The use of bacteriophages in the development of new alternatives in therapy

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Pathogenic and opportunistic bacteria have evolved in order to avoid the action of antibiotics. In this regard, the attention has been drawn to their natural co-evolving enemies: the bacteriophages. The classic investigations on phage therapy evaluate the use of complete viral particles, either from a single phage strain or a combination of them. However, the application of whole viral particles as therapeutic agents exhibits certain limitations. The focus of current research is aimed at the molecular functions used by phages to kill bacteria, like the viral proteins that target the bacterial envelope elements. These have shown effective antimicrobial activity in murine models of infection. Consequently, holins, lysins, endolysins, lysozymes, among other proteins involved in bacteriophage release, are now subjected to study. In addition, a current strategy in which one or several proteins are displayed on the surface of the viral particle by genetic fusion with the phage coat protein, has allowed the development of new technologies for pathogen elimination and production of antibodies. Therefore, new challenges and alternatives continue emerging with the study of phages.

Keywords: Bacteriophage; phage therapy; phage coat protein; phage release; lytic development; antimicrobial activity

1. Introduction

Pathogenic and opportunistic bacteria rapidly evolve in order to avoid any factors that may cause cell damage or death. Recently, the constant use, abuse and misuse of antibiotics in the clinical settings, as well as in animal farming and aquaculture, has resulted in a generation of bacteria that are resistant to diverse synthetic or natural antibiotics.

Bacterial viruses are known as bacteriophages or phages. Due to the increasing presence of multi-resistant bacteria and to the decay in the production of new antibiotics, which are also limited because of their increasing costs, phages have re-emerged as an alternative for treatment of bacterial infections. As the natural enemies of bacteria, these entities can regulate bacterial populations in the ecosystems. However, phages require the transcriptional and translational molecular machinery of their host in order to develop, as they cannot reproduce by themselves, and that is why they are considered as "non-living" organisms, as obligate intracellular parasites or simply as entities. Two types of phages are known according to their development: (1) Lytic phages, which infect bacteria, produce progeny and kill their host, and (2) temperate phages, which once they have infected the bacteria, they can either produce progeny, killing their host (as the lytic phages), or they can remain in a latency state within the host, and replicate together with the bacterial chromosome during cell division.

The four states of bacteriophages are presented in Figure 1. (1) The prophage state, in which the viral genome remains either like a plasmid or integrated into the bacterial genome (this state of the bacteria is termed lysogeny). When alterations in the environment stress the bacteria, the prophage can sense such conditions and turn into the state of replication. (2) The lytic cycle or replication state, in which the virus replicates its genome often using the molecular machinery of the host, and then expresses its own proteins, which mainly consist in structural components of virions. While these proteins are assembled to form viral particles, a holin protein is expressed in order to form of pores in the internal membrane. Through these pores another phagic proteins, the endolysins, cross from the cytoplasm into the periplasmic space, to degrade peptidoglycans (the cell wall structure), therefore lysing the cell and allowing the release of the progeny to the environment. (3) Cryptic state; this state occurs when the prophage establishes within the host genome, and is maintained for a long time period. In this condition, changes in the viral genome structure can happen, such as recombinations, deletions, insertions, etc., and as a consequence, the prophage loses its ability to split from the host. In other words, it becomes trapped as a cryptic element, forming part of the host genome. Normally, this state is considered to provide evolutionary advantages for the bacterial hosts, depending on the presence of accessory genes in the phage genome. And (4) pseudolysogeny state; this occurs when the phage DNA enters into a bacterial cell growing in a limited nutrient environment, which is the case most likely to occur in the natural conditions. In this pathway, the phage is incapable to replicate or integrate, and remains carried by its host in a passive or carrier state. However, it is able to develop a lytic cycle whenever the bacterial cell enters to a dynamic metabolic activity [1 - 4].

2. Phage utility

Entire phages or their isolated components have been used for therapy applications, as shown in the Figure 2. These phages have been characterized, purified and used in an individual form, or by mixing several components of them. The majority of phages that are used in therapy are lytic, and have been directly isolated from nature. The complete viral

particle is frequently used, however, the different components of the phage even with modifications for a better efficacy, have been recently tested.

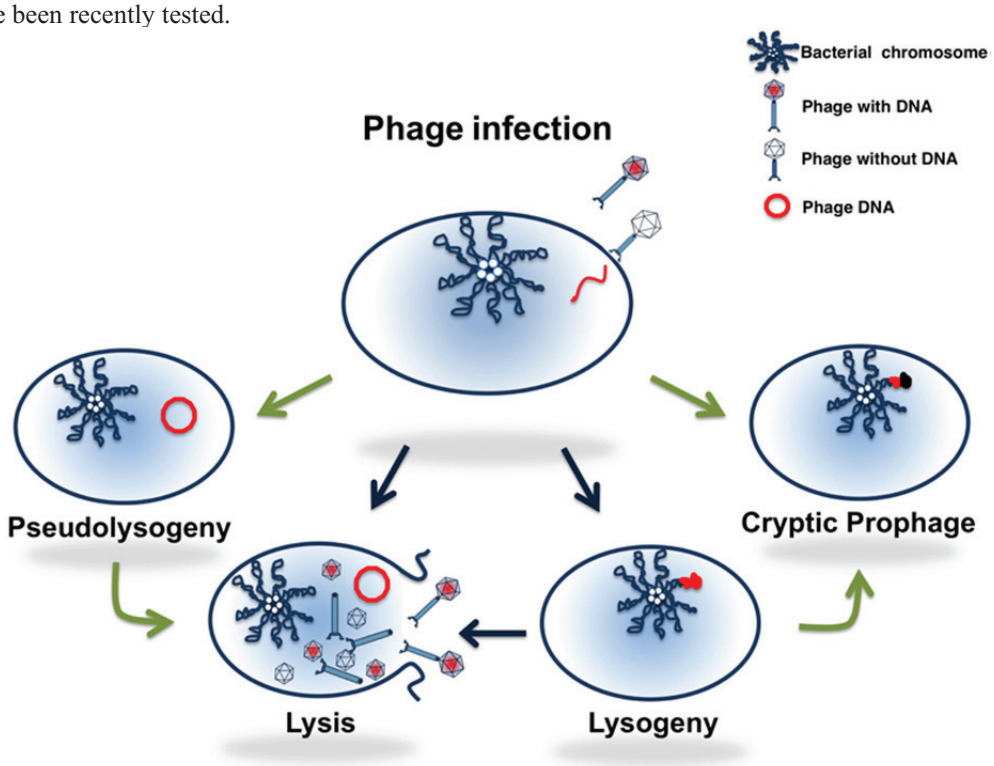

Fig. 1 States of phage development. Once that the phage infects the bacteria, its genome can establish in 4 different states: pseudolysogeny, lysis, lysogeny and cryptic state. The dark blue arrows show the most common states, which constitute the basis of the phage therapy studies. Green arrows indicate other states: the cryptic, which derives from lysogeny, and the recently described state of pseudolysogeny, which can derive into lysis if the environmental conditions are favorable.

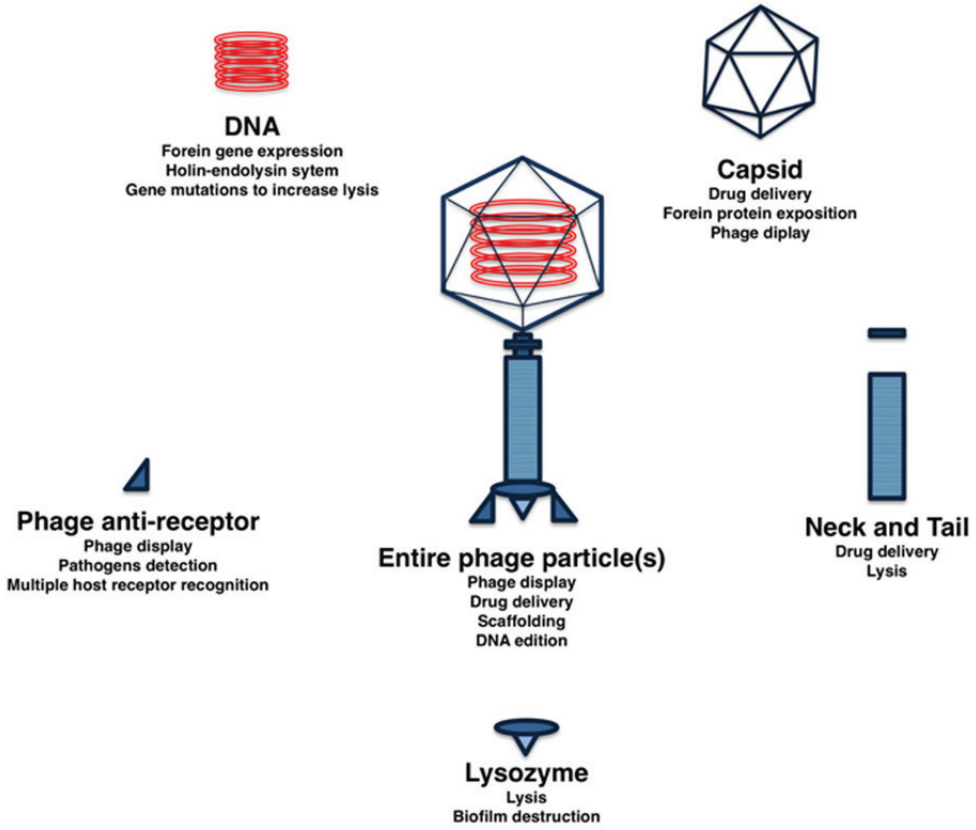

Fig. 2 Phage components used in therapy. Complete phage structures or individual phage components can be used in therapy. Specific components can yield the same efficacy as complete particles, and their modification can even provide more benefits.

2.1 Phage Therapy

During the last three decades, active research on this topic has been published. Bacteriophages have been used as therapeutic agents against pathogenic multi-resistant bacteria, including Gram-positives like *Staphylococcus aureus, Enterococcus* spp, and Gram-negatives like *Escherichia coli, Salmonella enterica, Klebsiella pneumoniae, Pseudomonas aeruginosa*, etc. Also, they have been used as a complement to antibiotic therapy. Generally, the source of these entities corresponds to the environments in which their hosts proliferate.

The means of administration for phage therapy have varied: oral, intradermal, muscular, intraperitoneal, and others [5, 6]**.** However, it is important to note that along with their advantages, they also present disadvantages when compared to antibiotics.

2.1.1 Attributes of the phages used in therapy

The main advantage of phages is their specificity against bacteria, as they do not infect nor colonize eukaryotic cells; hence, they are not toxic to humans or animals and also, by being species-specific, they do not affect the rest of the microbial population. Conversely, their specificity can be a disadvantage, as their action range is narrow when compared to the wider range of antibiotics; phage therapy is limited to the bacterial species that will be treated [7 - 9].

Another advantage is that phages can be isolated from any place, and preferably from where their host is found. The cost to set up a phage therapy laboratory is low, as well as the elaboration of a phage cocktail.

The specific characteristics of the phages used for therapy include the following:

- Phages must be lytic (they should always kill the bacteria).
- Their administration should preferably be as a cocktail of different phages. In the case that any resistant mutant appears, it is not probably that it would resist more than one phage strain; therefore, the bactericidal efficiency is expected to be higher if a phage cocktail is used.
- The phage preparation should not contain any contaminants from the bacterial lysate as possible, in order to avoid the innate immune response of the host, which is activated by the bacterial lipopolysaccharides (LPS).

2.1.2 Factors that affect the efficiency of phage therapy

Considerations regarding the phage concentration.- In order to determine the quantity of phages to be inoculated, the bacterial species must be taken into account, as well as the source of infection, the means of inoculation, the pH of the environment and the possible presence of antibodies that may neutralize or strengthen them. However, the empiric use of phages has ranged from 1 to 100 viral particles per bacterial cell, according to the reported assays [10].

Dosage and time of treatment.- In a similar way to that of antibiotics, it is necessary to know the dosage that should be administrated. A unique dose has been used in most of the cases, however it may not be sufficient. In general, the recommended dosage will be more than one dose, but it will depend on the time required by the infectious bacteria to establish. For instance, bacteria that display slower infection processes will require different doses. This can be a problem if no evident symptoms of the pathogenic bacteria are shown [10]**.**

Environmental conditions and phage resistance.- The phage activity can be affected by diverse physical and chemical factors, such as temperature, pH, salinity and presence of ions. Although phages seem to be considerably resistant to environmental conditions, it is necessary to consider the environment from which they were isolated, in order to better define the conditions for their development and usage. Phages seem to tolerate temperature fluctuations, resisting in some cases very high temperatures. However, it is not the same regarding to pH changes, as their infectivity increases when they are near to neutral pH. Despite this, there are phages that can tolerate acid environments. Regarding ions and salinity, phages can be sensible to osmolarity, but many of them have been isolated from environments in which high concentrations of salt is present, such as the marine [10, 11].

Access to target bacteria.- In general terms, phages are found in the same habitats as their bacterial hosts, hence their access to them is direct, and this situation can also be found in extracellular infections. However, several complications arise when bacteria display an intracellular living. In this way, the bacteria avoids the phage infection, not only because the access of phages is limited by the membrane barriers of the infected eukaryotic cell, but also because of the metabolic state of the bacteria inside the host cell, which can be minimal. These barriers can be surpassed, as it has been shown that when phages and bacteria are co-internalized, the former are capable to adsorb, infect and eliminate bacteria once the latter becomes metabolically activated [5, 10].

Means of administration.- Phage administration can vary depending on the site of the infection. Research generated for several decades has explored diverse routes. The direct or localized administration has yielded good results, as observed when the treatment was applied directly on the site in which the microorganism is found, like in burns or diabetic lesions. On the other side, the parenteral route of administration has been the most widely used. Recent studies show that sites of inoculation such as the intraperitoneal, subcutaneous and intramuscular, can result in a variable efficiency of the therapy. Intraperitoneal administration seems to be the most effective, as it can be maintained for longer periods, reaching any organ or tissue. It is even better than the intravenous route, in which phages tend to be neutralized by antibodies. Another frequently used route is the oral, which displays some issues. The simple path across acid environments such as the one found in the stomach, reduces the phage activity. In order to solve this problem, it is

recommended that the phages be administered encapsulated in polymers or together with an antacid. The use of phages as aerosols has also been effective [5, 6, 10].

3. Phage display

While lytic phages have been successfully used in phage therapy, the characteristics of other phages have been used in the development of other technologies, such as phage display. This technology emerged as a tool that allowed the identification of protein-protein and protein-nucleic acid interactions. It is based mainly in the genetic modification of viral envelope proteins. For instance, the capsid proteins are genetically fused to a series of peptides, allowing the exposition of a high number of these peptides on the surface of the viral particle, in a single experiment.

The system began with the use of filamentous phages of single stranded DNA, such as M13, fd and f1. George P. Smith firstly reported in 1985 a peptide display system using phage f1, which does not lyse its host. The experiment consisted in introducing a codifying fragment of the restriction enzyme *Eco*RI into the middle section of gene 3. This fusion did not affect the infectivity of the phage and allowed the correct conformation of the fused protein, which maintained its antigenic properties. From then, a series of variations of this system have emerged for the development of diverse technologies, including the arrangement of protein arrays on the capsid, and the employment of different kinds of phages. Currently, lytic phages as T4, T7 and the temperate phage λ have been incorporated [12-14].

3.1 Lytic versus filamentous phage display

The most important characteristic of the filamentous phages is that they do not lyse their host, hence they allow a constant production of phages and limit the release of contaminant proteins to the preparation. However, the size of the genetic fusion in these phages is limited. This situation demanded the exploration of new alternatives for larger inserts, and now the use of a wider spectrum of phages for this technology has increased. Another limitation of filamentous phages is that the protein fusion of the capsid has to be translocated through the cell membrane, but many of the cytoplasmic proteins are hydrophilic and therefore cannot be extruded. An additional problem is the fact that the filamentous phages depend on the viability of the host cell, which can be affected when the expressed peptides result toxic for the bacteria [15].

Lytic phages have not only compensated the limitations of filamentous phages, but also they offer other characteristics that enhance phage display systems, such as the expression of a higher number of proteins in the envelope surface and a better exposition of the fusion peptides [15].

3.2 Applications

The first application of this technology was in the determination of protein-protein interactions. Currently, diverse applications have been developed. Examples include the mapping of epitopes to determine enzyme-inhibitor specificity, the screening of agonist or antagonist receptors, the search for anticancer proteins or peptides capable to affect angiogenesis and tumor cell growth, the production of vaccines and the design of new antibiotics, among others. Nowadays, phage display coupled to chromatography is used for the purification of phages, allowing the elimination of bacterial proteins, DNA, LPS and even other phages. However, the classic purification technique using cesium or sucrose gradients has yielded good results [14].

Among the aforementioned applications, the use of phage display in cancer research is worth to be noted. In this case, phage display is combined with other techniques in order to facilitate the delivery of nano-encapsulated drugs (nanomedicines) to cancer cells using biopolymers. The unique characteristics of the capsid protein of filamentous phages are exploited in this application, taking advantage of its propensity to remain in the internal membrane during the infection, as well as its ability to spontaneously integrate into lipid bilayers *in vitro*. The strategy starts with the formation of micelles or liposomes that contain the drug of interest, and then the capsid proteins fused to the peptide that will recognize the target cell are incorporated. Finally, the particles are covered with polyethylene glycol to mask the liposome cargo, avoiding its recognition by the immune system [12].

The development of nanomedicines is not limited to the delivery of synthetic compounds or proteins with activity against cancer cells. A similar system has been developed in which small interfering RNAs (siRNAs) are delivered in order to suppress the action of specific genes by binding to their mRNAs. In this case, the capsid proteins do not function only as ligands for the target cell, as they also protect the small RNA, promoting its stability [12].

Another example in the development of vaccines is the use of phage T4. It has been shown that the display of antigens through this phage does not require adjuvants because it promotes a strong immune response. In addition, it can display more than one epitope on its surface, allowing the generation of multivalent vaccines. For instance, one or several combinations of proteins from VIH have been tested. In the murine model, both humoral and cellular immunity were successfully activated using this system [16]. Moreover, the use of phages in this technology is not limited to interaction assays and compound delivery. Filamentous phages have recently been used to substitute the extracellular matrix as scaffolding for tissue regeneration *in vitro*. The genetic addition of eukaryotic proteins that regulate the

differentiation, proliferation and adhesion of the cells that will constitute the tissue, makes this system an excellent option for these applications [17].

4. Phage proteins

Recently, the lysis mechanisms of phages are being exploited for therapeutic applications. One of such systems has been very useful: the holin-endolysin system (shown in Figure 3).

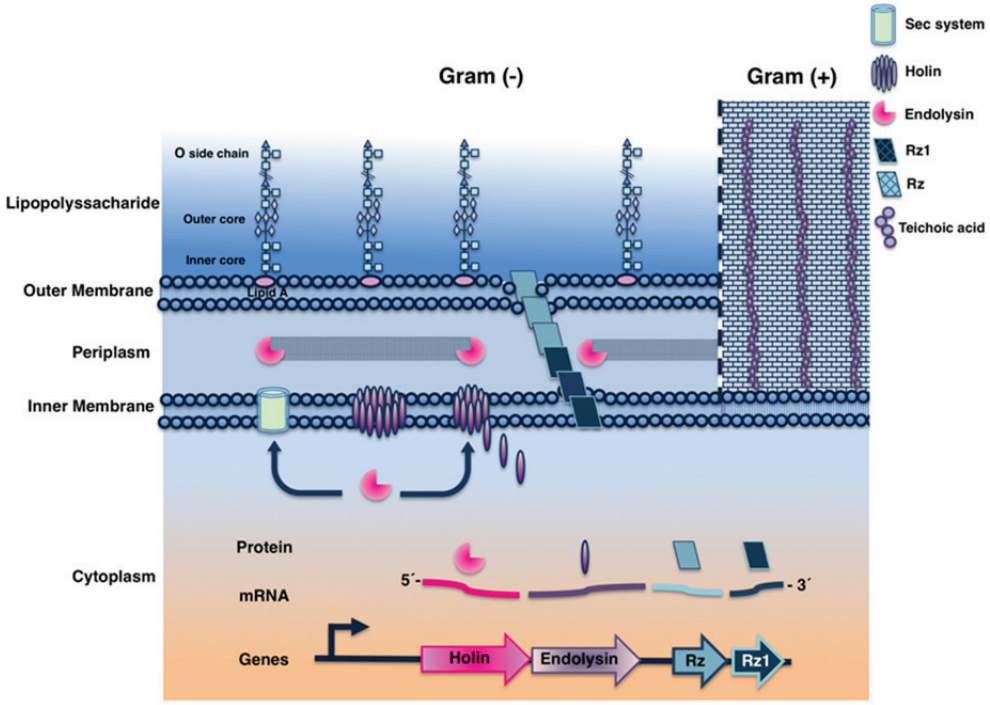

Fig. 3 Phage components involved in bacterial lysis. The holins and endolysins can act in combination or independent manner, but each one has a specific and different function that increases the efficiency of cell envelope disruption when they act in combination or with other accessory proteins such as Rz and Rz1.

The main lytic protein produced by phages is the peptidoglycan hydrolase, generically known as endolysin. Its function is to degrade peptidoglycan, leading the bacteria to a subsequent hypotonic lysis. Five functional types of endolysins exist: lysozymes (*N*-acetyl muramidase), endo-β-*N*-acetyl glucosaminidases, lytic transglycosylases, *N*-acyl-muramyl-$_L$-alanine amidases and the endopeptidases [18 - 20]. Other proteins named holins also participate in bacterial lysis. The function of holins is to form holes in the cell membrane. Initially, this protein was described as the responsible for the formation of pores that allow the transit of endolysins into the periplasmic space. However, endolysins that are capable to cross through the internal membrane using the general secretion system have been reported. This process is illustrated in Figure 3. Despite that these proteins can cross through the membrane without the use of holins as a hallway, they are not able to induce lysis *per se*, because they require the assembly of holins in the internal membrane in order to break the membrane potential. The participation of two additional proteins that alter the membrane architecture is shown in Figure 3. Protein Rz is distributed in the internal membrane, while Rz1 is in the external membrane, allowing the formation of a bridge-like complex that induces a mechanical stress from the internal to the external membrane, forcing the breakdown of the envelope [21].

Endolysins are highly effective against Gram-positive bacteria. For instance, their potential against the Methicillin-resistant *Staphylococcus aureus* (MRSA) has been demonstrated [20]. Despite this, endolysins are not efficient against Gram-negatives, because the external membrane constitutes a barrier for their action. This has guided the development of genetic modifications in order to translocate them into the periplasmic space and kill the host [20]. A second application of endolysins takes advantage of their action outside the bacteria and during the infection. In this case, enzymes are associated to the tail of phages to hydrolyse the peptidoglycan. Treatments with this kind of constructions are being explored, due to their potential against both Gram-positive and Gram-negative bacteria [19, 20, 22, 23].

4.1 Other phage proteins

Not only the lytic proteins can be used for therapy; the application of other parts of the phages is under development. The use of phage tails as bacterial elimination agents has been successful against *Staphylococcus* spp [24]. Also, the

fibers of the tail have been useful to provide specificity for a target, as in the case of phage T2, in which the fibers were substituted for those of phage PPO1, specific for pathogenic *E. coli* O157:H7 [25].

Finally, phages contain a high number of open reading frames (ORFs) with unknown functions, which may be useful in therapy. For example, the proteins of phage Sb-1 of *Staphylococcus* spp have a bactericidal effect on *E. coli* [26]**.** The polypeptides isolated by Liu *et al*. (2004) or Shibayama *et al*. (2011) follow the same mode of inhibitory activity in the bacteria overexpressing them [27, 28]. The Nun protein of lambdoid phage HK022 is toxic for *E. coli* when overexpressed [29]. These examples show that phage proteins may affect bacterial targets other than the cell envelope, and these can be involved in vital processes such as transcription or translation. In addition to therapy strategies using complete viral particles, the use of peptides is showing encouraging results.

5. CRISPR-Cas system

The CRISPR-Cas system has recently been used in therapy with success, and despite it is not a phage-derived system, it is related to phages. Throughout this chapter, we have described the perspective of the phage attacking the bacterial cell, but now we should mention that bacteria are capable to detect phage infection. In this regard, bacteria have developed several mechanisms to avoid such infection. It is worth to ask how this systems be used in our benefit? The answer to this will be described below.

In the case of bacteria, there are at least three basic mechanisms to avoid infection: (1) Restriction-modification system, in which a restriction endonuclease is expressed along with a methyl-transferase that will methylate the bacterial DNA. Generally, immediately after the DNA polymerase-III has replicated the DNA, it is methylated in order to avoid its fragmentation by the endonucleases that will cut and degrade any unmethylated foreign DNA. (2) The abortive infection system, which is orchestrated by specific bacterial proteins (like holin-antiholin systems) that abort the infection through cell death, preventing the infection of other bacterial cells [1, 30]. (3) The recently described CRISPR (clustered, regulatory interspaced, short, palindromic repeats)-Cas (CRISPR-associated) system, which is a global defense mechanism used to inactivate different mobile elements such as phages, plasmids and transposons. In general terms, this defensive system is a specific array in the chromosome, conformed by short repeated sequences (frequently palindromic) that flank short genetic material fragments from the invader (spacer). Along with the help of gene products adjacent to *cas,* they generate a small interfering RNA that initiates the degradation of the foreign genetic material. A general scheme of this system is illustrated in **Figure 4.** The Cas proteins are also involved in the acquisition of spacers (short sequences related to the foreign target, which are located between the repeats). Three different types of CRISPR-Cas systems have been described according to their mechanism, and at least 12 sub-types according to the mode of target recognition. The type-I system cuts and degrades DNA, while type-II system only cuts DNA. In both systems, at least two main factors are required in order to degrade the foreign genetic material: a) complementarity between crRNA (interfering RNA produced in the CRISPR system) and the sequence of the DNA target (proto-spacer), and b) a specific region adjacent to the proto-spacer, known as protospacer-adjacent motif (PAM). The type-III system is capable of cutting DNA and RNA, and similarly requires that recognition between the crRNA and the proto-spacer region [30 - 35].

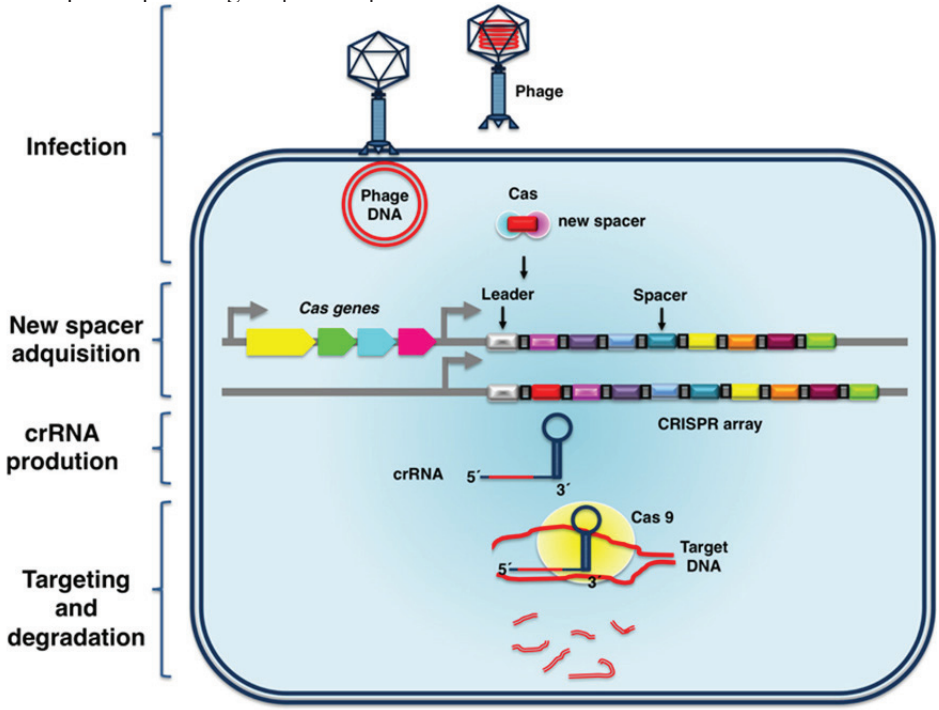

Fig. 4 CRISPR-Cas system. CRISPR-Cas is a defensive bacterial system that eliminates foreign DNA such as plasmids, phages and transposons. It functions by copying a short segment of the foreign genome, then adding it to an array of repeats that will allow the production of a small interfering RNA (crRNA). This crRNA binds to its target sequence, inducing strand breaks and/or degradation of the genetic material that bacteria do not recognize as their own.

Due to the characteristics of the CRISPR-Cas system, it has become an important tool for the edition of genetic material, having diverse applications such as the generation of mutations by insertion/deletion, substitution of sequences, arrangements or deletion of large regions such as chromosomal translocations. It has been useful in the localization of specific genomic sites by fusion of the Cas protein with GFP [36 - 38].

The focus of applications has been on the type-II CRISPR-Cas system, which uses the Cas9 protein along with the ciRNA of DNA edition [36]. For example, this system has been used in cell models to emulate several cancer types. This is achieved by introducing double-stranded cuts at defined positions in genomic DNA, causing rearrangements similar to those produced in certain types of cancer. Models such as myeloid leukemia, liver cancer and Ewing's sarcoma have been reproduced with this methodology.

Another relevant example giving hope to patients with hereditary disease, is the application of CRISPR-Cas system in the correction of dominant mutations producing cataracts. In this case, the mutated region was replaced with the wild type sequence. Another example involves the use of primary cultures of intestinal stem cells from patients with cystic fibrosis, in which the responsible gene was corrected by homologous recombination [30 - 41].

Paradoxically, the system that was originally discovered as part of the bacterial defense strategies, is now used against them. Its main advantage is that DNA sequences are used to eliminate the target genetic material with high specificity, allowing the selection of bacterial genus, species and even strains. In the case of antibiotics and phages, the number of bacteria to be eliminated cannot be controlled, therefore this systems eliminate entire microbial communities. In the case of CRISPR-Cas systems, they could be employed to selectively and quantitatively remove individual bacterial strains, which can be an advantage considering that the variation in specific populations of the intestinal microbiota can have an impact in the health of the individual [35].

6. Conclusions

The phages, as complete entities or in part, show a promising potential as therapeutic agents. Recently, phages have been recognized as a very important factor for human health, along with the human microbiota. Considering that the human intestine contains ~10^{15} viral particles, it has been estimated that these entities basically control the population dynamics of the different species present [42, 43]. Despite that there have been decades of study on phage therapy, we have little information regarding the Genetics and Molecular Biology of the used phages. The thorough study of these and new phages will provide more information that may be useful for the development of novel therapeutic alternatives.

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