integrons, and can carry lots of resistance genes (Gillings 2014; Hamidian et al. 2015). The mycelial structure of many soil microbes may greatly facilitate HGT and the exchange of antibiotic resistance elements (Berthold et al. 2016). Mobile elements (e.g., plasmids and transposons) are found in many bacteria and offer facile routes of HGT that often do not respect species or genus boundaries. As a result, antibiotic resistance elements had diversified and moved across environments over the millennia. Analysis of the resistance gene makeup of environmental microbes in comparison

to naive antibiotic pathogens clearly shows that resistance elements are highly enriched in the former and rarer in the latter (Surette and Wright 2017). Waksman was among the first to observe the antagonistic interactions between environmental microbes comprising primarily bacteria and fungus (Waksman 1941). A more systematic study of the resistome of ~500 actinomycetes collected from a variety of soils revealed that these bacteria show resistance toward 7–8 antibiotics at 20 μg/mL (D'Costa et al. 2006).

In contrast, a survey of pre-antibiotic era *Salmonella* strains identified numerous plasmids but no resistance genes (Jones and Stanley 1992). The differences in antibiotic resistance before 1940 between pathogens and environmental microbes reflect the more challenging and diverse chemical ecology experienced by environmental bacteria in comparison with pathogens, many of which are commensal. Resistance genes present in bacterial genomes may not be expressed, and as a result resistance genotype may not correlate with phenotype as measured by standard minimal inhibitory concentration (MIC) in the laboratory (Perry et al. 2014). For this chapter, we consider antibiotic resistance to be driven by genotype, i.e., a bona fide resistance gene is one that confers protection from the antibiotic (increase in MIC) when expressed. We do not consider mechanisms of antibiotic tolerance such as persistence, biofilm formation, and stochastic gene amplification, though these do contribute significantly to the global challenge of antibiotic resistance.

3 Soil: Sink of Antibiotics Resistome

The soil has an enormous diversification in terms of biological, chemical, and physical properties. With regard to the microbial ecology, there is an existence of large number of niches comprising individual organisms and their group (Misra et al. 2017). The residents of soils include microbes (bacteria, fungi, archaea, cyanobacteria, protozoans, phages, and other viruses), plants, and larger animals, including nematodes, arthropods, worms, and burrowing mammals. Earlier, Waksman (1945) expounded on the complexity of soil microbe communities over 70 years ago. He summarized the interactions between microbes as associative (symbiosis, growth promotion, the liberation of nutrients from complex forms, consumption of oxygen), competitive (for nutrients or space), or antagonistic (production of growth-inhibitory substances). The latter can be passive, e.g., change in the pH of the local medium, exhaustion of nutrients, or active, in the form of excretion of toxic compounds, pigments, or lytic enzymes. Using culture-based approaches, an effort was made to analyze microbe-mediated production of selectively toxic metabolites (Waksman 1941). This realization not only launched the golden era of antibiotic discovery but also identified some of the first anticancer and antifungal agents as well as multiple other drug classes—from immune suppressants to cholesterol-lowering agents. Soils indeed offered a wealth of leads for new medicines, and mining of soil microbes for such compounds dominated efforts in the pharmaceutical field for decades. Bioactive compounds from soil microbe area are boon to drug

discovery but are rarely investigated for their intrinsic roles in producers or their impact on the ecology of the soil. Thousands of such compounds reported in the literature represent only a small part of the chemical space available to these organisms (Bérdy 2005). Sequencing of the genomes of soil microbes including bacteria of the genus *Streptomyces* and other filamentous actinomycetes reveals that they have the genetic capacity to produce a dizzying spectrum of compounds. Each actinomycete genome has on average 20 to >30 genetic programs encoding bioactive compounds, many of which are antibiotics (Katz and Baltz 2016). Similarly, filamentous fungi are important producers of secondary metabolites, with approximately 40–80 biosynthetic gene clusters per *Aspergillus* genome, for example (Inglis et al. 2013). The precise roles of most of these compounds in soil environments are rarely known with confidence (O'Brien and Wright 2011). Certainly, many do have antimicrobial activity, but whether this is their primary role is disputed (Davies 2006). The killing activity of antibiotics is concentration dependent, and sub-MIC concentrations can have pleiotropic effects on bacterial gene expression and metabolite production (Surette 2013). The fact that antibiotic quantities in soils are difficult to measure, and likely generally much lower than concentrations that can be secreted in the lab, fuels doubt that cell killing is their primary activity. Imaging mass spectrometry experiments demonstrate that production of secondary metabolites, including antibiotic, is highly dependent on growth conditions and proximity to other microbes (Traxler et al. 2013). Not surprisingly, what is evident from these studies is that there is a gradient where antibiotic concentrations are high close to the producing cells diminishing as compounds diffuse out into the medium. Furthermore, it is well known to researchers purifying antibiotics and other secondary metabolites that these are very frequently physically associated with producing cells. Microbes live on the micron scale, so although antibiotics may have multiple effects on adjacent cell metabolism and gene expression not directly related to cell death, as the proximity of cells increases, antibiotics very likely do have antibiotic activity. This hypothesis was supported by the studies demonstrating genetically diverse and extensive soil resistome evolved to attenuate antibiotic killing activity. The similarity of the molecular mechanisms of resistance observed in the clinic to those in soil bacteria, in particular, the self-protection mechanisms of antibiotic-producing bacteria, had been made over the past four decades (Marshall et al. 1998). D'Costa et al. (2006) have systematically explored the soil resistome revealing antibiotic resistance in spore-forming bacteria having ubiquitous and diverse nature. Resistance mechanisms include several metabolic processes in clinical isolates and also the strategies that are not recognized in pathogens. In another study that explored a broader spectrum of bacterial genera, 412 strains isolated from a variety of soils were tested for resistance against a panel of 24 drugs (Walsh and Duffy 2013). The majority (80%) were multidrug resistant, confirming the diversity and frequency of resistance in soils. Efflux mechanisms were the primary source of the multidrug phenotype, but drug inactivation was also prevalent, in particular for penicillin resistance. Common β-lactamases circulating in the clinic were not detected, pointing to the importance of the intrinsic β-lactamases.

4 Antibiotic Resistance: Possible Risks to the Environment

The environmental resistome is vast, ancient, and mobilizable. As such, it presents a risk to the development of resistance in pathogens and subsequent drug failure. Reports are available demonstrating circulation of resistance elements in pathogens belong to gene families that have their origins in the environment. Given the immense numbers of environmental bacteria and associated resistance genes accumulated over geologic time, it is a reasonable assumption that environmental bacterial genomes are the source reservoir of antibiotic resistance. That said, there are few concrete examples where the direct link between genes found in the clinic and those found in the environment is unimpeachable. The emergence of the *qnr* fluoroquinolone resistance genes, now widely circulating on mobile elements in pathogens, likely originated in environmental *Shewanella* species (Poirel et al. 2005).

Similarly, the Extended Spectrum Beta Lactamases (ESBL) of the Cefotaximases (CTX-M) family is thought to have emerged from bacteria of the genus *Kluyvera* (Rodriguez et al. 2004). Examination of the genomic context of the majority of resistance elements in environmental microbes reveals that these are embedded in genomes and rarely associated with mobilization elements (transposases, integrases, inverted repeat sequences). The history of antibiotics is that resistance, often via mobile genetic elements, inevitably follows use. The environment is a reservoir for these genes, but the acquisition by pathogens is not facile. Indeed, it is unlikely that antibiotics would have emerged as therapeutic successes in the first place. One is therefore left with the hypothesis that the use of antibiotics in bulk provides a strong selection for the stochastic capture of resistance genes by mobile genetic elements that can eventually be acquired by pathogens. This gene capture is unpredictable and can occur relatively quickly after antibiotic development, such as in the case of the serine β-lactamases, or only after decades of use, such as with vancomycin resistance. The frequency of these capture events correlates with the gene diversity and burden in the environment. For example, most surveys of resistance genes and phenotypes in nonpathogenic environmental bacteria identify β-lactamases and aminoglycoside-modifying enzymes as highly prevalent, and these were recorded as the first elements showing resistance present on mobile elements in sensitive pathogenic bacteria (Davies and Davies 2010). It is not unreasonable to hypothesize that antibiotics that show less abundant resistance gene diversity and frequency in the environment will similarly be slower to show resistance in the clinic. This criterion should be a valuable screen in antibiotic discovery to identify which scaffolds to focus on in drug development. Their cognition that the environment is a nearly boundless reservoir of antibiotic resistance has resulted in several studies that seek to estimate the risk of gene transfer to pathogens (Bengtsson-Palme and Larsson 2015; Martínez et al. 2015; Manaia 2016). This examination identifies so-called hot spots of gene transfer (manure, wastewater treatment plants), where mixing of pathogens, mobile elements, and resistance genes is more likely and therefore has greater potential to affect health (Gaze et al. 2013). The absence of contact between pathogens and environmental bacteria and the lack of enabling mobilization genes and sequences, for example, are mitigating issues that decrease the risk of gene

transfer into pathogens. Establishing more sound policies to avoid unnecessary risk of gene transfer, in particular for new antibiotics, should be a priority. Nevertheless, the history of antibiotics has demonstrated time and again that even low-frequency events will occur and antibiotic resistance is inevitable.

5 Metagenomics: A Tool to Explore Soil Resistome

Metagenomic technique has been used from the past few decades to identify resistance genes among the isolates from environmental samples (D'Costa et al. 2006; Wright 2007). The identified genes are responsible for producing compounds having antimicrobial activity and signaling molecules within bacterial community (Linares et al. 2006). Antibiotic resistome signifies collection of genes contributing to antibiotic resistance in microorganisms, having their origin either from nonpathogenic or pathogenic bacteria. This term comprises not only the genes involved in antibiotic production but also the precursor gene that could be evolved to behave as resistance element under selective pressure of the natural environment. Most of the resistance genes show ambiguity and sometime not naturally expressed in isolates from environment (Wright 2007). D'Costa et al. (2006) were the pioneers to establish this antibiotic resistome concept by screening antibiotics and constructing a library of *Streptomyces* sp. strains isolated from different environmental samples. In this way, the metagenomic approach has increased its breadth by being applied to different natural environment exploring genes and genetic elements responsible for resistance or resistance gene transfer commonly termed as "antibiotic extended resistome" (D'Costa et al. 2007). This approach has been proved beneficial for exploring diversity in different ecosystems, including nosocomial transmission, and human hosts. Moreover, it is useful for predicting future evolution of antibiotic resistance (Martínez et al. 2007).

There are several metagenomic-based reports demonstrating functional or sequence-based approach for characterization of ARGs present in natural environments (Table 1). The studies evaluated soil, water, sludge, and other samples from different environmental sources to assess genes responsible for antibiotic resistance comprising β-lactams, amphenicols, macrolides, fluoroquinolones, sulfonamides, aminoglycosides, polymyxins, lincosamides, phosphonic acids, tetracyclines, polypeptides, and trimethoprim. The only difference between functional and sequence-based metagenomic approach is that the latter can be used to identify known ARGs using in silico analysis by comparing from ARG databases, e.g., Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al. 2013) and Antibiotic Resistance Database (ARDB) (Liu and Pop 2008). In contrast, functional metagenomic approach can explore novel resistance mechanisms or could allocate new roles to previously identified proteins by classifying and separating possible open reading frames (ORFs). Perhaps, Amos et al. (2014) mark off quinolone resistance in a water sample along with assigning new characters to recombinase A (RecA) and regulatory protein X (RecX), previously known to be as ARGs. The authors established the role of RecX in modulating RecA which in turn is responsible for DNA damage repair caused due to ciprofloxacin-mediated inhibition of DNA

Table 1 List of different sources evaluated for antibiotic-resistant gene(s)/clone(s) analysis

S. No.	Sources	Phylum/genera of microbiome associated with different sources	Gene(s)/Clone(s)	References
1.	Maize	*Proteobacteria*, *Actinobacteria*	Antibiotic resistance gene for transporters, β-lactamases	Li et al. (2014)
2.	Paddy	*Actinobacteria*, *Proteobacteria*	Bac A, Sub E, Upp P, Amp C, Cmx A, Tet A, Tet G, Van A, Van H, Van R	Xiao et al. (2016)
3.	Arable soil	*Pseudomonas* sp., *Janthinobacterium* sp., *Psychrobacter pulmonis*	$bla_{CEP\text{-}0}$, $bla_{CEP\text{-}01}$, $bla_{CEP\text{-}02}$, $bla_{CEP\text{-}03}$, $bla_{CEP\text{-}04}$, $bla_{CEP\text{-}05}$, $bla_{CEP\text{-}06}$	Udikovic-Kolic et al. (2014)
4.	Apple	*Chitinophaga*, *Pseudomonas, Pedobacter*	AOAmox2, AOCarb3, AOCefta2, AOAmox1, AOCefta11	Donato et al. (2010)
5.	Arable soil	*Streptomyces* sp.	*van a*, *van b*, *van d*, *bla_a*, *bla_b*, *bla_c*	Nesme et al. (2014)
6.	Arable soil	*Actinobacteria*, *Verrucomicrobia*, *Acidobacteria*	Antibiotic resistance gene for mfs_ transporter, β-lactamase	Forsberg et al. (2014)
7.	Wood composts, activated sludge	*Proteobacteria*, *Gammaproteobacteria*, *Enterobacterales*, *Enterobacteriaceae*	NHMcSpc1 (LC306682), mgSpc1 (LC306679), mgSpc2 (LC306680)	Miyazaki and Kitahara (2018)
8.	Mariculture system	*Proteobacteria*, *Bacteroidetes*	tet31, sul2, cml_e3, cml, qnrs, dfra 1	Wang et al. (2018)

gyrase. Other reports have also successfully established the role of functional metagenomics by identifying new ARGs to β-lactam and dioxygenase through screening soil metagenomic libraries (Allen et al. 2015; dos Santos et al. 2015). Allen et al. (2015) demonstrated a response regulator gene encoded in a 5169-bp ORF which is being isolated from a resistant metagenomic clone of Alaskan soil along with a putative metallopeptidase gene. Through phenotypic experiments, it has been analyzed that the resistance to carbenicillin was due to the response regulator gene which usually alters the gene expression encoding porins and efflux pumps in *Escherichia coli*. Similarly, dos Santos et al. (2015) evaluated resistance toward nine β-lactam antibiotics through screening metagenomic library of Brazilian Cerrado soil.

However, the two abovementioned studies established the importance of metagenomics in ARG research which useful to not only reveal novel and unpredicted mechanisms of antimicrobial resistance but also to provide understanding about novel ARGs functions in natural environments (Allen et al. 2015; dos Santos et al.

2015). List of the antibiotic-resistant gene(s)/clone(s) reported from different sources is summarized (Table 1). However, functional metagenomics approach is more beneficial as it is not limited to earlier known sequences. It has also advantage of exploring some ARGs which have low or no expression (cryptic resistance genes).

6 Conclusions

Developing resistance against antibiotics in microbes is a serious concern to human health. Therefore, an improved understanding toward mechanisms conferring resistance is required to control clinically important multidrug-resistant strains. Although resistance toward antibiotics in nosocomial strains is relatively well established, natural environment comprises an immense and large ARGs diversity unexplored. To accomplish this genetic wealth, culture-independent metagenomic approaches have demonstrated to be a vital technique. This technique may provide easy way for bioprospecting new antimicrobial products and developing novel drugs through identifying and describing new mechanisms toward resistance. Apart from clinical background, roles of ARGs and antimicrobial resistance could be supported by adding new elements responsible for resistance to ARG databases such as CARD and ARDB. In the present chapter, we discussed the reports highlighting extensive diversity of ARGs in natural environments, particularly in soil. Furthermore, functional metagenomics could be utilized to characterize and classify new ARGs along with those not earlier related with antibiotic resistance. This approach will provide us the required influence to combat against multiple drug-resistant microbes of clinical importance and to unravel the ecological aspects of new ARGs present in the environment.

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