Opinion

CellPress

Revisiting phage therapy: new applications for old resources

Franklin L. Nobrega, Ana Rita Costa, Leon D. Kluskens, and Joana Azeredo

CEB – Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

The success of phage therapy is dependent on the development of strategies able to overcome the limitations of bacteriophages as therapeutic agents, the creation of an adequate regulatory framework, the implementation of safety protocols, and acceptance by the general public. Many approaches have been proposed to circumvent phages' intrinsic limitations but none have proved to be completely satisfactory. In this review we present the major hurdles of phage therapy and the solutions proposed to circumvent them. A thorough discussion of the advantages and drawbacks of these solutions is provided and special attention is given to the genetic modification of phages as an achievable strategy to shape bacteriophages to exhibit desirable biological properties.

Phage therapy

Bacteriophages (phages) were independently discovered at the beginning of the 20th century by Frederick Twort (1915) and Félix D'Hérelle (1917). Since then, these bacteria-infecting viruses of high specificity have significantly contributed to the evolution of many fields of science, in particular the areas of molecular biology and bacterial genetics [1,2]. They have also been pursued as antimicrobial agents, but lack of knowledge of phage biology and the advent of antibiotics in the 1940s resulted in the disregard of this application. However, the alarming rise of multidrug-resistant bacteria and the consequent need for antibiotic alternatives has renewed interest in this application in the West. Despite the numerous successful therapeutic outcomes reported in Eastern European countries, phage therapy remains disregarded in the West. This is mainly due to the lack of a specific regulatory framework that meets the requirements of a flexible and patient-tailored model of phage therapy [3] and the demand for large-scale *in vivo* trials that provide efficacy and safety evaluation of a standardized phage product [4]. Due to these hurdles and the difficulty to obtain intellectual property rights for therapeutic phage products [5], large pharmaceutical companies remain reluctant to invest in phage therapy. In April 2014, the European Parliament proposed a motion for the resolution of antibiotic resistance asking member states of the Council of Europe to prioritize the development of phage therapy as a complement to antibiotic therapy [6]. This was an important sign of good will, but the impact is unknown and will probably take some time to have an effect.

The current limitations of phages, whether in terms of biological properties or patentability, may be circumvented by synthetic biology approaches. In this review we discuss the possibility of applying genetic manipulation of bacteriophage genomes to shape these viruses to exhibit desirable biological properties for therapeutic applications. As a patentable product, we believe this strategy would be appealing to the pharmaceutical industry and would attract potential investors to the field.

Bacteriophage properties, advantages, and limitations

Phages have many advantageous properties over antibiotics as antimicrobial agents; however, some of those properties can also be limiting in certain applications, as detailed below.

The main characteristic of phages is their high specificity of infection, typically recognizing a limited range of bacterial strains. This reduces the damage caused to the normal microbial community of the host but it also requires identification of the specific target pathogen and the selection of an effective phage [7,8], which may delay the treatment. Also, as the propagation of phages depends on their host, they replicate only at the site of infection, are self-limiting and self-dosing, and do not persist when their specific bacterial pathogen becomes absent.

Another advantage of phages is their general lower propensity to induce resistance and the absence of cross-resistance to antibiotics [9,10]. This makes phages an effective solution against multidrug-resistant bacteria and biofilms [11]. However, the development of phage-resistant bacteria may occur and some resistance mechanisms have already been identified. These include blocking of phage adsorption due to loss or mutation of the bacterial receptor [7,12] as well as horizontal acquisition of a restriction-modification system or development of adaptive immunity by interfering clustered regularly interspaced short palindromic repeats (CRISPR) sequences, both resulting in degradation of the injected phage DNA [12,13].

Another concern is that phages may carry antibiotic-resistance genes or other bacterial virulence factors, which can be transferred to the bacteria through generalized transduction [14,15]. As this is more common in phages able to infect bacteria lysogenically, only obligatory-lytic, non-transducing phages are used as therapeutics and even these should be propagated on hosts lacking virulence

Corresponding author: Azeredo, J. (jazeredo@deb.uminho.pt).

Keywords: bacteriophages; phage therapy; genetic modification; recombineering.

0966-842X/

© 2015 Elsevier Ltd. All rights reserved. http://dx.doi.org/10.1016/j.tim.2015.01.006

CrossMark

genes [16]. However, lytic phages can also be problematic, as the rapid lysis of a large number of bacteria *in vivo* may lead to the release of endotoxins and superantigens that may induce an inflammatory response, potentially causing serious side effects [7,17].

There is a conceivable concern about phage immunogenicity and consequent *in vivo* efficacy. Phages are perceived by the immune system as invaders and can be rapidly removed from systemic circulation, making it hard to sustain an effective phage concentration [17]. A recent study by Łusiak-Szelachowska *et al.* demonstrated induction of antiphage antibodies after phage therapy, with the activity being dependent on the route of administration and phage type [18]. Nevertheless, the authors considered that the detection of antiphage activity during and after phage therapy does not exclude a favorable result of the treatment [18].

Finally, from a development point of view, phages have the advantages of rapid isolation, lower development costs than antibiotics, and versatility of formulation and application [11,16]. However, although strictly lytic phages are easily obtained for major bacterial pathogens (e.g., *Escherichia coli*, *Salmonella*, *Campylobacter*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*), their isolation for certain bacterial species has proven to be difficult (e.g., *Mycobacterium tuberculosis*, *Clostridium difficile*) [17,19,20].

The few hundred phages currently described in public datasets represent a 'drop in the ocean' of the estimated 10^{31} virions present in nature. Such diversity confers them potential as one of the most promising therapeutic strategies identified to date. It is therefore expected that, with the identification of more phages, the application of phage therapy will become successful.

Strategies to overcome phage limitations

Many strategies are being pursued to overcome the limitations of phages as therapeutic agents. Among them, phage cocktails were the first to be considered. Using a cocktail of phage types of different but complementary features, the limited host range of a single phage may be circumvented, also allowing the use of presumptive (before pathogen identification) phage therapy [16,17,21]. However, this may result in a greater impact on non-targeted bacteria and higher costs [16]. Another advantage of phage cocktails is that having different types of phage infecting the same species and strains reduces the probability of emergence of phage-resistant bacteria [21,22]. Nevertheless, resistance to phage cocktails may eventually emerge, as was demonstrated by Tanji *et al.* [23].

A more recent strategy explores the antimicrobial synergy between phages and antibiotics [24,25]. Studies have shown that sublethal concentrations of certain antibiotics (typically cell division inhibitors) increase the biosynthetic capacity of bacteria, which the phage explores to increase its own production. This hastens cell lysis and ultimately allows the phages to spread more quickly [24,25].

Another approach uses phage gene products instead of the whole virion; for example, endolysins, which are considered a promising alternative in several applications [26–28]. Their use eliminates the risk of phages imparting toxic properties to bacteria [29] and reduces the risk of development of resistance [27,28,30]. However, like phages, endolysins that target Gram-positive pathogens are highly specific, whereas endolysins for Gram-negative bacteria, in their native state, are nonspecific, with the related advantages and disadvantages, and have an inherent limited application due to the impermeable outer membrane of Gram-negative bacteria. Moreover, endolysins for Gram-positive bacteria have been shown to stimulate a fast immune response resulting in a short half-life [31].

Also relevant is the use of drug-delivery technologies, such as polymer-based coatings, to enhance the systemic delivery of phages and reduce their inactivation and clearance by the immune system [32]. This enhancement was observed after the chemical modification of phages by conjugation of monomethoxy polyethylene glycol (mPEG) to its proteins. However, it also resulted in the loss of phage infectivity, proportionally to the degree of modification [32].

The strategies presented have indeed shown positive outcomes. However, they aim to circumvent specific phage limitations instead of eliminating or correcting their detrimental properties. To specifically address this point, we propose the genetic manipulation of phage genomes as a way to shape bacteriophages into safe and efficient biocontrol agents.

The dawn of a new era: bacteriophage genomic engineering

A range of genetic tools that have been used to study phage biology and function and to shape phages' biological properties toward the improvement of their antimicrobial effect.

Until recently, efficient targeted modification of phage genomes has been hindered by a lack of broadly applicable techniques that can be used for both temperate and virulent phages. Fortunately, a new *in vivo* technology was developed to introduce genetic changes in bacterial genomes – recombineering – which has been adapted for the efficient manipulation of temperate and lytic phages in bacteriophage recombineering of electroporated DNA (BRED). This technique was developed by Marinelli *et al.* for *Mycobacterium* phages [33] but is adaptable to other phages. For example, it has been applied by others for the modification of *E. coli* phages [34,35]. BRED uses bacterial overexpression of plasmid-encoded recombination genes to enhance the frequency of homologous recombination between phage DNA and the targeted DNA substrate [34,35]. The recombination systems used for BRED are typically those encoded by phage lambda and Rac prophage. The lambda Red system comprises three proteins: Exo, Beta, and Gam. Exo degrades one strand of double-stranded DNA, generating a single-stranded substrate that is annealed to the chromosomal target by the DNA-pairing enzyme Beta. Gam prevents the degradation of the double-stranded DNA by inhibiting the *E. coli* RecBCD and SbcD enzymes [36,37]. The Rac prophage system comprises RecE and RecT, which are functionally equivalent to lambda Exo and Beta, respectively [38,39]. Similar recombination systems exist for other

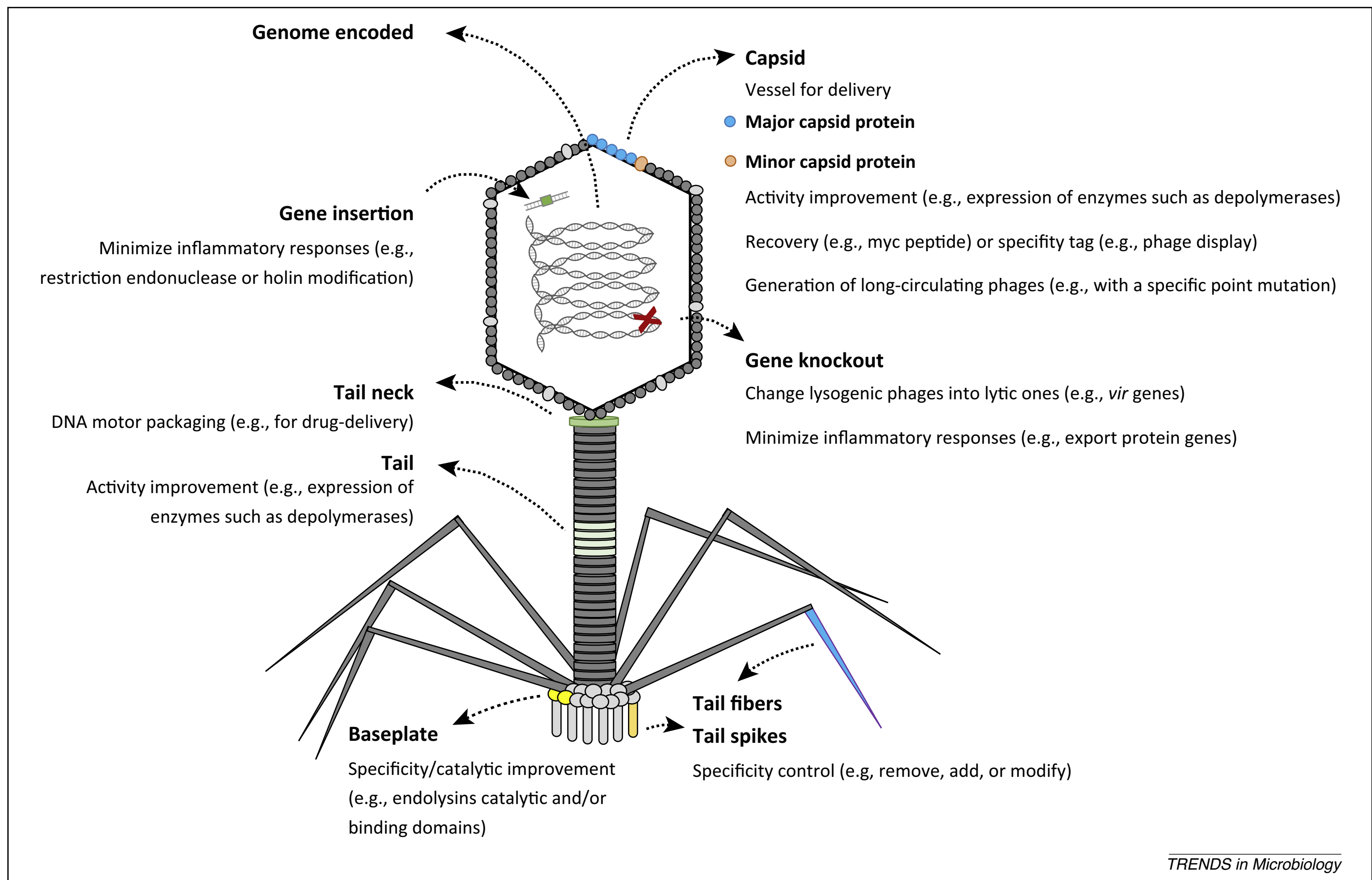

Figure 1. Schematic representation of current and future targets or possibilities for the genetic engineering of phages, using *Caudovirales* as an example.

phages and bacteria [40,41], such as the products of genes *60* and *61*, recently identified in the mycobacteriophage Che9c [42].

The recombination system can be integrated into the bacterial chromosome by modified temperate bacteriophages or can be added to the cells on plasmids. There are several plasmids currently available that contain the recombineering functions, generally under control of the arabinose promoter pBAD, alone or carrying a temperature-sensitive origin of replication to be cured from the cells after recombination [43].

It is important to note, however, that the existing recombineering systems and plasmids have been optimized for Gram-negative bacteria and may not give optimal results in Gram-positive organisms. Ideally, searches should be done in Gram-positive organisms and their phages for new recombineering systems to achieve higher efficiency of recombination [40].

Although it seems that electroporation has become the gold standard for phage genomic engineering, chemically based transformation techniques have also been evaluated. However, of the various protocols tested none has exceeded the efficiency of electroporation. Nevertheless, the use of an agent to condense DNA (e.g., polycations such as spermidine) can be helpful in the recombineering event using chemical methods since, by causing aggregation, these agents can help the linear DNA enter the pores formed in the host, which are smaller in chemical methods than in electroporation [44,45].

In a short time span, the genetic manipulation of phages has already been used in various manners to improve phage properties, and the possibilities seem endless. Figure 1 schematically represents these possibilities, using *Caudovirales* phages as an example since these account for about 96% of the known phages [46].

Of all features, the phage host range is one of the most obvious targets for manipulation. Scholl *et al.* extended the host range of a T7 phage by designing it for the expression of an endosialidase [47]. This enzyme degrades the K1 capsule present in some *E. coli* strains, thereby allowing the modified T7 to surpass this barrier, enhancing phage adsorption to efficiently infect K1 *E. coli*. Other studies focused on the modification or addition of phage components responsible for host binding. For example, Yoichi and colleagues were able to change the host specificity of phage T2 by substituting genes encoding putative host binding proteins (37 and 38) by genes with similar functions from phage PP01 (encoded on a plasmid) using homologous recombination [48]. As a result, the lytic spectrum of phage T2 was changed into that of PP01. A platform for the development of expanded host range phages would represent a faster, more economical, and more practical solution than the isolation and characterization of new phages for each bacterial strain.

Genetic manipulation of phages has been used to reduce cytotoxicity and immunogenicity. Several studies have developed lysis-deficient and non-replicative phages in an attempt to avoid toxin release (e.g., Gram-negative

endotoxins) caused by massive bacterial lysis *in vivo* and consequently minimize inflammatory responses. So far these studies have involved primarily the elimination of export protein genes, the insertion of restriction endonuclease genes, and the introduction of modified holin genes [49,50]. In addition, attempts have been made to extend phage survival after administration. For example, Merril *et al.* used a serial-passage technique to generate long-circulating phage mutants, which were found to have a mutation in the major capsid protein [51]. Later, Vitiello *et al.* used genetic-manipulation techniques to introduce this single specific mutation, obtaining phages with greater capacity to remain in the circulatory system [52].

The genetic modification of temperate phages to become permanently lytic has also been explored [53]. Transducing phages are more commonly found in bacteria, and are easier to isolate, than strictly lytic phages. Thus, this type of manipulation could be a feasible solution for the difficult isolation of the obligatory-lytic phages of some pathogens. The loss of lysogeny is accomplished by a mutation in the *vir* gene, which prevents the repressor protein from binding to the operator, allowing transcription and translation of phage DNA with subsequent lysis of the bacterial host [53].

Phages have shown promise for the treatment of bacterial states difficult to address by traditional antimicrobials, such as biofilms. However, due to the presence of a matrix of extracellular polysaccharides, some biofilms do not respond to phage therapy. This has led to work focusing on improving the activity of phages against biofilms, such as that reported by Lu and Collins, who engineered phage T7 to disrupt bacterial biofilms by expressing a biofilm-degrading enzyme, dispersin B, during infection [54]. This enzyme simultaneously attacks the glycocalyx of the bacterial cells of the biofilm and the matrix, resulting in a 100 000-fold higher efficiency of the modified T7 phage compared with the wild type T7 [54].

Genetic modification can also be used to enhance phage efficacy when combined with antibiotics. Lu and Collins engineered lysogenic phages to overexpress proteins that enhance antibiotic killing of wild type and antibiotic-resistant bacteria by targeting specific nonessential gene networks [55]. These engineered phages also exerted minimal evolutionary pressures, therefore reducing resistance development [55].

Recombinant techniques have been employed to increase the bacterial killing capacity of phages by association with antibacterial factors. An example is the lethal agent delivery system (LADS), which uses a phage-based *in vivo* packaging system to create a recombinant phage capable of delivering and naturally expressing the antibacterial genes [56,57]. Basically, a transfer plasmid containing those genes is maintained in a phage P1 lysogen that is unable to package its own DNA. Following delivery to the target bacterial cell, the plasmid recirculates and expresses the lethal agents resulting in cell death. Cell death occurs due to the activity of the toxic agent and not as a consequence of phage-induced lysis because the lytic machinery is absent in the phage constructs. In a similar approach, Fairhead [58] replaced the lysis genes of phages by a gene encoding a small acid-soluble spore protein (SASP), which binds irreversibly to the bacterial DNA stopping all cellular activity. However, it should be noted that when applying these approaches the beneficial effect of natural phage multiplication at the infection site is lost.

The phage genome has also been modified to express toxins while retaining lytic activity and completing the life cycle (http://www.rowland.harvard.edu/organization/past_research/bacteriophage/bacteriophage.html). In this approach, cell death may occur as a consequence of toxin activity and/or phage-induced lysis.

In addition, further techniques and approaches to modify phage genomes are being explored. Lu *et al.* proposed the insertion of the whole phage genome inside a yeast artificial chromosome (YAC) vector by homologous recombination to create a recombinant YAC that is then propagated in the vector host, which is not the phage host [59]. Yeast recombineering can then be applied for the modification of the phage genome inside the vector. The YAC containing the mutant phage can then be inserted into bacteria, followed by replication of the engineered phage. A similar approach can be explored using a bacterial artificial chromosome (BAC) instead of a YAC, which, with the well-developed cloning strategy, will allow the control of the lytic cycle of a phage within the bacterial host, enabling the manipulation of any phage in a nonpathogenic or even nonspecific host. Subsequent recovery can be achieved by the release of the phage genome in a linear form by Cre–Lox recombination (recovery by 'lysis from within'). Compared with recombineering (Figure 2), the YAC/BAC strategy has the advantage of not requiring a repetition of the whole process for any new modification within a particular phage. We foresee that this method, when optimized, could become the future of any phage engineering.

More recently, a few studies have shown that the CRISPR–Cas system may be used as an efficient and adaptable tool for the manipulation of lytic phage genomes. CRISPR–Cas is an adaptive immune system that protects microbial cells from DNA invasion. It comprises an array of repeated sequences called 'repeats' and flanking sequences called 'spacers'. Transcribed spacers guide specific proteins to the target DNA, called 'protospacer' by virtue of sequence homology, and cleave it. The break caused can be repaired by homologous recombination if a mutated template is provided in *trans*, resulting in precise genome editing [60]. Using this approach, the *E. coli* CRISPR–Cas type I-E system was used to isolate deletions of specific genes in a T7 genome [60]. Moreover, the *Streptococcus thermophilus* CRISPR–Cas type II-A system was used to engineer specific point mutations and small and large deletions as well as complete gene replacement in the lytic phage 2972 [61]. The main advantage of this system is its superior efficiency, which results in high percentages of mutant phages and consequently simplifies selection and recovery [60,61].

This approach requires a bacteriophage-insensitive strain that has acquired a spacer targeting the phage gene of interest. This can be easily obtained through the engineering of a strain with a low-copy plasmid containing the repeat–spacer–repeat sequence desired. The selection of a spacer is based on *in silico* analysis to target the mutation of interest [61].

As discussed here, the techniques for phage genome manipulation are quickly expanding and improving. It is

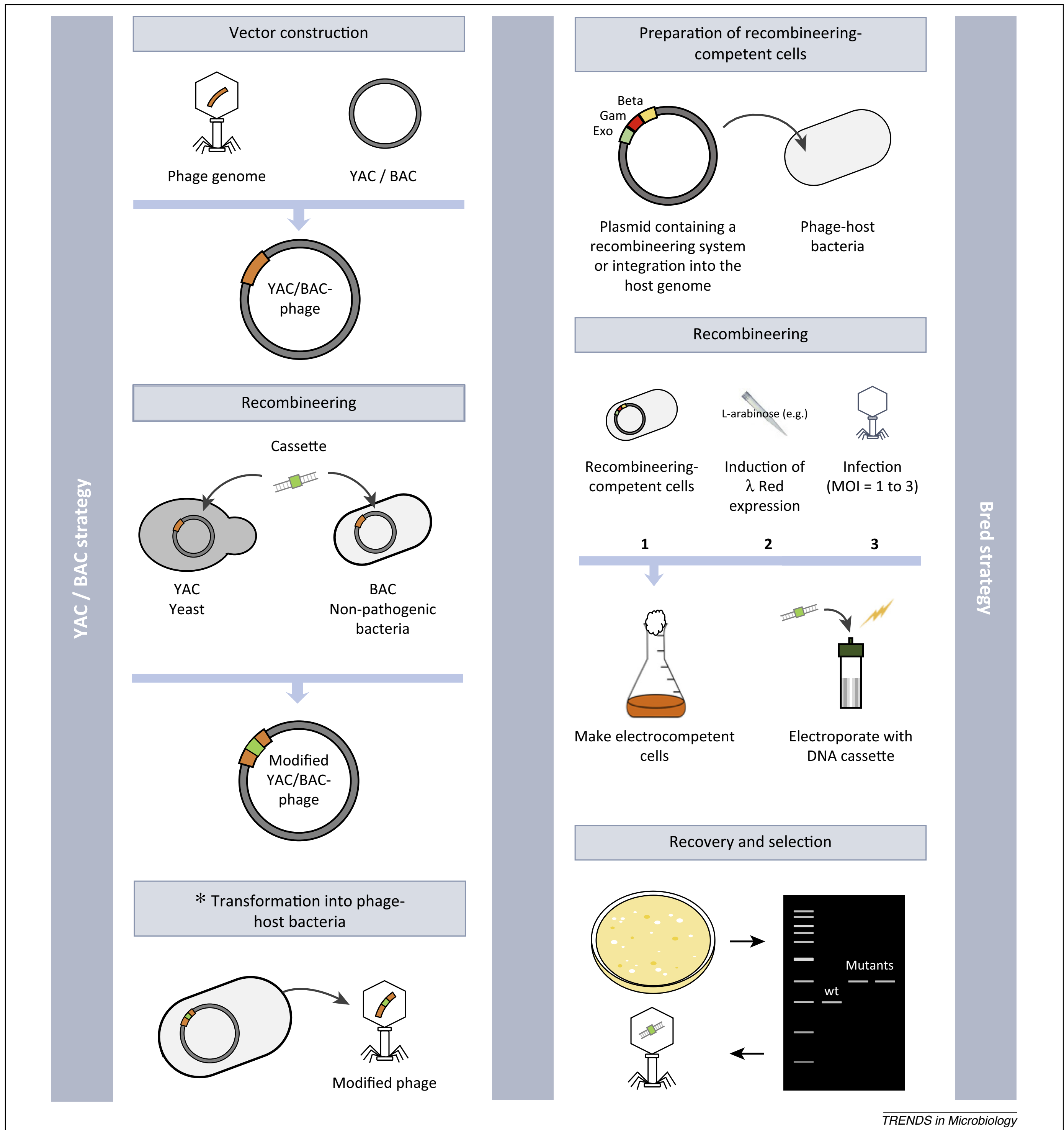

Figure 2. Step-by-step schematic comparison of yeast artificial chromosome (YAC)/bacterial artificial chromosome (BAC) and bacteriophage recombineering of electroporated DNA (BRED) strategies for phage engineering. The asterisk indicates that BAC does not require transformation (see text for more details).

now becoming feasible to modify, in a relatively simple manner, the genome of both temperate and lytic phages, creating great expectations for the improvement of phage properties for therapeutic applications.

Ethical and regulatory issues of modified and unmodified bacteriophages

The use of recombinant phages in therapy raises ethical and social questions due to the usually strong opposition of the public toward genetic manipulation. However, phages in their native form already face many regulatory hurdles as they are difficult to classify, being considered neither a chemical nor a living entity [8]. The regulatory agencies are struggling to find the best approach to regulate phage products because the current strategy of using traditional antibiotic regulatory protocols may render phage therapy prohibitively expensive and less effective [62].

During regulation of phage products, their inherent biological risks to human health and the environment need to be addressed (more on this subject can be found in [63]).

Box 1. Outstanding questions

- What is hindering the broad use of phage therapy as an alternative or complementary strategy to control infectious diseases?
- Is public acceptance going to be a major problem for phage therapy? What measures can be taken to change the public's view?
- Which could be the best approach for efficacy and safety evaluation of a phage product, either natural or genetically modified?
- What needs to be done to attract the interest of large pharmaceutical companies?
- Can we exclude the possibility of mutation during the production of natural phage cocktails due to the high mutation rate? Is this so different from targeted genetic modification of phages?

Introduced modifications can attenuate or even eliminate some of the risks; for example, by deleting virulence genes, reducing the capability of phages for gene transfer, or controlling the host range. The elimination of such risks would make regulation easier. Moreover, recent work by Hammerling *et al.* suggests the possibility of controlling genetically modified phages in a 'semantic' manner; that is, through the modification of the codon code of the host (engineering of the host to have non-natural amino acids) [64]. By doing so, it is possible to 'addict' the organisms to the unique codon code of the engineered host, which may be a means to control their capacity for replication (this means that the phage would replicate only inside the engineered host, losing the ability to kill other hosts) and thereby achieving 'biocontainment' [64].

Ultimately, the main problem for the acceptance of modified phages may be the negative opinion of the public toward genetic modification. Moreover, the idea of using viruses as therapeutic agents *per se* may already be not well received. In this context, perhaps knowing that the virus is genetically manipulated to become safe will make phage therapy more attractive and acceptable to the public. Furthermore, and quoting Henein, 'with patients dying because of infections not treatable with conventional antibiotics, is it even ethical not to pursue phage therapy?' [65]. This therapeutic option may be the future of antimicrobial therapy or at least an important addition to current strategies. Thus, it is essential to explore all of the alternatives available to overcome the limitations presently faced by phage therapy, which includes the genetic engineering of phage genomes.

Concluding remarks

Phage therapy as a realistic alternative to antibiotics depends on our capacity to overcome the hurdles faced by this therapeutic option (Box 1). The narrow host range and the possibility of cytotoxicity and immunogenicity are indicated by regulatory entities and investors as major drawbacks of phage therapy. Furthermore, patenting and regulatory issues hinder the interest of pharmaceutical companies in investing in these products. Many strategies have been proposed to overcome phage biological limitations, one of which is the genetic manipulation of phages. In the past, modification of phage genomes, particularly of those that are strictly lytic, has faced some challenges, but the recently developed recombineering techniques (BRED) as well as other recently reported approaches anticipate the easier manipulation of both virulent and temperate phages. These techniques can be used to shape bacteriophages' biological properties to increase their efficacy and safety. Furthermore, genetically modified phages solve the problem of patenting and increase the interest of large pharmaceutical companies in these products. We are not advocating that this therapeutic alternative should be solely based on this option (we also support the use of cocktails of natural phages), but due to the difficulties faced by phage therapy we believe that we are at the dawn of a new era, where genetically modified phages can open new perspectives toward the success of phage therapy.

Acknowledgments

The authors thank the FCT Strategic Project PEst-OE/EQB/LA0023/2013 and the Project 'BioHealth – Biotechnology and Bioengineering approaches to improve health quality, Ref. NORTE-07-0124-FEDER-000027' cofunded by the Programa Operacional Regional do Norte (ON.2 – O Novo Norte), QREN, and FEDER. F.L.N. also acknowledges the FCT for grant SFRH/BD/86462/2012.

References

1 Daniel, A. *et al.* (2010) Synergism between a novel chimeric lysin and oxacillin protects against infection by methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 54, 1603–1612
2 D'Herelle, F. (2007) On an invisible microbe antagonistic toward dysenteric bacilli: brief note by Mr. F. D'Herelle, presented by Mr. Roux. 1917. *Res. Microbiol.* 158, 553–554
3 Verbeken, G. *et al.* (2014) Taking bacteriophage therapy seriously: a moral argument. *Biomed. Res. Int.* 2014, 8
4 Brüssow, H. (2012) What is needed for phage therapy to become a reality in Western medicine? *Virology* 434, 138–142
5 Reardon, S. (2014) Phage therapy gets revitalized. *Nature* 510, 15–16
6 Parliamentary Assembly (2014) *Phage Therapy, a Public Health Issue – Motion for a Resolution*, pp. 1–2, Council of Europe
7 Drulis-Kawa, Z. *et al.* (2012) Learning from bacteriophages – advantages and limitations of phage and phage-encoded protein applications. *Curr. Protein Peptide Sci.* 13, 699–722
8 Vandamme, E.J. and Miedzybrodzki, R. (2013) Phage therapy and phage control:. . .to be revisited urgently!! *J. Chem. Technol. Biotechnol.* 89, 329–333
9 Carlton, R. (1999) Phage therapy: past history and future prospects. *Arch. Immunol. Ther. Exp.* 47, 267–274
10 Ormälä, A. and Jalasvuori, M. (2013) Phage therapy: should bacterial resistance to phages be a concern, even in the long run? *Bacteriophage* 3, e24219–e24221
11 Loc-Carrillo, C. and Abedon, S.T. (2011) Pros and cons of phage therapy. *Bacteriophage* 1, 111–114
12 Hyman, P. and Abedon, S.T. (2010) Bacteriophage host range and bacterial resistance. *Adv. Appl. Microbiol.* 70, 217–248
13 Labrie, S.J. *et al.* (2010) Bacteriophage resistance mechanisms. *Nat. Rev. Microbiol.* 8, 317–327
14 Kutter, E. *et al.* (2010) Phage therapy in clinical practice: treatment of human infections. *Curr. Pharm. Biotechnol.* 11, 69–86
15 Gill, J. and Hyman, P. (2010) Phage choice, isolation, and preparation for phage therapy. *Curr. Pharm. Biotechnol.* 11, 2–14
16 Chan, B.K. *et al.* (2013) Phage cocktails and the future of phage therapy. *Future Microbiol.* 8, 769–783
17 Goodridge, L. (2010) Designing phage therapeutics. *Curr. Pharm. Biotechnol.* 11, 15–27
18 Łusiak-Szelachowska, M. *et al.* (2014) Phage neutralization by sera of patients receiving phage therapy. *Viral Immunol.* 27, 295–304
19 Balogh, B. *et al.* (2010) Phage therapy for plant disease control. *Curr. Pharm. Biotechnol.* 11, 48–57
20 Hargreaves, K.R. and Clokie, M.R.J. (2014) *Clostridium difficile* phages: still difficult? *Front. Microbiol.* 5, 184
21 Chan, B.K. and Abedon, S.T. (2012) Phage therapy pharmacology: phage cocktails. *Adv. Appl. Microbiol.* 78, 1–23
22 Gu, J. *et al.* (2012) A method for generation phage cocktail with great therapeutic potential. *PLoS ONE* 7, e31698

23 Tanji, Y. *et al.* (2004) Toward rational control of *Escherichia coli* O157:H7 by a phage cocktail. *Appl. Microbiol. Biotechnol.* 64, 270–274
24 Ryan, E.M. *et al.* (2012) Synergistic phage–antibiotic combinations for the control of *Escherichia coli* biofilms *in vitro*. *FEMS Immunol. Med. Microbiol.* 65, 395–398
25 Comeau, A.M. *et al.* (2007) Phage–antibiotic synergy (PAS): β-lactam and quinolone antibiotics stimulate virulent phage growth. *PLoS ONE* 2, e799 (http://dx.doi.org/10.1371/journal.pone.0000799)
26 Fischetti, V. (2005) Bacteriophage lytic enzymes: novel anti infectives. *Trends Microbiol.* 13, 491–496
27 Nelson, D.C. *et al.* (2012) Endolysins as antimicrobials. *Adv. Virus Res.* 83, 299–365
28 Schmelcher, M. *et al.* (2012) Bacteriophage endolysins as novel antimicrobials. *Future Microbiol.* 7, 1147–1171
29 Hermoso, J.A. *et al.* (2007) Taking aim on bacterial pathogens: from phage therapy to enzybiotics. *Curr. Opin. Microbiol.* 10, 461–472
30 Borysowski, J. *et al.* (2006) Bacteriophage endolysins as a novel class of antibacterial agents. *Exp. Biol. Med.* 231, 366–377
31 Fischetti, V.A. (2008) Bacteriophage lysins as effective antibacterials. *Curr. Opin. Microbiol.* 11, 393–400
32 Kim, K. *et al.* (2008) PEGylation of bacteriophages increases blood circulation time and reduces T-helper type 1 immune response. *Microbiol. Biotechnol.* 1, 247–257
33 Marinelli, L.J. *et al.* (2008) BRED: a simple and powerful tool for constructing mutant and recombinant bacteriophage genomes. *PLoS ONE* 3, e3957
34 Marinelli, L. *et al.* (2012) Recombineering: a powerful tool for modification of bacteriophage genomes. *Bacteriophage* 2, 5–14
35 Fehér, T. *et al.* (2012) Bacteriophage recombineering in the lytic state using the lambda red recombinases. *Microb. Biotechnol.* 5, 466–476
36 Murphy, K.C. (2007) The λ Gam protein inhibits RecBCD binding to dsDNA ends. *J. Mol. Biol.* 371, 19–24
37 Court, R. *et al.* (2007) The crystal structure of λ-Gam protein suggests a model for RecBCD inhibition. *J. Mol. Biol.* 371, 25–33
38 Gottesman, M. *et al.* (1974) Characterization of bacteriophage lambda reverse as an *Escherichia coli* phage carrying a unique set of host-derived recombination functions. *J. Mol. Biol.* 88, 471–487
39 Zhang, Y. *et al.* (1998) A new logic for DNA engineering using recombination in *Escherichia coli*. *Nat. Genet.* 20, 123–128
40 Datta, S. *et al.* (2008) Identification and analysis of recombineering functions from Gram-negative and Gram-positive bacteria and their phages. *Proc. Natl. Acad. Sci. U.S.A.* 105, 1626–1631
41 Swingle, B. *et al.* (2010) Recombineering using RecTE from *Pseudomonas syringae*. *Appl. Environ. Microbiol.* 76, 4960–4968
42 Kessel, J. and Hatfull, G. (2008) Mycobacterial recombineering. In *Chromosomal Mutagenesis* (Davis, G. and Kayser, K., eds), pp. 203–215, Humana Press
43 Datsenko, K.A. and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* 97, 6640–6645
44 Wagner, E. *et al.* (1991) Transferrin–polycation–DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. *Proc. Natl. Acad. Sci. U.S.A.* 88, 4255–4259
45 Staedel, C. *et al.* (1994) High-efficiency transfection of primary human keratinocytes with positively charged lipopolyamine:DNA complexes. *J. Invest. Dermatol.* 102, 768–772
46 Ackermann, H.W. (2001) Frequency of morphological phage descriptions in the year 2000. Brief Review. *Arch. Virol.* 146, 843–857
47 Scholl, D. *et al.* (2005) *Escherichia coli* K1's capsule is a barrier to bacteriophage T7. *Appl. Environ. Microbiol.* 71, 4872–4874
48 Yoichi, M. *et al.* (2005) Alteration of tail fiber protein gp38 enables T2 phage to infect *Escherichia coli* O157:H7. *J. Biotechnol.* 115, 101–107
49 Hagens, S. *et al.* (2004) Therapy of experimental *Pseudomonas* infections with a nonreplicating genetically modified phage. *Antimicrob. Agents Chemother.* 48, 3817–3822
50 Matsuda, T. *et al.* (2005) Lysis-deficient bacteriophage therapy decreases endotoxin and inflammatory mediator release and improves survival in a murine peritonitis model. *Surgery* 137, 639–646
51 Merril, C.R. *et al.* (1996) Long-circulating bacteriophage as antibacterial agents. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3188–3192
52 Vitiello, C.L. *et al.* (2005) An amino acid substitution in a capsid protein enhances phage survival in mouse circulatory system more than a 1000-fold. *Virus Res.* 114, 101–103
53 Rapson, M. *et al.* (2003) *Bacteriophages Useful for Therapy and Prophylaxis of Bacterial Infections*, University of Warwick
54 Lu, T.K. and Collins, J.J. (2007) Dispersing biofilms with engineered enzymatic bacteriophage. *Proc. Natl. Acad. Sci. U.S.A.* 104, 11197–11202
55 Lu, T.K. and Collins, J.J. (2009) Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4629–4634
56 Norris, J. *et al.* (2000) Prokaryotic gene therapy to combat multidrug resistant bacterial infection. *Gene Ther.* 7, 723–725
57 Westwater, C. *et al.* (2003) Use of genetically engineered phage to deliver antimicrobial agents to bacteria: an alternative therapy for treatment of bacterial infections. *Antimicrob. Agents Chemother.* 47, 1301–1307
58 Fairhead H. Sample6 Technologies, Inc. Small Acid-soluble Spore Protein and Uses Thereof WO2013049121
59 Lu, T.K.T. *et al.* (2013) *Recombinant Phage and Methods*, Sample6 Technologies
60 Kiro, R. *et al.* (2014) Efficient engineering of a bacteriophage genome using the type I-E CRISPR–Cas system. *RNA Biol.* 11, 42–44
61 Martel, B. and Moineau, S. (2014) CRISPR–Cas: an efficient tool for genome engineering of virulent bacteriophages. *Nucleic Acids Res.* 42, 9504–9513
62 Keen, E. (2012) Phage therapy: concept to cure. *Front. Microbiol.* 3, 238
63 Verheust, C. *et al.* (2010) Contained use of bacteriophages: risk assessment and biosafety recommendations. *Appl. Biosaf.* 15, 32–44
64 Hammerling, M.J. *et al.* (2014) Bacteriophages use an expanded genetic code on evolutonary paths to higher fitness. *Nat. Chem. Biol.* 10, 178–180
65 Henein, A. (2013) What are the limitations on the wider therapeutic use of phage? *Bacteriophage* 3, e24872 (http://dx.doi.org/10.1038/nchembio.1450)