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Bacteriophages in *Escherichia coli* antimicrobial resistance

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ABSTRACT

The continuous battle between humans and the multitude of pathogenic microorganisms in the environment has sought relief in the form of antimicrobials. But the counter attack by pathogenic organisms in the form of multidrug resistance, acquired by various mechanisms such as transformation, transposition, conjugation and transduction is a major reason for concern. Bacteriophages have contributed in a significant way to dissemination of genes encoding antimicrobial resistance, heavy metal resistance and virulence factors through the phenomenon of transduction. This review aims at compiling information about the different mechanisms by which bacteriophages aid in transferring genes involved in antimicrobial resistance to *Escherischia coli* in various environments.

Keywords: Bacteriophages; *Escherischia coli*; Antimicrobial Resistance

1. INTRODUCTION

Transduction is a phenomenon by which genes are transferred from one bacterium to another, using viruses as vectors. These viruses called bacteriophages, infect a bacterial cell and their mode of reproduction is to harness the replicational, transcriptional, and translation machinery of the host bacterial cell to make numerous virions, or complete viral particles, including the viral DNA or RNA and the protein coat. They are the most abundant life form on the globe and drive the diversity and abundance of bacteria in the environment, including, in many instances, the pathogenic profiles of many of mankind's most feared bacterial pathogens [1]. Bacteriophages act as facilitators of two types of horizontal genetic exchange. Generalized transduction is a process by which any gene within a donor is transferred to a recipient strain by a lytic or temperate bacteriophage. The recipient DNA can either get degraded or integrated within the host genome. Specialized transduction is a more efficient process that specifically involves transition from prophage to lytic cycle [2]. Many bacteriophages incorporate their genome into a specific point in the host genome and upon induction the integrated prophage usually excises itself precisely as a whole unit. Sometimes, however, this excision is imprecise and part of the host genome becomes excised also, facilitating the transfer of genetic material to new bacterial hosts by the transducing phage [3]. Transduction is especially important because it explains one of the major modes by which antimicrobial drugs become ineffective due to the transfer of antimicrobial-resistance genes between bacteria. The packaging of bacteriophage DNA has low fidelity and small pieces of host bacterial DNA, along with the bacteriophage genome, may become packaged into the bacteriophage genome. This unintentional phenomenon is a mean of transfer of many genes including toxin encoding genes, heavy metal resistance genes and antimicrobial resistance genes between bacteria of different species or within strains of the same species. One of the most glaring examples of gene transfer via transducing phages is the transfer of Shiga toxin genes from Shigella to *E. coli*, converting the recipient *E. coli* into stx toxin producers. It is believed that the gene coding for Shiga-like toxin comes from a toxin-converting lambdoid prophage, such as H-19B or 933W, inserted into the bacteria's chromosome via transduction [4].

Escherichia coli encompasses an enormous population of bacteria that exhibit a very high degree of both genetic

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and phenotypic diversity. Virulent strains of *E. coli* can cause gastroenteritis, urinary tract infections, and neonatal meningitis. In rarer cases, virulent strains also cause hemolytic-uremic syndrome, peritonitis, mastitis, septicemia and Gram-negative pneumonia [5]. The genome of *E. coli* is known for its plasticity owing to the ability to constantly evolve by mutation, conjugation, transduction and transposable elements. These properties have conferred a high fitness quotient to the bacteria enabling it to thrive robustly in various environments under different selection pressures. The evolution of multidrug resistant *E. coli* is of growing concern because it makes it very difficult to combat infections caused by these strains. Experimental evidences have demonstrated that transduction has an important role in genetic exchanges among environmental microbiota, especially in freshwater [6]. The range of mobile genetic elements (MGEs) involved in the evolution and adaptation of bacteria through horizontal gene transfer (HGT) is continuously updated and re-classified according to a better understanding of HGT mechanisms. It has been proposed, for example, to unify the heterogeneous classes of MGEs, such as conjugative transposons, integrative "plasmids", genomic islands, and numerous unclassified elements into integrative and conjugative elements [7]. The basis for this re-classification was that these elements share similar characteristics, such as the excision by site-specific recombination, transfer by conjugation, and integration by recombination between a specific site of elements and a site in the host's genome. Thus, ICEs combine the features of other MGEs, such as bacteriophages (integration into and excision from the host chromosome but no transmission by conjugation), insertion sequences (ISs), and simple transposons [8]. The aim of this review is to highlight the role of bacteriophages in conferring antimicrobial resistance to *E. coli* and also to document the genomic evolution of *E. coli* aided by the bacteriophages.

2. CRYPTIC PROPHAGES

The term prophage was coined in by André Lwoff in 1952, who along with François Jacob and Jacques Monod, discovered that phages use lysogeny (integration into the host chromosome) as a means to replicate, and who believed that lysogeny is the key factor defining the relationship between bacteria and phages. Although bacteria have compact genomes, it has been found that due to the their high prevalence rate, prophages get easily incorporated into bacterial genomes, constituting about 20% of the DNA and contribute most to the horizontal gene transfer. In fact phage-like elements have been identified in almost all sequenced pathogenic and non-pathogenic bacterial genomes [9]. A particular interest with respect to transduction includes the cryptic prophages, who over a period of time become inactive in terms of lysis [10]. Interestingly, the well-studied *Escherichia coli* K-12 has gained 1600 kbp of novel DNA (18%) since its divergence from *Salmonella* sp. 100 million years ago and contains nine cryptic prophage elements, constituting 3.6% of its genome [11] Recently, Wang *et al.*, 2010 [12] studied the cryptic prophage genes of *E. coli*. They created a single *E. coli* strain, Δ9, devoid of all nine cryptic prophages (CP4-6, DLP12, e14, rac, Qin, CP4-44, CPS-53, CPZ-55 and CP4-57); each prophage was removed precisely using a method that leaves one attachment site and mimics natural excision. Two systems of biology tools, phenotype and DNA microarrays, were then used to identify the altered phenotypes and to elucidate the important prophage genes responsible for the changes in the phenotypes. They found that these cryptic phages were associated with increased growth, provided protection for quinolone and *β*-lactam antimicrobiotics, increased resistance to osmotic, oxidative and acid stress, as well as influenced biofilm formation, conferring profound benefits to the host bacterium. Their study showed that the emergence of resistance to both antimicrobials as well as to environmental stress arises in some cases from the acquisition of phage DNA. The benefits conferred to the host by such cryptic prophages are very significant because the changes brought to the genome by these cryptic prophages are permanent as they have lost the ability to lyse or excise from the host genome as compared to the temperate phages which, on the other hand, can reverse the traits conferred as they can revert to the lytic form at some point of time. Another study by Kenzaka *et al.*, in 2007, [13] employed cycling primed *in situ* amplification-fluorescent *in situ* hybridization (CPRINS-FISH) and investigated the movement of the ampicillin resistance gene among *E. coli* cells mediated by phage at the single-cell level. Their experiments showed that the difference in the number of viable cells carrying the transferred gene and the number of cells capable of growth on the selective medium was 3 to 4 orders of magnitude, indicating the unexpectedly high frequency of occurrence of phage-mediated exchange of DNA sequences among bacteria. This convincingly proved that lateral gene transfer by bacteriophages has contributed significantly to the genetic diversity of bacteria. Although factors affecting the transduction rates *in vivo* are not well known, it is well established that bacterial stress and induction of the SOS response could facilitate phage movement *in vitro*.

Bacteriophages are highly ubiquitous and are found in a wide range of environments such as aquatic, veterinary, food and clinical environments. This is easily explained by the fact that they are obligate parasites and hence their occurrence is concurrent with the survival efficiency of their host bacteria in various environments. The environ-

mental and nutritive conditions are important factors that help the phage in choosing between a lytic and a lysogenic life cycle. The following section of this review deems it pertinent to discuss bacteriophage distribution in various environments and how their respective environments influence their ability to transduce.

3. TRANSDUCING PHAGES IN ENTEROHEMORRHAGIC *ESCHERICHIA COLI* O157:H7

Bacteriophages act as key genetic factors promoting horizontal gene transfer (HGT) between bacteria. Their roles in dynamic bacterial genome evolution have been increasingly highlighted by the fact that many sequenced bacterial genomes possess multiple prophages carrying a wide range of genes. Enterohemorrhagic *Escherichia coli* O157:H7 is the most well known case [14]. Shiga toxin (Stx)-producing *Escherichia coli* (STEC) O157:H7 (STEC O157:H7) is the most prominent STEC serotype, causing hemorrhagic colitis and hemolytic-uremic syndrome in many countries [15]. Cattle colonized with STEC O157:H7 are important animal reservoirs of this pathogen, which is frequently transmitted to humans through manure-contaminated foods, water, and other environmental sources [16]. Antimicrobial resistance (AMR) can potentially contribute to enhanced survival of STEC O157:H7 in cattle, particularly under intensive rearing conditions where antimicrobials are frequently used for disease prevention and growth promotion [17]. According to a recent study on cattle by Ziebel *et al*., in 2011 [18] the multidrug resistance to streptomycin, sulfonamide, and tetracycline (AMR-SSuT) was identified in 156 of 171 isolates of *Escherichia coli* O157:H7 of phage types 23, 45, and 67. Another study by Asadul-Ghani *et al*., in 2009 [19] on a sequenced strain of O157:H7 called the Sakai strain revealed the presence of 18 prophages that possessed the genes for virulence as well as multidrug resistance.

Temperate bacteriophages have been implicated in the evolution of foodborne pathogens to a great extent. According to Brabban *et al*., 2005 [20], bacteriophages have contributed to the evolution and shaping of emerging foodborne pathogenic bacteria through the dissemination of virulence and antimicrobial resistance genes. For example, the genome sequences of Shigella dysenteriae, *E. coli* O157:H7, and the Stx encoding bacteriophages indicate the key role of bacteriophage-mediated gene transfer events in the evolution of these high-profile human pathogens. They have reviewed the basic genetic exchange mechanisms mediated by temperate bacteriophages and how these mechanisms played a major role in the dissemination of virulence genes, such as toxins and antimicrobials from one species to another (the shiga-like toxins, and multiple antimicrobial resistance dissemination in Salmonella are used as specific examples). Data demonstrating the role of bacteriophages in the spread of antimicrobial resistance in bacteria, including interspecies transduction, are also presented and it is postulated that bacteriophages will also play a vital role in food safety. Although STEC strains are food-borne pathogens, there are no data on the potential transduction that could occur in food. Some food treatments can increase the rate of induction of prophages and the number of transduction events [21]. In order to guarantee food quality, it is necessary to evaluate whether transduction by Stx phages can occur in food and the conditions that could favor *stx* transfer in food samples. Food products can be subjected to conditions that hinder the growth of some bacteria but prolong their persistence in the food in an inactive state. In these conditions, bacterial populations suffer stress, which can trigger the induction of prophages to enter their lytic cycle and therefore increase the number of free phage particles [20]. Certain treatments used for the processing of food or water samples, such as chlorination, thermal treatment, radiation with ^{60}Co, or high hydrostatic pressure fail to inactivate Stx phages. Indeed, some of these treatments may even increase the induction of the lytic cycle of certain Stx phages, which will persist after the application of the treatment [22].

4. PHAGES IN DISSEMINATION OF ANTIMICROBIAL RESISTANCE IN AQUATIC ECOSYSTEMS

Antimicrobial resistance has been recognized as a worldwide problem for quite some time, very little focus was on the role played by bacteriophages, until recent findings have convincingly established their vital role. Bacteriophages have been implicated as a major source of transmission of antimicrobial resistance in the aquatic environment. Phages persist better in aquatic environments than their bacterial hosts [23] and, due to their structural characteristics, better than free DNA [24]. This higher survival and the abundance of phages carrying antibiotic resistance genes (ARGs) in animal and human wastewater [25] support the notion that phages are vehicles for mobilization of the environmental pool of ARGs that contribute to the maintenance and emergence of new resistances. Lluch *et al*., in 2011, [25] studied antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. They studied the genes encoding beta lactam resistance namely blaTEM and blaCTX-M9 in bacteriophage DNA isolated from environmental water samples. The three genes were quantified in the DNA isolated from bacteriophages collected from 30 urban sewage and river water samples, using quantitative PCR amplification. They found both the genes in high copy number in the phage DNA indicating that phages could

be suitable candidates as intermediates between the original bacteria and the clinical isolate and that that phages are reservoirs of resistance genes in the environment. Another recent study by the same research group evaluated the occurrence of bacteriophages carrying antibiotic resistance genes in animal environments. The bla_{TEM} and $bla_{CTX\text{-}M}$ (clusters 1 and 9) were quantified by quantitative PCR in 71 phage DNA samples from pigs, poultry, and cattle fecal wastes. This study was conducted with archived fecal wastes collected from several slaughterhouses and farms in Spain. Densities of 3 to 4 $\log_{10}$ gene copies (GC) of bla_{TEM}, 2 to 3 $\log_{10}$ GC of $bla_{CTX\text{-}M}$ per milliliter or gram of sample were detected, suggesting that bacteriophages can be environmental vectors for the horizontal transfer of antibiotic resistance genes. Biyela *et al.*, in 2004, [26] reported the incidence of antimicrobial resistance in enteric bacteria isolated from the Mhlathuze River and the distribution of genetic elements that may be responsible for the observed antimicrobial resistance. They found that integrons and bacteriophages harboured by the bacteria were responsible for transferring genes responsible for resistance to beta-lactam, aminoglycoside, sulfonamide and quarternary ammonium antibiotic agents. The environment, and especially freshwater, constitutes a reactor where the evolution and the origin of new resistances occur. In water bodies such as wastewater effluents, lakes, and rivers or streams, bacteria from different sources, e.g., urban, industrial, and agricultural waste, probably selected by intensive antibiotic usage, are collected and mixed with environmental species. This may lead to two effects on the development of antibiotic resistances: first, the contamination of water by antibiotics or other pollutants leads to the rise of resistances due to selection processes, for instance, of strains over expressing broad range defensive mechanisms, such as efflux pumps. Second, since environmental species are endowed with intrinsic antibiotic resistance mechanisms, the mixture with allochthonous species is likely to facilitate genetic exchange [27]. In this context, phages seem to play a significant role in dissemination of resistance genes in the aquatic environment.

5. BACTERIOPHAGES CONFERRING ANTIMICROBIAL RESISTANCE IN ACTIVATED SLUDGE

Activated sludge in wastewater treatment plants is an open system with a dynamic and phylogenetically diverse microbial community [28]. Parsley *et al.*, 2010 [29] conducted a study on identification of diverse antimicrobial resistance determinants carried on bacterial, plasmid, or viral metagenomes from an activated sludge microbial assemblage using function and sequence based metagenomic approaches. Using sequence based studies, they found that phages in the activated sludge systems carried partial genes that may be responsible for resistance to several antimicrobials, including tetracycline, ampicillin, acriflavine, and bleomycin, as well as efflux systems that may mediate resistance to additional antimicrobials. These studies have convincingly proven that transduction events may be responsible for the propagation of antimicrobial resistance genes in these environments. Another study by Jury *et al.* in 2010 [30] suggests that high bacterial density in activated sludge and sewage treatment plants provides a conducive environment for bacteriophages to transduce antimicrobial resistance genes. The conditions in wastewater treatment plants are such that they favour the growth of degradative microorganisms and also provide an opportunity for intensive interaction between the microorganisms that in turn favors genetic exchange in between them. Another study by Muniesa *et al.*, 2004 [31] clearly documented the role of bacteriophages in dissemination of beta lactamase genes in sewage. They evaluated the presence of various β-lactamase genes within the bacteriophages in sewage and their results showed the occurrence of phage particles carrying sequences of $bla_{OXA\text{-}2}$, $bla_{PSE\text{-}1}$ or $bla_{PSE\text{-}4}$ and bla_{PSE}-type genes and hence proved that phages were the main source of conferring antimicrobial resistance in the *E. coli* strains found in sewage. It is important at this point to discuss the fact that some bacteriophages have a broad host spectrum that taxonomically includes very distant species, such as *Sphaerotilus natans*, *E. coli*, and *Pseudomonas aeruginosa* [32]. Consequently, infection of these bacteria by phages could be the way the genes, or groups of genes, are able to move over great phylogenetic distances [33]. Another recent study by Dolejska *et al.*, 2011 [34] has found multidrug-resistant *E. coli* and *Klebsiella* species carrying extended-spectrum β-lactamases in municipal waste water treatment plant effluents in the Czech Republic and associate the resistance genes to have originated from transducing bacteriophages. Similarly Seyfried *et al.*, 2010 [35] have found phage mediated transfer of tetracycline resistance genes in aquaculture facilities in north-western Wisconsin, USA.

All the above findings clearly document the fact that the aquatic environment offers a very favorable milieu for the bacteriophages to survive, replicate and in the process transduce and disseminate virulence factors and antimicrobial resistance genes [36]. According to D'Costa *et al.*, 2011 [37], antimicrobial resistance has a very ancient origin followed by recent mobilization from a gene pool, the so-called resistome. Thus, the previous dogma that resistance genes have evolved mainly as a result of the recent clinical use and misuse of antimicrobials is now seriously questioned, although our understanding of the origins and diffusion of antimicrobial resistance in the microbial community remains low. However, recent

studies such as those discussed in this review clearly point towards bacteriophages as one of the key elements that aid ion achieving the evolution of antimicrobial resistance in the microbial community.

6. BACTERIOPHAGES IN ANTIMICROBIAL RESISTANCE OF UROPATHOGENIC *E. COLI*

Uropathogenic *E. coli* (UPEC) is the primary cause of urinary tract infections (UTIs), and treatment of these infections is estimated to cost in excess of 1.6 billion dollars annually in the United States [38]. UPEC is genetically tractable via homologous recombination methods, such as lambda Red mutagenesis [39], a technique which uses exogenous expression of the phage lambda genes bet, exo, and gam to induce a hyper-recombinative state within the host cell that aids the replacement of a chromosomal region with an antimicrobial resistance cassette [40]. A study by Battaglioli in 2011 [41] described the isolation and characterization of transducing bacteriophages for UPEC strain CFT073. Seventy-seven phage isolates were acquired from effluent water samples collected from a wastewater treatment plant in Madison, WI and four of these phages were found to possess the ability to transduce antimicrobial resistance genes. Recently, Bien *et al*., 2012 [42], while reporting the role of UPEC in UTIs and subsequent kidney damage, have suggested that transducing bacteriophages could play a major role in aiding UPEC in acquiring virulence factors and antimicrobial resistance.

7. BACTERIOPHAGES IN ANTIMICROBIAL RESISTANCE OF *E. COLI* CAUSING NOSOCOMIAL INFECTIONS

Hospitalized patients are at unusually high risk of infections, and the hospital environment favors the acquisition of resistance to antimicrobial agents, complicating the treatment of nosocomial infections due to drug-resistant pathogens. More recently the importance of Gram-negative bacteria has increased since the advent of broad-spectrum antibiotics because these organisms often carry multiple antimicrobials resistance [43]. *E. coli* is the most common cause of nosocomial urinary tract infections and many of these strains are multidrug resistant [44]. Lluch *et al.*, in 2011 [25] have suggested that transfer of beta lactamase genes amongst bacteria is a major mode of spread of resistance genes in nosocomial infection causing bacteria and that phage transduction could play a key role in dissemination of the resistance genes. Another study by Karbasizaed *et al.*, in 2003 [45] was carried out on nosocomial infection causing *E. coli* strains that were multidrug resistant and also heavy metal resistant and this study also implied the probable role of bacteriophages as transducing agents that confer the ability to tolerate heavy metals as well as multiple antimicrobials in these strains. It is important here to recall that as early as 2000, Blahova *et al.*, [46] reported the role of bacteriophages in conferring resistance to antimicrobials imipenem, aztreonam and ceftazidime in nosocomial strains of *Pseudomonas aeruginosa*. These reports clearly implicate that transducing phages could be a major cause of emergence of multidrug resistant nosocomial pathogens. The hospital environment seems to favor the dissemination of resistance genes due to two main factors: one, the exposure of patients to multidrug resistant organisms and secondly the immuno-compromised nature of the patients that puts them at a higher risk of acquiring infections and disseminating infectious strains.

8. PHAGE THERAPY AND ANTIMICROBIAL RESISTANCE

The alarmingly potential crisis in increasing prevalence of resistant bacteria has provided impetus to develop new class of antimicrobials to treat bacteria that became resistant to older class of antimicrobials like ampicillin, kanamycin, tetracycline and chloramphenicol. Clinicians and the large Pharmaceutical industry leaders are alarmed by the prospect that effective novel classes of antimicrobials may not be available to treat seriously ill patients in the near future and phage therapy may have to be exploited to destroy antimicrobial resistance bacterial strain. For over 70 years, this approach has been extensively employed as a therapeutic agent in many countries especially in Russia and Georgia [47]. The efficacies of the therapeutic use of phages and few latest ongoing phage therapy projects have revealed that phages were used topically, orally or systemically [44]. The overall success rate found in these studies was 80% - 95% with few gastrointestinal or allergic side effects. Interestingly, several British studies have also demonstrated significant efficacy of phages against *Escherichia coli*, *Acinetobacter* spp., *Pseudomonas* spp. and *Staphylococcus aureus* [48]. While the therapeutic use of lytic bacteriophages to treat pathogenic bacterial infections is enormous [49], the acquisition of antimicrobial resistance through integron and transposon jumping into the phage genome will pose a serious threat to the wide usage of such approaches. The application of such bacteriophages in a time sensitive and biodegradable manner in controlled surfaces may be a way to go forward as proposed by Jikia *et al*., in 2005 [50].

9. THE PHAGE PARADOX—FRIENDS OR FOES?

Evolution of bacterial antimicrobial resistances, and its spread and emergence, represent one of the most threat-

ening health care problems with worldwide proportions. All the known antimicrobial resistance mechanisms, acquired by opportunistic and pathogenic bacteria, evolve by means of Darwinian forces, *i.e*., mutations occurring in pre-existing genes of the bacterial chromosome positively selected by environmental forces [51]. However, adaptation to the selective pressure of antimicrobials accelerates acquisition of antimicrobial resistance genes by lateral transfer from donor species [52]. Although the role of plasmids and transposons in HGT has been much spoken about and well documented, until recently there has been little focus on the role of phages in HGT. This review has specifically enumerated the role of transducing phages as well as resident prophages in transfer and dissemination of antimicrobial resistance genes in a wide range of environments in the pathogenic *E. coli*. While discussing the role of bacteriophages as key agents of antimicrobial resistace gene transfer, it becomes mandatory to throw light on the other side of the phage paradox, which is use of phages in combating antimicrobial resistance. While on one hand phages are considered to be the cause of evolution of pathogenic bacteria, on the other hand, the era of using bacteriophages against multidrug resistant bacteria has finally dawned. Bacteriophages have several characteristics that make them potentially attractive next-generation therapeutic agents, with several benefits over conventional antimicrobials [53]. Phages are highly specific and, therefore, do not disturb normal flora; replicate in their host, facilitating effective treatment by delivery of a low-phage dose; are amplified at the site of infection; and are capable of rapid adaptation to combat emergence of bacterial resistance. Bacteriophages, therefore, hold significant promise as a therapeutic approach in the face of widespread antimicrobial resistance [54]. Although phages have been implicated in conferring virulence and pathogenecity to bacteria over the course of evolution, an alternate approach to using these natural pathogens of bacteria is the basis of phage therapy and surely promises to go a long way in handling the problem of emergence of multidrug resistant pathogens.

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