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Minireview

Bacteriophages and genetic mobilization in sewage and faecally polluted environments

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Summary

Bacteriophages are one of the most abundant entities on the planet and are present in high concentrations within humans and animals, mostly in the gut. Phages that infect intestinal bacteria are released by defecation and remain free in extra-intestinal environments, where they usually persist for longer than their bacterial hosts. Recent studies indicate that a large amount of the genetic information in bacterial genomes and in natural environments is of phage origin. In addition, metagenomic analysis reveals that a substantial number of bacterial genes are present in viral DNA in different environments. These facts support the belief that phages can play a significant role in horizontal gene transfer between bacteria. Bacteriophages are known to transfer genes by generalized and specialized transduction and indeed there are some examples of phages found in the environment carrying and transducing genes of bacterial origin. A successful transduction in the environment requires certain conditions, e.g. phage and bacterial numbers need to exceed certain threshold concentrations, the bacteria need to exist in an infection-competent physiological state, and lastly, the physical conditions in the environment (pH, temperature, etc. of the supporting matrix) have to be suitable for phage infection. All three factors are reviewed here, and the available information suggests: (i) that the number of intestinal bacteria and phages in faecally contaminated environments guarantees bacteria–phage encounters, (ii) that transduction to intestinal bacteria in the environment is probable, and (iii) that transduction is more frequent than previously thought. Therefore, we suggest that phage-mediated horizontal transfer between intestinal bacteria, or between intestinal and autochthonous bacteria in extra-intestinal environments, might take place and that its relevance for the emergence of new bacterial strains and potential pathogens should not be ignored.

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Introduction

Viruses are one of the most abundant entities in all known ecosystems, such as seawater and surface freshwater, marine and freshwater sediments, and soils (Bergh *et al*., 1989; Danovaro *et al*., 2001; Williamson *et al*., 2003). Most of the viruses found in different biomes are assumed to be bacteriophages because they show a characteristic phage morphology (Bergh *et al*., 1989) and because environmental viral DNA sequences have been identified as phage DNA by metagenomic analysis (Breitbart *et al*., 2003; Willner *et al*., 2009). High concentrations of phages are also found within humans and animals, mostly in the gut, which holds by far the most important microbial community in animals (Cann *et al*., 2005; Letarov and Kulikov, 2009).

Bacteriophages seem to be significant mediators of horizontal gene transfer in their natural habitats. The relevant role of bacteriophages as mobilizing elements is supported by recent studies showing that: (i) a large amount of the genetic information in bacterial genomes is of phage origin (Brüssow and Hendrix, 2002; Weinbauer and Rassoulzadegan, 2004), and (ii) the DNA in the viral fraction of certain biomes contains a significant percentage of bacterial genes (Wagner *et al*., 2002; Otawa *et al*., 2006; Del Casale *et al*., 2011). Metagenomic, molecular and cultural studies have found that bacteriophages move between different biomes (Breitbart and Rohwer, 2004; Sano *et al*., 2004; McDaniel *et al*., 2010). The question addressed here is whether by moving between different biomes bacteriophages can assist in the transfer of genes between autochthonous bacteria from different habitats, such as for example between intestinal bacteria of different species, or between intestinal bacteria and bacteria from activated sludge, or sediments in rivers or lakes.

Phages infecting intestinal bacteria are released by defecation, either as free phages or as prophages of the faecal bacteria. Those released as free phages and those released by induction of prophages outside the intestine remain free in extra-intestinal environments, where they usually persist for longer than their bacterial hosts (Muniesa *et al*., 1999; Durán *et al*., 2002). This may lead to genetic exchange in natural extra-intestinal environments and in human-altered environments such as those that result from intensive farming, the meat industry, sewage works, hospital waste, outfalls of raw or poorly treated wastewater, and the formation of sediments near wastewater outfalls. These human-altered environments may favour genetic exchange between intestinal bacteria from humans or animals, and between intestinal and non-intestinal bacteria whose natural niches are either in or near intensely human-altered environments. This is important since historically the containment of intestinal microbiota may have created barriers to gene flow between different species of intestinal microbiota or between populations of the same species that have maintained no or little contact for long periods of time. The enormous increase in the human population, globalization and the profusion of the abovementioned human-altered environments, are potential causes of an increase in this genetic exchange between different biomes.

Until very recently, the importance of phage-mediated horizontal gene transfer in nature has been widely neglected (Miller and Day, 2004). There may be several reasons for this, among them the following: (i) the identification of only a limited number of transducing phages, (ii) the threshold numbers of host bacteria and phages reported to be needed for phage infection, and consequently for gene transfer to occur, and (iii) the low transduction frequencies described recently. When considering intestinal bacteria, other reasons, such as the poor physiological conditions of the cells outside the gut, can be added to the list. However, the idea of a limited occurrence of transduction in the environment needs to be reconsidered in the light of recent scientific developments.

Here, we examine the likelihood that intestinal bacteriophages play a role in horizontal gene transfer in extra-intestinal environments. The potential transfer of genes from intestinal bacteria mediated by phages should be considered important, especially if we bear in mind that some of the genes mobilized can affect human and animal health (including virulence genes and/or antibiotic resistance genes). Most of the available information refers to *Proteobacteria* and *Firmicutes*, which are not the majority in the gut (Qin *et al*., 2010) but whose proportions might increase very significantly in human-altered environments that receive faecal wastes, such as conventional biological wastewater treatment plants (Wagner *et al*., 2002; Del Casale *et al*., 2011). Recent studies (Del Casale *et al*., 2011) have identified 16S rRNA genes from *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Actinomycetales* and *Firmicutes* in bacteriophage DNA from a wastewater treatment plant, indicating that transduction occurs in this environment between several bacterial groups, although not all species are involved to the same extent. The information that is available regarding free bacteriophages infecting *Bacteroides* in wastewaters (Blanch *et al*., 2006), prophages in the genomes of prevalent bacteria in the intestine (Xu *et al*., 2007), lysogenic strains of ruminal bacteria (Klieve and Bauchop, 1988) and transduction in *Methanococcus* (Eiserling *et al*., 1999) suggests that the behaviour of phages and bacteria from the most abundant groups in the intestine is similar. Therefore, phage-mediated DNA transfer in these groups may be of considerable importance.

Acquisition of DNA in the environment through transduction

Foreign DNA, understood as DNA that resembles more that of other bacterial species than the DNA of the receiving host species (Langille *et al*., 2010), can be introduced into bacteria through horizontal gene transfer via either homologous or illegitimate recombination. Foreign DNA can remain in the recipient cells as independent replicons or plasmids, or it can become integrated into the host chromosome. In the host chromosome, foreign DNA can be identified as prophages, pathogenicity islands, integrated plasmids or transposons (Ochman *et al*., 2000). Bacteriophages mediate horizontal gene transfer through transduction, which is the process of transporting bacterial DNA in the capsid of transducing phages from one cell to another. Transduction can be either generalized or specialized (Miller and Day, 2004), depending on the life cycle of the particular bacteriophage.

Generalized (or general) transduction

Generalized transducing bacteriophages can mobilize chromosomal host markers that will be incorporated into the recipient through homologous recombination (Miller and Day, 2004). They can also mobilize virulence genes as phage CTXΦ of *Vibrio cholerae* does (Boyd and Waldor, 1999); plasmids (Nedelmann *et al*., 1998; Schickmaier *et al*., 1998; Hertwig *et al*., 1999); transposons (Nedelmann *et al*., 1998); and insertion elements (Nedelmann *et al*., 1998) which are incorporated into the recipient genome through independent replication, integration or illegitimate recombination. Generalized transduction is carried out by temperate phages such as P1 of *Escherichia coli* or P22 of *Salmonella typhimurium* but also by virulent bacteriophages such as phage T4 of *E. coli*.

Therefore, generalized transduction appears to constitute a formidable tool for horizontal transfer of genetic material.

Many generalized transducing bacteriophages have been described in recent years and many of them infect bacterial species that inhabit animal gut. Examples are bacteriophages that infect subclasses of *Gammaproteobacteria* (Jensen *et al*., 1998; Schickmaier *et al*., 1998; Boyd and Waldor, 1999; Hertwig *et al*., 1999; Evans *et al*., 2009), phages that infect *Betaproteobacteria* (Jensen *et al*., 1998) and phages that infect Bacilli species (Ruhfel *et al*., 1984; Nedelmann *et al*., 1998). Most of these bacteria (i.e. *E. coli* and other *Enterobacteriaceae*, *Pseudomonas*, *Acinetobacter, Arcobacter*, *Sphaerotylus*, *Staphylococcus* and *Bacillus*) are also present either as planktonic or as biofilm-associated bacteria in environments that are heavily contaminated with faecal waste, such as wastewater treatment plants (Wagner *et al*., 2002; Leroy, 2008). In addition, many of these phages are responsible for generalized transduction events between species (Ruhfel *et al*., 1984), between genera (Schickmaier *et al*., 1998; Evans *et al*., 2009) and even between classes, as reported between the *Betaproteobacteria* and *Gammaproteobacteria* classes (Murooka and Harada, 1979; Jensen *et al*., 1998). Moreover, as indicated before the progeny particles of bacteriophages that infect *Gammaproteobacteria* (*Shigella*, *Proteus*, *E. coli* and *Pseudomonas*), *Betaproteobacteria* (*Sphaerotilus natans*) and *Alphaproteobacteria* (*Rhodospirillum rubrum*) carry 16S rDNA genes from different genera of bacteria (Sander and Schmieger, 2001; Beumer and Robinson, 2005; Del Casale *et al*., 2011). This indicates an ability to carry bacterial genes, probably mobilized by generalized transduction.

Specialized transduction

Specialized transduction, in contrast to generalized transduction, results from an imprecise prophage excision from the bacterial genome. It causes the transfer of only those genes flanking the attachment site of an integrated prophage and this suggests that only a limited number of genes can be transferred in this way. The number of genes transported may increase if the chromosomal genes adjacent to the attachment sites are rearranged with other genes located far away from the attachment sites (Waldor *et al*., 2005).

The types of sequences that can be transferred by specialized transduction seem to be restricted, for this reason it appears that specialized transduction is a minor contributor to gene transfer in the environment compared with generalized transduction. On the other hand, these genes have the advantage that can be moved repeatedly from one bacterium to another, in otherwise replication-competent phages. This would increase the number of these specialized phages moving through the environment. To date, the genes that have been studied and which are mobilized through specialized transduction contribute to bacterial fitness or virulence (see Miller and Day, 2004; Waldor *et al*., 2005, for a review). Many of these bacterial genes are toxin genes carried by phages such as those encoding diphtheria toxin, Shiga toxin (*stx*), cholera toxin (*ctx*), cytolethal distending toxin, staphylokinase, enterotoxin A, exfoliative toxin and Panton-Valentine leucocidin, among others. The advantages bestowed on bacteria that incorporate these genes leads to them being positively selected for transfer and acquisition. Why these virulence genes remain phage-associated rather than becoming incorporated into the bacterial genome is still unknown.

Occurrence of genes mobilized through horizontal transfer in the free phage populations

Culture-independent methods for the detection of DNA in phages provide an indication of the abundance of viral particles carrying bacterial genes in the viral fraction of faeces and water environments contaminated with faecal residues. Metagenomic studies of phages in faeces indicate that VLPs (virus-like particles) carrying bacterial genes are very abundant. Breitbart and colleagues (2003) reports that almost 50% of the sequences found in VLP in human faeces correspond to bacteria; Cann and colleagues (2005) estimates this percentage to be 20% in horse faeces; and Parsley and colleagues (2010) reported a 60% homology with bacterial sequences in the viral metagenomic analysis of activated sludge. Furthermore, 16S ribosomal DNA corresponding to *Aeromonas*, *Acinetobacter* and *Arcobacter* was detected by PCR in the viral fraction of sewage (Sander and Schmieger, 2001).

Genes coding for Shiga toxin and cholera toxin have been found in the viral fraction of contaminated water and soils (Casas *et al*., 2006); *stx* was identified in the viral fraction of sewage, but also, infectious phages carrying *stx* have been identified and isolated in sewage and river water (Muniesa and Jofre, 1998; Muniesa *et al*., 2004a). Other studies detected phages carrying *stx* in wastewater and river water (Dumke *et al*., 2006), and it has been estimated that one phage carrying *stx* is present for each 1000 phages infecting *E. coli* O157:H7 in sewage (Tanji *et al*., 2003). Phage-mediated intergeneric transfer of toxins encoded in staphylococcal pathogenicity islands, transferred from *Staphylococcus aureus* to *Listeria monocytogenes* at the same high frequencies as they transfer within *S. aureus*, has recently been reported (Chen and Novick, 2009). Sequences corresponding to different β-lactamase genes were also reported in the viral fraction

Table 1. Estimation of virus-like particle (VLP) concentrations in different intestinal samples.

Sample	Method employed	Amount of virus like particles (VLP)	References
Ruminal fluid	TEM	5×10^7 VLP ml^{-1}	Paynter *et al.* (1969)
Ruminal fluid of sheep and cattle	TEM	10^7–10^8 VLP ml^{-1}	Klieve and Bauchop (1988)
Sheep ruminal	PFGE of total viral DNA and laser scanning densitometry	1.4×10^{10} VLP ml^{-1}	Klieve and Swain (1993)
Horse faeces	Calculated from yield of viral DNA	10^{10}–10^{11} phage particles per gram of faeces	Cann *et al.* (2005)
Surface of human gut mucosa in healthy humans	Epifluorescence microscopy	10^8 VLP per mm^2 of biopsy	Lepage *et al.* (2008)
Surface of human gut mucosa in patients with Crohn's disease	Epifluorescence microscopy	4×10^9 VLP per mm^2 of biopsy	Lepage *et al.* (2008)

of municipal sewage (Muniesa *et al.*, 2004b). Densities of antibiotic resistance genes in phages isolated from sewage evaluated by quantitative real-time PCR reached 10^4 gene copies (GC) of beta-lactamase TEM, 10^2 GC of beta-lactamase CTX-M and 10^2 GC of *mec*A gene, responsible for methicillin resistance in *S. aureus*. Moreover, the antibiotic resistance genes carried by phages were able to generate bacterial resistance in susceptible host strains (Colomer-Lluch *et al.*, 2011).

Phage particles containing bacterial genes in environments contaminated with faecal waste may have their origin in the intestine and be excreted via faeces, or they may be induced in the environment from defecated bacteria, or more probably both. In fact, conditions outside the intestine usually induce lysogenic phages in bacteria whose habitat is the intestine, through activation of the SOS response (Beaber *et al.*, 2003). Nutritional stress, UV irradiation and some chemicals that can be found in municipal wastewaters are all known to activate prophages. For example, the concentrations of ciprofloxacin present in municipal wastewater (Miao *et al.*, 2004) appear to be high enough to induce the SOS response (Beaber *et al.*, 2003). In addition, activation of prophages seems to be connected to the maturation processes of biofilms (Resch *et al.*, 2005), which are present in sewer pipes, wastewater treatment plants and other water environments contaminated with faecal waste.

Concentration of bacteriophages and susceptible bacteria in the gut, in municipal sewage and in other faecally contaminated environments

Intestinal content samples from humans and animals contain high densities of phages (Table 1). Consequently, enormous amounts of these intestinal phages are released into the environment via defecation by humans and animals. Municipal sewage is one of the matrixes with the greatest faecal load. The densities of both infectious phages in municipal raw sewage detected by cultural methods and phage particles detected by direct observation have been evaluated (Table 2). The most abundant information refers to the occurrence and densities of two groups of DNA phages: somatic coliphages and phages infecting *Bacteroides*. The latter have been studied as potential indicators of water quality in water environments affected by faecal waste. The available data indicate that somatic coliphages (i.e. those infecting *E. coli* through the cell wall) are the most abundant.

Not only human but also animal faecal pollution must be taken into consideration here. Concentrations of animal

Table 2. Concentrations of some abundant bacteria and phages in municipal raw sewage.

Organism	Genera	Values	References
Bacteria	*E. coli*	10^4–10^5 cfu ml^{-1}	Mandilara *et al.* (2006)
	Faecal streptococci	10^3–10^4 cfu ml^{-1}	Mandilara *et al.* (2006)
	Pseudomonas spp.	10^4–10^5 cfu ml^{-1}	O'Farrill *et al.* (2003)
	Acinetobacter spp.	10^4 cfu ml^{-1}	LaCroix and Cabelli (1982)
	*Staphylococc*us spp.	10^3–10^4 cfu ml^{-1}	Garcia and Becares (1997)
	Aeromonas spp.	10^4–10^5 cfu ml^{-1}	Bahlaoui *et al.* (1997)
	Spores of sulfite-reducing clostridia	5×10^2–5×10^3 cfu ml^{-1}	Garcia and Becares (1997)
	Uncultured bacteria[a]	1.3×10^8 bacteria ml^{-1}	Wu and Liu (2009)
Virus	Somatic coliphages	10^3–10^4 pfu ml^{-1}	Mandilara *et al.* (2006)
	Phages infecting *Bacteroides*	10^1–10^2 pfu ml^{-1}	Blanch *et al.* (2006)
	Stx bacteriophages[a]	10–10^3 GC ml^{-1}	Imamovic *et al.* (2010)
	Uncultured viruses[b]	7×10^8 VLP ml^{-1}	Wu and Liu (2009)

a. Evaluated by quantitative real-time PCR.
b. Evaluated by epifluorescence microscopy.
cfu, colony-forming units; pfu, plaque-forming units; GC, gene copies; VLP, virus-like particles.

Table 3. Concentrations of bacteriophages in different faecally polluted environments.

Sample	Phage type	Method employed	Maximum concentration	Reference
Cattle wastewater	Somatic coliphages	Culture	2.6×10^4 pfu ml^{-1}	Imamovic *et al.* (2010)
	Stx phages	Real-time PCR	6.0×10^7 GC ml^{-1}	Imamovic *et al.* (2010)
	Lamboid phages	Real-time PCR	6.5×10^6 GC ml^{-1}	Rooks *et al.* (2010)
Pig slurry	Somatic coliphages	Culture	2.5×10^5 pfu ml^{-1}	Hill and Sobsey (1998)
	Somatic coliphages	Culture	3.1×10^5 pfu ml^{-1}	Imamovic *et al.* (2010)
	Stx phages	Real-time PCR	3.5×10^8 GC ml^{-1}	Imamovic *et al.* (2010)
Poultry wastewater	Somatic coliphages	Culture	3.1×10^4 pfu ml^{-1}	Imamovic *et al.* (2010)
	Stx phages	Real-time PCR	2.0×10^2 GC ml^{-1}	Imamovic *et al.* (2010)
Poultry processing wastewater	Somatic coliphages	Culture	2.6×10^4 pfu ml^{-1}	Havelaar *et al.* (1984)
Animal slurry	Somatic coliphages	Culture	3.0×10^7 pfu ml^{-1}	Blanch *et al.* (2006)
	Phages infecting *Bacteroides*	Culture	1.0×10^4 pfu ml^{-1}	Blanch *et al.* (2006)
Polluted river water	Somatic coliphages	Culture	$> 2.0 \times 10^5$ pfu ml^{-1}	Borrego *et al.* (1987)
Sediments in river	Phages	TEM	10^6 phage particles g^{-1}	Leroy (2008)
	Somatic coliphages	Culture	3.5×10^5 pfu ml^{-1}	Araujo *et al.* (1997)
Activated sludge	Phages	Epifluorescence microscopy	10^9 phage particles ml^{-1}	Otawa *et al.* (2006)
Estuarine water	Somatic coliphages	Culture	$> 2.0 \times 10^5$ pfu ml^{-1}	Borrego *et al.* (1987)
Marine area affected by municipal sewage	Phages	Nucleic acid staining	5.3×106 VLPs ml^{-1}	Shepard *et al.* (2008)
Mussels collected in polluted sites	Somatic coliphages	Culture	2.0×10^3 pfu g^{-1}	Lucena *et al.* (1994)
	Somatic coliphages	Culture	6.3×10^3 pfu g^{-1}	Muniain-Mujika *et al.* (2002)

faeces have increased drastically in recent decades due to intensive livestock farming and huge meat industries. The densities of the phages in samples that are heavily contaminated with animal faeces, such as slaughterhouse wastewater or slurries, polluted river and river sediments (Table 3), are similar or slightly greater than those in municipal sewage (Blanch *et al.*, 2006).

Bacteriophage particles, which probably correspond to phages that infect non-enteric bacteria, are also very abundant in other environments, such as sediments, activated sludges or marine areas contaminated with sewage (Table 3). Few quantitative results are available regarding the densities of phages carrying bacterial genes in the environment. Those reports that focus on Stx-converting bacteriophages (Muniesa and Jofre, 1998; Imamovic *et al.*, 2010), confirm their abundance in sewage waters.

The potential hosts for these bacteriophages are intestinal bacteria released into the environment through faecal wastes. To give just a rough idea of the occurrence and densities of these potential hosts, concentrations of some of the most abundant cultivable and uncultured bacteria are shown in Table 2. Again, values for faecal bacteria in samples heavily contaminated with animal faeces, such as slaughterhouse sewage or samples derived from meat industries, are similar to or slightly greater than those for municipal sewage (Blanch *et al.*, 2006).

Comparison of the densities of phages and their hosts conducted with somatic coliphages and *E. coli* indicate that the densities of somatic coliphages in municipal raw sewage collected around the globe are lower than *E. coli* (around one-fifth the density of *E. coli*) (Table 2). When environments contaminated with faecal wastes are analysed, the available information indicates larger proportions of somatic coliphages with respect to *E. coli*. This increase in the densities of phages with regard to *E. coli* could be because these groups of DNA bacteriophages originating in the gut persist longer in the environment than their bacterial hosts do (Muniesa *et al.*, 1999; Sinton *et al.*, 1999; Durán *et al.*, 2002). The different inactivation of bacteriophages compared with their hosts to natural inactivation factors has been reported for intestinal phages, such as Stx phages (Muniesa *et al.*, 1999), phages infecting *Bacteroides*, F-specific RNA phages and somatic coliphages (Sinton *et al.*, 1999; Baggi *et al.*, 2001; Durán *et al.*, 2002). All group of phages were more persistent in the environment that their bacterial hosts and showed larger densities in faecally polluted environments than their hosts.

Conditions needed for transduction in the environment

In spite of the great abundance of intestinal bacteria and phages in many extra-intestinal environments successful transduction requires certain conditions. The first requirement is that bacteriophages and bacterial hosts must meet. Bacteria and phage concentrations must exceed certain threshold concentrations to ensure encounter, phage infection and subsequent gene transduction. Phage–bacterium interaction could be guaranteed by the densities of potential host bacteria, which could be intestinal bacteria released into the environment through faecal waste or naturally occurring bacteria in the recipient environments, and the densities of phages infecting these bacteria. Most studies on the threshold densities needed for bacteriophage infection are based on the

detection of phage progeny. The threshold numbers of *E. coli* and phages required to ensure infection have been calculated as the sum of the $\log_{10}$ units of the number of phages and the $\log_{10}$ units of the number of bacteria (Wiggins and Alexander, 1985; Muniesa and Jofre, 2004). This calculation indicates that the threshold number to ensure infection ranges from 6 to 7 under optimal conditions, and that it can vary between strains and phages (Wiggins and Alexander, 1985; Muniesa and Jofre, 2004). Contrary to this assumption, a theoretical study complemented by experiments using reporter phagemid (chimeric phage/plasmid vectors) systems indicates that phage infection of *E. coli* occurs at lower densities than that indicated by the experiments based on the detection of phage progeny. Bacteriophage infection depends on the bacterium–bacteriophage pair and the receptors in the bacterial cell surface specifically used by each bacteriophage (Kasman *et al*., 2002). Likewise, the threshold densities reported in freshwater for *Pseudomonas*, an autochthonous microorganism, and its bacteriophages (Kokjohn *et al*., 1991) is about 10 times lower than the threshold reported above for *E. coli.*

Considering only the values of cultivable bacteria reported in many faecally polluted environments, such as municipal raw sewage (Table 2), the numbers of viruses and bacteria could easily reach the minimum threshold necessary to guarantee phage–bacteria interactions. In fact the number of infectious bacteriophages infecting all susceptible strains are surely underestimated, since the viable but non-cultivable bacteria always overwhelm the values of the cultivable bacteria and the bacteriophage densities are based on enumeration in a single host bacterium.

A second requirement for transduction is that the host bacteria must be in adequate physiological condition to act as a successful recipient host. Limited nutrient availability or starving conditions, or non-optimal temperatures can affect the bacterial host and therefore hamper phage infection. This is a limiting factor for phage infection of *E. coli* in the extra-intestinal environment when detected through phage replication (Muniesa and Jofre, 2004), but it has been demonstrated that phages that infect bacteria that occur naturally in freshwater, such as *Pseudomonas*, can even infect starving host cells (Kokjohn *et al*., 1991). Phage-mediated gene transfer studied at the single cell level by cycling primed *in situ* amplification-fluorescent *in situ* hybridization indicates that gene transfer to hosts occurs in non-optimal physiological conditions, both to intestinal *Enterobacteriaceae* and to freshwater indigenous bacteria (Kenzaka *et al*., 2010). The physiological conditions cannot be a limitation for transduction when referring to environmental bacteria, since the intrinsic characteristics of environmental bacteria, such as the genomic flexibility shown in some marine microbial populations, facilitates more than it limits environmental genetic transfer, by adapting to changing environmental conditions (McDaniel *et al*., 2010).

The characteristics of the environmental matrix where phage and host meet form the third requirement, as this has also a strong influence on the phage–bacteria interaction and hence on horizontal gene transfer. Reports indicate that the presence of suspended particles or other bacterial hosts increases the critical densities necessary for phage infection of *E. coli* (Wiggins and Alexander, 1985; Muniesa and Jofre, 2004). In contrast, the presence of inhibitory factors, such as particulate material, facilitates the interaction of *Pseudomonas* and bacteriophages (Ripp and Miller, 1995); again it should be borne in mind that *Pseudomonas* naturally inhabits freshwater, where it moves around searching for food. In the same way as occurs with *Pseudomonas*, other environmental bacteria–phage interactions could benefit from certain environmental conditions of the matrix (suspended particles or other bacteria) rather than being hampered by them.

Phage transduction is usually studied by detecting transductants using conventional plating on selective media, and mostly using non-lysogenic strains as recipients, as it is thought that lysogeny generates phage immunity. Most results obtained in this way indicated low transduction frequencies, with 10^{-7}–10^{-9} transductants per colony-forming unit of the recipient cell being the most common values. Due to these low transduction frequencies obtained in the laboratory, low frequencies were also envisioned for the environment (Miller and Day, 2004; Kenzaka *et al*., 2010). However, recent observations indicate that transduction efficiency may be a lot greater than previously thought under both laboratory and natural conditions.

First, phage-mediated gene transfer studied at the single cell level by *in situ* DNA amplification methods showed transduction frequencies three or four orders of magnitude greater than those detected by conventional plating on selective media; the frequencies are as high as 10^{-2} per recipient cell, determined as total direct counts (Kenzaka *et al*., 2007; 2010). Second, even using the conventional method of detecting transduction, highly efficient generalized transduction of plasmid-borne antibiotic resistance between *Serratia* and *Pantoea* was recently reported (Evans *et al*., 2009). Third, some reports indicate that it is easier to generate lysogens with strains that have been previously lysogenized by another phage, and these are more frequently recovered (from 10 to 100 times more) than lysogens of host strains which do not contain prophages. This has been observed for phages that encode *stx* genes (Serra-Moreno *et al*., 2008) and for chromosomal genes (Saye *et al*., 1990). Lysogeny is extremely common among bacteria (Paul, 2008), and it has been estimated that 60–70% of the bacterial genomes sequenced to date contain prophages (Casjens

and Hendrix, 2005) and that far more than 50% of natural isolates of bacteria are lysogenic (Ackermann and DuBow, 1987), including the bacteria present in extra-intestinal environments contaminated with faecal wastes.

Phage-mediated horizontal DNA transfer between intestinal bacteria in the environment

Due to the characteristics and numbers of phages excreted as free viruses or as prophages, and the evidence that transduction could take place under suitable conditions, the question is how important they are as active and passive players in the horizontal transfer of genes between intestinal bacteria of different origins, and between intestinal bacteria and autochthonous bacteria from contaminated environments (sewage, wastewater treatment plants, sewage sludge, sewage-affected water bodies, sediment, slurries, livestock wastewater, etc.). Unfortunately, the frequency of transduction in the environment is difficult to asses, except in experiments performed in a microcosm. However, indirect evidence may also provide some insight.

Generalized and specialized transduction in *Pseudomonas, E. coli* or *Shigella,* all bacteria found in the gut and in faecally contaminated water, has been reported in freshwater, drinking (mineral) water, and food and soil microcosms (Zeph *et al*., 1988; Saye *et al*., 1990; Ogunseitan, 2008; Imamovic *et al*., 2009). The reported frequencies of these horizontal gene transfers vary widely; ranging from 10^{-2} to 10^{-10}, the most common values ranging from 10^{-5} to 10^{-9} transductants per recipient bacteria, depending on the environmental matrixes, the physiological state of the recipient bacteria and the bacterium–bacteriophage pair itself.

Although not related to intestinal bacteria, transduction has been assessed in freshwater and in marine systems and transduction frequencies of from 10^{-8} to 10^{-5} have been shown (Saye *et al*., 1990; Jiang and Paul, 1998). In contrast, more recent studies conducted with marine bacteria in coastal and oceanic environments showed much higher frequencies (10^{-1} to 10^{-3}) (McDaniel *et al*., 2010).

Chun and colleagues (2009) compared the genomes of 23 strains of *V. cholerae* isolated over the past 98 years. The genetic content of these strains reveals several prophage and prophage-like elements, and pathogenic islands, which result in variation and the emergence of new variants. They found that the strains responsible for the current cholera pandemic, which started in 1961, are descendants of a single strain and evolved mainly through horizontal gene transfer between cholera strains and environmental *V. cholerae* strains. This study strongly suggests that this evolution has occurred in the environment, probably in certain estuarine environments, which is the natural habitat of both epidemic and non-epidemic cholera strains.

Beutin and colleagues (1997) studied the presence of strains of *E. coli* producing Shiga toxins in two separate animal populations, consisting of a herd of cattle and a flock of sheep, over a period of 6 months. A great diversity of serotypes between the Shiga toxin-producing *E. coli* (STEC) strains isolated in each population was found. However, in contrast to the genetic diversity of the bacteria, the *stx* genes of most STEC strains isolated from the same animal population were identical. It is not clear with the data available whether the Stx phages had been moving between bacteria belonging to the different serotypes in the environment, and therefore the populations of animals acquired the genes by ingesting free phages; or whether all the steps involved in horizontal gene transfer between bacteria occurred in the gut of the animals.

There is now little doubt that certain, mostly anthropogenic, environments contaminated with faecal waste contain great densities of bacteriophages that infect both human and animal intestinal bacteria as well as bacteria whose natural habitat are these environments. Some of these phage species are able to infect bacteria from the different biomes as well as to perform DNA transfer between bacteria from the different biomes. Culture-independent studies of the viral fraction of faeces, and natural and anthropogenic environments, show that a fraction of phage particles contains bacterial DNA and other genes which are not strictly of phage origin. Phages therefore contribute to the mobilization of genes, such as toxin genes (Brüssow and Hendrix, 2002) or the mobilization of other genetic elements, such as pathogenicity islands (Chen and Novick, 2009).

According to the data reviewed in this study, the densities of host bacteria and phages in faecally polluted environments are high enough to guarantee bacteria–phage encounters and hence transduction. The condition of the cells and the presence of disturbing particles should not weaken transduction to the autochthonous bacteria, although these factors may weaken transduction to intestinal bacteria that usually are not active in extra-intestinal environments. It can be assumed that in these habitats the flux of genetic information mediated by phage transduction will be asymmetric, with the greatest flux being from intestinal bacteria to the autochthonous ones.

Irrespective of that, intestinal content together with natural and anthropogenic environments contaminated with human and animal faecal wastes, make up a sort of ecological continuity where the complex network of human faecal bacteria, animal faecal bacteria, autochthonous bacteria and phages infecting all of these is the perfect scenario for gene interchange mediated by bacteriophages. The study of phages carrying bacterial genes in the environment opens interesting perspectives for determining the role of phages in the storage and transfer

of these genes between different strains and different species of bacteria under natural conditions.

Taking into consideration the data regarding transduction frequencies, phage-mediated horizontal transfer of intestinal bacteria in extra-intestinal environments should not be ignored. This gene flux may be important in bacterial evolution and could lead to the emergence of new waterborne human and animal pathogens if the genes incorporated encode for virulence determinants, for antibiotic resistances or increase the fitness of the lysogenized bacteria. This scenario must be considered, and it would be advisable to contain the transfer of virulence genes in the environment, by avoiding the mixture of different biomes containing high densities of microorganisms. Concerning bacteriophages as a genetic vehicle, many treatments and protocols applied in faecally polluted waters aim to eliminate pathogenic bacteria effectively, but they fail to inactivate phages. The evidence presented in this study may lead to us reconsidering the present-day practices of urban sewage and slurry treatment and disposal, as well as food management. It is also important to increase our knowledge on the transduction mechanisms behind the spread of genes in the environment to reduce the genetic transfer and the emergence of new pathogenic strains before they become a public health problem.

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