Trends in Microbiology

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Opinion

Bacteriophages as Environmental Reservoirs of Antibiotic Resistance

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Although antibiotic resistance represents a significant and growing public health concern, the contribution of bacteriophages (phages) to the mobilization of antibiotic resistance genes (ARGs) in the environment has not been extensively studied. Recent studies, however, suggest that phages play an important role in the acquisition, maintenance, and spread of ARGs than previously expected. This Opinion article offers an update on the contribution of phages to environmental antibiotic resistance. A better understanding of the mechanisms and factors that promote antibiotic resistance may significantly contribute to the implementation of control strategies.

Highlights

Given the growing global concerns about antibiotic resistance, it becomes essential to explore the mechanisms and pathways by which this phenomenon evolves and spreads.

Phages, viruses that infect bacteria, have emerged as prime suspects in bacterial adaptation and evolution by facilitating the exchange of genetic material.

Recent studies based on high-throughput sequencing approaches suggest that environmental phages play a more important role in the acquisition, maintenance, and spread of antibiotic resistance genes than previously expected.

Despite recent advances, further studies are needed to establish the extent to which phages contribute to the mobilization of ARGs in environmental settings.

The Antibiotic-Resistance Crisis

Since Alexander Fleming's discovery of penicillin in 1928, antibiotics have saved millions of lives because of their use in treating bacterial infections [1]. However, the development of antibiotic resistance soon after their discovery and application has led to the reduction or elimination of their effectiveness. Despite great efforts to combat antibiotic resistance by developing novel antibiotics between the 1960s and 1980s, the advance of new antibiotics was outpaced by the emergence of antibiotic-resistant bacteria, with the result that only a limited number of antibiotics without resistant counterparts are currently available [2]. Consequently, antibiotic resistance has made bacterial infection a serious threat to global public health.

Several reasons have contributed to the current antibiotic resistance crisis, including the overuse and misuse of antibiotics in human and veterinary medicine and the decreased effort that the pharmaceutical industry makes to develop novel antibiotics [3]. To meet the demand of the ever-increasing world population, antibiotics have been widely applied in the intensive breeding industry to treat and/or prevent diseases. This has resulted in a large amount of antibiotics being released into the environment through animal manure, which may exert selective pressure on the indigenous microbial community, leading to the selection and spread of antibiotic-resistant bacteria and their resistance genes. Moreover, previous studies have reported that antibiotic residues in the soil could clearly lead to the migration of **antibiotic-resistance genes** (**ARGs**; see Glossary) from soil to the edible part of vegetables along the soil–food chain, resulting in potential risks to public health due to exposure to antibiotic-resistant pathogenic bacteria [4,5]. It is reasonable therefore for regulatory agencies to implement stricter rules of antibiotic application for either therapeutic or preventive purposes. Meanwhile, measures should be taken to encourage the development of novel antibiotic agents. Given the extent and seriousness of the current crisis, it becomes essential to explore the mechanisms and factors that promote antibiotic resistance.

Bacteriophages – The Role of an Old Acquaintance

Bacteriophages (also known as phages) are viruses that infect bacteria and represent the most abundant and diverse entities in the biosphere [6]. The recent technological approaches applied to different ecosystems have highlighted their prevalence and importance in bacterial population control, biogeochemical cycles, and bacterial evolution [7]. In fact, it is estimated that around 20%

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of total bacterial genomes have been acquired by phage-like elements, underlining their evolutionary relevance [8]. Despite the growing evidence that supports their global importance, their functional implications are not yet well defined [9].

There is an extensive diversity among phages according to their genomic and morphological characteristics. Broadly speaking, they may be grouped according to their life cycle: lytic (virulent) or lysogenic (temperate) phages. In the lytic cycle, virulent phages use the host machinery to replicate and generate new progeny, which are released from the infected cell. In contrast, temperate phages can either multiply via the lytic cycle or enter the lysogenic cycle by integrating their genome into the host chromosome [10].

The interactions between temperate phages and bacteria can be beneficial in evolutionary terms, as many bacterial species harbor putative **prophages** in their genomes, providing benefits that can improve bacterial survival and their genetic exchange according to ecological conditions [11,12]. In other cases, prophage insertion is detrimental to host fitness because of the potential inactivation of functional genes [8,13]. Under specific conditions (environmental stress or spontaneous prophage induction), the bacterial SOS response is induced and the prophages activate the lytic cycle [14,15]. The size of phage genomes may vary from a few to 100 kb of either single- or double-stranded DNA or RNA, which generally encodes phage components, specific replicase genes and, in certain cases, bacterial genes [16]. A plethora of auxiliary metabolic and functional genes have been identified in phage genomes, suggesting their fundamental role in the contribution of phages to prokaryote evolution. For instance, phages can encode toxins, superantigens, effectors translocated by a type III secretion system, intracellular survival/host cell attachment proteins, and ARGs [17,18]. Taken together, there is no doubt that bacteria–phage interactions represent a complex synergistic relationship that drives ecological and evolutionary processes in the microbial world.

There are different mechanisms that drive the packaging of host genes into phages or phage-like particles, and their subsequent transmission to a bacterial host through horizontal gene transfer (HGT) (Figure 1). The phage-mediated transfer of foreign DNA is known as

Glossary

Antibiotic-resistance genes (ARGs): genes responsible for intrinsic or acquired resistance to antibiotics.
Bacteriophages (phages): viruses that infect and replicate within bacterial cells.
Gene transfer agents (GTAs): phage-like entities that package random DNA and facilitate horizontal gene transfer.
Metagenomics: sequencing-based analysis of genomes recovered from environmental samples.
Microbiome: a collection of microorganisms (i.e., bacteria, fungi, and viruses) in a particular environment.
Prophage: a bacteriophage genome inserted into the bacterial chromosome or plasmid.
Transduction: a process of horizontal gene transfer mediated by a bacteriophage.
Virome: genomes of all the viruses inhabiting a specific organism or environment.

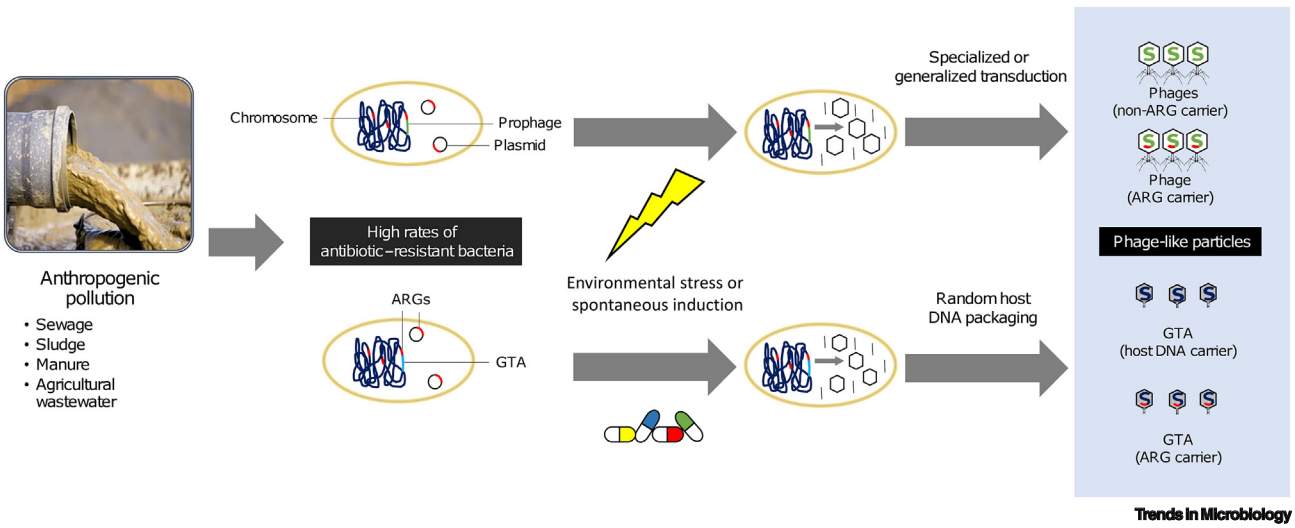

Figure 1. Phage-Mediated Horizontal Gene Transfer (HGT) Mechanisms. High rates of antibiotic-resistant bacteria are usually related to anthropogenic pollution (human or animal origin). Exposure of these resistant bacteria to environmental/chemical stressors (e.g., antibiotics) or spontaneous induction may trigger gene transfer between bacteria via phages (specialized or generalized transduction) or gene transfer agents (GTAs) (random host DNA packaging).

transduction; transduction can be divided into two types: specialized and generalized. Specialized transduction consists of the transfer of host genes in a limited number, resulting from the incorrect excision of a lysogen that also carries neighboring genes from the host chromosome [19]. In contrast, generalized transduction involves the mobilization of random segments from the bacterial DNA into capsids [20]. Another interesting genetic exchange process is carried out by **gene transfer agents** (**GTAs**): phage-like particles, produced by the host cell, that can carry a random section of their previous host cell; however, their DNA does not contain enough structural GTA genes so that their subsequent replication is limited [21]. It is estimated that the frequencies of GTA-mediated transfer are considerably higher in comparison with the frequencies observed in transformation and transduction [22]. Although the efficiency of GTA transduction highlights the importance of GTAs in the exchange of genetic information in prokaryotes, their complete mechanisms are yet to be elucidated.

By October 2018, the NCBI database included the genomes of 2200 phages (filtered by their bacterial hosts) [23]. However, they represent an insignificant fraction of the estimated population of phages in the biosphere (~ 10^{31} organisms) [24]. Over the past decades, advances in next-generation sequencing techniques have opened up the possibility of studying different organisms and their surrounding environments, and has helped us to understand better the different ecological processes. One of the advantages of the analysis of whole viral communities is that it can avoid any bias related to culture-based methods [25]. Currently, **metagenomics** data from viral sequences show a large diversity of phages, emphasizing their relevance; however, the data deposited in the public databases are still limited, impeding direct contig assemblages and functional annotation [26].

Although the number of sequenced **viromes** is limited, existing data have highlighted the relevance of phages in bacterial diversity, ecology, and evolution. Recent studies have revealed the role of human gut phages in maintaining the structure and functions of the gut

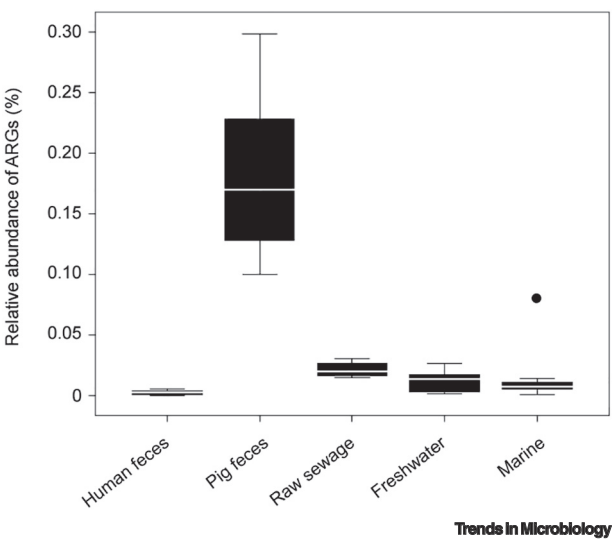

Figure 2. Relative Abundance of Antibiotic-Resistance Genes (ARGs) in Viromes from Different Habitats. Data are normalized to the total annotated sequences and are expressed as percentages. The horizontal line in each box plot represents the mean of the relative distribution in each of the five environments (human feces, pig feces, raw sewage, freshwater, and marine). Data have been adapted from Lekunberri *et al*. [42], Wang *et al*. [43], and Calero-Cáceres and Balcázar [44]. It should be noted that all viromes were screened (presence of 16S rRNA genes) to discard bacterial DNA contamination.

Key Table

Table 1. ARGs Detected Using PCR-based Methods in the Phage DNA Fraction of Environmental Samples

Drug class	Genes	Resistance mechanism[a]	Matrices	Locations	Refs
Aminoglycosides	*strA* *strB* *aaD*	Antibiotic inactivation	Fertilized soils (with dairy manure or municipal biosolids)	Canada	[45]
	aph(3')-IIIa	Antibiotic inactivation	River water	China	[46]
	armA	Antibiotic target alteration	Urban wastewater	Spain	[47]
	armA	Antibiotic target alteration	Soils; vegetables	Spain	[48]
β-Lactams	bla_{OXA-20}	Antibiotic inactivation	Fertilized soils (with dairy manure or municipal biosolids)	Canada	[45]
	$bla_{CTX-M-1}$	Antibiotic inactivation	Pig feces	China	[49]
	$bla_{CTX-M-15}$	Antibiotic inactivation	Sewage	India	[50]
	bla_{TEM} bla_{OXA-2}	Antibiotic inactivation	Animal farm soils; poultry slaughterhouse wastewater	India	[51]
	$bla_{CTX-M-1}$ $bla_{CTX-M-9}$ bla_{TEM}	Antibiotic inactivation	Cattle feces and slurry; pig and poultry slaughterhouse wastewater; river water and sediments; sewage sludge; soils; urban wastewater; vegetables	Spain	[47,48,52–56]
	mecA	Antibiotic target replacement			
	bla_{KPC} bla_{NDM} bla_{TEM}	Antibiotic inactivation	River water	Spain	[57]
Glycopeptides	*vanA*	Antibiotic target alteration	River water	Spain	[57]
Macrolides	*ermB*	Antibiotic target alteration	Pig feces	China	[49]
	ermF	Antibiotic target alteration	River water	China	[46]
	ermB	Antibiotic target alteration	River water	Spain	[57]
Phenicols	*fexA* *floR*	Antibiotic efflux	Pig feces	China	[49]
Quinolones	*qnrA*; *qnrS*	Antibiotic target protection	Pig feces	China	[49]
	aac-(6')-Ib-cr	Antibiotic inactivation			
	aac-(6')-Ib-cr	Antibiotic inactivation	River water	China	[46]
	qnrA *qnrS*	Antibiotic target protection	River water and sediments; sewage sludge; soils; urban and animal wastewater; vegetables	Spain	[47,48,54,55]
	qnrA *qnrB* *qnrS*	Antibiotic target protection	Hospital wastewater	Spain	[56]
Sulfonamides	*sul1*	Antibiotic target replacement	Fertilized soils (with dairy manure or municipal biosolids)	Canada	[45]
	sul1 *sul2*	Antibiotic target replacement	Pig feces; river water	China	[46,49]
	sul1	Antibiotic target replacement	River water and sediments; sewage sludge; soils; urban wastewater; vegetables	Spain	[47,48,54,55,57]
Tetracyclines	*tetM*	Antibiotic target protection	Pig feces	China	[49]

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Table 1 (*continued*)

Drug class	Genes	Resistance mechanism[a]	Matrices	Locations	Refs
	tetW	Antibiotic target protection	River water	Spain	[57]
	tetA	Antibiotic efflux	Animal farm soils; poultry slaughterhouse wastewater	India	[51]
	tetW	Antibiotic target protection			
	tetC *tetE* *tetG*	Antibiotic efflux	Greenhouse soils	China	[58]
	tetM	Antibiotic target protection			

[a]Mechanism of antibiotic resistance defined according to the Comprehensive Antibiotic Resistance Database (https://card.mcmaster.ca).

microbiome [27,28]. It has also demonstrated that the effect of certain stressors (environmental or metabolic) can alter the microbiome by prophage induction [29]. Interestingly, Modi *et al*. [30] showed that antibiotic treatment leads to the packaging of diverse ARGs in phage-like particles, altering the structure of the microbiome. These studies undoubtedly represent the basis for understanding the complex mechanisms that drive HGT and the role of different environments that could act as reservoirs and vehicles of ARGs and other mobile gene elements (MGEs). For this purpose, it is important to consider the potential biases related to nucleic acid extraction methods, library preparation, sequencing technologies, and bioinformatics analysis that could influence the diversity and abundance of ARGs in samples. In fact, a recent study suggests that the abundance of ARGs in viromes could be overestimated by bacterial contamination and recommends the use of conservative thresholds to avoid false positives [31].

Despite these limitations, phages have emerged as prime suspects in bacterial adaptation and evolution by facilitating the exchange of genetic material. Considering that phages are the most abundant biological entities, and have the potential to transfer genetic material between bacterial hosts, the contribution of phages to environmental antibiotic resistance should not be underestimated.

Exploring the Contribution of Phages to Antibiotic Resistance

The use, overuse, and persistence of antibiotics worldwide have accelerated the evolutionary dynamics of antibiotic resistance in bacterial communities. Although the problem was initially considered from the clinical perspective, its complex epidemiology requires a more extensive analysis that includes humans, animals, food, and the environment [32]. Recent studies have highlighted the role of the environment as the main hotspot for the evolution and spread of new antibiotic-resistance determinants [33,34]. Although ARGs have been detected in different environments, including nonanthropogenically influenced ecosystems, there are many reservoirs that contain a considerable diversity and abundance of ARGs, most of them related to bacteria from human and animal origins [35,36]. For instance, pollution sources such as urban and industrial sewage, manure, sludge, and agricultural waste contain vast numbers of ARGs that can be disseminated through different routes (bioaerosols, manure fertilization, water sources) [37,38]. Consequently, water can contribute to the dissemination of anthropogenic pollution in rivers, lakes, streams, oceans, and crop fields [39,40]. The effects of this pollution could trigger bacterial mutations of defensive mechanisms, promote the selective pressure favoring the prevalence of resistant bacteria, or facilitate the suitable conditions for HGT events between environmental bacteria and potential pathogens [35,36]. However, a quantitative model that could estimate the risk of evolution and spread of resistant bacteria and ARGs in environmental settings is still unknown, owing to the complex ecological variables that influence these phenomena [41].

Taking into account the abundance and global distribution of phages, their influence in bacterial evolution cannot be underestimated. As an example, a comparative analysis of data available from previous studies revealed that viromes from both anthropogenically impacted and nonimpacted environments contain a large reservoir of ARGs (Figure 2). Moreover, an increasing amount of literature suggests that environmental phages harboring ARGs are correlated to the proximity of anthropogenically influenced areas, especially those influenced by animal and urban wastewaters (Table 1, Key Table). These studies, based on genomic and metagenomic approaches, have revealed that environmental phage particles can harbor ARGs belonging to different groups.

Based on current evidence, wastewater treatment plants (WWTPs) represent the main hotspot of antibiotic resistance, considering the abundance and diversity of microorganisms, residual antibiotics and their metabolites that could support HGT episodes and mutations [47,54,59]. Traditionally, wastewater treatment processes aim to obtain a significant reduction in bacterial densities [60]; however, their efficiency does not imply significant reductions either in the number of resistant bacteria or in ARGs [61,62]. Different treatment approaches (i.e., UV irradiation, chlorination, Fenton and ozone oxidation, photocatalytic processes, wetland technology, coagulation, biodegradation, nanoparticles, and biochar), which are applied to reduce the microbial load, have a variable effect on resistant bacteria and ARGs, depending on the matrix, pollutant densities, and operational conditions [63–66]. In the case of phages harboring ARGs, the ARGs show a considerable persistence under disinfection treatment (i.e., chlorination, ozonation, and UV irradiation) and natural attenuation due to their protection inside the protein capsid [67]. These characteristics offer them an increase in HGT events among bacteria. Little attention has been paid, however, to the effect of advanced wastewater treatment technologies and combined disinfection methods in the removal of phages and phage-like particles harboring ARGs.

Concluding Remarks

Phages and phage-like particles play a key role in bacterial HGT and the subsequent evolution of bacteria. These characteristics make phages suitable vehicles for the acquisition, maintenance, and spread of ARGs, considering their abundance and their versatile DNA-packaging mechanisms. Given this, the prevalence, persistence, and diversity of phages and phage-like particles harboring ARGs in different ecosystems highlight the importance of including them in surveillance and monitoring programs aimed at the evolution and spread of antibiotic resistance.

This Opinion article offers an update on the contribution of phages to environmental antibiotic resistance. Although recent studies suggest that phages and phage-like particles play a more important role in the mobilization of ARGs than previously expected, additional studies are needed to elucidate the factors and mechanisms that promote dissemination of antibiotic resistance in the environment via phages and phage-like particles (see Outstanding Questions). Moreover, considering the potential link between anthropogenic pollution and increased numbers of both phages and phage-like particles harboring ARGs, it is necessary to develop technological approaches that may eliminate or reduce such agents in WWTPs. Finally, recent advances in high-throughput sequencing technologies, such as metagenomics and metatranscriptomics, may offer the possibility to analyze viromes from different ecosystems. It is therefore necessary to standardize sampling procedures, sequencing, and bioinformatics analyses in order to avoid potential biases related to these methodological procedures.

Outstanding Questions

To what extent do phages and phage-like particles contribute to antibiotic resistance?

What factors facilitate the transfer of ARGs via transduction?

Can anthropogenic activities promote the mobilization of phage-encoded ARGs?

Should phages and phage-like particles be included in the surveillance of potential risks associated with antibiotic-resistant bacteria?

Is the standardization of genomic and metagenomic techniques necessary for reducing biases related to genomic extraction, sequencing, and bioinformatics analyses?

How do phage-encoded ARGs persist under advanced WWTP technologies and combined disinfection methods?

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