Potential impact of environmental bacteriophages in spreading antibiotic resistance genes

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Summary

The idea that bacteriophage transduction plays a role in the horizontal transfer of antibiotic resistance genes is gaining momentum. Such transduction might be vital in horizontal transfer from environmental to human-body–associated biomes and here we review many lines of evidence supporting this notion. It is well accepted that bacteriophages are the most abundant entities in most environments, where they have been shown to be quite persistent. This fact, together with the ability of many phages to infect bacteria belonging

to different taxa, makes them suitable vehicles of gene transfer. Metagenomic studies confirm that substantial percentages of the bacteriophage particles present in most environments contain bacterial genes, including mobile genetic elements and antibiotic resistance genes. When specific genes of resistance to antibiotics are detected by qPCR in the bacteriophage populations of different environments, only ten-fold lower numbers of these genes than those found in the corresponding bacterial populations are observed. In addition, the antibiotic resistance genes from these bacteriophages are functional and generate resistances to the bacteria when these genes are transfected. Finally, reports about the transduction of antibiotic resistance genes are on the increase.

Introduction

The World Health Organization (WHO) has identified the increasing antibiotic resistance among bacteria as a major problem for public health on a global scale. The causes of this increase in resistance are frequently attributed to overuse and incoherent application of antibiotics in humans together with the use of antibiotics in animal husbandry [1]. But, at the same time, a growing body of evidence points to the potentially important role of environmental microorganisms from ecosystems in which the presence of antibiotics produced by humans is expected to be very low or completely absent. Such ecosystems are as varied as soil [2], a microcave isolated for over four million years [3], and pristine waters [4].

Environmental bacteria seem to be an unrestricted source of resistance genes, probably because they have emerged in bacteria that produce antibiotics, which are mainly found in

environments with limited nutritional resources. There are also resistance genes in bacteria that share habitats with antibiotic producers. Finally, many antibiotic resistance genes are not primarily resistance genes, but can easily be converted to antibiotic resistance genes, and are thus known as the hidden resistome [2]. Bearing in mind that the production of antibiotics is considered a competitive advantage for microorganisms living in environments with scarce nutritional resources, it seems likely that antibiotic resistance genes are more abundant in the microbiomes of non-contaminated ecosystems than in the microbial communities of humans and animals not suffering the pressure of antibiotics. It seems clear nowadays that environmental bacteria are an unlimited source of genes that may act as resistance genes when transferred to pathogenic microorganisms through horizontal gene transfer.

Moreover, bacteria in environments that are not contaminated with antibiotics from anthropogenic practices share antibiotic resistance genes, or resistomes, with human and animal pathogens [5,6]. A study by Tacao *et al*. [6] focused on Extended-spectrum β-lactamase (ESBL) and cefotaxime-hydrolyzing β-lactamase (CTX-M) compared resistomes in polluted and unpolluted rivers, and found that: i) the level of diversity among CTX-M-like genes from unpolluted rivers was much greater than in polluted ones; ii) the majority of CTX-M like genes found in polluted waters were similar to chromosomal ESBL such as β-lactamase $bla_{\text{RAHN-1}}$; iii) diversity was much lower in the polluted river, revealing the presence of different genetic mobile platforms previously described for clinical strains. A good example is found when looking at β-lactamases and *Enterobacteriaceae.* Available information reveals that many β-lactamases nowadays present in genetically mobile platforms are originally chromosomally located in strains of the *Enterobacteriaceae* family, which are considered environmental bacteria. These include different species of *Kluyvera*, *Rahnella aquatilis*, *Klebsiella oxitoca*, *Citrobacter diversus*, *Proteus penneri*, *Serratia*

fonticola, and *Raoultella planticola*, among others [7]. Whether and how these chromosomally located genes from environmental bacteria, some with little or no contact with β-lactam antibiotics, reach the pathogens and appear in genetically mobile platforms remains to be elucidated. Similar observations and questions arise for other antibiotics and bacterial groups.

Some consensus is developing regarding the view that horizontal transfer and subsequent integration into mobile platforms of resistomes found in environmental bacterial populations are two different events that may occur independently at different stages. The horizontal transfer occurring anywhere at random and the assembly of genetically mobile platforms more likely to occur in environments where there is a selective pressure, like human and animal bodies associated biomes or wastewater treatment plants [7, 8], where phage and bacteria are very abundant and there is an exposure to antibiotic that makes wastewater treatment plants a good source of antibiotic resistance genes and their spread in the environment.

Mobilization of antibiotic resistance determinants

Horizontal gene transfer by conjugation, transformation or bacteriophage transduction is thought to provide the single most important mechanism with which to accelerate the dispersal of antibiotic resistance genes among bacterial populations. Genetically encoded resistance determinants are inherited vertically, but also through horizontal transfer, which means that the spread of the resistance genes is not restricted to microorganisms of the same species but also occurs between different bacterial species or even genera. This process may occur both between pathogens and between pathogenic and non-pathogenic

strains. Within a human or animal body this transfer could occur in the microbiomes in which these bacteria reside, for example the microbial populations of the gut or the lungs.

Resistance genes are then usually spread by mobile genetic elements (MGEs). Previously described MGEs for the horizontal dissemination of antibiotic resistance determinants are diverse: plasmids, transposons, bacteriophages, genomic islands and integrons could be included in this group, since they agglutinate different genes in a single genetic platform [7-9]. Many integrative MGEs incorporate a method of getting into and out of genomes, involving integrases or related enzymes. Integration could occur in the chromosome, but also within a plasmid present in the recipient strain. Incorporation into the bacterial genome seems to be necessary for the survival of the recently acquired element and the antibiotic resistance genes that it contains.

Some studies have evaluated the role of MGEs in aquatic environments. Sengelov and Sorensen [10] reported that plasmid transfer from a donor to a recipient cell occurs in environments such as bulk water, although at a low frequency. In contrast, integrons, particularly class 1 integrons, play a crucial role in the evolution of antibiotic resistance in clinics [7]. Indeed, class 1 integrons are not only platforms for gene aggregation, leading to the establishment of multi-drug resistance, but their localization on MGEs such as plasmids and transposons favors the spread of several genes in a unique transfer event. Integrons of class 1 are largely found in the environment and there is evidence that the clinical class 1 integrons originated from environmental bacterial communities [11]. Thus, conjugation, which requires cell-to-cell contact, has been considered to play a major role in the horizontal transfer and consequent spread of antibiotic resistance. Probably due to their higher incidence in clinical settings and the methodological complexities involved in the study of phages, much effort has been devoted to the study of plasmids, integrons and

transposons. Additionally, free exogenous DNA can also be captured by natural transformation [7] .

More recently, several reports [8,12-14] have proposed that the role of phages in the horizontal transfer of antibiotic resistance genes is much more relevant than previously thought [12]. However, information on the actual involvement of phages in the spread of antibiotic resistance genes remains scarce.

Bacteriophages transduction

Bacteriophages, or phages, are viruses that infect bacteria. Bacteriophages are extremely abundant in nature, probably the most abundant life form on Earth. Their role in microbial ecology is nowadays widely accepted. On the one hand, by infecting and lysing infected bacteria, they contribute remarkably to bacterial mortality, for example up to 15% in the case of bacterioplankton [15]. Consequently, they regulate the numbers of certain bacteria in a given environment and by releasing organic compounds through cell lysis they have an important impact on the cycling of organic matter in the biosphere at a global level. On the other hand they control microbial diversity. They achieve this by selecting some types of bacteria that are resistant to their attack [16], thus changing the proportions of bacterial species or strains in a community, and consequently influencing the evolution of bacterial genomes through horizontal gene transfer by transduction.

As viruses, they can only replicate in a susceptible host cell. Basically, bacteriophages present two different life cycles, the lytic and the lysogenic. In the lytic cycle, following infection the bacteriophage redirects the host metabolism towards the production of new

phages that are released by lysis of the host cell. Bacteriophages that can only follow the lytic cycle are known as virulent bacteriophages. Other bacteriophages, known as temperate bacteriophages, can follow the lysogenic cycle, in which the genome of the temperate phage remains in the host, replicating along with the host, either integrated in the cell chromosome or as an independent replicon. At this stage, the bacteriophage is known as a prophage, which can be induced to follow the lytic cycle. Induction occurs either spontaneously or when stimulated by inductors. Lysogenic inductors can be natural, such as host starvation and UV light, or introduced by human activity in the environment (e.g., some antibiotics, the best known being the quinolones) [17].

In their extracellular phase, bacteriophages basically consist of a nucleic acid molecule, the genome, surrounded by a protein coat called the capsid. Many phages also contain additional structures such as tails and spikes. These extracellular viral particles are named virions. Because of their simple structure and composition, virions persist quite successfully in the environment and are quite resistant to natural and anthropogenic stressors [18,19]. Their persistence in comparison to their host depends on the habitat of the host. It is likely that phages infecting bacteria indigenous in a given habitat are less persistent than the bacterial host [20]; in contrast, in habitats in which the host bacteria are aliens, they persist much better than the bacteria [18,19]. Neither the persistence of the bacteriophage virions in a given habitat nor their high resistance to stressors seems to depend on the habitat in which their hosts live. Due to the structural characteristics of phages, their persistence in the environment is also much higher than that of free DNA, which is more sensitive to nucleases, temperature and radiation. These survival capabilities make bacteriophages especially suited for movement between different biomes [21].

Indeed, many but not all phages can mobilize genetic material among different host bacteria in a process known as transduction. Typically, transduction has been associated with temperate bacteriophages, probably because their method of replication facilitates the detection of transductants, but virulent bacteriophages can also transduce [22]. Via transduction, genetic material can be introduced into a bacterium by a phage that has previously replicated in another bacterium, in which it packaged random DNA fragments (generalized transduction) or the DNA adjacent to the prophage attachment site (specialized transduction). The size of the DNA fragments that can be packaged into a bacteriophage particle is limited by the size of the phage capsid, but can reach upwards of 100 kilobases (kb). Transduction by bacteriophages includes any sort of bacterial DNA, including linear chromosome fragments and all sorts of mobile elements such as plasmids, transposons and insertion elements [23,24]. Recent metagenomic studies of the viral fraction of activated sludge liquor show that the viral fraction contains a significant percentage, 8.2%, of mobile genetic elements [25]. Transduction does not require “donor” and “recipient” cells to be present at the same place or even at the same time, and for this reason bacteriophages have been contemplated as the optimum way of transferring genetic information among different biomes [26]. Transduction has so far been considered as a rare event occurring around once every 10^7–10^9 phage infections [27]. Even given this low frequency, considering the numbers of phages and hosts in many environments, gene transfer by transduction will take place an exceptional number of times per second in any one place. Moreover, it has recently been reported that transduction might occur at frequencies several orders of magnitudes greater than previously thought [28-30]. Since phage-encapsulated DNA is protected from degradation and phages may survive in special environments without the loss of their infectious capabilities, gene transfer by transduction might well be more important than previously thought, which supports the notion that the contribution of phages to gene transfer in non-human–associated microbial

communities and in human-generated environments is greater than that of plasmids. In clinical settings, though, plasmids are probably the most relevant MGEs for horizontal antibiotic resistance transfer.

On the other hand, the spectrum of bacteria (referred to as the host range) that can be infected by a given bacteriophage depends firstly on the presence of bacterial receptors recognized by the phage. Many bacteriophages have a narrow host range infecting a limited number of strains of a given species. But others, known as polyvalent bacteriophages, have been reported to have a wide host range that crosses the boundaries of different taxa. The transduction by polyvalent phages, although thought of as relatively rare, has been reported between: i) different bacterial species in a genus, for example *Enterococcus* [31]; ii) different genera in a family, for example *Enterobacteriaceae* [29,32], *Actinomycetaceae* [33] and *Synechococcaceae* [34]; iii) bacteria belonging to different orders, for example *Lactobacillales* and *Bacilalles* [35], and *Pasteurelalles* and *Enterobacteriales* [36]; iv) bacteria belonging to different classes, for example *Gammaproteobacteria* and *Betaproteobacteria* [37]; v) and even between Gram+ and Gram- bacteria [38]. Furthermore, transduction has also been described between bacteria belonging to different taxa [31,35]. In addition, similar prophages have been detected in bacteria of different species of *Clostridium* and *Bacillus* spp, [39].

Once inside the new cell, the acquired sequences must escape degradation by the bacterial restriction systems of the cell, and then be incorporated into the recipient's genome. Incorporation can be achieved either by homologous recombination or by integration, or by becoming associated with, or being itself, an autonomous replicating element (i.e., a plasmid). Other than the characteristics of the sequence transduced, the persistence of transduced sequences will depend on several factors (*e.g* the host cell, the

growth rate, environmental factors, etc), that will determine the final frequency of transduction.

Ubiquity and abundance of bacteriophages

Extracellular bacteriophages, or virions, are ubiquitous, with a global abundance that exceed that of bacteria and archea. At a given time, a significant fraction of bacterial cells can be infected by a lytic phage (up to 5% of the bacterial population) [40]. Moreover, relevant percentages of lysogeny, and hence of bacteria with inducible bacteriophages, have also been described in some environments; thus inducible fractions ranging from 4 to 68% have been described in different types of soil [41].

High numbers of bacteriophages have been detected in all sorts of environment, with variable numbers that seem to depend on bacterial abundance and activity (Table 1). Indeed, they have been detected in high numbers in marine, freshwater and soil systems [42], in human- and animal-associated microbial communities [43], in microbial communities associated with the plant phyllosphere and rhizosphere [44], in anthropogenic environments such as wastewater treatment plants [45], and even in extreme environments [46]. The concentrations of bacteriophages detected in different environments are summarized in Table 1.

Relevant information for evaluating the chances of bacteria–bacteriophage interactions, at least to guarantee the encounters needed for infection, is the concomitant presence of adequate or minimal numbers of host bacteria that can ensure such interactions. The concept bacteriophage (virus) to bacteria ratio (VBR) can be used to infer the numerical

relationship between bacteriophages and bacteria in a given setting. This ratio depends on the source of the samples and is variable in time. Reported values range from 0.01 to 100; but the predominant situation is that VBRs range between 1 and 10 [42] in most microbiomes, indicating that on most occasions phages outnumber bacteria by a factor ranging from 1 to 10.

While not all phages will find and infect a bacterial host, the ubiquity, abundance and persistence of phages in the environment makes them ideal genetic vehicles for the transfer of genes between bacteria, be they from different taxa or biomes (Fig. 1).

Bacterial genes in viral communities

The inability to culture most of the bacteriophages present in natural viral communities and the limitations of the traditional techniques used in virology and the study of bacteriophages have hampered the study of aspects of viral communities such as their diversity and the potential contribution of these populations to horizontal gene transfer in natural environments. However, in the last few years, very powerful tools for genomic analysis have provided some insight into these aspects. On the one hand metagenomic analysis of these viral communities has provided a huge amount of information on the characteristics of the genetic material included in the viral particles that constitute the viral communities of the biomes of natural environments, anthropogenic environments such as wastewater treatments plants, and in the microbial communities associated with human and animal bodies. On the other hand, the application of qPCR specific for the amplification of certain sequences has allowed the abundance of a number of genes in different viromes to be determined.

Other than confirming that most viruses in the viral fraction of most environments are bacteriophages, metagenomic studies have shown that a large proportion of the viral particles contain bacterial DNA sequences. Searching the metagenomic libraries obtained using DNA of the viral fractions corresponding to different environments yields variable, but high percentages of sequences assigned to bacteria. Thus, these percentages ranged from 14 to 72% in different oceanic regions [47]; 54% in marine sediments [48]; 56% in an activated sludge microbial assemblage [49]; from 7.9 to 28% in stool samples of infants [50,51]; and between 30 and 35% in well and reclaimed water [52]. Significant fractions have also been reported for sewage [53] and respiratory tract communities [54].

Various explanations contribute to the large number of bacterial genes found in viral DNA fraction, but perhaps the most relevant is revealed by sequencing the genome of phages from environmental samples, that shows that some carry bacterial genes (8). These genes are fully-functional and can be transcribed and translated by the host. While phages do not need these genes for their replication, they likely give phages or their host a selective advantage. Examples of such genes include *psb* (photosynthesis), *pho* (phosphate acquisition), *spe* (exotoxin A), *stx* (Shiga toxin), *ctx* (Cholera toxin) or *hns* (histone-like protein for transcription regulation), and functional analysis of the bacterial sequences detected in phage genomes reveals genes implicated in all cellular functions. However, the composition varies depending on the environment. Whereas the prevalence of genes related to DNA metabolism displays little variation, the incidence of many genes varies according to habitat [55,56]. In addition, there is a clear correlation between the functional composition of viral and cellular metagenomes [57]. The bacterial DNA seized by the viral particles in a number of biomes, other than bacterial genes implicated in all cellular functions, contains prophages, mobile genetic elements and integrases, transposases and

recombinases [52]. Thus, the percentage of reads similar to prophages ranges from 12% in an infant gut [51] to 26% in near-shore marine sediments [48]. Mobile genetic elements have also been detected in the viromes of marine sediments, infant guts, activated sludge and fermented foods [25,48,51,58], with values ranging from 15 to 22%.

All the bacterial genes and genetic elements contained in the viral communities of most biomes studied indicate that both specialized and generalized transduction occur frequently.

Antibiotic resistance determinants in viral communities

Metagenomic studies of viral communities indicate that sequences corresponding to antibiotic resistance genes were detected in the viral communities of the human gut [59] and in an activated sludge wastewater treatment plants [25]. Sequences corresponding to drug efflux pumps, streptogramin acetyltransferases, lipoprotein, TetC protein, glyoxilase/bleomycin resistance protein and β-lactamases have been identified in these studies. Fancello *et al*. [60] found many short sequences in cystic fibrosis sputum viromes putatively encoding resistance to antimicrobials, and only three in the non-cystic fibrosis sputum. Of these, they confidently identified 66 efflux pump genes, 15 fluoroquinolone resistance genes and 9 β-lactamase genes. Phylogenetic analysis of these genes demonstrated different origins of these genes within the cystic fibrosis bacteriophage community.

A few years before the first studies on viral metagenomics in 2002, detection by Polymerase Chain Reaction (PCR) of specific genes in the viral fraction of raw municipal

wastewater was described. Indeed, Muniesa *et al*. [61] reported the abundance of bacteriophages infecting *Escherichia coli* O157:H7 and carrying the Shiga Toxin 2 gene in municipal wastewater and, a couple of years later, Sander and Schmieger [62] detected phages carrying 16S rRNA of different bacterial species in the viral community of the mixed liquor of an activated sludge plant. In addition, Muniesa *et al*. [13] reported the occurrence of viral particles carrying sequences of $bla_{\text{OXA-2}}$, $bla_{\text{PSE-1}}$ or $bla_{\text{PSE-4}}$ and *bla*PSE-type genes in the viral fraction of raw municipal wastewater.

The emergence of highly sensitive quantitative-PCR has enabled the quantification of viral particles carrying a given gene in all sorts of samples. Thus, Colomer-Lluch *et al.* [14] used quantitative real time PCR to quantify the number of viral particles carrying sequences corresponding to bla_{TEM} and $bla_{CTX\text{-M}}$, as well as *mec*A, in the bacteriophage DNA fraction of raw municipal wastewater and river water impacted by anthropogenic contamination, with concentrations of bla_{TEM}, $bla_{CTX\text{-M}}$ and *mec*A ranging from 3 to 4, 1 to 2 and 1 to 2 $\log_{10}$ units in raw municipal wastewater and 2 to 3, 0 to 1 and 1 to 2 $\log_{10}$ units in river water respectively. In both cases, the values in the viral fraction were about 10 times lower than those in the bacterial fraction. In addition, densities of 3 to 4 $\log_{10}$ gene copies (GC) of bla_{TEM}, 2 to 3 $\log_{10}$ GC of $bla_{\text{CTX-M}}$, and 1 to 3 $\log_{10}$ GC of *mecA* per milliliter or gram of sample were detected in the viral community of fecal waste from cattle, pigs and poultry [63], with the samples corresponding to cattle being unlikely to have had any contact with anthropogenically introduced β-lactam antibiotics. Again, the ratio of genes carried by bacteria to genes carried by bacteriophages was relatively constant and of the same order of magnitude as that found in the samples of wastewater and the river. Table 2 shows a summary of antibiotic resistance genes found in viral comunities or as a part of phage genomes.

To date, to the best of our knowledge, it has not been possible to detect the transduction of antibiotic resistance determinants using phages partially purified from the different microbial communities studied. This may be due to experimental difficulties in the preservation and identification of the potential transductants (see Fig. 2). *In vitro* though, the *bla* genes have been successfully transfected from phage DNA to host bacteria, which became resistant to ampicillin [14].

All these studies strongly indicate that bacteriophages are a reservoir of antibiotic resistance genes in different habitats. The studies reported in this section raise two questions: i) the potential of bacteriophages to mobilize antibiotic resistance determinants, either by generalized or specialized transduction, and ii) the presence of antibiotic resistance genes in viruses that have not been in contact with antibiotics for which they carry resistance determinants.

Transduction of antibiotic resistance genes into/from pure cultures

An increasing number of phages induced from lysogenic bacteria, most of them isolated in clinical studies, as well as a few isolated from natural samples, have been reported to transduce genes of resistance to antibiotics. These examples reinforce the role of phages in the mobilization and spread of antibiotic resistance.

In *Streptococcus pyogenes*, there are some early descriptions of drug-resistant strains that were treated with mitomycin C to induce phages, along with the transduction of drug resistance by means of the phages so induced. Transduction of tetracycline resistance or multiresistance acquisition to chloramphenicol, macrolide antibiotics, lincomycin and

clindamycin via phages occurred [64]. Also in *S. pyogenes*, a bacteriophage from clinical isolates harbouring resistance to erythromycin caused transduction of the resistance, yielding transductants resistant to relatively high concentrations of erythromycin [65]. Moreover, also in *S. pyogenes*, the *mefA* gene, which encodes a macrolide efflux protein, is associated with a 58.8-kb chimeric genetic element composed of a transposon inserted into a prophage [66].

In *Bacillus anthracis*, prophage Wβ encodes demonstrable fosfomycin resistance, and the authors suggest that this could have occurred by the well-documented ability of bacteriophages to acquire prophage genes from their host via recombination, thus creating chimeric forms [67].

In *Pseudomomas aeruginosa*, wild-type phages induced from a strain resistant to imipenem, cefotaxime, kanamycin and streptomycin showed a high frequency of transduction for kanamycin and particularly for cefotaxime resistance determinants, followed by imipenem determinants [68]. The resistance determinants to anti-pseudomonal antibiotics (imipenem, aztreonam and ceftazidime) could be separated by transduction. Thus, the resistance to these antibiotics was presumably coded by different genes [68].

Actinobacillus actinomycetemcomitans (Aa) strain ST1 carries the tetracycline (Tc) resistance transposon Tn916 and the Aa phi ST1 prophage. High-titer phage preparations induced from this strain by mitomycin C were used to transduce the Tc resistance determinant to susceptible recipient strains [69]. In addition, the same bacteriophage, Aa phi ST1, and another one named Aa phi 23, were capable of transducing the chloramphenicol (Cm) resistance marker of a plasmid (pKT210). This plasmid in the recipient strains was indistinguishable from the same plasmid found in the donor strain.

In Enterococcus, three bacteriophages isolated from environmental samples of pig host strains of *Enterococcus gallinarum* and *Enterococcus faecalis* were used to transduce tetracycline resistance from *Ent. gallinarum* to *Ent. faecalis* and gentamicin resistance from *Ent*. *faecalis* to *Enterococcus faecium*, and from *Enterococcus hirae*/*durans* to *Enterococcus casseliflavus* [31] .

Varga *et al*. [70] recently reported a high frequency of transduction of penicillinase and tetracycline resistance plasmids within methicillin-resistant *Staphylococcus aureus* clone US300, one of the *S. aureus* clones with the greatest spread worldwide. This study proves that transduction is an effective mechanism for spreading plasmids within a single clone that could evolve faster.

In *Salmonella* there are some examples of transduction of antibiotic resistance genes. Schmieger and Schicklmaier [23] reported phage-mediated transfer of ampicillin, chloramphenicol and tetracycline resistance among *S. enterica* Typhimurium DT104. Moreover, transduction of bla_{CMY-2}, *tet*A, and *tet*B was achieved with phages induced from *S. enterica* serovar Heidelberg to *S. enterica* serovar Typhimurium, indicating that transduction of antibiotic resistance genes can happen between serovars and that this is common in *Salmonella* of bovine origin, since many of these phages demonstrate a broad host range. This is not surprising given data that indicate that about 95% of strains of *S. enterica* serovar Typhimurium examined to date contained complete inducible prophage genomes and that 99% of these phages were capable of generalized transduction of chromosomal host markers and plasmids [71]. The occurrence of generalized transduction in this study was supported by the fact that β-lactam resistance and tetracycline resistance were not co-transduced and the transduction frequency for β-lactam resistance was the

same as that for tetracycline resistance. The core resistance genes in *S. enterica* serovar Typhimurium DT104 are chromosomally encoded in a tight cluster as part of *Salmonella* genomic island I (43 kb), which is well within the size that a bacteriophage could package and transduce [23,72].

Conclusions

All this information suggests that phages play a much more important role in mobilizing determinants of resistance to antibiotics than was thought a few years ago, when horizontal transfer of these antibiotic resistance determinants was almost exclusively thought to be due to plasmids. This conclusion is based on a number of facts that have been reviewed throughout the paper and that are summarized below.

The ubiquity of phages, their great abundance, and resistance to environmental stressors means that they can move between different biomes, and since they can transfer genetic information by transduction, they are good candidates for the transfer of genetic information between biomes. They can transfer both individual antibiotic resistance genes and resistance genes linked to mobile genetic platforms, by both generalized and specialized transduction. Hence, they may play an important role in transferring antibiotic resistance genes between biomes and within biomes.

They can also transfer genetic information between bacteria belonging to different taxa. Bacteriophages are numerous in many environments, mostly in aquatic environments. Their numbers are high enough to guarantee phage–bacteria encounters and hence guarantee infection and transduction.

Recent genomic studies of viromes (viral communities), with bacteriophages being in the majority, indicate that large proportions of viral particles carry bacterial genes, among them antibiotic resistance genes, pointing to greater probabilities of transduction than previously thought.

Based on the above, we hypothesize that phages might play a crucial role in the early stages of transfer of the chromosomally located resistomes of environmental bacteria, as a random event probably through generalized transduction, to commensal bacteria of the microbial communities of human and animal bodies (which have recently been described as potential reservoirs of resistance genes [73]), and ultimately to pathogens. In addition, since they can also transfer plasmids and other mobile genetic elements, their participation in the horizontal transfer of these platforms between members of different microbial communities including those of human and animal bodies is quite likely (Fig. 1).

Future perspective

Information on this topic remains scarce and much work remains to be done to confirm some of the hypothesis discussed, and how to act to minimize the transfer of genes in natural, mostly water-borne, environments to human and animal commensals and pathogens. Key aspects to investigate are:

- Better characterization of the resistomes of environments, both natural and anthropogenically managed, like wastewater treatment plants, that are not contaminated with present and future antibiotics used in medicine and veterinary practice.

- Determine whether bacteriophages participate in their spread to human and animal microbial communities.
- Determine which biomes are most highly implicated in the origin and transfer of antibiotic resistance genes to human and animal pathogens.

Finally, all the information generated should be used to minimize the transfer of antibiotic resistance genes from biome to biome.

Executive summary

- The World Health Organization (WHO) has identified increasing antibiotic resistance among bacteria as a major problem for public health on a global scale.
- Bacteria in environments not contaminated with antibiotics by anthropogenic practices share antibiotic resistance genes with human and animal pathogens. The key question is how do these genes move from environmental bacteria to those found in clinical settings?

<u>Mobilization of antibiotic resistance determinants</u>

- Resistance genes are usually spread by mobile genetic elements (MGEs).
- Horizontal gene transfer by conjugation, transformation or bacteriophage transduction is thought to provide the single most important mechanism for accelerating the dispersal of antibiotic resistance genes among the bacterial population.

<u>Bacteriophages</u>

- Because of their simple structure and composition, virions persist quite successfully in the environment and are quite resistant to natural and anthropogenic stressors.
- Phages can mobilize antibiotic resistance genes through generalized or specialized transduction and convert susceptible hosts to clones with resistance to a given antibiotic.

Ubiquity and abundance of bacteriophages

- Extracellular bacteriophages, or virions, are ubiquitous, with a global abundance that seems to exceed that of bacteria and archea.
- High numbers of bacteriophages have been detected in many different environments.

Bacterial genes in viral communities

- Metagenomic analysis of these viral communities as well as the application of qPCR specific for the amplification of certain sequences has enabled measurement of the abundance of a number of bacterial genes in different viromes.

Antibiotic resistance determinants in viral communities

- Metagenomic studies of viral communities indicate that sequences corresponding to antibiotic resistance genes are detected in the viral communities of different biomes.
- Bacteriophages may be a reservoir of antibiotic resistance genes in different habitats.

Transduction of antibiotic resistance genes into/from pure cultures

- An increasing number of phages induced from lysogenic bacteria, most of them isolated in clinical studies, as well as a few isolated from natural samples, have been reported to transduce genes coding resistance to antibiotics.

- It has not yet been possible to detect the transduction of antibiotic resistance determinants using phages partially purified from the different microbial communities studied.

Conclusions

- Because of their physical characteristics and resistance to environmental stressors, phages can move between different biomes, and transfer genetic information by transduction.
- Phages might play a crucial role in the early stages of transfer of the chromosomally located resistomes of environmental bacteria, as a random event probably though generalized transduction, to commensal bacteria of the microbial communities of human and animal bodies (which have recently been described as potential reservoirs of resistance genes), and ultimately to pathogens.

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Reference Annotations

**D'Costa et al. 2006.

This paper reports how the soil is a reservoir of all sorts of resistance determinants from where they can be mobilized into different microbial communities.

**Lupo et al. 2012.

This paper reviews how the environment, and specifically freshwater, constitutes a reactor where the rise, evolution and mobilization of antibiotic resistance determinants occur.

*Saylers et al. 2004

The authors review the importance of commensal bacteria in human and animal body communities as reservoirs and intermediaries in the mobilization of antibiotic resistance determinants from environmental bacteria to pathogens and vice versa.

*Chibani-Chennoufi et al. 2004.

This is a review paper about the bacteriophages and their host interactions, mostly from an ecological perspective. It contains interesting information on the role of bacteriophages in gene mobilization, numbers and persistence of phages in nature, polyvalence of phages, etc.

**Weinbauer, 2002.

This is a very long, but very useful review that contributes a lot of information essential to assess the potential of phages as tools of genetic interchange in nature and that is feasible the movement of phages between different biomes.

*Parsley et al. 2010.

This is one of the many papers, published in the last few years, referring how the viral fraction of a given biome, in this case the population of a activated sludge water depuration plant, contains abundant and diverse bacterial sequences that code for all functional capabilities, as well as mobile genetic elements so frequently associated to antibiotic resistance.

*Schmieger and Schickmaier. 1999.

This is an example of the numerous papers that report the transfer by transduction of determinants of antibiotic resistance.

**Colomer-Lluch et al. 2011.

To our knowledge this is the first paper to report quantitative data on the numbers of phage particles carrying antibiotic resistance genes in a pair of water biomes. As well it compares the numbers of these genes in phages and in the bacterial fraction. As well it reports how these genes are functional since they we transfected to host bacteria.

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Figures

Figure 1.- Schematic representation of the mobility of bacteriophages between biomes and between commensal and pathogenic bacteria.

Figure 2.- Difficulties in the process of generation and detection of transductants with bacteriophages from an environmental pool. To guarantee successful transduction, an infectious phage should encounter its bacterial host, overcome defense systems of the host to integrate its genome within the host genome. Moreover, the presence of lytic phages in the same phage pool could cause the lysis of the transductants generated before they could have been selected on an agar plate.

Table 1. Ubiquity and abundance of bacteriophages**.**

Origin	Concentration	Reference
Deep sea environments	10^4-10^5 VLP/ml	[74]
Coastal environments	10^6-10^7 VLP/ml	[74]
Productive lakes or estuarine waters	10^8 -10^9 VLP	[75]
Limnetic and marine sediments	>10^8 -10^9 VLP	[76]
Soil or rizosphere	10^7-10^8 VLP/g	[77]
Intestinal content	5x10^7-10^{10} VLP/g	[43]
Sputum of patient with bronchopulmonary infections	10^3-10^7 PFU on *Pseudomonas aeruginosa*	[78]
Plant's microbial communities	>10^6 PFU/g of leave tissue of phages infecting *Erwinia*	[79]
Activated sewage sludge	>10^9 VLP/ml	[45]
Raw municipal wastewater	10^8 VLP/ml	[52]
Potable and well water	10^5-10^6 VLP/ml	[52]

VLP: virus like particles; PFU: plaque forming units

Table 2.- Antibiotic resistance genes described within the genome of bacteriophages, phage-related elements or in the viral DNA fraction of diverse biomes.

Resistance gene or protein	Antibiotic	Source (natural reservoir, or bacteria)	Reference
Genes			
bcrA	bacitracin	swine fecal microbiomes, human gut viromes, cystic fibrosis sputum microbiota	[17, 59, 60]
*bla*OXA-2, *bla*PSE-1, *bla*PSE-4, *bla*(PSE)-type genes	β -lactam antibiotics	sewage	[13]
bla_{TEM}, $bla_{CTX\text{-}M}$	β -lactam antibiotics	sewage water, river water, animal wastewater	[14]
$bla_{CTX\text{-}M\text{-}10}$	β -lactam antibiotics	*Enterobacteriaceae*	[80]
*bla*CMY-2,	ampicillin	*S. enterica*	[23]
dfrAa	trimethoprim	swine fecal microbiomes	[17]
fluoroquinolone resistance genes	fluoroquinolones	cystic fibrosis sputum microbiota	[60]
macB	macrolides	swine fecal microbiomes	[17]
mecA	methicillin	Sewage water, river water, animal wastewater	[14, 63]
mef(A)	macrolides	*S. pyogenes*	[17, 66]
*tet*A, *tet*B	tetracylcline	*S. enterica*	[23]
tet(W)	tetracycline	swine fecal microbiomes, human gut viromes	[17, 59]
tet37	tetracycline	swine fecal microbiome	[17]
Genes not anotated	β -lactam antibiotics	human gut viromes, cystic fibrosis sputum microbiota	[59, 60]
vancomycin resistance genes	vancomycin	swine fecal microbiomes, human gut viromes	[17, 59]
genes not anotated	fosfomycin resistance	prophage Wβ *B. anthracis*,	[67]
genes not anotated	tetracycline, gentamicin	*Enterococcus*	[31]
ND	erythromycin	*S. pyogenes*	[65]
ND	tetracycline, chloramphenicol, macrolide antibiotics, lincomycin, clindamycin	*S. pyogenes*	[64]
ND	imipenem, cefotaxime, ceftazidime, aztreonam, kanamycin, streptomycin	*P. aeruginosa*	[68]
Predicted proteins			
Acriflavin resistance protein	acriflavin	viral metagenomes from an activated sludge microbial assemblage	[25]
class A β-lactamase	β -lactam antibiotics	viral metagenomes from an activated sludge microbial assemblage	[25]
Drug resistance transporter Bcr/CflA	ND	viral metagenomes from an activated sludge microbial assemblage	[25]
glyoxalase/bleomycin resistance protein	ND	viral metagenomes from an activated sludge microbial assemblage	[25]
TetC protein	tetracylcline	viral metagenomes from an activated sludge microbial assemblage	[25]
Tc resistant transposon Tn916	tetracylcline	phage Aa phi ST1 in *A. actinomycetemcomitans*	[69]
Cm resistance marker Of plasmid pKT210	chloramphenicol	phages Aa phi ST1 and Aa phi 23 in *A. actinomycetemcomitans*	[69]
Streptogramin acetyltransferase	streptogramin	human gut viromes	[59]

ND. Not determined