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Biotechnological applications of bacteriophages: State of the art

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ABSTRACT

Bacteriophage particles are the most abundant biological entities on our planet, infecting specific bacterial hosts in every known environment and being major drivers of bacterial adaptive evolution. The study of bacteriophage particles potentially sheds light on the development of new biotechnology products. Bacteriophage therapy, although not new, makes use of strictly lytic phage particles as an alternative in the antimicrobial treatment of resistant bacterial infections and is being rediscovered as a safe method due to the fact that these biological entities devoid of any metabolic machinery do not have affinity to eukaryotic cells. Furthermore, bacteriophage-based vaccination is emerging as one of the most promising preventive strategies. This review paper discusses the biological nature of bacteriophage particles, their mode(s) of action and potential exploitation in modern biotechnology. Topics covered in detail include the potential of bacteriophage particles in human infections (bacteriophage therapy), nanocages for gene delivery, food biopreservation and safety, biocontrol of plant pathogens, phage display, bacterial biosensing devices, vaccines and vaccine carriers, biofilm and bacterial growth control, surface disinfection, corrosion control, together with structural and functional stabilization issues.

1. Introduction

Bacteriophages (or phages, viruses that infect bacteria) are the most abundant entities on our planet, being harmless for all organisms including humans except for their target bacterial hosts, infecting every type of bacterium in every known environment and being among the major drivers of bacterial adaptive evolution (Santos et al., 2014; Brussow and Kutter, 2005; Chibani-Chennoufi et al., 2004; Brussow and Hendrix, 2002; Bergh et al., 1989; McCallin et al., 2013; Abedon, 2015; Pirnay et al., 2015). Due to both the vast number of bacteriophages and the even larger number of unexplored genes that these metabolically inert particles carry, research is of utmost importance to fully understand and benefit from the biology and biotechnological potential of such entities. Bacteriophages are highly diverse and infect essentially all bacteria on earth (Catalão et al., 2013). Their genomes encode proteins that have been useful for biotechnology applications including food safety diagnostics, antibiotherapy of infections caused by antibiotic-resistant bacterial strains, DNA delivery vehicles and many more relevant technologies. Phages infect one bacterial cell, replicate, and then new virions are released and infect another cell. As a consequence, phages control bacterial population numbers, and on the other hand they contribute to moving genes from one bacterium to another. The study of bacteriophage particles provides insights into the evolution of genomes, bacterial adaptative evolution, and the way DNA is expressed and copied, and potentially sheds light on the development of new biotechnology products. Worldwide, the unstoppable increase of bacterial resistance to conventional chemical antibiotics is becoming a renewed driving force for bacteriophage (or phage) therapy, making use of lytic phage particles, since these biological entities devoid of any metabolic machinery do not possess affinity to eukaryotic cells (Chan and Abedon, 2012; Dąbrowska et al., 2005). Additionally, the utilization of bacteriophage particles in phage-based vaccination is emerging as one of the most promising preventive strategies, together with plant and animal gene transfer methods, phage display-based selection for biological affinity molecules, bacterial biosensing devices, gene delivery, food biopreservation and safety, biocontrol of plant pathogens, biofilm control, surface disinfection, and corrosion control. The issue of structural and functional stabilization acquires a special relevance in

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the context of phage particles. Since these are of proteinaceous nature, stabilization from both structural and functional points of view is directly related to rigidification of their three-dimensional structure (Balcão and Vila, 2015). This is a critical feature that will be discussed further below in this review paper.

1.1. Bacteriophage particles

Bacteriophages are viruses that infect solely bacterial cells, being biological entities known for over a century. To answer the question "what is life?", bacteriophages were elected as the simplest and most logical biological systems, with research based on them becoming the cradle of molecular biology (Chibani-Chennoufi et al., 2004). Despite all the basic biological research involving bacteriophages, a special interest in bacteriophages has now re-emerged, viewing them as potential alternatives and/or complements to conventional chemical antibiotherapy mainly because of their high-specificity and unique properties to fight multi-resistant bacterial strains (Summers, 2012; Rios et al., 2016). Bacteriophages are (biological) entities totally devoid of any metabolic machinery, thus being obligate intracellular parasites requiring a bacterium for their replication *via* their genetic material, taking over the biochemical machinery of the bacterial cells (Hyman and Abedon, 2010; Hermoso et al., 2007; Skurnik and Strauch, 2006; Kokjohn et al., 1991). Due to the important role that bacteriophages play in influencing the evolution of bacterial genomes, also inducing the development of bacterial pathogenicity, these metabolically inert particles may provide potential tools to face the current antibiotic resistance crisis (Chibani-Chennoufi et al., 2004). Over the last few years, a clear shift from the reductionist approach focusing on selected bacteriophages in carefully controlled laboratory conditions, towards the study of many different bacteriophages in the complexity of real-life situations can now be perceived. Indeed, bacteriophages harness the potential to be utilized in many different biotechnological applications, ranging from human antibiotherapy to environment disinfection. The vast majority of bacteriophages discovered so far interact with bacterial cells that express specific membrane surface receptors. However, if a bacterial cell does not expose a specific receptor for a particular bacteriophage at its surface, then the bacteriophage cannot infect it, which demonstrates the naturally high specificity of a bacteriophage to a particular bacterial host. Estimates suggest about ten different bacteriophages for every bacterial cell, some of which are highly specific for their bacterial host (either monophages (recognizing only one type of receptor) or polyphages (displaying a broader host range and recognizing more than one type of receptor)) (Skurnik and Strauch, 2006; Hyman and Abedon, 2010; Chan and Abedon, 2012). From the morphological point of view, bacteriophage particles exhibit a well-defined three-dimensional structure, the vast majority presenting an icosahedral protein capsid enclosing the genetic material in its core, a spiral contractile sheath (or tail) (surrounding a core pipe) and, usually, six tail fibers connected to a baseplate containing the receptor-binding proteins responsible for recognizing specific molecules at the surface of the bacterial membrane, as graphically illustrated in Fig. 1.

In relation to the type of genetic material they harness within the capsid's core, bacteriophage particles can be divided into four major groups (see Fig. 2): single stranded DNA phages (ssDNA), double stranded DNA phages (dsDNA), single stranded RNA phages (ssRNA), and double stranded RNA phages (dsRNA).

Over the last fifty years, more than 5100 bacteriophages have been identified and studied, with more than 90% of them possessing tails and belonging to the *Myoviridae*, *Siphoviridae* and *Podoviridae* families (Wittebole et al., 2013; Ackermann, 2007; Hanlon, 2007; Dąbrowska et al., 2005) (see Table 1). More than 2200 complete bacteriophage genomes can be found in the NCBI Genome database (as verified on October 7th 2017).

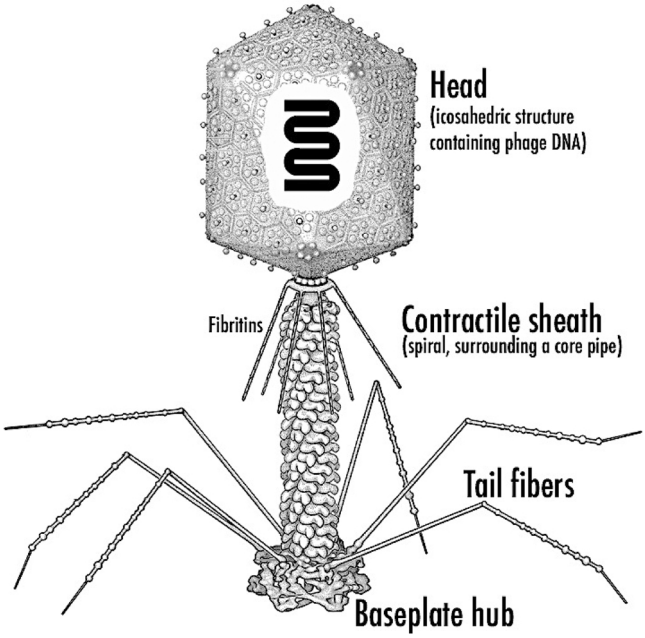

Fig. 1. Schematic representation of a prototypical bacteriophage particle. The bacteriophage DNA is protected by the icosahedral capsid, which is attached to the contractile sheath, a highly specialized and extremely efficient phage component required for infecting its host. The hexagonally shaped baseplate is situated at the distal end of the contractile sheath, and coordinates the movement of the tail fibers that initially sense the presence of the host, the short tail fibers that unfold from underneath the baseplate to firmly anchor on its bacterial host surface, and the spiral contractile sheath surrounding a core pipe that contracts, ejecting DNA into the bacterial host.
Adapted from Rossmann et al. (2005).

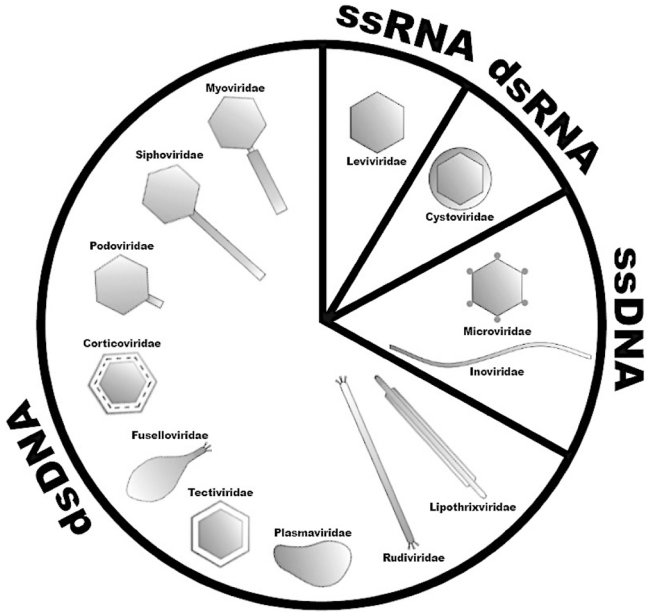

Fig. 2. Classification of bacteriophages according to their morphology, genetic material and major characteristics.
Adapted from Ackermann (2007) and Hanlon (2007).

1.1.1. Bacteriophage-bacterial host surface interactions

The baseplate in the bacteriophage three-dimensional structure coordinates both bacterial host recognition and attachment, with tail sheath contraction (if applicable), a movement initiated at the baseplate and propagated through the entire sheath in a wave-like fashion.

Table 1
Major types of bacteriophages.
Modified from Ackermann, 2007).

Bacteriophage group	Nucleic acid type	Distinctive features of the virion	Example
Myoviridae	linear dsDNA	contractile sheath/tail	T4
Siphoviridae	linear dsDNA	non-contractile long and flexible sheath/tail	λ
Podoviridae	linear dsDNA	non-contractile short sheath/tail	T7
Microviridae	circular ssDNA	isometric particles without sheath/tail	&x3D5;X174
Corticoviridae	circular dsDNA	isometric particles without sheath/tail	PM2
Tectiviridae	linear dsDNA	isometric bacteriophage particles without sheath/tail	PRD1
Leviviridae	linear ssRNA	isometric bacteriophage particles without sheath/tail	MS2
Cystoviridae	enveloped segmented dsRNA	spherical bacteriophage particles without sheath/tail	&x3D5;6
Inoviridae	circular ssDNA	filamentous bacteriophage particles without sheath/tail	fd
Plasmaviridae	enveloped circular dsDNA	pleomorphic bacteriophage particles without sheath/tail	MVL2

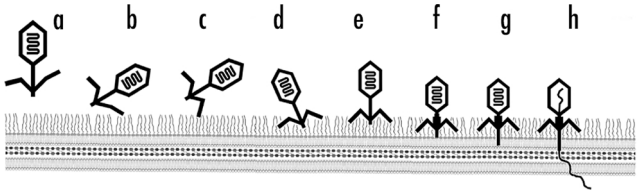

Fig. 3. A putative mechanism for the bacteriophage-driven breaching of the bacterial host envelope, during the infection process. The inserted arrows in Fig. 3a, b, c and d indicate bacteriophage particle drifts (*viz*. Brownian movements, thermally induced, convective currents) induced by solvent movement, whereas the inserted arrows in Figs. 3e, 3f, 3 g and 3 h indicate the bacteriophage capsid movement (driven by sheath contraction) towards the bacterial host surface. When the bacteriophage particle is free in aqueous solution (see Fig. 3a) its tail fibers move freely depending on solvent movement patterns; upon approaching a bacterial host (see Fig. 3b), the bacteriophage particle binds to its surface with one or two of its tail fibers, a binding that functions as a tether to restrict the movements of the bacteriophage particle; after the initial tethering at the bacterial host's surface, the bacteriophage particle drifts in such a way that the (already bound) tail fiber acquires a conformation (see Fig. 3c) from which it cannot switch back to that of the free bacteriophage particle, establishing new interactions with the baseplate proteins, and leading to initiation of the baseplate three-dimensional conformational change; the bacteriophage particle continues its kind of a "hula–hula dance" on the bacterial host's surface (see Fig. 3d), while being tethered to it *via* the bound tail fiber, and, eventually, the other tail fibers come into contact with their binding partners on the bacterial host's surface; now that all of its tail fibers are bound to bacterial host's surface receptors, the bacteriophage drifts in such a way that all of its tail fibers point toward the host cell (see Fig. 3e), with the conformational change of the baseplate continuing to proceed; The three-dimensional conformational change of the baseplate initiates contraction of the sheath (see Fig. 3f), driving the capsid and the tail tube toward the bacterial host's membrane, and the outer bacterial host's membrane is punctured with the help of the baseplate central spike protein; after puncturing the outer bacterial host's membrane, the spike dissociates from the tail tube tip and opens the tail tube channel (see Fig. 3g), with the tail-associated glycosidase creating a small opening in the peptidoglycan layer; in the final stage of infection, the bacteriophage particle's tail tube interacts with the bacterial host's inner membrane (see Fig. 3h), which is further pushed toward the tail tube by the osmotic pressure of the cytoplasm in the small region now lacking the peptidoglycan layer, and the bacteriophage DNA is released within the bacterial host's cytoplasm.
Adapted from Inamdar et al. (2006) and Leiman and Shneider (2012).

The tail fibers, extending from the bacteriophage baseplate, constitute the primary determinants of bacterial host specificity (Leiman and Shneider, 2012). The successful binding to a bacterial host receptor triggers a conformational change in the baseplate that ultimately leads to sheath contraction. Hence, the signal from binding to the bacterial host must be transmitted through the entire fiber to the baseplate, a transmission that occurs *via* changing the orientation of the fibers relative to the baseplate. When the bacteriophage particles are free in (aqueous, buffer) solution, their tail fibers do not exhibit a fixed orientation, pointing roughly sideways or even towards the capsid (Leiman and Shneider, 2012). The bacteriophage particle's tail fibers bound to the surface of the bacterial host point towards it. Two possible mechanisms for the bacteriophage infection of a specific bacterial host have been accepted nowadays, explaining how and why such changes in tail fiber orientation occur only on the bacterial host surface, but not when the bacteriophage particle is free in (aqueous) solution (see Fig. 3) and, remarkably, without any use of chemical energy in fiber reorientation and baseplate triggering.

The first postulated and most widely accepted mechanism is based on the rationale that, when the bacteriophage particle encounters a susceptible bacterial host, only one or two (at most) of its tail fibers bind to specific bacterial host surface receptors, hence subjecting the attached bacteriophage particle to the influence of all solvent movements originating from bacterial cell swimming and other types of molecular motions (*viz*. Brownian, thermally induced, and convective-current movements). The bacteriophage particle is now tethered to the bacterial host *via* binding between its tail fiber(s) and specific receptors on the surface of the bacterial host, while being constantly shaken by the solvent (Leiman and Shneider, 2012). At a certain point, other tail fibers of the bacteriophage particle establish connections with other receptors on the surface of the bacterial host, causing all the tail fibers to be pointed towards the surface of the bacterial host with concomitant positioning of the bacteriophage particle perpendicular to the membrane of the bacterial host. With the tail fibers pointing towards the surface of the bacterial host, more interactions are formed in the baseplate causing it to switch to a lower free energy conformation, unlocking the sheath and allowing it to contract (Inamdar et al., 2006). However, spontaneous contraction of the tail does not occur, since the orientations of the tail fibers associated with both the extended and contracted three-dimensional conformations of the sheath are separated by a significant variation in Gibbs free energy (ΔG). The only way to overcome such high ΔG, with the tail fibers immobilized on the surface of the bacterial host, is for the bacteriophage particle to be constantly agitated by the solvent movements (Leiman and Shneider, 2012; Kostyuchenko et al., 2005; Leiman et al., 2004).

Another possible explanation for how bacteriophage tail fiber reorientation may be coupled to sheath contraction upon attachment of the bacteriophage particle to the surface of the bacterial host arises from the fact that many bacteriophage particles often require calcium for infection of bacteria. Certain molecules on the surface of bacterial cells (*e.g.* polysaccharides and proteins) bind divalent cations such as calcium, greatly increasing their concentration in the immediate neighborhood of the bacterial surface. Such a high concentration of divalent cations may promote a change in the three-dimensional conformation of the baseplate with concomitant extension of the tail fibers so as to bind to the surface of the bacterial host, followed by unlocking of the sheath. This second possible mechanism is similar to that proposed by Sciara et al. (2010) for bacteriophage p2, specific for the Gram-positive lactic acid bacterium *Lactococcus lactis*, whose infection process requires calcium.

A further mechanism that may regulate phage ability to interact

with the surface of bacteria is the ionic strength of an environment. Szermer-Olearnik et al. (2017) identified ionic strength as a triggering factor for bacteriophage virions aggregation/disaggregation. As demonstrated by scanning electron (SEM) and atomic force (AFM) microscopy, bacteriophage particles can form viral packages, each containing 20-100 virions. In this form, bacteriophages show weakened infectivity, but they are more likely resistant to environmental factors. In a higher ionic strength solution, which is characteristic for the surroundings of bacteria, bacteriophages disaggregate and restore their full infectivity. Ionic fluxes are typical for living bacteria, as part of cation exchangers in bacterial membranes. Thus, bacteriophage particles are able to detect bacterial cells in their proximity and to change their arrangement in a way that facilitates infection.

In fact, there is a possibility that all these mechanisms work at the same time. As mentioned before, the tail fibers become oriented towards the surface of the bacterial host as a result of solvent-induced bacteriophage drifts and, concomitantly, the baseplate becomes more prone to a change in its three-dimensional conformation in the presence of certain “surface-associated” ions. With sheath contraction the capsid becomes closer to the surface of the bacteria, at the same time that the tail tube protrudes from below the plane of the baseplate. The central hub in the baseplate complex, onto which the tail tube is initially assembled, is dislodged from the baseplate to form the tip of the tube. This complex is the membrane-piercing “spike” that perforates the bacterial outer membrane driven by the sheath's energy (Leiman and Shneider, 2012) and allows the bacteriophage genetic material to exit through the tail tube into the cytoplasm of the bacterium. The large amount of energy stored in the folded conformation of capsid-packaged bacteriophage DNA is not used in the bacterial membrane penetration process. This principle is supported by observations of tailocins, i.e. bactericidal proteins resembling bacteriophage tails that are functional without an associated phage head. They are able to disrupt the bacterial envelope with concomitant cell death as DNA-free complexes (Scholl et al., 2009; Ghequire and De Mot, 2015). Also, tail sheath contraction and DNA ejection phenomena are not linked, since bacteriophages with contracted tails can be fairly stable and do not release their DNA (Leiman et al., 2004). Hence, it can be hypothesized that the bacterial cytoplasmic membrane must contain a specific receptor, at least as abundant as the bacteriophage receptor on the bacterial surface, for opening up the bacteriophage tail tube and triggering DNA release. The tail tube channel opening is likely to be triggered by certain lipid molecules within the cytoplasmic membrane, with translocation of bacteriophage DNA into the bacterial host through the tail tube being the next step in the infection process.

As discussed above, bacterial infection by its natural predator (bacteriophage) is initiated when the bacteriophage particle(s) is adsorbed onto the surface of its bacterial host (Shao and Wang, 2008; Dąbrowska et al., 2005). After the initial contact between the bacteriophage particle(s) and bacterium (Smith and Trevino, 2009), *via* diffusion and Brownian motion, the bacteriophage particle establishes reversible and nonspecific binding to the bacterium surface mainly *via* electrostatic forces, followed by irreversible binding between the bacteriophage's capsid protein and a bacterial surface receptor which, depending on the type of bacteriophage, may be: (i) a glycoprotein, (ii) a lipopolysaccharide, (iii) an amino acid, (iv) a teichoic acid, or (v) pili (Wittebole et al., 2013; Maura and Debarbieux, 2011; Shao and Wang, 2008; Skurnik and Strauch, 2006; Miernikiewicz et al., 2016).

1.1.2. Bacteriophage-induced degradation of the host cell's envelope components

The onset of the bacterial cell infective process by the bacteriophage particles is hampered by the need to penetrate through a complex bacterial host cell envelope, a multilayered structure that protects these organisms from their unpredictable and often hostile environment. The bacterial cell envelope falls into one of two major categories. The envelope of Gram-negative bacterial cells usually encompasses an inner cell membrane, a thin periplasmic peptidoglycan layer and an outer membrane containing lipopolysaccharides (LPS). The envelope of Gram-positive bacterial cells lacks an outer membrane, but it possesses a much thicker peptidoglycan layer densely functionalized with long anionic glycopolymers called wall teichoic acids (Silhavy et al., 2010). In addition, many bacteria express an outermost protective coat, the S-layer, which is composed of a single protein that totally encases the organism, or these cell-envelope structures may be surrounded by other protective structures such as capsular polysaccharides (*e.g.* α-2,8-linked polysialic acid (PSA) and/or hyaluronic acid (HA)), or mycolic acids (Silhavy et al., 2010). Bacteriophage virions must therefore be equipped with tools that allow them to penetrate through all these protective layers. Hence, bacteriophages carry enzymes capable of perforating the capsule and peptidoglycan layer(s) of their specific bacterial hosts (Rodríguez-Rubio et al., 2016; Salmond and Fineran, 2015; Moak and Molineux, 2004). Bacteriophage virions that bind to lipopolysaccharides (LPS) usually degrade these polysaccharide chains immediately following attachment. Pires et al. (2016) presented an extensive review on the diversity of bacteriophage-encoded depolymerases, used by bacteriophages to overcome bacterial protective layers and thus reach the bacterial cytoplasm.

1.1.2.1. Bacteriophage-induced degradation of the bacterial host capsule. Many bacterial cells are protected by a network of polysaccharides globally called the glycocalyx, a carbohydrate-enriched coating that covers the outside of many eukaryotic and prokaryotic cells, particularly bacteria, comprised of tiny viscous fibers. When loosely covering the outer surface of bacterial cells, such a network is called a slime layer, facilitating bacterial movement. When it is in a condensed form that is relatively tightly associated with the underlying cell wall, the glycocalyx is referred to as a capsule. There are two main functions of the glycocalyx. The first is to enable bacteria to become more difficult for the immune cells (phagocytes) to surround and engulf, since the presence of a glycocalyx increases the effective diameter of a bacterium and it also covers up components of the bacterium, thus making it less “visible” and less stimulatory for the immune systems of animals and humans.

The second function of a bacterial glycocalyx is to promote the adhesion of the bacteria to living and inert surfaces, with the subsequent formation of adherent, glycocalyx-enclosed bacterial aggregates commonly referred to as biofilms. Bacteria in biofilms can become resistant to a variety of antibacterial factors, partly due to the presence of the glycocalyx material. Many persistent infections in the human body can be attributed to bacterial biofilm formation. Examples are chronic lung infections in patients afflicted with certain forms of cystic fibrosis and infected with glycocalyx-producing *Pseudomonas aeruginosa* (Glonti et al., 2010). The glycocalyx layer (with a thickness $\geq$400 nm) protects the cell from hostile environments and, at the same time, prevents bacterial viruses from reaching their entry receptor at the cell surface (Bazaka et al., 2011). Bacteriophages that infect bacterial cells encompassing a capsule (condensed glycocalyx tightly associated with the underlying cell wall) carry exopolysaccharide (EPS)-degrading enzymes (depolymerases) in their tail spikes. These enzymes allow bacteriophages to hydrolyze the bacterial outer sugar coat so that they can reach either the outer membrane or the peptidoglycan layer (Yan et al., 2014; Cornelissen et al., 2012). The glycocalyx-based bacterial capsule can be composed of various EPS, such as alginates, polysialic acids (PSAs) and hyaluronic acid (HA), whereas EPS depolymerases include enzymes such as glycanases, lyases and capsule-specific deacetylases.

1.1.2.2. Bacteriophage-induced degradation of the bacterial host lipopolysaccharides. Some bacteriophage particles contain enzymes able to hydrolyze the lipopolysaccharides (LPS) of Gram-negative bacteria, an enzymatic activity that is often associated with the tail spike proteins. LPS hydrolysis is likely a strategy utilized by the

bacteriophage particles to clear a path through the bacterial LPS, thus allowing them to gain access to the entry receptor(s) on the bacterial cell surface. On the other hand, and very interestingly, LPS-degrading bacteriophages can also use LPS as attachment receptors, and thus LPS degradation may act to release bacteriophage progeny particles from bacterial cell debris at the end of a lytic cycle (Drulis-Kawa et al., 2012). EPS depolymerases include enzymes such as LPS-specific glycanases and LPS-specific deacetylases.

1.1.2.3. Bacteriophage-induced degradation of the bacterial host peptidoglycans. Endolysins are the lytic enzymes used by phages at the end of the replication cycle to degrade bacterial peptidoglycan (PG) from within, resulting in a rapid host lysis and the release of phage progeny. Virion-associated lysins (VALs) and depolymerases are linked to the virion particle and serve at the beginning of infection to overcome bacterial cell surface barriers. VALs are responsible for PG degradation required for phage genetic material injection to the infected host cell, whereas depolymerases degrade polysaccharide molecules such as capsule, lipopolysaccharide (LPS), or biofilm matrix (Casey et al., 2018; Maciejewska et al., 2018; Criscuolo et al., 2017; Drulis-Kawa et al., 2015; Drulis-Kawa et al., 2012; Meng et al., 2011b; O'Flaherty et al., 2009). This hydrolytic activity is necessary for the bacteriophage to reach the bacterial host plasma membrane and inject its genetic material into the host's cytoplasm. Such hydrolysis is local, with the virion drilling a hole large enough to have its tail or sheath pass the cell wall. Depending on the enzymatic specificity, bacteriophage lysins (murein hydrolases) are divided into five main classes: (i) lysozymes (*N*-acetyl-β-D-muramidases), (ii) lytic transglycosylases, (iii) N-acetyl-muramoylamidases, (iv) N-acetyl-β-D-glucosaminidases, and (v) endopeptidases.

1.1.3. Lytic and lysogenic modes of action

Subsequently to the binding of the bacteriophage particle to the bacterium surface, a spike at the bottom of the baseplate in the bacteriophage structure penetrates into the bacterial host, followed by a complete release of the bacteriophage's genetic material into the host's intracellular environment.

Either a lytic (see Fig. 4a) or a lysogenic (see Fig. 4b) cycle can take place following bacterial infection, depending on the type of bacteriophage. Considering the lytic pathway, promoted by strictly lytic bacteriophages (also commonly termed virulent bacteriophages), the bacterial host's metabolic machinery is taken over and re-targeted to the production of new bacteriophage particles. This encompasses replication of the bacteriophage genome in the bacterial cytoplasm with concomitant synthesis of viral proteins (*viz*. capsid, tail) and assembly of more lytic bacteriophage virions in cycles of ca. 30 min each which, with the help of bacteriophage-synthesized holins and lysins, causes lysis of the bacterium (see Fig. 4a) (Dąbrowska et al., 2005) and releases a wave of newly formed virions. Simply put, the bacteriophage particle hijacks the bacterium, turning it into a bacteriophage-producing factory. To facilitate this, genomes of many bacteriophages are natively methylated by a DNA adenine-methylase that introduces methyl groups in the carbon 5 of the pyrimidine ring in cytosine residues. Methylation protects exogenous bacteriophage DNA from the destructive restriction endonucleases encountered in the bacterial cytoplasm (Wittebole et al., 2013; Maura and Debarbieux, 2011; O'Flaherty et al., 2009; Dąbrowska et al., 2005; Weinbauer, 2004).

The lysogenic cycle (see Fig. 4b) characteristic for temperate bacteriophages is a cycle with delayed propagation of the bacteriophage, since the viral genome becomes integrated within the bacterial genome. In this case, the bacterium becomes immune to attacks of other bacteriophage particles of the same strain. This may potentially make bacteria more virulent and resistant to some bacteriophages (Wittebole et al., 2013; Maura and Debarbieux, 2011; O'Flaherty et al., 2009; Weinbauer, 2004). Such a lysogenic bacterium contains an integrated prophage in its genome, and the prophage remains in a latent state even in many bacterial cell divisions, until it is activated by stress or cellular damage processes of the bacterial host. These factors turn the bacteriophage cycle to the replication *via* a lytic pathway (after excision from the bacterial genome) with concomitant release of the new virions (Wittebole et al., 2013; Maura and Debarbieux, 2011; Lu and Collins, 2009; Hanlon, 2007; Weinbauer, 2004).

In either the lytic or lysogenic cycle, after the intracytoplasmic synthesis of the bacteriophage proteins and enzymes responsible for both capsid formation and genome packaging, the assembly and formation of new virions occurs. To release newly formed virions, the cell membrane is disrupted by holins that perforate the membrane and facilitate translocation of lysins to the peptidoglycan layer (importantly, holins are products of the "old" bacteriophage particles since they are expressed from their genome; newly formed virions do not do it). Peptidoglycan is degraded, leading to lysis of the bacterium with concomitant expulsion of the newly formed virions to the extracellular environment (Wittebole et al., 2013; Maura and Debarbieux, 2011; Smith and Trevino, 2009; Ackermann, 2007).

1.1.3.1. Bacteriophage inter-particle communication for death or salvation. As stated above, temperate bacteriophage particles may become dormant in their host bacterial cells, in a process called lysogeny. In every infection cycle, such particles have to "decide" between lytic and lysogenic cycles, i.e., whether to replicate and lyse their host, leading to its death, or to lysogenize and keep the host viable (Erez et al., 2017). Here a question arises: how does a bacteriophage particle "know" when to kill or save an infected bacterial host cell? The mechanism of this decision has long been unclear but, in a very recent study by Erez et al. (2017), a mechanism for how bacteriophage particles make such a decision has been reported. It is actually a form of inter-virus communication (Davidson, 2017; Erez et al., 2017; Gerritsen, 2017). Using *Bacillus subtilis* as a model, these researchers identified a small-molecule communication system involving a hexapeptide moiety released into the medium that appeared to protect bacterial cells from lysis after infection by a bacteriophage particle. In fact, such peptide (called arbitrium by Erez et al. (2017)) turned out to originate from bacteriophages after they entered a lysogenic cycle, and instead of protecting bacteria *per se*, it directed other bacteriophage particles to lysogenic instead of lytic cycles. This arbitrium system enables a descendant bacteriophage virion to "communicate" with its predecessors, that is, to estimate the amount of recent previous infections and hence decide whether to employ the lytic or lysogenic cycle (Erez et al., 2017; Gerritsen, 2017). This is, ultimately, the first known mechanism of intracellular communication between viral particles, and may be a way for these bacterial viruses to continue infecting future generations of bacteria. It turns out that, when a bacteriophage particle is faced with the problem of how to infect a bacterial cell, natural selection prevails (Erez et al., 2017). The biological rationale behind this arbitrium system is in fact quite clear: when a single bacteriophage particle encounters a susceptible bacterial colony, there exists ample prey for the bacteriophage progeny produced from the first lytic cycles of infection. However, the number of viable bacterial cells is reduced in each lytic cycle to a level where newly released bacteriophage virions are at risk of no longer having a bacterial host to infect. According to Abedon (2017), bacteriophages extend their infections presumably to more fully exploit increasingly rare bacterial host cells, using either lysogenic cycles or lysis inhibition. Therefore, it is logical for bacteriophages to switch into lysogeny to preserve their chances for viable reproduction. Hence, the arbitrium system provides an elegant mechanism for a bacteriophage particle to estimate the number of recent prior infections and decide whether to pursue the lytic or lysogenic pathway. Regulation of lytic-lysogenic cycles in phages may have a profound impact on the possibility to use some phage strains for therapeutic purposes. Specifically because in some important (for therapeutic applications) phage groups still no obligatory lytic phages have been found, *e.g.* for *C. difficile* phages.

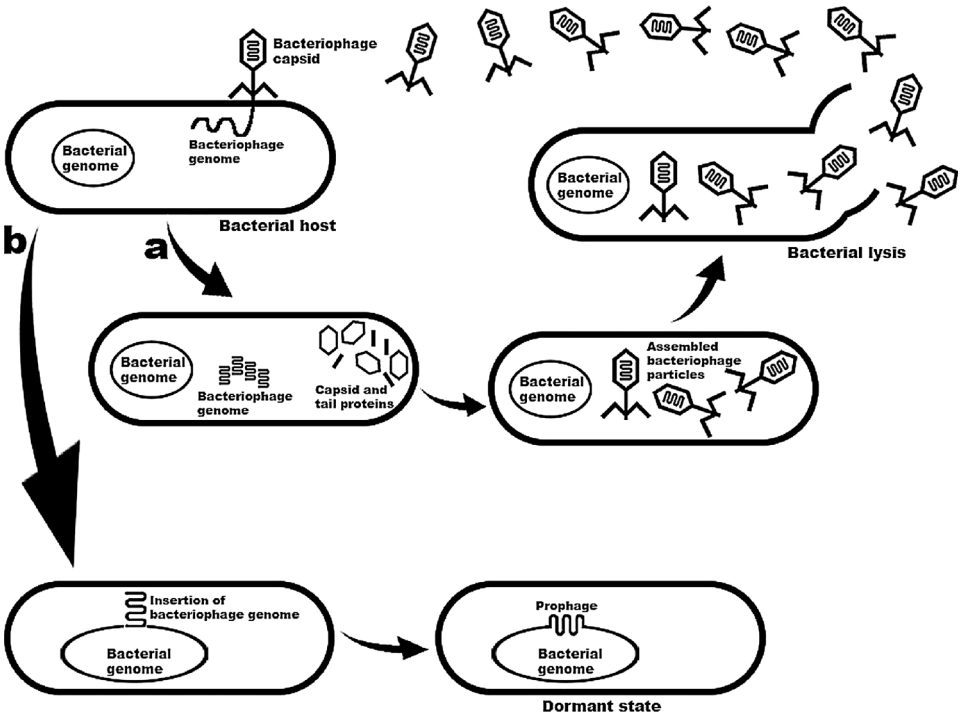

Fig. 4. Schematic diagram of the lytic (a) and lysogenic (b) infectious cycles of a bacterial predator (i.e. a bacteriophage). The process of bacterial infection by its predator begins when the bacteriophage adsorbs onto the surface of its bacterial host cell in a complex process. There is initially a contact between bacteriophage and bacterium by diffusion and Brownian motion after which the bacteriophage particle establishes reversible, nonspecific binding *via* electrostatic forces, followed by irreversible binding between the capsid protein of the bacteriophage particle and specific receptors on the bacterial surface. After binding of the bacteriophage to the bacterial cell surface, a portion of its structure penetrates into the bacterial host followed by a complete ejection of the genetic material into the host's intracellular environment. Depending on the type of bacteriophage, either a lytic or a lysogenic cycle can occur following bacterial infection. In the lytic response (promoted by strictly lytic bacteriophages), the metabolism of the bacterial host is assaulted and re-targeted to the production of new bacteriophage particles, by replicating the genetic material of the virus into the cytoplasm, leading to the synthesis of more lytic bacteriophage particles which, aided by produced holins and lysins, subsequently causes lysis of the bacterium. In the lysogenic response (promoted by temperate bacteriophages), the bacteriophage replication occurs at a later stage (since the viral genetic material is integrated into the genome of the bacterium) and the bacteriophage is replicated without lysing the host, with the bacterium becoming immune to attacks of other bacteriophages of the same strain (becoming a lysogenic, and usually more virulent, bacterium).

2. Structural and functional stabilization issues

The issue of structural and functional stabilization acquires a special relevance in the context of bacteriophage particles, if these metabolically inert proteinaceous entities are to be utilized in any biotechnological applications. Since they are of proteinaceous nature, stabilization from both structural and functional points of view is directly related to rigidification of their three-dimensional structure (Balcão and Vila, 2015). Attachment or embedment of a protein entity such as a bacteriophage particle in any solid matrix promotes a change in the water activity of its immediate (and surrounding) nanoenvironment, within which the motions of (any) water molecules are so restricted that they do not crystallize even at very low temperatures but instead transition into a highly viscous state, stabilizing the protein entities by hyper-increasing their rotational, translational and vibrational viscosities. Overall, the thermodynamic (or conformational) stability of the bacteriophage particle can be correlated with a change in the thermodynamic conditions of the nanoenvironment surrounding each (bio) particle, since the movements of (aqueous) solvent molecules in their immediate vicinity become seriously reduced. Because there is a direct relationship between the molecular motions of a protein entity and the molecular motions of its immediate vicinity, when we attach a (bio) particle such as a bacteriophage onto a solid matrix we are virtually eliminating the motions of the (aqueous) solvent molecules in its immediate vicinity (thereby increasing the bacteriophage particle's rotational, translational and vibrational viscosity), leading to a more rigid three-dimensional architecture with concomitant decrease of entropy and producing stabilization (Balcão and Vila, 2015).

3. Phage therapy

We currently face a dramatic increase in the levels of bacterial resistance to conventional antibiotherapy, with a real danger of entering a new "pre-antibiotic era". According to the World Alliance against Antibiotic Resistance (WAAR), conventional antibiotics are on the brink of completely losing their effectiveness, mostly due to a combination of self-medication, irrational prescription and overuse. These factors have led to the emergence of multi-resistant bacterial strains, even those resistant to all currently available antibiotics (Rios et al., 2016; Kutter et al., 2015; Kaźmierczak et al., 2014). The increased use of antibiotics is partially related to the growing numbers of people needing healthcare, as a result of population ageing with concomitant increase of chronic diseases and healthcare-associated infections (Fair and Tor, 2014). The overuse of antibiotics in humans leads to selection of resistant bacterial specimens also from the normal microbiota, hence contributing to environmental dissemination of resistance genes (Nitsch-Osuch et al., 2016). Additionally, the overuse of antibiotics for therapeutic, prophylactic or growth-promoting purposes in farm animals induces further the selection of pathogenic and/or commensal bacteria resistant to antibiotics (Aarestrup, 2015; Boerlin and Reid-Smith, 2008; Donabedian et al., 2003; Wegener, 2003; Aarestrup et al., 2000). Subtherapeutic dosages induce phenotypic changes in bacteria contributing to the emergence of bacterial resistance (Viswanathan, 2014).

Klebsiella pneumoniae carbapenemase (KPC), an enzyme that degrades carbapenem antibiotics, was historically largely associated with *Klebsiella pneumoniae*, and later isolated from *E. coli* all over the world (Liu et al., 2016; Nordmann et al., 2011; Stoesser et al., 2017). The ways pathogenic bacteria can resist to antibiotics are related to different mechanisms of resistance, namely (i) a marked decrease in bacterial uptake of the antibiotic molecule, (ii) production of hydrolases (*e.g.* β-lactamases) that inactivate the antibiotic, (iii) modification of the antibiotic receptor, (iv) a marked decrease in the intracellular concentration of the antibiotic by means of efflux pumps present in the bacterial membranes, (v) alteration of the enzymatic pathway(s) with concomitant decrease of bacterial susceptibility to the antibiotic, and (vi) deprivation of the intracellular enzymes needed for activation of prodrug(s) (Rios et al., 2016; Ojala et al., 2013; Rouveix, 2007; Stavri et al., 2007; Piddock, 2006).

The Gram-negative bacterium *Pseudomonas aeruginosa*, which can

infect a wide range of animal and plant hosts, has become a superbug (Breidenstein et al., 2011). Krylov et al. (2016) extensively reviewed the potential applications of bacterial viruses (bacteriophage therapy) aimed at eradication of antibiotic-resistant *Pseudomonas aeruginosa* in children with cystic fibrosis (CF). A transmissible plasmid has been isolated encoding MCR-1, an enzyme that transforms the bacterial lipid A in the outer membrane to a colistin-resistant state (Liu et al., 2016). This plasmid has been found in *Escherichia coli* strains, but given the relative ease of interspecies plasmids migration, it was almost inevitable that some strains of *Pseudomonas aeruginosa* infecting patients with CF would acquire additional transmissible resistance to colistin. Hence, the increased reliance on colistin for treating multidrug-resistant Gram-negative bacterial infections has resulted in the emergence of colistin-resistant *Pseudomonas aeruginosa* (López-Causapé et al., 2017; Lee et al., 2016b). The mechanisms through which *Pseudomonas aeruginosa* acquires and loses resistance to colistin have implications for the treatment options that can be applied against *Pseudomonas aeruginosa* infections, with respect to improving bactericidal efficacy and preventing further resistance to antibiotics.

These facts clearly demonstrate the need for developing effective and viable alternatives to current chemical antibiotherapy, aimed at protecting and promoting planetary public health (Rios et al., 2016; Dąbrowska et al., 2014; Oldfield and Feng, 2014; WHO, 2015). New actions and alternative strategies should thus be implemented not only at the community level but also at health care centers. Bacteriophages are natural antimicrobials that hold the potential to serve as viable alternatives to conventional antimicrobial chemotherapy (Dąbrowska et al., 2014; Pirnay et al., 2012; Cairns et al., 2009; Górski et al., 2016). The use of bacteriophages in the treatment of bacterial infections presents numerous advantages over treatment with conventional antibiotics. First, bacteriophage treatment does not interfere with the normal microbiota because it is highly specific. Second, bacteriophage treatment does not require successive administrations of the agent, as the bacteriophage begins to replicate on its own in the host and ceases its action when its host no longer exists. At that point, therapeutic success is achieved by virtue of the extinction of the etiological agent (Shlezinger et al., 2017). In the Republic of Georgia (the Eliava Institute and the Center for Phage Therapy, in Tbilisi) and in Poland (the Institute of Immunology and Experimental Therapy, in Warsaw), bacteriophage therapy has been extensively (and successfully) applied over the last decades in the treatment of bacterial infections (compassionate use) (Kutter et al., 2010; Górski et al., 2016). Notwithstanding the immense potential of bacteriophages for eradicating infections caused by antibiotic-resistant bacterial strains, only a limited number of clinical trials have been authorized by public health authorities (such as the US Food and Drug Administration (FDA) and European Medicines Agency (EMA)) and performed so far in humans. The issues related to therapeutic use of bacteriophages and bacteriophage registration have been recently extensively discussed by several authors from the field of bacteriophage therapy (Maura and Debarbieux, 2011; O'Flaherty et al., 2009; Debarbieux et al., 2016; Górski et al., 2016; Sarker and Brüssow, 2016).

3.1. Phage kinetics and phage dynamics

The pharmacokinetics of bacteriophages (herein proposed as “phage kinetics”) describes the amount of bacteriophage particles available in a living system with its changes within time. Pharmacodynamics of bacteriophages (herein proposed as “phage dynamics”), in turn, describes and measures the physiological effects that bacteriophages exert in a living system. Phage kinetics and phage dynamics are interrelated with one another. They represent the reciprocal interactions and impact that the human or animal body may have with a bacteriophage in a therapeutic action, being crucial for our understanding of therapeutic failures or successes. However, the paradigms associated with therapies based on conventional antibiotics cannot easily be transferred to bacteriophage-based therapies, which has partially contributed to problems with the acceptance of bacteriophagess as substitutes for antibiotics. In particular, predictions of certain threshold phenomena not normally encountered in the pharmacokinetics and pharmacodynamics of conventional antibiotics play a central role in bacteriophage therapy (Cairns et al., 2009). Phage kinetics and phage dynamics are more complex than “classic” drug pharmacokinetics, since bacteriophages propagate throughout bacterial host populations. The bacteriophage mode of spreading throughout macrobiological populations is similar to epidemics: they infect susceptible bacterial host cells, replicate (which also means that they multiply), and subsequently infect other susceptible bacterial host cells (Cairns et al., 2009). In the same way, the rate of bacteriophage propagation is completely dependent on the bacterial host population, since the bacteriophage population can only increase in the presence of sufficiently high numbers of bacterial host cells.

One should be aware that *in vitro* replication of bacteriophages may be quite different from what actually occurs *in vivo*, due to many factors that cannot be effectively transferred from a living organism to *in vitro* systems. In addition, the *in vivo* “phage kinetics” and “phage dynamic” processes change according to the bacteriophage particle in question. Hence, phage kinetics in phage therapy is dependent on several critical parameters: (i) adsorption rate; (ii) latency period; (iii) initial bacteriophage dosage; (iv) time point; (v) clearance rate of the bacteriophage particles from the body fluids; (vi) ability of the phage to replicate *in situ* including bacterial potential to form phage resistance; (vii) animal or human anatomophysiology; (viii) environmental conditions; and (ix) phage distribution in the human body, including specific effects of the immune system (Skurnik and Strauch, 2006; Dąbrowska et al., 2006; Payne and Jansen 2001; Levin and Bull, 2004; Hodyra-Stefaniak et al., 2015).

These universal factors can work differently in different routes of application. Dosing can be topical, or parenteral, or *via* per os delivery. The goal is to achieve relatively high bacteriophage particle concentrations within the immediate vicinity of infecting bacteria. To be effective, bacteriophages must adsorb to bacteria and successfully infect a large fraction of bacterial cells in order to clear bacterial infection.

3.2. Inhalational delivery of bacteriophages

Pulmonary infections are among the most common antibiotic-resistant infections (Rios et al., 2018; Balcão et al., 2014a; Matinkhoo et al., 2011). Bacteriophages capable of combating these infections have been tested *in vitro* and in animal models, showing promising results, especially when the phages are administered *via* nebulization in aerosol form (Borie et al., 2009; Cao et al., 2015; Carmody et al., 2010; Cooper et al., 2014; Golshahi et al., 2011; Saussereau et al., 2014; Henry et al., 2013). In respiratory tract infections, antibacterial agents such as bacteriophages and antibiotics, can be administered locally to the lung tissues as aerosols. This allows for higher concentrations at the site of infection, avoiding dispersion of antibacterial agents in other sites where such activity is not necessary, thus significantly increasing their activity *in situ* and reducing potential adverse effects (Rios et al., 2018; Balcão et al., 2014a; Golshahi et al., 2011; Matinkhoo et al., 2011). In the year 2008, the use of a commercial bacteriophage preparation (Pyophage) in a 5-year-old child diagnosed with cystic fibrosis who did not respond to standard antibiotic treatment was reported. The infection was associated with the presence of *P. aeruginosa* and *S. aureus*. The treatment was applied as nasal phage nebulization three times per day. After six days of treatment, the overall condition of the child improved significantly, and after twenty days of treatment a weight gain (1 kg) was observed, whereas no weight gain had been observed for a year before the treatment. After 3 courses of treatment, the last of which included tetracycline, *S. aureus* and *P. aeruginosa* were undetectable in the sputum (Kutateladze and Adamia, 2008). This is in line with the fact that antibacterial activity of *Pseudomonas*

bacteriophages was demonstrated *in vitro* in sputum of cystic fibrosis bacteriophages (Saussereau et al., 2014).

In an animal model, Morello et al. (2011) isolated multi-resistant *P. aeruginosa* from a patient with cystic fibrosis and inoculated mice intranasally with the bacteria to induce pneumonia. The progress of the infection was assessed by quantification of bacteria, inflammatory markers, and cytotoxicity (cell death and lysis) levels. The P3-CHA bacteriophage was used. Two doses (3.0×10^7 and 3.0×10^8 plaque-forming units [PFU] per mouse) of bacteriophage were tested in animals that received lethal doses of *P. aeruginosa*. Twenty hours after the start of treatment, the number of bacteria was quantified; it decreased by more than two orders of magnitude in the high bacteriophage dose-treated group when compared to the group that received no treatment. There was also a strong reduction of cytokines and lactate dehydrogenase (a cell death marker) in the bacteriophage-treated group in comparison to the group that received no treatment. The same favorable results were found in histological analyses of the animals' lungs. Intranasal aerosol of the same bacteriophage when applied prior to infection was used to prevent pneumonia. Immunohistochemistry of lungs from mice pre-treated with P3-CHA bacteriophages gave results that were similar to those observed after curative treatment (Morello et al., 2011).

Another group of pathogens that significantly affects cystic fibrosis patients is the Gram-negative *Burkholderia cepacia* complex (BCC). Two anti-BCC bacteriophages (KS4-M and F ϕKZ) were lyophilized and tested against BBC and *P. aeruginosa* in an *in vitro* model. These bacteriophages were found to be active and could disperse as an aerosol. Thus, lyophilization should facilitate the delivery of bacteriophages *via* inhalation (Golshahi et al., 2011). Also, other bacteriophages and bacteriophage cocktails (KS4-M, KS14, ϕKZ/D3 and ϕKZ/D3/KS4-M) were lyophilized and demonstrated as viable and appropriate for dispersion as aerosols (Matinkhoo et al., 2011). In an animal model of pneumonia caused by BBC (*B. cepacia* complex-K56-2) in immunocompromised mice, the authors tested the activity of the KS-12 bacteriophage that was administered intraperitoneally or as an aerosol. The aerosol therapy was much more effective in treating pneumonia than intraperitoneal administration. In a study by Semler et al. (2014), a conclusion that aerosol-based phage therapy appears to be an effective method for treating highly antibiotic-resistant bacterial respiratory infections, including those caused by BCC bacteria, was reached. Another study was conducted in 2015 to evaluate phage therapy against *P. aeruginosa* hemorrhagic pneumonia *in vitro* and *in vivo* in minks; in this study atomization of phage preparations by ultrasonic treatment was investigated. The authors used a lytic *Podoviridae* phage: vB_PaeP_PPA-ABTNL (PPA-ABTNL) isolate from hospital sewage. The phage was tested against five strains of *P. aeruginosa* that were isolated from minks with hemorrhagic pneumonia. *In vitro* testing showed that the phage was very effective in destroying the bacteria. The phage also proved to be very safe in subsequent *in vivo* tests performed in rats. The experiment again showed the possibility of using this route of administration to combat pneumonia caused by this microorganism (Cao et al., 2015).

Recently, Krylov et al. (2015) tackled the conditions for the safe, long-term use of phage therapy against various infections caused by *Pseudomonas aeruginosa*, describing how to select the most suitable phages, their most effective combinations and some approaches for the rapid recognition of phages unsuitable for use in therapy against this pathogen. The benefits and disadvantages of the various different approaches to the preparation of phage cocktails were also considered by these researchers, together with the specific conditions required for the safe application of phage therapy in general hospitals and the possibilities for the development of personalized phage therapy. More recently, Krylov et al. (2016) discussed the potential application of bacteriophages aimed at the eradication of antibiotic-resistant *Pseudomonas aeruginosa* in children with cystic fibrosis (CF).

Enteric *Salmonella* is a major problem in the production of poultry and eggs (Mead, 2000). Measures such as antibiotics, probiotics and vaccination are often used without the expected success (Borie et al., 2009). A novel approach to the use of phages as a coarse spray was proposed by Borie et al. (2009). Bacteriophages alone and in combination with probiotics were used to combat *Salmonella enteritidis* in infected chickens. Three experimental groups were tested: bacteriophages specific for *S. enteritidis* were used in group I, probiotics (*Enterococcus* spp. (1.0×10^9 colony-forming units [CFU]/g), *Lactobacillus* spp. (2.3×10^7 CFU/g), and nonpathogenic coliform bacteria (4.4×10^9 CFU/g) were used in group II and a combination of bacteriophages and probiotics was used in group III. The chickens were infected with *S. enteritidis* and administered one of the three treatments, with both probiotics and bacteriophages administered *via* coarse spray. The results showed that the group that received only probiotics showed a reduced infection rate of 75.7% (compared to the control group, which had an infection rate of 100%). The bacteriophage-treated group exhibited a reduced infection rate of 80%. However, the combination of the two agents reduced the infection rate to only 38.7% ($p < 0.0001$).

Phage spray was also used in experimental *E. coli* infections in chickens, where two bacteriophages (designated SPR02 and DAF6) were used against poultry isolates of *E. coli*. The infection was induced by a relatively low dose of bacteria (10^4 CFU per bird injected into the thoracic air sac). The mortality of birds was significantly reduced (from 50% to 20%) when phages were given immediately after the challenge but had little treatment efficacy when administered 24 h or 48 h after the challenge (Huff et al., 2003). Bacteriophages were also effective when applied before bacteria, i.e. they had a good protective effect (Huff et al., 2002). Later, however, the same group found that neither a coarse nor a fine spray protected birds from infection induced by intratracheal (IT) challenge with *E. coli* in a higher dose: 10^8 CFU per bird. Effective protection was achieved when bacteriophage was also administered intratracheally. This suggests that, at least in some models, administration of bacteriophages to the site of a bacterial infection is more effective than a spray. The authors propose that this decided the positive effect of phage treatment (IT) or its lack (spray) (Huff et al., 2013).

3.3. Gastrointestinal application of bacteriophages

Bacteriophage count in the human gut is estimated at 10^{15} particles (Dalmasso et al., 2014), which probably represents the highest concentration of biological entities on Earth. The three most abundant virus families in the human gut are *Siphoviridae*, *Myoviridae* and *Podoviridae* (Babickova and Gardlik, 2015). As in any natural environment, there is an ecological interplay between elements of the gut microbiota, so bacteriophages influence the microbial community and its relationships with the human host very strongly (Lucas Lopez et al., 2017). Early reports of phage therapy in enteric infections came from the Eliava Institute of Bacteriophages, Microbiology and Virology at the Republic of Georgia in the 1930s. Early reports of the use of bacteriophages showed good results in fighting cholera infections in India and Eastern Europe. Although the scientific reports from that time are few and poor, reports on prophylactic bacteriophage treatment against dysentery in Soviet soldiers reveal a 10-fold lower incidence of dysentery episodes in comparison to soldiers who did not receive phage therapy (Kutter et al., 2010). Other reports of clinical use in civilians in Georgia evaluated tableted phage therapy against dysentery in more than 30,000 children in the 1960s. The treated and control groups were separated based on the street on which they lived (i.e., on one side of the street, the children were treated with phage therapy, while the children across the street received placebo). The results showed a 3.8-fold reduction in episodes of dysentery among treated children (Kutter et al., 2010).

IntestiPhage preparations are cocktails of bacteriophages that are used for the treatment and prophylaxis of intestinal infections caused by *Shigella*, *Salmonella*, *Proteus*, *Staphylococcus*, *E. coli* and *Pseudomonas*. In 2008, Kutateladze and Adamia reported interesting results of the

IntestiPhage use in children from 1976 to 1982. Out of 580 children, 452 children were treated with the IntestiPhage preparation, 100 children were treated with antibiotics and 28 children were treated with a combination of antibiotics and the IntestiPhage preparation. The group treated with antibiotics exhibited clinical improvement after an average of 29 days. The group treated with the phage-antibiotic combination showed clinical improvement after 15 days, and the group that was treated with the IntestiPhage preparation alone recovered after only 9 days on average (Kutateladze and Adamia, 2008).

Studies performed in animals revealed strong activity of bacteriophages against enteropathogens. Specific phages used to combat diarrhea caused by enteropathogenic *E. coli* in calves, piglets and lambs were very effective in combating diarrhea. Calves treated with phages continued to excrete the phages in the stool as long as *E. coli* was detected in the feces (Smith and Huggins, 1983), which demonstrates that a positive therapeutic effect was related to phage ability to propagate within the gastrointestinal tract.

In 2011, in Germany, a large outbreak of hemolytic uremic syndrome and bloody diarrhea caused by Shiga-toxin producing *E. coli* O104:H4 was reported. The bacteria were resistant to all penicillins and cephalosporins, making treatment very difficult. Bacteriophages were demonstrated to be effective in combating this bacterium, thus offering an alternative for combating infections caused by this antibiotic-resistant pathogen (Merabishvili et al., 2012; Muniesa et al., 2012).

Sarker et al. (2012) tested the efficacy and safety of bacteriophage cocktails in a randomized, double-blind and placebo-controlled trial. A 9-phage cocktail (nine independent isolates of T4-like *E. coli* phages, including four phages closely related to the T4D reference phage) was given to 15 healthy adults from Bangladesh. Two doses were used in the subjects: 3.0×10^7 PFU per person and 3.0×10^9 PFU per person. The doses were diluted in mineral water (150 mL) and administered 3 times per day (50 mL). The placebo group was treated only with mineral water. No treated volunteers showed any clinical adverse effects as evaluated by self-reports, clinical examination, or laboratory tests for liver and kidney functions or for hematology (Sarker et al., 2012).

Another major cause of food poisoning, including gastroenteritis, is *Campylobacter jejuni*, which infects at least 2 million people each year in the United States (Siringan et al., 2011). The consumption of undercooked or contaminated meat or poultry appears to be the main source of contamination by this microorganism (Wagenaar et al., 2013). One important factor in the pathogenicity of *C. jejuni* is its ability to form biofilms, which makes antibiotics even less effective (Ica et al., 2012; Lu et al., 2012) and makes the eradication of *C. jejuni* infections particularly difficult. Therefore, bacteriophage therapy may be an important alternative. This possibility prompted Siringan et al. (2011) to test the effect of two phages (CP8 and CP30) on biofilms formed on a glass surface by *C. jejuni* strains NCTC 11168 and PT14. Phages reduced the number of viable bacteria from 1 to 3 log10 CFU/cm^2. The authors reached the following conclusion: “The viruses within the biofilm could not only effectively target and lyse campylobacters but were also able to disperse the extracellular matrix forming the biofilm” (Siringan et al., 2011).

A specific challenge in phage therapy against gastrointestinal infections concerns the route of administration, as gastric acidity can destroy phages. Polymer microencapsulation has proven to be very effective for the oral administration of these phages (Stanford et al., 2010). Another treatment option for dysbiotic disorders of the human gut involves the association of bacteriophages with probiotics. In 1981, Tolkacheva et al. evaluated this association in leukemic patients with dysentery. The researchers evaluated 59 patients with this condition, using four different treatments. The first group received oral phage therapy (*E. coli-Proteus* phage or *Pseudomonas* phage). The second group was treated with a probiotic (bifidobacteria). The third experimental group received a combination of bacteriophages and bifidobacteria. The fourth and last group received antibiotics as conventional oral treatment. The best clinical results were observed in the group that received the combined therapy that included both bacteriophages and probiotics (Alisky et al., 1998). Due to their bacteriolytic action, bacteriophages have also been widely used against enterobacteria, in food production, in animal husbandry, in product processing and in food preservation (Endersen et al., 2014; Gutierrez et al., 2016; Sillankorva et al., 2012; Zaczek et al., 2015). Although consuming foods treated with viruses remains controversial for consumers, there are no reports that these foods have caused any harmful effect on any individual who consumed the treated food (Mahony et al., 2011).

3.4. Dermal and transdermal delivery of bacteriophages

Wounds and burns cause breaches in the natural protective skin barrier, making it susceptible to infection. Topical solutions (*e.g.* ointments, creams, lotions) containing strictly lytic bacteriophage particles could be used in cosmetic and pharmaceutical formulations to treat skin infections. For purulent chronic wounds, bacteriophage particles may be applied directly in the wound, either *via* injection into (or around) the wound, *via* soaked bandages, or *via* impregnation into biodegradable polymeric hydrogels with controlled-release characteristics (Oliveira et al., 2015). *Pseudomonas aeruginosa* is a frequent etiological agent in wound infections, and the emergence of multiple antibiotic resistant strains has created significant problems in the treatment of infected wounds (Krylov et al., 2013). The first clinical trial in the world for the effectiveness of phage therapy in burn wounds infected with *P. aeruginosa* is about to be completed by Pherecydes Pharma in France, Belgium and Switzerland (http://www.phagoburn.eu/). Safety of *Pseudomonas* phage use in patients with leg ulcers was demonstrated by the group of Sulakvelidze (Rhoads et al., 2009). Active phage proliferation and successful bacterial kill (Mendes et al., 2013; Basu et al., 2015) as well as markedly increased animal survival resulting from phage treatment (Holguín et al., 2015) were demonstrated in rodents.

Skin infections can also be caused by *Mycobacterium marinum*, *Mycobacterium szulgai*, or *Treponema pertenue* (Oliveira et al., 2015). A major challenge in the treatment of skin infections caused by these intracellular bacteria consists in developing appropriate strategies for the targeted delivery of bacteriophage particles into mammalian cells (Oliveira et al., 2015; Chacko et al., 2012). Since several intracellular pathogens do have a transient extracellular living form, they can be tackled by bacteriophages at that stage; one example is the mycobacteriophage D29 efficient in therapy against *M. ulcerans* (Oliveira et al., 2015; Trigo et al., 2013). *Pseudomonas aeruginosa* is a frequent participant in wound infections, and the emergence of multiple antibiotic resistant strains has created significant problems in the treatment of infected wounds (Krylov et al., 2013).

Parenteral delivery is commonly considered the most efficient method of administration of bacteriophages. However, the parenteral administrations, which are in fact injections, have numerous drawbacks, such as the requirement of a health professional assistance and increased possibility of cross-contamination (Ryan et al., 2012). Transdermal delivery offers a potential means of overcoming many of the problems associated with systemic delivery of bacteriophages (Ryan et al., 2012). However, typical bacteriophage particles (*e.g. Caudovirales*), being hydrophilic viruses that differ greatly from small relatively lipophilic drug molecules, do not satisfy the criteria for efficient transdermal absorption. Two early reports of Keller (1958) and Bennett and Foster (1966) revealed that in animal models phage penetration through the skin was possible but not fully efficient, i.e. an active phage was noted in tissues of only some of the animals. To date, transdermal delivery of bacteriophages has not been widely considered, except for the novel microneedle technologies developed by Ryan et al. (2012). In their paper, they reported for the first time the design and evaluation of a novel hollow polymeric microneedle device for the transdermal delivery of bacteriophage particles. Such a hollow microneedle device successfully delivered viable T4 bacteriophages transdermally both *in vitro* and *in vivo*. The microneedle-mediated transdermal delivery

punctures the skin and bypasses the stratum corneum to create transient aqueous transport pathways of micron dimensions which, in turn, enhances transdermal permeability. The study demonstrated that this method allows for successful systemic phage absorption (Ryan et al., 2012).

3.5. Tooth decay biocontrol

The oral cavity is one of the largest ecosystems in the human body, with approximately 6 billion inhabiting bacteria and at least 35 times more viruses. Parasitic relationships between bacteria and bacteriophages can occur in two ways: through bacteriolytic behavior, by which viruses infect bacteria and kill them quickly to release their offspring, and through commensal lysogenic behavior, by which viruses integrate into the genome of the host and participate in the genetic functions of these bacteria (Edlund et al., 2015) (see also Section 1.1.3.). Because of this dual behavior, bacteriophages play a role in the control of bacterial communities, affecting the balance between health and disease in the oral cavity (Canchaya et al., 2003). Through adhesion and biofilm formation, bacteria in the oral cavity can colonize various oral surfaces, such as the gingival epithelium, the teeth and the oral mucosa, leading to pathological processes, such as caries, periodontal disease and endodontic (dental pulp) infections (Shlezinger et al., 2017). Biofilms are formed *via* the adhesion of bacteria to surface adhesins, increasing the force of adhesion to the surface *via* irreversible binding. After adhesion, larger colonies form and become interlaced in a polymer matrix of peptidoglycan, allowing the biofilm to achieve maturation (Salli and Ouwehand, 2015). Under the protection of the mature biofilm, bacteria are inaccessible to chemical agents (chlorhexidine and antibiotics), are able to escape from the oral immune system, and are able to act as a reservoir of microorganisms to promote chronic diseases (Khalifa et al., 2015). Due to the difficulty of reaching aggregated bacteria in biofilms and the ineffectiveness of antibiotic agents against resistant bacteria, dental infections have become very difficult to treat. Thus, there is a need for alternatives to combat these infections, and bacteriophage therapies have become an option for combating orodental infections (Parasion et al., 2014).

3.5.1. Dental caries

Dental caries is a widespread and common bacteria-related disease in humans worldwide. These infections result from the adhesion of *Streptococcus mutans*, among other microorganisms, to the dental surface, the formation of dental plaque and the subsequent erosion of the tooth. The pathogenicity of this organism results from the presence of sucrose in the diet, which leads to fermentation, lactic acid formation and demineralization of the enamel, with the consequent formation of caries (Dalmasso et al., 2015; Struzycka, 2014). Although phage therapy is highly developed for the treatment of several infections, this approach to treating dental caries caused by *S. mutans* is still very new. A few studies have aimed to isolate specific phages able to counteract caries caused by *S. mutans* with bacteriophages (Dalmasso et al., 2015; Maal et al., 2015; Delisle et al., 2012; van der Ploeg, 2007), while the isolation of bacteriophages or their lytic enzymes could be used to selectively remove *S. mutans* from dental plaque and promote the fight against dental caries (van der Ploeg, 2007). In a recent study, Dalmasso et al. (2015) isolated and characterized a phage that is specific for *S. mutans* (ϕAPCM01). The phage was isolated from a saliva sample and belonged to the *Siphoviridae* family with a B1 morphology. Lysogenic potential of the phage and its ability to penetrate and degrade biofilms were also evaluated; the phage exhibited a strong lytic effect against *S. mutans* and weak lysogenic activity (0.03%). The activity of the bacteriophage against *S. mutans* was evaluated after 18 h of bacterial contact with the phage at multiplicity of infection values (MOIs) ranging from 2.5×10^{-5} to 250. The results showed at least a 2-fold reduction in optical density at 600 nm (OD600) between the control culture and cultures of *S. mutans* grown with the bacteriophage at all of the tested MOIs. These results showed that the phage was very effective in destroying *S. mutans*, even at smaller MOIs. This anti-*S. mutans* phage activity was confirmed by a decrease of at least 5.6 log CFU/mL between the control culture and the cultures grown with ϕAPCM01 at MOIs lower than 2.5×10^{-3}. The lytic activity of the phage against the bacteria was also evaluated in artificial saliva. The phage decreased the OD600 by at least 1.5-fold when compared to cultures grown without the bacteriophage. The phage activity against *S. mutans* attached to biofilms was evaluated after 48 h at various phage concentrations. At phage doses near 10^2 PFU/well, there was a significant reduction of the biofilm metabolic activity after 24 h and it failed to increase again after 48 h. At doses between 10^5 and 10^9 PFU/well, the bacteriophage caused complete inhibition of the metabolic activity of the biofilm. The authors concluded that despite its narrow host range, the newly isolated *S. mutans* phage exhibited promising antimicrobial properties. The isolation of bacteriophages specific to *S. mutans* opens the possibility of using these phages alone or in combination with prophylactic measures, such as water fluoridation, to combat dental caries, thus preventing this major cause of human tooth loss (Dalmasso et al., 2015).

3.5.2. Endodontic infections

Endodontic disease is a biofilm-mediated infection of the dental pulp. The main objective of treatment in endodontic disease is to eliminate the bacterial biofilm from the root system and to disinfect it. The treatment involves mechanical cleaning measures in combination with the topical application of chemical agents, with the aim of combating the infection (Jhajharia et al., 2015). Despite accessing the site of infection and applying the chemical treatment directly at the site of infection, treatment failure with worsening endodontic disease occurred in approximately 1/3 of treated patients. These failure rates are credited to the increasing rates of bacterial resistance to etiologic agents and to the formation of root canal and extraradicular biofilms (Waltimo et al., 2005). The difficulties of treating endodontic infections have led researchers to seek new therapeutic, either chemical or biological approaches to combat these infections; one of the investigated strategies is phage therapy. Thus, efforts have been made to isolate bacteriophages specific for *Enterococcus faecalis*, which is a major cause of endodontic infections and exhibits high levels of resistance against antimicrobial agents that are regularly used in dentistry (Paisano et al., 2004; Stevens et al., 2009).

Khalifa et al. (2015) investigated bacteriophages in the treatment of root canals infected by *E. faecalis* (ATCC 700802). Bacteriophage was isolated from a sewage treatment facility and it was named EFDG1. The phage activity was tested against *E. faecalis in vitro*, against previously formed biofilms, and in a root canal treatment model. The results obtained in the *E. faecalis* culture showed that at MOIs of 10^{-2}–10^{-4}, limited bacterial growth occurred and it was followed by a rapid lysis. At phage-bacteria rates greater than 10^{-2}, the bacteriophage almost completely prevented bacterial growth. The bacteriophage significantly reduced the activity and dispersed a 2-week-old 600-μm-width biofilm of *E. faecalis*. The biofilm biomass evaluation was performed using crystal violet and revealed a 5-fold reduction in the samples treated with the phage within 7 days. Biofilms that were not treated with the phage were stable, and no reduction could be observed. To evaluate the antibacterial activity of EFDG1 in root canal infections, the authors used an *ex vivo* two-chamber bacterial leakage model of human teeth. The results showed that in chambers containing teeth treated with the phage turbidity was not observed. Thus, irrigation of obturated root canals with EFDG1 resulted in reduced bacterial leakage from the root apex in comparison to the control group. The authors concluded that phage therapy may be an important ally in the fight against *E. faecalis* in root canal biofilms for which conventional therapies have shown no results (Khalifa et al., 2015). The same group further concluded in a review on phage use in dental infections: “In the future, phages such as EFDG1 and other phages of *E. faecalis* like phiEF24C, IME-EF1, and EFLK1 can be used either as cocktails or as combinations with

antibiotics to combat VRE (vancomycin-resistant *enterococci*) *E. faecalis* in dental biofilms" (Khalifa et al., 2016).

Paisano et al. (2004) in their study conducted in Brazil tested an *E. faecalis*-specific bacteriophage isolated from a stream in the city of São Paulo, Brazil. The authors inoculated *ex vivo* human dental roots with suspensions of *E. faecalis* (ATCC 29212) and with the phage at three rates: MOI 0.1, 1 and 10. All three were effective in inhibiting bacteria after 3 h of incubation. The teeth were also inoculated with the bacteria six days before treatment with the bacteriophage to ensure the penetration of the bacteria into the tooth tubules (Paisano et al., 2004). One, two and three days after incubation with the phage, no viable bacteria were found, showing that phage therapy was highly effective in dental roots and in the dental tubules. The authors concluded their work with the following statement: "Phage therapy might be especially helpful for the elimination of bacteria resistant to antibiotics, being a second front in the fight against multidrug resistant bacteria" (Paisano et al., 2004). Phee et al. (2013) studied specific phage therapy (JBD4 and JBD44a) against biofilms produced by *Pseudomonas aeruginosa* (PA14) in prepared root canals of extracted human mandibular incisors treated with bacteriophages with potential biofilm-degrading activities. The results showed a significant reduction in *P. aeruginosa* biofilm biomass at 24 h and 96 h after treatment (Phee et al., 2013).

4. Bacteriophage display

Bacteriophage particles exhibit a high level of organization and thus can be viewed as elaborate nanomachines. Filamentous bacteriophage particles can be easily genetically manipulated and the tremendous attention they received by the scientific community allowed the development of bacteriophage display techniques (Henry and Debarbieux, 2012; Onodera, 2010; Petty et al., 2007; Clark and March, 2006; Benhar, 2001).

Bacteriophage display is now widely used in various domains including antiviral research, design of novel therapeutics and vaccines, cancer research, targeted delivery or nanotechnology (Huang et al., 2012; Hyman, 2012; Ronca et al., 2012).

The use of bacteriophage particles to display exogenous (heterologous) peptides or proteins has numerous applications, including epitope identification, antigen delivery, drug discovery, vaccine design, targeting of eukaryotic cells, bioimaging and biosensing, enzyme display or design of nanomaterials (Henry and Debarbieux, 2012).

Bacteriophage display has been the subject of several scientific reviews published in specialty journals (Benhar, 2001; Onodera, 2010), and involves the engineering of bacteriophage particles so that they become surface-decorated with exogenous (heterologous) peptides or proteins: (i) *via* molecular manipulation resulting in translational fusion with a surface protein, (ii) *via* conjugation techniques, or (iii) *via* competitive incorporation of foreign phage protein fusions into the capsid, while the fusions are produced by an engineered bacterial host of the phage (Henry and Debarbieux, 2012; Pande et al., 2010; Clark and March, 2006; Benhar, 2001; Sidhu et al., 2000; Ceglarek et al., 2013; Majewska et al., 2015; Hodyra and Dąbrowska, 2015).

In the first and the most common approach, the genetic fragment that encodes the envisaged polypeptide is fused with bacteriophage coat protein genes, and the desired protein is presented on the surface of the bacteriophage particle (Haq et al., 2012; Onodera, 2010; Petty et al., 2007). This results in novel bacteriophage particles with a wide range of potential uses.

Molecular manipulation or chemical modification of the bacteriophage genome is now widespread and has been implemented for display using major bacteriophage systems, *viz*. T4, T7, M13, λ, and others (Adhya et al., 2014; Haq et al., 2012; Henry and Debarbieux, 2012; Teesalu et al., 2012; Beghetto and Gargano, 2011; Meng et al., 2011; Rakonjac et al., 2011; Hemminga et al., 2010; Onodera, 2010; Petty et al., 2007; Clark and March, 2006). In the molecular manipulation of bacteriophages, the exogenous gene is spliced into the bacteriophage genome as a fusion to a phage gene coding for a structural protein. This foreign gene codes for a foreign peptide that is eventually presented on the bacteriophage surface as a fusion to a structural protein. As a result, the bacteriophage particle becomes surface-decorated with the product of the expression of the foreign gene. Bacteriophage display is widely used due to its potential to carry out large scale screening, since the spliced genetic information may be randomly generated to produce a library, to achieve targeted delivery or to study molecule interactions (Henry and Debarbieux, 2012). Within the framework of libraries, the bacteriophage display technique allows one to screen a wide array of molecules based on their specificity or binding affinity to various compounds, allowing selection of ligands to virtually any target (Haq et al., 2012). The application of the bacteriophage display technique involves, in the first step, immobilization of the antigen moiety of interest on a surface that is then exposed to the engineered bacteriophage library. Those bacteriophage particles presenting molecules with affinity to the immobilized antigen moiety will therefore bind, while the rest of the library will be washed away. The bound bacteriophage fraction can then be eluted and amplified, resulting in a concentrated mixture of bacteriophage particles presenting the relevant molecule. Repetition of this procedure, in a process called "biopanning" (or affinity selection), allows identification of molecules exhibiting a high binding affinity to the antigen moiety of interest (Vodnik et al., 2011; Benhar, 2001). Both the size and charge of the peptide displayed on the surface of the bacteriophage particle must be taken into account, since they influence the number of peptide moieties that can be displayed on the surface of a bacteriophage particle. In order to maintain good bacteriophage production during propagation, the normal processing of the bacteriophage capsid proteins should not be hindered by the fused peptide moiety so as to allow for the correct expression of the capsid proteins and to let them fulfill their biological roles in the bacteriophage replication process (Henry and Debarbieux, 2012; Imai et al., 2008).

The second approach to engineering bacteriophage particles for the display of foreign elements is chemical modification, in a process termed bioconjugation (Patel and Swartz, 2011). In this biotechnological approach, attachment of specific molecules onto the surface of the bacteriophage particle allows decoration of both the outside and the inside of the capsid protein shell, allowing the use of the viral particles as carriers (Kovacs et al., 2007).

This approach allows for presentation of foreign elements on a phage surface without genetic modifications of the phage. This means that bioconjugation helps to escape the necessity to construct genetically modified organisms (GMOs). From the practical point of view, this can be an important advantage for phage-derived products developed this way.

The third alternative for phage display is "competitive phage display". This method allows for presentation of foreign peptides or proteins on the phage surface without any modifications in the phage genome, since protein fusions are expressed by an engineered bacterial host, *e.g.* from expression vectors. In the same bacterial cell, when infected by a target phage, wild types phage proteins are also produced. Wild type and fusion proteins are randomly (competitively) incorporated into the phage capsid. This is again a method that allows one to avoid the necessity of GMO construction, but it also offers more specific control of the capsid site that is decorated with a foreign element. This is because these elements are fused only to selected, predefined phage surface proteins, like in the classic phage display (Ceglarek et al., 2013; Majewska et al., 2015).

5. Vaccine carriers

Two distinct approaches have been developed so far for antigen delivery with phage vaccines, termed "phage display vaccines" and "phage DNA vaccines" (Rodríguez-Rubio et al., 2016; Adhya et al., 2014; Clark and March, 2004). Phage display vaccines aim at producing

immunogenic bacteriophage particles. Their surfaces are decorated with foreign antigens, which is achieved by expression of antigens as fusion products of one of the selected structural proteins of the bacteriophage virion (Majewska et al., 2015). Bacteriophage DNA vaccines, in turn, are produced by incorporating foreign antigen genes in the bacteriophage genome under control of strong eukaryotic promoters (Adhya et al., 2014; Golkar et al., 2014; Haq et al., 2012; Clark and March, 2004, 2006). In bacteriophage DNA vaccines, the bacteriophage particle acts as a passive carrier to transfer the foreign DNA into mammalian cells where the antigen gene is then expressed. These two approaches are often combined to produce bacteriophage particles that carry a foreign antigen gene and that display a protein or peptide on their surface. Hence, bacteriophages have the potential to be used as vaccine carriers *via* two distinct ways, *viz*. (i) by direct vaccination with bacteriophage particles carrying vaccine antigens decorating their surface, or (ii) by using the bacteriophage particles to deliver a DNA vaccine which is an expression cassette incorporated into the bacteriophage genome (Adhya et al., 2014; Golkar et al., 2014; Haq et al., 2012; Clark and March, 2004, 2006). In phage display-based vaccination, bacteriophage particles may be engineered to display a specific antigenic peptide or protein on their surface or, alternatively, bacteriophage particles displaying peptide libraries may be screened with a specific antiserum aiming at isolating protective antigens or mimotopes, i.e. peptides mimicking both the secondary structure and the antigenic properties of a protective carbohydrate, protein or lipid moiety, despite possessing a different primary structure (Clark and March, 2006). In a handful of cases, however, whole bacteriophage particles displaying antigenic proteins have been used as vaccines in animal models (Wang and Yu, 2004; Irving et al., 2001).

Instead of producing a transcriptional fusion to a capsid coat protein, antigenic molecules can also be artificially conjugated at the surface of bacteriophage particles after propagation, further increasing the range of antigens that can be displayed (Haq et al., 2012; Clark and March, 2006). Since bacteriophage particles contain potential adjuvants like phage proteins and DNA (Haq et al., 2012; Clark and March, 2004, 2006; Dąbrowska et al., 2014), a specific antigen present on the bacteriophage capsid would not need separate protein purification and subsequent conjugation to a carrier molecule before immunization. However, it has been shown more recently that unmodified bacteriophage particles may be used to deliver DNA vaccines more efficiently than standard plasmid DNA (Haq et al., 2012; Clark and March, 2006), with the vaccine gene (under control of a eukaryotic expression cassette) being cloned into a standard lambda bacteriophage particle and concomitant injection of purified (whole) bacteriophage particles into the host (Haq et al., 2012). The bacteriophage capsid protects the DNA from degradation and at the same time it targets the vaccine to the antigen-presenting cells. According to Clark and March (2006), there is also a possibility for producing a “hybrid bacteriophage vaccine”, where a eukaryotic promoter-driven DNA vaccine is integrated within the bacteriophage particle and a bacteriophage display variant of the same antigen is integrated on the bacteriophage particle's surface, aiming at efficiently targeting both the humoral and cellular arms of the immune system (Adhya et al., 2014; Clark and March, 2006). Additionally, it might also be possible to modify the surface of a bacteriophage particle-based vaccine with specific protein or peptide sequences aiming at the preferential targeting of the particle to particular immune cell types. Phage display has been used mostly for the identification of immunogenic epitopes or mimotopes on displayed peptides which, in turn, could become lead molecules for the design of peptide-based vaccines (Rodríguez-Rubio et al., 2016; Adhya et al., 2014; Haq et al., 2012; Benhar, 2001).

6. Gene delivery

The DNA packaging systems of bacteriophages have been identified as a special type of nano-vehicle for gene delivery (Karimi et al., 2016; Haq et al., 2012; Clark and March, 2006). While the DNA packaging motor of bacteriophage Phi29 was shown to be an efficient machine for packaging viral ds-DNA, its RNA had a high tendency to form dimers, trimers and hexamers (Lee et al., 2009). This motor that uses ATP to push DNA into the procapsid requires only three components, the gp10 (connector) and gp16 proteins and a small packaging RNA (pRNA) (Henry and Debarbieux, 2012). By engineering this molecular machinery of bacteriophage Phi29 RNA, Hao et al. (2014) produced a nanocage. In particular, the special anisotropic shape of bacteriophage M13 (Karimi et al., 2016) together with its long rod-like architecture displays liquid-crystal-like properties. Due to the high aspect ratio of this bacteriophage particle, its penetration into the targeted cells is improved due to a higher number of ligand-receptor interactions. In contrast to spherical-shaped bacteriophages, their filamentous counterparts have a tendency to migrate toward the blood vessel walls when administered *in vivo*, and therefore have a better chance to interact with their receptors (Karimi et al., 2016; Bakhshinejad et al., 2015). Bacteriophage particles can thus be used as designed nanocarriers (Karimi et al., 2016; Henry and Debarbieux, 2012) for the targeted delivery of both therapeutic agents and genetic sequences, representing a new tool of nanotechnology in drug delivery systems. The phage coat protects the DNA from degradation after injection, and the ability to display foreign molecules on the phage coat also enables targeting of specific cell types (Clark and March, 2006). Surface decoration of the bacteriophage particle with targeted ligands provides versatility and high specificity in this regard.

7. Food biopreservation and safety

Temperate bacteriophages (*i.e.*, lysogenic) are generally considered unsuitable for the development of biocontrol strategies. However, their virulent counterparts (*i.e.*, strictly lytic viruses) are very much appropriate for biocontrol (downstream) applications. Biocontrol of pathogens in food products may in fact represent an economically viable field for bacteriophage-based biocontrol (Henry and Debarbieux, 2012). Several systems have already been approved by authorities for use on food products, *viz*. ListShield (from Intralytix) or LISTEX (from Micreos Food Safety) for the control of *Listeria monocytogenes*, EcoShield (from Intralytix) for targeting *Escherichia coli* O157:H7 and SALMONELEX (from Micreos Food Safety) for the control of Salmonella (Rodríguez-Rubio et al., 2016; Henry and Debarbieux, 2012). Bacteriophages were also found to be efficient for the decontamination of livestock raised for the food industry, therefore limiting the risk of pathogens reaching the food chain. They were also used to detect pathogens in animal-derived alimentary products (Henry and Debarbieux, 2012). Phage-mediated biocontrol has successfully been applied against many pathogens that cause economic losses in food production (see Table 2).

Phages infect bacteria that cause important losses in all of these areas of food and agriculture. Studies of their biology and biotechnology, as well as of useful phage gene products (*e.g.* endolysins), have the potential to improve food safety and agricultural yields. There are several useful reviews summarizing current as well as envisaged applications of bacteriophages in the entire food chain. They also address the concerns of consumers about chemical preservatives due to both safety and toxicity (Jhamb, 2014; Haq et al., 2012; Sillankorva et al., 2012). Additionally, outbreaks of food-borne infections associated with the increasing prevalence of antibiotic-resistant microorganisms has led to a search for novel food (bio)preservation techniques. Pre- and postharvest bacteriophage research has targeted many life-threatening bacteria including *Escherichia coli* O157:H7 as thoroughly discussed by Sillankorva et al. (2012) (see Table 2).

Shigella is one of the most important waterborne and foodborne bacterial pathogens in the world, usually related to the ingestion of contaminated water and food. Recently, Jun et al. (2016) reported on the potential of a virulent *Myoviridae* bacteriophage (pSs-1) isolated from environmental water in South Korea as a biocontrol agent that can

Table 2
Phage-mediated biocontrol of pathogens that cause economical loss in food production.
Modified from Haq et al. (2012), Sillankorva et al. (2012), Adriaenssens et al. (2012) and Fujiwara et al. (2011).

Pathogen	Loss in food production
Aeromonas spp	fish in aquaculture
Paenibacillus larvae	honeybee
Xanthomonas pruni	peaches, cabbage and peppers
Xanthomonas campestris	tomatoes
Pseudomonas tolaasii	mushrooms
Salmonella	poultry products, pork, processed foods (ripened cheese), fresh-cut melon and apple, frankfurters, raw and cooked beef, fresh tomatoes, mung bean sprouts and alfafa seeds, and chocolate milk
Campylobacter	poultry products and beef
Listeria monocytogenes	beef, fresh melons and apples, honeydew melon, red-smear soft cheese, cooked ham, salmon fillet, catfish fillets, and chicken
Escherichia coli O157:H7	poultry (broiler chicken), lamb, sheep, cattle and steer meat, tomato, spinach, lettuce and cantaloupe
Staphylococcus aureus	dairy cattle, raw milk whey, milk curd, pasteurized milk, and cheese
Dickeya solani	potato
Ralstonia solanacearum	Potato, tomato, aubergine (egg plant), banana, ginger, tobacco, sweet pepper, olive, soybean

be used to control contaminated waters with *Shigella flexneri* and *Shigella sonnei* (Jun et al., 2016). Three major conclusions arise from the literature survey: (i) bacteriophage therapy in animals is able to reduce levels of foodborne pathogen and control the pathogen load upon entry at the slaughterhouses; (ii) the bacteriophage-based biocontrol of pathogens in foods significantly reduces dangerous pathogens and appears to be a promising alternative to traditional food preservation techniques; (iii) bacteriophages used in agricultural settings are at least as efficient as the agents conventionally used to control the growth of plant-related bacterial pathogens.

The company Omnilytics (www.omnilytics.com) developed the bacteriophage product Agriphage for the control of bacterial spot caused by *Xanthomonas campestris* or bacterial speck caused by *Pseudomonas syringae*. The effectiveness of this product in protecting crops against these pathogens is reflected by the report of a marked increase in yield. The control of pathogens of fruits and vegetables is highly important, since these foods cannot be further processed to kill any pathogens present without the risk of imparting damage.

8. Bacterial biosensing devices

Rupture of the host bacterial cell induced “from within” is the last step in the bacteriophage lytic replication cycle, being mediated mainly by two special proteins produced in the last stage of the virion progeny assemble, *viz.* holins (which perforate the inner side of the cytoplasmic membrane) and endolysins (which pass through the newly formed pores and access the peptidoglycan layer, degrading it and resulting in destabilization of the murein sacculus). In addition to the liberation of the virion progeny particles, lysis of the host bacterial cell also results in the release of intracellular components, some of which may be exploited as markers for biodetection purposes (Ahmed et al., 2014; Zourob and Ripp, 2010). Bacterial cytoplasmic markers utilized in bacteriophage-mediated bacterial detection include (but are not limited to) detection of released ATP, adenylate kinase, bacterial β-D-galactosidase, and α- and β-glucosidase (Schmelcher and Loessner, 2014; Zourob and Ripp, 2010).

Bacteriophage particles are also suitable tools for specific impedimetric detection of bacteria, since addition of bacteriophage particles to a sample results in retardation of changes in impedance if the target organism is present. This is due to changes in conductivity of the growth medium that are caused by the growth of microorganisms, mostly through transformation of large uncharged metabolites (*e.g.* carbohydrates) to smaller charged molecules (*e.g.* acids) (Schmelcher and Loessner, 2014; Zourob and Ripp, 2010). Zourob and Ripp (2010) provided an extensive overview of the techniques, methodologies and assays that employ bacteriophage particles in biosensing applications.

Further, bacteriophage particles recognize specific molecular motifs expressed on the bacterium surface, to which they bind and inject their genetic material (Ahmed et al., 2014; Sorokulova et al., 2014; Gervais et al., 2007; Clark and March, 2006). The high specificity of this recognition constitutes a remarkable tool to provide a rapid biodetection result ($\leq$1 day). This is of utmost importance when an emergency detection is needed, *e.g.* in an outbreak situation (Sorokulova et al., 2014; Henry and Debarbieux, 2012). Additionally, in the context of hospitals, health care centers (Ahmed et al., 2014; Sorokulova et al., 2014; Smietana et al., 2011) and food industries (Singh et al., 2013), the rapid identification of the presence of bacterial pathogens is vital in order to prevent the spread of infections by implementing appropriate control strategies (Henry and Debarbieux, 2012; Smartt et al., 2012). Common methods for detection and identification of pathogens in food products (Schmelcher and Loessner, 2014; Singh et al., 2013), drinking water supplies and hospitals continue to rely mostly on conventional microbiological culture techniques (Gervais et al., 2007). However, only a few diagnostic kits for pathogen detection in human samples (*e.g.* FastPlaqueTB (BIOTEC Laboratories Ltd, *Mycobacterium tuberculosis*) assay, KeyPath (MicroPhage Inc., *Staphylococcus aureus* MRSA/MSSA) test, xTAG Gastrointestinal Pathogen Panel (GPP) (Luminex Molecular Diagnostics Inc., *S. typhimurium*, and MRSA/MSSA Blood Culture Test) have reached the market so far (Schmelcher and Loessner, 2014; Sorokulova et al., 2014; Henry and Debarbieux, 2012; Zourob and Ripp, 2010). The constantly increasing number of disease-causing bacteria that are resistant to one or more anti-bacterial drugs utilized for therapy is the driving force for the development of early and speedy biodetection systems for these pathogens. Traditional pathogen detection techniques include microbiological and biochemical assays that are both time-consuming and labor-intensive, whereas antibody or DNA-based techniques require substantial sample preparation and purification. On the other hand, biosensing devices based on bacteriophage particles have demonstrated over the last few years a remarkable potential to surpass the aforementioned restrictions, offering rapid, efficient and highly sensitive biodetection techniques for (antibiotic-resistant) bacteria (Ahmed et al., 2014).

Currently, the applications of bacteriophage particles in the detection of pathogens can be divided into two categories: (i) those based on inert bacteriophage particles or isolated bacteriophage proteins, and (ii) those that require a non-going infectious cycle (Schmelcher and Loessner, 2014; Henry and Debarbieux, 2012). One may simply use the lytic nature of the bacteriophage as the “sensor” to detect the presence of its bacterial host. This is the principle of the FASTPlaqueTB assay for the detection of *Mycobacterium tuberculosis* in sputum, in which bacteriophages infecting the slow-growing *Bacillus tuberculosis* are subsequently detected when forming plaques on a fast-growing strain (Webster, 2010; Rees and Loessner, 2005). Balcão et al. (2013, 2014b) reported on the development of carbohydrate hydrogels for the stabilization of bacteriophage particles aiming at bacterial biosensing, and thoroughly studied the diffusion of bacterial cells from the surface of the hydrogel into its core. Besides using native, unaltered phage particles for bacterial detection, molecular cloning techniques make it possible to engineer bacteriophage particles to carry a specific reporter gene (Sorokulova et al., 2014; Zourob and Ripp, 2010; Ripp et al., 2006). Upon infection of a target host cell, the gene is expressed and allows detection of its product, *e.g.*, by measuring bioluminescence, fluorescence, or enzymatic conversion of a chromogenic substrate (Schmelcher and Loessner, 2014; Ripp et al., 2006). Bacteriophage particles carrying a luciferase gene for bacterial detection account for the predominant uses (Schmelcher and Loessner, 2014; Zourob and

Ripp, 2010; Ripp et al., 2006), with major advantages of using luciferase genes as reporters being the highly sensitive detection of the bioluminescent signal they generate. Additionally, several other reporter genes have been used to construct reporter bacteriophage particles, of which genes encoding fluorescent proteins such as green fluorescent protein (GFP) (Vinay et al., 2015; Lee et al., 2013; Zourob and Ripp, 2010) have been found to be particularly suitable. GFP combines many desirable properties such as high stability, low toxicity, and the fact that fluorescence is triggered by excitation light (Vinay et al., 2015), abolishing the requirement for adding a substrate as required for luciferases (Schmelcher and Loessner, 2014).

Methods that have been used over the last two decades to detect pathogenic bacteria include (but are not limited to) using bacteriophage particles specifically to deliver reporter genes (*e.g.* lux (Schmelcher and Loessner, 2014; Petty et al., 2007; Kodikara et al., 1991) or green fluorescent protein (Schmelcher and Loessner, 2014; Funatsu et al., 2002)) that are expressed after infection of the target bacterial host, using bacteriophage particles with a fluorescent dye covalently attached to their protein capsids and detecting specific adsorption (Goodridge et al., 1999), the detection of released cellular intracytoplasmic components (*e.g.* adenylate kinase) (Corbitt et al., 2000) after specific bacteriophage-mediated lysis, and using bacteriophage particles displaying peptides or antibody fragments that specifically bind bacterial pathogens or toxins (Petrenko and Vodyanoy, 2003).

The chemical attachment of genetically biotinylated T4 bacteriophage particles (engineered to express a biotin-binding domain on a capsid protein using a bacteriophage display technique) onto streptavidin-coated (derivatized) gold surfaces (Gervais et al., 2007) or streptavidin-coated magnetic beads and microcrystalline cellulose beads (Lee et al., 2013; Tolba et al., 2010) has also been reported. Chemical attachment of bacteriophage particles onto (bio)sensor surfaces could significantly improve both the stability and performance of the overall biodetection platform. The chemical biotinylation of bacteriophage particles has already been shown to increase the efficiency of bacteriophage-based biosorbents when compared to simple physical adsorption (Lee et al., 2013; Zourob and Ripp, 2010; Sun et al., 2001). Such genetic biotinylation opens the possibility of leveraging the affinity of the streptavidin/biotin system for the attachment of the bacteriophage particles onto surfaces (Petty et al., 2007). Additionally, as opposed to bacteriophage particles biotinylated *via* chemical procedures, the biotin is in these cases exclusively present on the bacteriophage capsid and not on its tail, potentially allowing the oriented attachment of the bacteriophage particle onto surfaces (Sorokulova et al., 2014; Webster, 2010; Gervais et al., 2007; Sun et al., 2001). Bacteriophage particles bound from their head capsid protein onto the (bio)sensor surface would also allow the tail fibers to face the medium, enabling more efficient capture of their host bacterium (Tolba et al., 2010; Gervais et al., 2007). The oriented immobilization of bacteriophage particles onto specific surfaces results in highly active biosorbents that can simultaneously capture and detect a target bacterium with high sensitivity (Tolba et al., 2010). Hence, bacteriophage particles represent potential tools for (bio)diagnostic assays due not only to their very high specificity for bacterium hosts, but also to their signal-amplifying properties (Lee et al., 2013). Every single phase of the bacteriophage lytic replication cycle, from the initial recognition of its specific host cell to the final cell lysis events, may be harnessed in different ways for the purpose of bacterial detection (Sorokulova et al., 2014; Lee et al., 2013). In addition to intact bacteriophage particles, bacteriophage-derived affinity molecules such as cell wall binding domains (CBDs) and receptor binding proteins may serve for this purpose (Rodríguez-Rubio et al., 2016; Schmelcher and Loessner, 2014).

Generally speaking, a biosensing device integrates several components, including a solid surface to which the recognition elements are attached; a transduction element that can be electrochemical (*e.g.* impedimetric), optical (*e.g* based on surface plasmon resonance) or mass-based (*e.g.* magnetoelastic); a signal amplifier; a signal detector; and a signal display (Schmelcher and Loessner, 2014; Sorokulova et al., 2014; Hiremath, 2013; Singh et al., 2013; Webster, 2010; Zourob and Ripp, 2010). All these biosensing components were described in detail elsewhere (Zourob and Ripp, 2010). Vital for the performance of a bacteriophage-based biosensor is the effective immobilization of the bacteriophage particles or bacteriophage-derived recognition elements on the biosensor solid surface (Webster, 2010; Zourob and Ripp, 2010). Therefore, strategies used for immobilization of those entities include physical absorption, covalent (undirected) immobilization *via* chemical functionalization, and oriented immobilization *via* genetic modification of the recognition element (Hosseinidoust et al., 2014; Sorokulova et al., 2014; Singh et al., 2012; Webster, 2010). Regretfully, bacteriophage immobilization *via* physical absorption is often hampered by the weak binding produced and inconsistent density of recognition elements attached to the biosensor surface, but this may be overcome by chemical coupling (Webster, 2010; Singh et al., 2009). Self-assembled monolayers encompassing chemisorbed elements play an important role in immobilizing bacteriophage particles. Several researchers have reported that modification of solid surfaces with cysteine followed by treatment with 2% (v/v) glutaraldehyde, resulted in a 37-fold enhancement of bacteriophage particle attachment compared with plain physical adsorption (Sorokulova et al., 2014; Singh et al., 2009). The strategy of oriented bacteriophage immobilization offers an additional advantage since the binding sites/domains are exposed and therefore available for capturing target cells (Schmelcher and Loessner, 2014). Bacteriophage particles integrate several properties that are highly desirable in designing biodetection systems for bacterial pathogens (Zourob and Ripp, 2010): (i) bacteriophage particles are highly specific for their target host cells; (ii) bacteriophages can discriminate between viable and non-viable bacterial host cells; (iii) bacteriophage particles function under a wide array of environmental conditions (some of them harsh); (iv) strictly lytic bacteriophages act as signal amplifiers; and (v) bacteriophage particles are inexpensive and easy to isolate and propagate (Singh et al., 2013; Petty et al., 2007). In addition, bacteriophage particles offer a virtually unlimited selection of tools (including complete bacteriophage particles and bacteriophage-derived affinity molecules such as cell-wall binding domains and receptor-binding proteins (Singh et al., 2013)) that can be harnessed for different biodetection strategies.

9. Biofilm and bacterial growth control: surface disinfection

An important challenge in health-care and food industry environments is related to the presence of bacterial biofilms on medical devices and industry surfaces (Rodríguez-Rubio et al., 2016; Hosseinidoust et al., 2014). It is now commonly accepted that most bacterial species live in communities and thrive in complex biostructures called biofilms. Biofilms consist of extracellular polymeric materials (i.e. the matrix) surrounding bacterial cells, and they are directly related to serious health problems caused by bacterial infections in diseases such as vaginosis, cystic fibrosis, and dental plaque and in transmission of bacterial infections associated with medical devices. Planktonic, free-floating bacterial cells express different phenotypes as compared with their counterparts inside biofilm communities. The study of these differences is of utmost importance in order to achieve a better understanding of why bacterial cells in biofilms are usually more pathogenic than their planktonic counterparts. Bacteria embedded in biofilms are considerably less susceptible to antibiotics and disinfectants than their planktonic counterparts, due to both a reduced growth rate and limited access of the antibacterials to bacterial cells in a biofilm (Rodríguez-Rubio et al., 2016; Davies, 2003). Since bacterial biofilms pose considerable health threats, innovative antimicrobial approaches using bacteriophage-derived products or engineered bacteriophage particles are being developed (Salmond and Fineran, 2015; Hosseinidoust et al., 2014). Considerable research has therefore been focused on endolysins,

peptidoglycan hydrolases involved in bacterial cell lysis during bacteriophage replication (Roach and Donovan, 2015; Salmond and Fineran, 2015; Briers et al., 2014; Rodríguez-Rubio et al., 2013).

Endolysin-based antibacterials efficiently kill Gram-positive bacteria upon contact by specific cell wall hydrolysis. However, a major hurdle in their exploitation as antibacterials against Gram-negative pathogens is the impermeable lipopolysaccharide layer surrounding their cell wall. Hence, Briers et al. (2014) developed and optimized an approach to engineer these enzymes as outer membrane-penetrating endolysins (coined Artilysins), rendering them highly bactericidal against Gram-negative pathogens, including *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

Loss of the structural integrity of the bacterial cell wall upon hydrolysis of the peptidoglycan layer causes lysis *via* osmotic imbalance. Additionally, in Gram-negative bacteria the outer membrane must also be breached by protein complexes (spanins) that fuse outer and inner membranes (Salmond and Fineran, 2015). Bacteriophage endolysins encompass a diverse range of hydrolytic enzymes. Most of them are species-specific, while some are more promiscuous. Typically, endolysins contain an enzymatically-active domain and a cell wall-binding domain (Roach and Donovan, 2015; Salmond and Fineran, 2015). The first commercially available endolysin, Staphefekt (Micreos Human Health, The Netherlands), is available for the treatment of human skin infections caused by *Staphylococcus aureus*.

Another enzyme-based approach makes use of bacteriophage-derived tail spikes or diffusible polysaccharide-depolymerizing enzymes (Salmond and Fineran, 2015). These enzymes reduce the levels of surface polymers such as exopolysaccharides (EPS) and lipopolysaccharides (LPS) (Roach and Donovan, 2015; Salmond and Fineran, 2015). Instead of lysing bacterial cells, the removal of polysaccharides may lead to disruption of the biofilm matrix, reducing bacterial virulence and assisting in bacterial clearance by the host immune systems (Salmond and Fineran, 2015). Lu and Collins (2007) engineered bacteriophage T7 so that it could express an EPS-degrading enzyme during infection of an *Escherichia coli* biofilm. Bacterial cell lysis released the EPS-degrading enzyme, facilitating further bacteriophage infections and increasing the anti-biofilm effect (Lu and Collins, 2007). Hence, problems associated with bacterial biofilms could be overcome by using endolysins and virion-associated peptidoglycan hydrolases (Rodríguez-Rubio et al., 2016). Sass and Bierbaum (2007) and Son et al. (2010) demonstrated that recombinant endolysins from bacteriophages phi11, phi12 and SAP-2 were able to remove biofilms of *Staphylococcus aureus* formed on polystyrene surfaces, and Domenech et al. (2011) reported that bacteriophage endolysins Cpl-1 and Cpl-7 were highly effective in removing biofilms of *Streptococcus pneumoniae*. In the last few years, Shen et al. (2013) reported that endolysin PlyC directly lysed *Streptococcus pyogenes* cells within the biofilm matrix due to the ability of this endolysin to diffuse through the extracellular material constituting the bacterial biofilm.

10. Bacteriophage tethering

Most steps in the control of gene expression depend on bi-functional RNA-binding proteins, since they have the ability both to bind to RNA and to interact with other proteins in a functional complex (Keryer-Bibens et al., 2008; Coller and Wickens, 2007). A powerful approach developed to study *in vivo* the functional properties of such bi-functional proteins, independently of their RNA-binding capability, is to tether (or attach) them to specifically engineered reporter mRNAs whose fate can be easily followed (Keryer-Bibens et al., 2008). Two tethering systems have been mainly used in eukaryotic cells, namely the MS2 bacteriophage capsid protein system and the lambda bacteriophage N-B box system (Keryer-Bibens et al., 2008; Coller and Wickens, 2007).

Another potential use of bacteriophage tethering involves the controlled covalent attachment of bioactive bacteriophage particles in medical devices so that these become resistant to the development of biofilms (Hosseinidoust et al., 2014; Donlan et al., 2013). In the patent application described by Donlan et al. (2013), bacteriophage particles are tethered to the surface of an indwelling medical device or a hydrogel-type coating on the surface of the device by covalent binding. Bacteriophage lytic activity is maintained in this process, thus preventing biofilm formation on the surface of the device when in use, leading to increased safety.

Uyanga (2015) described two novel pathways for engineering M13 bacteriophage aiming at cancer therapy applications, by exploiting the uniquely malleable biology of the M13 filamentous bacteriophage (Khalil et al., 2007). By engineering filamentous bacteriophages of shorter lengths *via* constructing a proprietary set of small viral ssDNA that are packaged by M13 capsid proteins (Uyanga, 2015), the small bacteriophage particles retained the M13 major and minor coat proteins previously manipulated to serve as tethers to carry various therapy and imaging agents and target specific cancer sites. The incorporation of bacteriophage particles into medical materials so as to provide an antibacterial effect upon contact of that material with the target bacterium would be of utmost importance in health-care environments. Such biomaterials could respond to an emergent infection in advance before clinical signs become evident, with potential to greatly improve patient prognosis. Hence, bacteriophage-impregnated (bio)materials could be used as medical implants and in applications relevant to hospital hygiene. Another example could be bacteriophage-tethered wound dressings and dermal substitutes for targeted therapy that would allow production of infection-responsive biomaterials such as biodegradable and biocompatible films and highly porous cryogels, designed for the management and repair of skin injuries, particularly burns and ulcers, where the risk of infection is high. Bacteriophages have high specificity toward bacteria, which makes them an attractive natural bioreceptor. In a recent study by Tlili et al. (2013), bacteriophages were chemically tethered to SAM-functionalized gold electrodes to quantify *Escherichia coli* cells.

11. Bacteriophage-based nanomaterials

A growing number of nanotechnological applications are driving many researchers towards exploitation of bacteriophage particles (Rios et al., 2018) as vectors or as matrices for new nanodevices, with a wide array of applications in nanomedicine (Karimi et al., 2016; Scibilia et al., 2016; Farr et al., 2014; Fan et al., 2012; Henry and Debarbieux, 2012; Hyman, 2012; Rakonjac et al., 2011). Filamentous, MS2, lambda and T bacteriophage particles can be used as designed nanocarriers for the targeted delivery of both therapeutic agents and diagnostic reporter molecules, hence representing a new aspect of nanotechnology in drug delivery systems (Karimi et al., 2016). The bacteriophage capsid consists of several protein subunits assembled together. Although this protein scaffold is already extensively used for the display (surface decoration) of molecules, its structure can also be used as a nanocage to entrap biomolecules of interest (Karimi et al., 2016; Lee et al., 2016a). Each type of bacteriophage has a particular shape, a different capacity for loading cargo, a specific production time, and its own mechanisms of supramolecular assembly, which have enabled them to act as tunable carriers (Karimi et al., 2016).

In their review paper, Karimi et al. (2016) extensively tackled the major bacteriophage species that have been used in drug and gene delivery systems, together with biological applications of bacteriophages as novel targeting ligands and targeted therapeutics. Ideally, a protein construct must deliver relevant biomolecules upon command to a specific target while avoiding neutralization by the immune system. With this goal in mind, modification of the internal and external surfaces of the icosahedral single-stranded RNA coliphage MS2 capsid was carried out by Kovacs et al. (2007). Their goal was to turn the capsid into an efficient (cage-encasing) drug delivery system. They performed extensive PEGylation of bacteriophage MS2 capsids, producing a

stealth-like effect, to effectively reduce neutralization by antibodies. This was followed by binding a ligand to the distal terminations of the PEG chains (making it possible to attach targeting moieties, to direct the cargo to a specific target) and encaging large "drug-like" molecules. In another (more recent) study, the synthesis of MS2 particles able to specifically deliver a number of molecules such as nanoparticles, drugs, siRNAs or toxins, to hepatocellular carcinoma cells was described (Ashley et al., 2011) *via* the co-display of a targeting peptide and a fusogenic peptide promoting endosomal escape.

The structural features of bacteriophages (*viz.* genomic and proteomic information in the same body) associated with the ease of their genetic engineering to accommodate various target genes and/or proteins make them very useful in the design of novel drug delivery carriers (Scibilia et al., 2016; Farr et al., 2014; Merzlyak and Lee, 2006). The use of bacteriophage particles in drug delivery has three potential major applications: gene therapy, drug targeting and target imaging. Since in gene therapy it is vital that a specific gene is delivered efficiently to a specific tissue or cell type, bacteriophage particles can be used to achieve such specificity *via* engineering targeting peptide sequences on the surface of their capsids (Scibilia et al., 2016; Farr et al., 2014; Merzlyak and Lee, 2006). Additionally, bacteriophage particles are also good gene delivery vectors because they protect DNA from degradation. Very recently, Scibilia et al. (2016) reported on the formation of silver nanoparticle (AgNPs)-bacteriophage networks that can find application in the biomedical field of advanced biosensing and targeted gene and drug delivery.

Several characteristics render filamentous bacteriophages (and especially M13 bacteriophage particles) ideal for nanotechnology applications (Bakhshinejad and Sadeghizadeh, 2014). M13 bacteriophage virions have the ability to self-assemble into nanoscale structures, meaning that the major part of the structural information present in the bacteriophage capsid is carried by capsid proteins themselves and does not require the involvement of other proteins (Bakhshinejad and Sadeghizadeh, 2014). One of the most important applications of bacteriophage nanoparticles in neural regeneration is the development of biomimetic scaffolds for tissue engineering purposes and, indeed, over the last few years, M13 bacteriophage nanoparticles have received much attention for the design and synthesis of nanomaterial scaffolds for neural tissue engineering (Bakhshinejad and Sadeghizadeh, 2014).

12. Corrosion control

For a long time, bacteria were known to cause corrosion due to the chemical change they induce on metal surfaces. A biological method of preventing corrosion is the use of lytic bacteriophages as bactericidal agents (Zarasvand and Rai, 2014). The growth of microorganisms within biofilms contributes to serious issues in oilfield systems, including reservoir souring and corrosion (Baldwin and Summer, 2012). Current methods of biofilm control within the oil and gas industries include physical removal and treatment with chemical biocides. Although these methods can be effective, incomplete biofilm removal or poor diffusion of chemicals into the biofilm may allow for microbial regrowth once treatment is halted (Baldwin and Summer, 2012). Hence, a new and more effective approach to controlling biofilms could make a significant contribution to reducing the damage caused by microbially influenced corrosion. Bacteriophages have the ability to degrade the exopolysaccharide matrix that is a key constituent of biofilms and plays an important role in protecting the bacterial cells within a biofilm from chemical treatments (Baldwin and Summer, 2012). As mentioned previously, the potential of bacteriophage particles for tackling bacterial biofilm growth on medical devices has been demonstrated, as well as the success of bacteriophages in degrading biofilm polysaccharides and infecting cells. A similar performance in the oil and gas industries would allow to use bacteriophages to be used as a more effective and targeted means of biofilm treatment than currently deployed methods. Petroleum reservoir souring, caused by microbially induced production of hydrogen sulfide and other sulfur compounds, and the associated corrosion, could be remediated by isolating specific bacteriophage particles for the problematic (target) bacteria and adding an effective amount of such bacteriophage particles to the water introduced into, or resident in, the reservoir, to kill at least some of the target bacteria (Baldwin and Summer, 2012). The invention reported by Baldwin and Summer (2012) relates to control of bacterial contamination, corrosion, fouling and souring of oil and gas wells and reservoirs that result from injecting bacteria-contaminated water into a well, especially bacteria producing acid and/or sulfur compounds that cause reservoir souring, fouling and corrosion, using naturally occurring bacteriophage particles, lytic for the targeted bacteria, particularly sulfate-reducing bacteria and acid-producing bacteria. This biological contamination process would allow the capital costs of creating new wells to be decreased by maintaining sweet gas production, mitigating the need for sour service pipes and hydrogen sulfide removal apparatus (Baldwin and Summer, 2012). Similarly, bacterial contamination of industrial water systems leads to biofouling by biofilms and corrosion from bacterially induced corrosion (Summer and Summer, 2012). The invention reported by Summer and Summer (2012) relates to control of bacterial contamination, corrosion and fouling, using naturally occurring bacteriophage particles, lytic for the targeted bacteria. As the elimination of corrosion-causing bacteria is necessary for corrosion inhibition, the bacteriophage lytic cycle is a good alternative to using a biocide. An advantage of bacteriophage particles over biocide use is its self-replication ability, which increases the bacteriophage dosage over the course of treatment (Zarasvand and Rai, 2014). Sulfate-reducing bacteria are one of the causes of microbially induced corrosion and filamentous bacteria such as *Gordonia* cause bulk foaming and process upsets (Summer and Summer, 2012). The most widespread are members of the delta subgroup of the Proteobacteria, including *Desulfobacterales*, *Desulfovibrionales*, and *Syntrophobacterales* (Summer and Summer, 2012). Bacteria selected for bacteriophage treatment may include all members of the sulfate-reducing bacteria, including *Desulfovibrio*, *Desulfotomaculum*, *Desulfobacter*, and *Desulfuromonas* (Summer and Summer, 2012). Specific members include *Desulfovibrio vulgaris* and *Desulfovibrio desulfuricans*. These bacteria are also known to mediate corrosion through interactions with the hydrogen film on water-exposed iron. Bacteria selected for bacteriophage treatment also include those that produce acidic metabolites. This specifically includes sulfur-oxidizing bacteria capable of generating sulfuric acid, *viz*. *Thiobacilli thiooxidans* and *Thiobacilli denitrificans* (Summer and Summer, 2012).

13. Conclusions and future prospects

With the worldwide increase in research data pertaining to bacteriophage particles, the number of potential biotechnological applications of these metabolically inert entities increases everyday. In fact, humanity takes advantage of the natural predators of bacteria to fight pathogens and to increase the quality of life. Bacteriophages are the most abundant biological entities in our biosphere, infecting specific bacteria in every known environment, and their genetic diversity is stunning. Due to their ability to transmit infection from one cell to another, they play an important role in gene transfer among bacterial populations and in maintaining ecological balance in microbial communities. Studies of bacteriophages provide insights into genome evolution, bacterial adaptation to new conditions, DNA expression and replication, and they potentially provide new biotechnology products.

Compared to bacterial genomics, bacteriophage genomics has advanced slowly, and a higher-resolution picture of the phagosphere is still only emerging. Phage genomes encode products that have been useful for biotechnology applications including food biopreservation and safety, diagnostics and therapeutics, bacterial biosensing, plant and animal gene transfer methods, antimicrobial therapy, vaccine carriers, antibiotic resistant strain therapeutics, DNA delivery vehicles, strain

construction, gene delivery, biocontrol of plant pathogens, bacteriophage display, biofilm control, nanotechnology, and corrosion control, just to mention a few. Nowadays, through genetic engineering and recombinant DNA technology, it is possible to develop transgenic bacteriophage particles, lysins and other antibacterial phage proteins, in order to increase their ability to infect biofilms and make them more specific and stable, with a wider spectrum of action and increasing both their potency and efficacy. Among phage-based technologies, we find two that seem to be the cutting edge in the field. First, phage display technology, that constantly delivers novel therapeutics and therapeutic strategies for medicine. Second, phage-derived enzymes. These enzymes are not only simple proteins capable to lyse bacteria, but also a source of bacteria-binding domains, biofilm degrading products, and other active elements that in the future can play a role of useful bioblocks for further engineering.

This review was written with the goal of showing the increasing diversity of bacteriophage applications in modern society. However, one must not forget that the initial interest in bacteriophage particles arose primarily from the study in 1917, in Paris, by Felix d'Herelle who applied them to treat infectious diseases in the era before the discovery of antibiotics. The consistent discoveries of all new antibiotics and their successful uses led to both a belief that they could be used indefinitely and to the oblivion of the idea of bacteriophage therapy. However, the rapid emergence of problems with the search for, and introduction of, new chemical antibiotic molecules, brought back the memory of phage therapy. Hence, it is of utmost importance to develop a code of use of bacteriophages in medical phage therapy, indirectly or directly restricting some non-medical applications of bacteriophages. For example, the use of bacteriophages to disinfect contaminated medical devices and wash floors in hospitals should be avoided at all costs, as it would inevitably lead to the emergence and spread of bacteriophage-resistant pathogenic bacteria. It is also necessary to bear in mind the potential for the emergence of new bacterial pathogens as a consequence of the co-conductance of bacteriophages in bacteria, for example, as a follow-up to the interspecies migration of the genomes of moderate bacteriophages with a wide spectrum of lytic activity. This will allow preservation of the possibility to use phage therapy in cases of full bacterial resistance to available antibiotics. Due to the vast number of existing bacteriophages, and the even larger number of unexplored genes that they carry, further research is of utmost importance to deepen the current knowledge about these metabolically inert protein entities and fully understand and benefit from the biology and biotechnology of bacteriophages.

Transparency declarations

None to declare.

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