Phage cocktails and the future of phage therapy

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Viruses of bacteria, known as bacteriophages or phages, were discovered nearly 100 years ago. Their potential as antibacterial agents was appreciated almost immediately, with the first 'phage therapy' trials predating Fleming's discovery of penicillin by approximately a decade. In this review, we consider phage therapy that can be used for treating bacterial infections in humans, domestic animals and even biocontrol in foods. Following an overview of the topic, we explore the common practice – both experimental and, in certain regions of the world, clinical – of mixing therapeutic phages into cocktails consisting of multiple virus types. We conclude with a discussion of the commercial and medical context of phage cocktails as therapeutic agents. In comparing off-the-shelf versus custom approaches, we consider the merits of a middle ground, which we deem 'modifiable'. Finally, we explore a regulatory framework for such an approach based on an influenza vaccine model.

Bacteriophages, or simply phages, are viruses with an ability to infect and, in many cases, kill bacterial cells. As with most viruses, these infections begin with virion binding to specific cell-surface receptors, which is then followed by intracellular replication. Over 90% of phages have tailed icosahedral heads that, after inserting their nucleic acid into the bacterial cell, terminate their infections by lysing the cells they have infected. Depending on the life cycle of the phage, this lysis can occur either soon after the initiation of infection (lytic cycle) or instead following lengthy periods of delay (lysogenic cycle) [1]. In addition to permanently shutting down bacterial metabolism, lysis also releases phage progeny into the surrounding environment, allowing them to infect similar bacteria found nearby.

Similarly to antibacterial agents such as antiseptics and antibiotics, a crucial aspect of phage functioning as biological antibacterials is their potential to be applied directly to living tissues without causing harm, that is, they demonstrate selective toxicity. Though not always emphasized, especially historically [2], an important component of selective toxicity is an ability to avoid harming the often useful normal microbiota that are associated with mammalian bodies [3,4]. Therefore, displaying a narrow spectrum of activity can be a useful property for an antibiotic or equivalent antibacterial. Furthermore, the host range of phages, as equivalent to their spectrum of activity, tends to be relatively narrow, often consisting of only a subset of strains making up a single bacterial species [5]. This same characteristic can be limiting, however, in terms of the ability of specific phage products to impact bacterial infections.

Using phages to treat bacterial infections (commonly termed phage therapy) dates back to the early 1900s, after their codiscovery by Frederick Twort [6] and Felix d'Hérelle [7]. D'Hérelle in particular used phage suspensions to treat infections such as dysentery, which at the time had no other consistently effective treatment. His success led to a period of widespread enthusiasm for the phage therapy of humans [8]. Although eclipsed in much of western medicine upon the advent of antibiotics, the use of phages as the treatment of choice for bacterial infections has persisted in various regions of the world [9]. This includes, most notably, the former Soviet Republic of Georgia, where phages are often used as the standard of care for bacterial infections. In addition, there is the phage therapy center of Wrocław, Poland, where phages are used to treat especially chronic bacterial infections that are proven to be resistant to antibiotic treatment [10].

As a pioneering medical innovation, phage therapy came to be implemented well before the underlying science was even rudimentarily understood. The use of phages as therapeutic agents consequently had many problems stemming especially from a profound lack of understanding of phage biology [11]. In particular, as with any antimicrobial therapy, it is crucial to employ agents that have some potential, first *in vitro* and then *in vivo*, to serve as effective

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antagonists to target organisms, and that are also safe in their application. These and other critical characteristics, which we now know should be required of phages before they can be considered as suitable candidates for therapeutic use, were underappreciated in the early days of phage therapy. In addition to the need for therapeutic phages to be both active and sufficiently antagonistic against the bacteria being targeted, it is imperative to employ phages that are incapable of infecting bacteria lysogenically and that neither encode nor are capable of transducing bacterial virulence factor genes. Also worth mentioning is the necessity for phage preparations to be adequately purified (e.g., to remove bacterial debris). Such purification should be substantial (e.g., to remove most bacterial components, including endotoxins), particularly when phages are to be delivered directly to an animal's systemic circulation [12,13].

The primary criticism of phage therapy is generally that a paucity of modern, double-blinded, Phase III (efficacy) clinical trials in humans have been undertaken and, therefore, a substantial uncertainty exists within western medical practice regarding the potential for phage therapy to cure disease. A number of recent reviews have investigated the question of phage therapy efficacy explicitly in humans [8–10,14]. For the most part, phage therapy has not yet met the 'gold standard' of double-blind efficacy determination. The lack of such studies, however, is primarily a consequence of these types of studies not being undertaken owing to a relative lack of funding for such endeavors rather than because they have been undertaken and failed to show evidence of efficacy. Phage therapy consequently exists as an older technology that seems to have shown great promise, both in terms of commercial use both for human treatment (e.g., Pyophage and Intestiphage sold in the former Soviet Union) and in the form of biocontrol products (as sold by OmniLytics [UT, USA] and Micreos Food Safety [The Netherlands]). Nonetheless, at present most phage products have not been subjected to sufficiently rigorous analysis, particularly in terms of the clinical treatment of humans. In this article we thus consider the use of phages as antibacterial agents as a maturing technology.

We first focus on more recent research, investigating the use of phage cocktails to broaden the spectrum of activity of therapeutic phage formulations. We then consider commercial development and related issues of regulation, especially of polyphage cocktail use as antibacterial drugs. We do not present information on monophage therapy since this involves using single-phage preparations and previous reviews have covered this area extensively [15,16]. Overall, we are fairly confident of the medical potential of phages to treat antibiotic-resistant bacterial infections given the Georgian, Polish and other experiences. We are less certain, however, of the potential of phage therapy to become well integrated into most western models of drug development, regulation and clinical implementation.

Phages & phage therapy basics

Numerous authors have suggested that phages are the most abundant and also potentially most diverse semiautonomous genetic agents on Earth, with estimations of total phage numbers ranging to in excess of 10^{30} virions [17]. Owing to these qualities, it is often relatively simple to isolate phages against major bacterial pathogens, such as *Escherichia coli* O157:H7, *Salmonella*, *Campylobacter*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The host range of these phages can range from quite narrow (just a subset of strains within a single species) to quite broad (with host ranges spanning multiple bacterial genera) [5]. For certain bacterial hosts, however, this relative ease is less true, such as for the isolation of suitable phages for the biocontrol of certain plant pathogens [18]. In addition, different phage isolates often possess different modes of antibacterial activity, such as the mechanisms used to enter the bacterial cells or in terms of takeover of host functions. Consistently, study into the interaction of phage proteins with bacteria has been proposed as a means of identifying novel targets for antibacterial activity towards discovery of novel small-molecule antibacterial agents [19]. These mechanisms of action are generally also considered to not be equivalent to those observed with antibiotics or other small-molecule antimicrobial agents [10]. Indeed, although typically phage-induced bacterial lysis is considered to represent the phage bactericidal effect during phage therapy, in most cases bacteria are genetically dead upon successful phage infection long before the physical destruction of the bacterial cell. Overall, it can be argued that phages have been crafted by billions of years of evolution to be highly specialized deliverers of diverse bactericidal agents to the cytoplasms of the bacteria they target [20].

The process of using phages for therapy is conceptually simple, although to a degree with that simplicity comes consequences of what can otherwise involve rather complex pharmacokinetics.

Specifically, this complexity stems from the phages' ability to increase their number, and resulting antibacterial activity, precisely where target bacteria are present and vulnerable to phage attack. As with any antimicrobial agent, the key is for phages to reach bacterial targets in sufficient abundance to ensure that a minimum inhibitory concentration is exceeded [21,22]. This can be achieved either through the direct application of phages to the infection site or via systemic delivery. In both cases, successful phage penetration to target bacteria can be aided by a combination of the phage potential to lyse bacteria (such as during clearance of bacterial biofilms [23]) along with the ability to increase in number after infecting target bacteria (a pharmacokinetic property that can be listed under the heading of 'metabolism' [24]). Note, however, that not all bacterial pathogens under all circumstances can be reached by phage virions to allow for initiation of such local amplification of phages following systemic delivery and, furthermore, chronic bacterial infections can require months of treatment before clearance is achieved [10].

The host range of the phages used in phage therapies in particular will have a direct impact on these potentially dose-independent pharmacokinetics that may be required for successful clinical outcomes (i.e., that are dependent on such *in situ* phage population growth) [5,25,26]. To assure that phage formulations provide a phage that possesses a host range that includes target bacteria, multiple phage types possessing a diversity of host ranges are often combined into mixtures called 'phage cocktails' [27,28]. Additional pharmacological issues for phage therapy also exist [22], many of which, such as avoidance of elimination by the host immune system, can be addressed, at least in part, through informed phage isolation [12], subsequent phage modification [27] or by employing alternative routes of phage delivery, such as oral administration [29]. In Box 1 we provide a checklist outlining parameters that should be considered when designing experimental phage therapy studies. The references cited after the bullet points are good examples of experimental designs used to best highlight how to set up a particular parameter; refer to the methods section in the individual articles for more information. See [30] for a complementary discussion.

Polyphage therapy

Strategies of phage therapy can be distinguished in terms of the number of phage types used during treatment. Monophage therapy involves the application of only a single phage type, whereas polyphage therapy [28] is the application of a phage cocktail, that is, therapy involving the simultaneous use of more than one phage type (also see 'multiphage' [31,32]). Monophage therapy is used for the sake of proof of concept during development of phage therapy experimental models, such as in certain circumstances where sufficiently wide host range phages are available [33] or clinically, following careful matching between pathogens and individual phage isolates. This approach has the advantage of simplifying treatment since only a single phage preparation and purification effort is needed and there is less potential for complications stemming from phage immunological interaction with the treated subjects. Matching phages are often obtained from phage banks, which are collections of previously characterized phage isolates, though with the caveat that *in vitro* phage activity is not always

Box 1. Checklist towards successful phage therapy experimental development.

Relevant for all approaches

- Phages should be lytic and nontemperate (unable to display lysogenic infections)
- Phages should be able to lyse representative strains of target bacteria [34,78,79]
- Phage preparations should be purified to a level that is appropriate for the model used [80]
- Models should be sufficiently representative of relevant 'real-world' aspects [81]
- Efforts should be made to test surviving bacteria for phage resistance, retention of pathogenicity and genetic relationship to originally targeted bacteria [10,31,82]
- Combinations of phages should be tested towards cocktail optimization [31,83]
- Efforts should be made to avoid phage contact with bacteria during bacteria enumeration

Relevant for* in vitro *models

- Phage purification by filtration can be adequate for these studies
- Peak phage titers should reflect what can be achieved in the vicinity of target bacteria during the 'real-life' scenario being modeled [39,84]

Relevant for* in vivo *and/or* in situ *models

- Highly purified phage preparations are desirable to avoid formula-associated *in vivo* immune responses as well as experimental results other than those stemming from phage-mediated action
- For topical application, filtered phage lysates may suffice, although a phage-minus mock formulation may be necessary to distinguish phage- from nonphage-mediated effects
- Timing of administration should be considered to avoid especially unrealistic positive results that can stem from premature phage application following bacterial challenge [85]
- Phage persistence ability *in vivo,* in the absence of target bacteria, should be determined [34,86]
- *In vivo* toxicity testing of phage formulation should be performed
- Various *in vivo* routes of administration should be evaluated for effectiveness [87]
- Bacterial counts should be sampled from sites where bacteria can contribute to disease [88]

The terms in vitro, in vivo *and* in situ *refer to experimentation using laboratory cultures, model organisms (e.g., animal models) and real-world circumstances (e.g., in the clinic, farm or kitchen), respectively. References cited provide helpful illustrations.*

predictive of *in vivo* therapeutic efficacy [22,34–36]. In terms of efforts towards commercial development of phages as either therapeutic or, more generally, biocontrol agents of bacteria [37], it is typically either broad host range monophages that are employed (e.g., Listex™ P100 [Micreos], an antilisteria food additive [33]) or phage cocktails. In this section, we discuss recent results stemming from experimental phage application using the latter approach.

Research using phage cocktails started to increase at the start of the millennium owing to a number of insufficient-to-modest outcomes obtained while evaluating single phage preparations [16]. This is not to imply that monophage therapy successes have not been seen, but rather that a number of strategies exist by which phage therapy outcomes may be improved [38]. One such strategy is phage formulation into cocktails. A number of research groups have recently published phage therapy experiments using phage cocktails and we summarize their results in Table 1. Although some studies report relatively impressive results (e.g., [31,39–43]), we also note a number of studies in which reductions in bacterial load are only in the range of approximately tenfold (i.e., 1 log reduction).

The treatment of *P. aeruginosa*-associated chronic otitis as reported by Wright *et al.* is particularly noteworthy because it is the first controlled double-blind Phase I/II clinical trial for a phage cocktail [44]. The phage cocktail containing six phages at 10^5 PFU was applied directly into the ear, and patients were observed for 6 h posttreatment then at days 7, 21 and 42. Although significant clinical improvements were reported in the phage-treated group compared with the placebo group, it should be noted that those improvements were measured using a visual analog scale, whereby the attending physician reported on their subjective findings, and even the microbiological analysis was based on semiquantitative methods involving swabbing of the treated ear. Nevertheless, the investigators observed a mean reduction of 50% in the 'visual analog scale' for the phage-treated group versus the placebo-treated group (mean reductions of 20%).

The phages used in the cocktails for all the studies highlighted in Table 1 were propagated individually, purified to a required degree (based on its application) and mixed prior to use. The only group noting viral inference problems with some of their cocktails was Hall *et al.* [31], although the other investigators did not specifically look for this issue during their studies. Various interference phenomena between different phage types have been observed by other authors, such as the 'depressor effect' [45] (their potential to interfere with cocktail-mediated phage therapy has been discussed elsewhere [22,24,28]).

The use of phage cocktails can be conceptually differentiated into: efforts that seek to broaden the utility of phage formulations, that is, what diseases or fraction of potential etiologies of a given disease may be treated; and as a means of preventing the development of phage-resistant bacterial mutants during individual treatments. These different approaches can be viewed as a means of increasing the medical or commercial applicability of a given phage formulation versus increasing the robustness of a formulation's curative power against any one infection. Although this distinction is made within publications, it nonetheless is not as relevant as it may first appear. In particular, it is not entirely clear exactly what modifications to a phage cocktail might result in one of these two outcomes but not the other.

Efforts towards cocktail optimization

Several studies have proposed techniques to improve the phage cocktails being developed. Kelly *et al.* developed a procedure, based on *Staphylococcus* phage K, involving multiple passages on previously phage-resistant strains to enrich for broad host-range spontaneous phage mutants [46]. Phage resistance systems were identified by these authors in 29 *S. aureus* strains [5,47]. Of these, 24 had restriction modification mechanisms, three had an adsorption inhibition mechanism and for two, the underlying resistance mechanisms were not identified (see [5,47] for further discussion of bacterial mechanisms of phage resistance). Six of the most potent phage derivatives, along with the original phage K, were chosen to make up the resulting cocktail, which was then tested against a panel of *S. aureus* strains to confirm the breadth of their combined spectrum of activity.

Similarly to the selective approach used by Kelly and colleagues, Gu *et al.* developed a phage cocktail by isolating phages using both wild-type bacteria and phage-resistant variants as hosts [40]. Their three-phage cocktail was tested for efficacy by treating mice suffering following a lethal dose of *Klebsiella pneumoniae*. A single intraperitoneal dose administered 1 h postbacterial inoculum resulted in 100% recovery, which was reproducible for a delay of 3 h if a higher phage dose was administered. *In vitro*, the phage cocktail was found to lyse 88% of *K. pneumoniae* strains tested.

Table 1. Recent publications investigating phage cocktails for bacterial treatment control.

Target	Entity	Characteristic	Dosing	Phages[†]	Reduction[‡]	Comments	Ref.
Pseudomonas aeruginosa	Murine	Lung	in.	m × 1, p × 1	$10^{0.5}$	Bioluminescent quantification	[89]
Klebsiella pneumoniae	Murine	Bacteremia	ip.	m × 1, s × 2	–	100% recovery with 3 h delay from lethal dose	[40]
P. aeruginosa	Wax moth	–	–	m × 3, p × 1	–	Doubling of lifespan	[31]
Vibrio cholerae	Rabbit	Enteric	Oral	u × 5	10^{5}	6 h delay	[41]
Escherichia coli	Murine	Enteric	Oral	m × 1, p × 1, s × 1		Biofilm reduction	[34]
E. coli	Food	–	–	u × 3	10^{1}	Relative to bacterial growth	[90]
Listeria monocytogenes	Food	–	–	u × 3	$10^{1.7}$	Relative to bacterial growth	[90]
Salmonella enterica ser. Typhimurium	Pig	Skin	–	m × 2, s × 2	10^{1}	Pig skin model	[91]
Enterococcus faecalis^{V}	Surfaces	–	–	m × 1, s × 1 or s × 1, s × 1	$<10^{1.5}$	Reductions on glass, cotton and polyester	[92]
E. coli	Leafy greens	–	–	u × 8	10^{5}	*E. coli* O157:H7 strain	[39]
E. coli	Surfaces	–	–	u × 8	10^{3}	*E. coli* O157:H7 strain	[84]
Campylobacter jejuni	Poultry	Colonization	Oral	m × 3	10^{2}	Reduction in feces greater with phage delivered in feed	[87]
P. aeruginosa	Catheter	Biofilm	–	u × 3	$<10^{2}$	Relative to bacterial growth	[81]
P. aeruginosa	Dog	Otitis	Topical	u × 6	$<10^{2}$	Chronic disease, 24 h post-phage treatment	[93]
Clostridium perfringens	Poultry	Necrotic enteritis	Oral	u × 5 (s or m)	–	92% reduced mortality with phage treatment	[78]
E. coli	Poultry	Colibacillosis	Oral	m × 2, s × 1	–	Several-fold reduction in flock mortality due to natural infections	[42]
E. coli	Cattle	Colonization	Oral	m × 1, s × 1	–	Significant reduction in *ex vivo* but not in *in vivo* model	[94]
S. enterica ser. Typhimurium	Pigs	Colonization	Oral	m × 6, s × 9	$10^{1.4}$	Microencapsulation of phages for delivery	[88]
E. coli	Sheep	Colonization	Oral	m × 3	–	*E. coli* O157:H7 strain; reduction to zero in 60 versus 0% without phages	[85]
None	Murine	None	Oral	m × ?	NA	No adverse effects	[95]
K. pneumoniae	Murine	Burn wound	ip.	u × 5	–	Survival increase from 6 to 94%	[86]
P. aeruginosa	Murine	Burn wound	ip.	u × 5	–	No significant impact on survival	[96]

Entries are listed in decreasing date and then author alphabetized order.
[†]*Lettering indicates different phage types* – Myoviridae *(contractile tails),* Podovirdae *(short tails),* Siphoviridae *(long noncontractile tails) and uncertain – with one letter for each phage used in a cocktail. Standard practice is to propagate cocktails as individual phage stocks that are then mixed together.*
[‡]*Indicated is maximum average reduction seen.*
Enterococcus faecalis^{V}*: Vancomycin-resistant* Enterococcus faecalis*; im.: Intramuscular; in.: Intranasal; ip.: Intraperitoneal; m:* Myoviridae*; NA: Not applicable; p:* Podovirdae*; s:* Siphoviridae*; sc.: Subcutaneous; ser.: Serovar; u: Uncertain.*

Table 1. Recent publications investigating phage cocktails for bacterial treatment control (cont.).

Target	Entity	Characteristics	Dosing	Phages†	Reduction‡	Comments	Ref.
E. coli	Cattle	Colonization	Oral	m × 4	–	Marginally positive reductions	[97]
P. aeruginosa	Human	Otitis	Topical	u × 6	10^{1}	Phase VII clinical trial; indication of efficacy with no adverse effects	[44]
E. coli	Food	–	–	m × 3	–	Reductions up to 100%	[79]
Salmonella enteritidis ser. Enteritidis	Poultry	Colonization	Spray	u × 3	$10^{1.7}$	Phage treatment prior to bacterial inoculation	[98]
S. enteritidis ser. Enteritidis	Poultry	Colonization	Oral	u × 4 or u × 45	–	Substantial early reductions in colonization (45–100%) but less later (15%)	[99]
P. aeruginosa	Murine	Burn wound	im., ip. or sc.	u × 3	–	12% mortality with phage treatment (ip.) and 94% without	[43]

Entries are listed in decreasing date and then author alphabetized order.
†*Lettering indicates different phage types* – Myoviridae *(contractile tails),* Podovirdae *(short tails),* Siphoviridae *(long noncontractile tails) and uncertain – with one letter for each phage used in a cocktail. Standard practice is to propagate cocktails as individual phage stocks that are then mixed together.*
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Using a more molecular approach to determine phage receptors, in a case of *Yersinia pestis*, Filippov *et al.* applied site-directed mutagenesis and transcomplementation to nine phages [48]. They identified six receptors for eight of the phages in the lipopolysaccharide core, postulating that a combination of these phages could be formulated into a therapeutic cocktail. Testing in mice showed that bacteria that had mutated to develop resistance against these engineered phages had become attenuated, resulting in a higher 50% lethal dose and longer survival times. For additional discussion associated with genetically or chemically modifying phages for phage therapy, see Goodridge [27], in addition to proposals for phage development provided by Verbeken *et al.* [49].

Integrating phages & phage cocktails into clinical medicine

The strengths of phages as therapeutic agents [22,50] include:

- Their modes of antibacterial action tend to not be affected by mechanisms of bacterial resistance to antibiotics;
- Properly formulated and applied phages have sufficient potential to cure bacterial infections, which supports their use as antibiotic substitutes [8,9,51];
- In many cases, phages are numerous, diverse, easily isolated and readily characterized;
- A substantial fraction of phages are not inherently toxic to life forms other than their target bacteria [20];
- Collateral damage to normal microbiota, which can be associated with the use of less-specific chemical antibacterials [4], is avoided.

Nonetheless, there are several challenges that must be addressed prior to widespread adoption of phage therapy. In this section, the authors consider various strategies of phage formulation, including strategies mixing into cocktails, and do so predominantly from the perspectives of both commercialization and regulatory approval.

Striking a balance in terms of activity breadth

The potential of bacteriophages to remove unwanted bacteria, while not disrupting native microbiota, is an appealing property for a modern antibacterial agent [20,24,50,52]. However, the benefits of using phages or other narrow-spectrum antibacterials [4] must be weighed against the costs associated with identifying the phage susceptibility of pathogens prior to initiation of treatment. The resulting effort contrasts with the relative ease of presumptive treatment, which can be employed when using broader-spectrum

antibacterials. In particular, although many pathogens can be rapidly identified to the species level, bacteriophages are often not effective against all strains, even within a single bacterial species [5]. By contrast, activities spanning multiple genera are typical of antibiotics, including various drugs that have entered the 'pipeline' within the last decade [53]. For these reasons, the typical narrowness of a phage's 'spectrum of activity' can be viewed as a key issue to be considered during both development and subsequent medical application of phage therapeutics.

Formulation of phages into cocktails increases their potential to be used presumptively, that is, prior to identification of pathogens (e.g., in terms of phage susceptibility), and the more phages that are included, the greater the potential that there will be sustainable levels of medical as well as commercial demand for a given formulation. However, having too many phages in a cocktail could result in a greater impact on nontarget bacteria, although in most cases this impact is still less than that expected of typical commercial antibiotics. Too many phages per formulation can also result in higher development and manufacturing costs. Figure 1 provides a summary of the burdens associated with overly complex cocktails as a phage therapy strategy (e.g., >50 distinct phages) compared with more personalized monophage therapy. As also indicated in Figure 1, less complex cocktails, for example, two to ten distinct phages, potentially inhabit a middle ground between these two extremes.

To summarize, while it may be possible to design phage cocktails that are applicable to all possible bacterial targets, including targets that may vary over time, in practice, for a variety of reasons it is usually preferable to generate less complex cocktails. The coverage of less complex cocktails may be incomplete, however, particularly given the limited host range of many phage isolates, as the phage susceptibility of prominent pathogenic strains of bacteria may change over time; for example, as one can observe within the context of phage typing [54].

Phage cocktails & personalized medicine

The specificity of a therapeutic phage formulation can either be fixed at the point of drug approval or instead can be subject to ongoing development to allow for drug reformulation. More generally, these differences represent distinctions between the 'one-size-fits-all' approach to medical practice versus a more personalized medicine approach [10,55]. In phage therapy terms, this has been described as 'prêt-à-porter' versus 'sur-mesure' [56], which translates to 'ready-to-wear' and, idiomatically, 'custom-made'. Alternatively, a middle ground, which we refer to as 'modifiable', can exist between such 'off-the-shelf' versus 'bespoke' [57] strategies, as we consider in this section (see also the related discussion provided by Verbeken *et al.* [49]).

The recent push for 'personalized medicine' has resulted in numerous 'omics-based diagnostics that are gradually making their way into clinical practice [58–61]. These technologies can provide a plethora of information with respect to disease predisposition, allowing for the creation of detailed, personalized prevention and treatment plans [62]. In principle, phage therapy could also utilize 'omics technologies, as applied to presumptive bacterial pathogens isolated from patients, to formulate sur-mesure cocktails for those patients. Owing to limitations in our knowledge of phage–bacterial interactions, such an approach could currently only provide a suggestion of which phages may be effective, rather than proof of actual activity. Therefore, in the nearer term, phage-based methods of bacterial identification [54,63] may be used instead [22]. Unless drawn from a well-developed phage bank

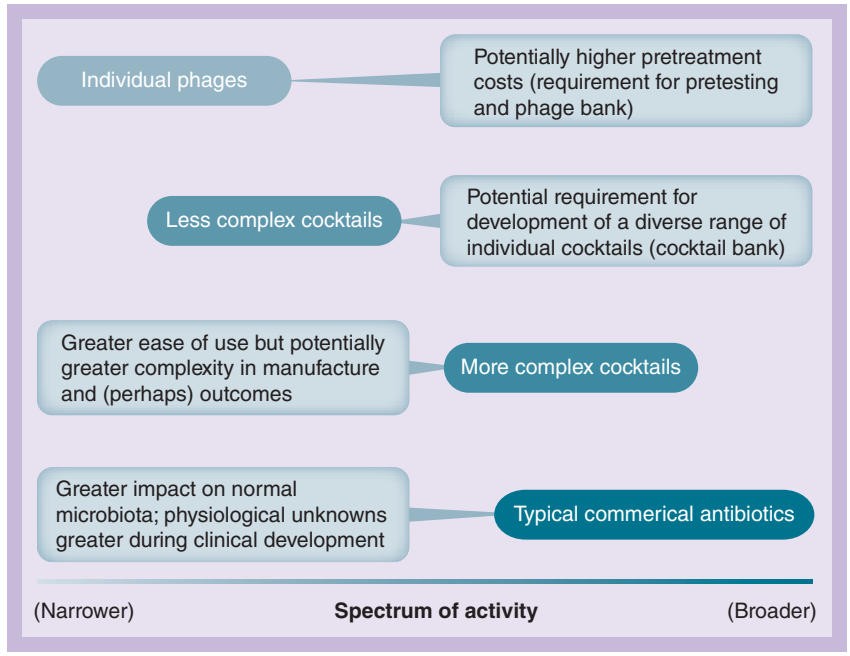

Figure 1. Summary of costs and benefits associated with various phage-based formulations versus typical commercial antibiotics, each as a function of associated spectra of activity. The suggestion of greater complexities of outcomes refers to the potential for side effects, which, although relatively low on a per-phage-type basis, should be inherently more likely the more phages that are included per formulation. Note that the figure is not intended to imply substantial overlap between the spectrum of activity of more complex cocktails, nor the substantial 'collateral damage' (i.e., bacterial dysbiosis) that can especially result from the use of broader-spectrum chemical antibiotics.

or otherwise using rapid identification means, such sur-mesure phage therapy can take time to initiate, in the order of days [56].

Table 2 provides a summary of various general approaches for the treatment of bacterial infections. The phage bank-derived monophage or sur-mesure cocktails are equivalent to personalized phage therapy as mentioned earlier. Preformulated – that is, prêt-à-porter single cocktails – represent the extreme alternative. Here, only a single product is provided for a given infection type. This product would ideally contain a sufficient diversity of phages to have an overall spectrum of activity to include those bacterial pathogens generally known to cause the type(s) of infections being treated. Without personalized sur-mesure approaches, or other alternative phage formulations as back up, however, a lack of target bacteria susceptibility to single cocktails could result in treatment failure without recourse. This prêt-à-porter strategy nonetheless represents what the authors would label as a 'western pharmaceutical model of development' of phage therapy (Figure 2), as it is thought to best fit a combination of constraints imposed by drug regulation and typical medical practice. The prêt-à-porter approach is especially appealing when consistency in drug formulation over time and in different locations is deemed to be particularly desirable.

More than one middle ground exists between these two extremes. The first could involve the preformulation of more than one phage cocktail. Rather than a single cocktail formulated to simultaneously combat multiple species or even genera of possible etiologies of a given infection type, multiple cocktails could be formulated, each with a spectrum of activity that includes, for example, only a single pathogen species. In this case, treatments could be initiated presumptively against the most likely cause(s) of an infection but then could also be followed up with cocktails against different bacterial species if initial treatments were proven to be ineffective. This approach could also be personalized for specific patients; however, rather than individual phages chosen from a phage bank, individual cocktails would be chosen from a 'cocktail bank'. The utility of this cocktail bank approach would ideally be the use of fewer phage types overall in the course of treatment. The costs, however, would be a greater development expense, as well as the production and distribution costs of multiple cocktails.

Table 2. Models of formulation for antibacterial treatment.

Type of formulation	Approach	Antibacterial types used per formulation	Personalized medicine	Prior to use characterization of infection†	Breadth of spectrum of activity	Flexibility (to bacterial resistance)‡	Flexibility (in spectrum of activity)§
Phage bank (monophage)	Sur-mesure	1	+++	+++	+	+++	+++
Personalized cocktail	Sur-mesure	>1	+++	+++	++	+++	+++
Cocktail bank	Sur-mesure via prêt-à-porter	>1	++	++	+++	++	+
Single cocktail	Prêt-à-porter	>1 or >>1	–	+	+++	–¶	–
Typical antibiotic	Prêt-à-porter	1	–	+	++++	–	–
Narrow-spectrum antibacterial#	Prêt-à-porter	1	+	≥+	≥+	–	–
Single cocktail	Prêt-à-porter but modifiable	>1 or >>1	+	+	+++	+††	++

†*Degree to which etiologies must be identified to achieve reasonable likelihood of treatment success.*
‡*Potential to respond, in a clinic, to treatment failures resulting from infection resistance to a given phage formulation.*
§*Potential for modification of formulation used for treatment over subyear time scales.*
¶*Should phages prove able to adapt* in vivo *to resistant bacteria, then this designation may be modified towards increased flexibility; see* [28] *for discussion of this potential.*
#*Such as monoclonal antibodies* [100]*, enzymes* [101] *or bacteriocins* [102]*; proposed characteristics assume that the antibacterial diversity of such agents is small relative to that of phages.*
††*This designation refers to an inability to respond to resistance in specific patients when using a fixed cocktail, but nonetheless that such patients may be subsequently treated given sufficiently rapid updating of otherwise modifiable cocktail formulations by manufacturers.*

The second middle ground dispenses with the complexity of multiple cocktails as well as that of strictly personalized medicine, opting instead for a single cocktail, but one that has a formulation that is modifiable over time. This latter approach is exemplified by the experience in the former Soviet Republic of Georgia [8,57], where prêt-à-porter products are used that are relatively fixed per formulation, but nonetheless are not held completely static over time. These products include 'Pyophage', which contains phages that target *E. coli*, *Proteus*, *Pseudomonas*, *Staphylococci* and *Streptococci* and is used to treat infections, such as those from wounds. A different product that is used to treat gastrointestinal infections, 'Intestiphage', instead targets over a dozen gastrointestinal pathogens. These cocktails may be updated twice annually, for instance, by the addition of phages targeting the most prevalent circulating pathogenic strains (in Figure 2C this is what completes the dashed-line loop, thus allowing for ongoing formulation development). In addition to obtaining new phages to include in cocktails, existing phages are also adapted to otherwise phage-resistant strains of target bacteria (see [56] and also above), a process that can be viewed as a means of extending the useful lifespan of otherwise well-characterized and effective phage isolates. To a large extent, these products are also allowed to vary from region to region and producer to producer.

Are 'modifiable' antibacterial strategies permissible in western medicine?

"Ninety years of phage therapy have shown that after a while phage preparations become less effective and need to be updated."

– Pirnay *et al.* [56]

Is it inherently necessary for steep regulatory costs, as associated with reformulation, to cause phage cocktails to remain static over long time frames? Or, instead, should bacteriophages be treated using a different model from that associated with typical antibacterial drug development? Historically, US FDA regulations have required safety and efficacy testing of each component of drug cocktails [64]. Nonetheless, one alternative model for drug development – which bears similarity to the single cocktail-modifiable approach previously discussed – is employed in the annual reformulation of trivalent influenza vaccines [56,65]. This approach in particular has the utility of not requiring *de novo* regulatory approval. In this model, individual influenza isolates, at a first approximation, are assumed to behave pharmacologically in a manner that

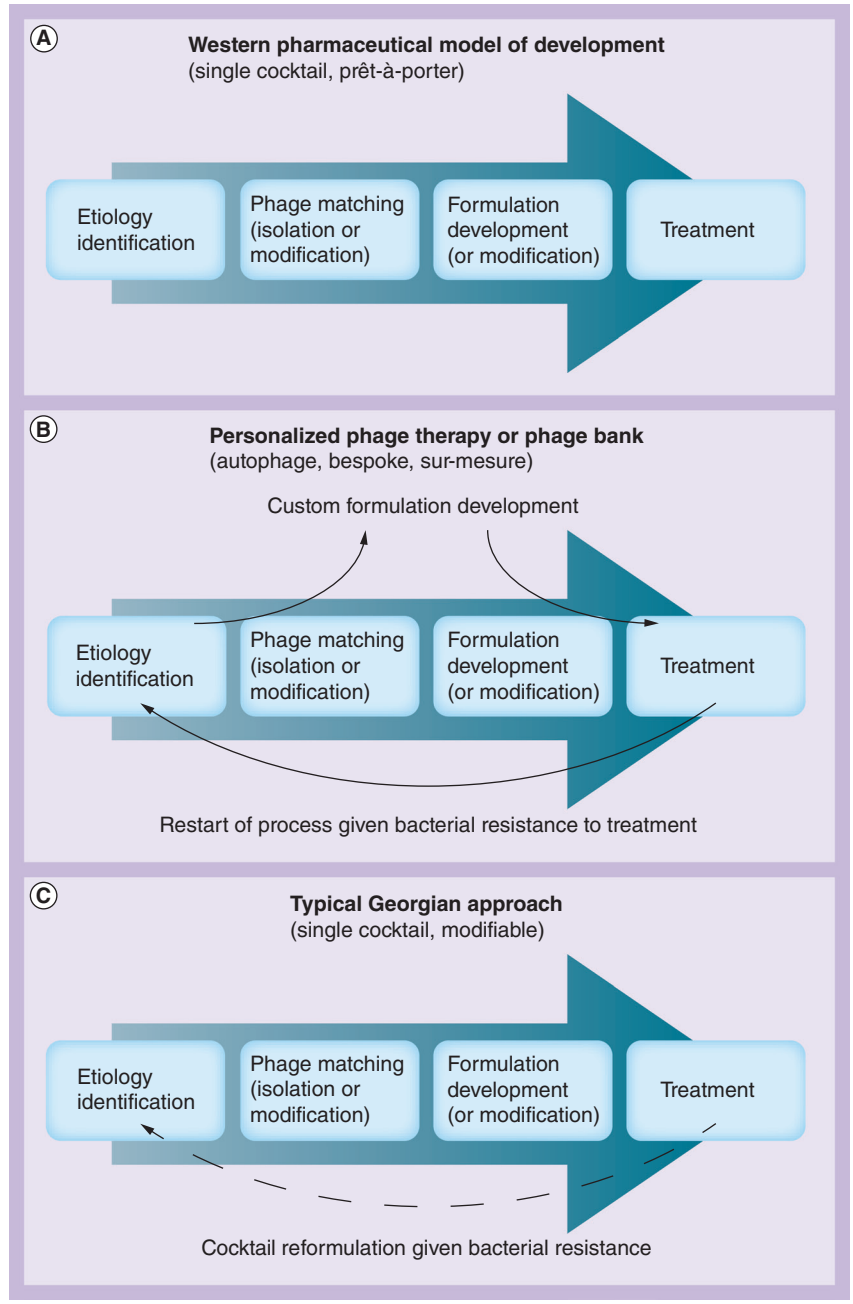

Figure 2. Models for implementation of phage therapy. These consist of either **(A)** fixed-formulation phage cocktails, **(B)** cocktail banks or individual phages, or **(C)** nonfixed formulation cocktails. **(A)** The fixed-formulation approach ('western pharmaceutical model of development') inherently requires less effort at all stages following formulation regulatory approval as both phage matching and formulation development or modification are avoided. The same approach, however, is also inherently less flexible in terms of responding to bacterial resistance and can be a problematic model towards phage therapy regulatory approval [49]. **(B)** The phage bank approach is highly flexible but also somewhat labor intensive, especially immediately prior to the commencement of treatment. **(C)** A single cocktail but modifiable approach represents a middle ground that allows for flexibility in formulation in response to bacterial resistance, but otherwise inherently requires less effort during use. See [56] for additional discussion of many of these concepts.

is similar to previously employed influenza vaccines. With phages, this assumption cannot be strictly applied since the genetic variability within phage communities can be vastly greater than that seen even among the rapidly evolving influenza viruses. In addition, while the predominant form of the influenza vaccine is inactivated [66,67], phages in phage therapy are applied in a live, typically genetically unmodified form. Nevertheless, phages possess two characteristics that might allow for therapeutic development based on an influenza vaccine-like model.

The first of these characteristics is that most phages are not inherently human pathogens and therefore are less in need of either attenuation or inactivation prior to their safe use as drug equivalents. Instead, in many circumstances phages may be viewed as intrinsically nonharmful owing to their long history of replication using hosts that are not humans, mammals, animals or even eukaryotes [20]. The inherently benign behavior of many phages can also be viewed as representing one of the underlying biological reasons that the P100 phage found in the Listex food additive was granted a 'Generally Regarded as Safe' designation by the FDA in 2006 [201] as well as for the FDA approval of LMP-102, now known as ListShield™ (Intralytix, MD, USA), also for food treatment [202].

Not all phage isolates are innocuous [68,69]. Consequently, phages still require substantial characterization prior to their use as therapeutics. The second relevant phage characteristic, therefore, is the potential for bioinformatic methods to rapidly predict phage properties that should be avoided, if possible, in phages used for phage therapy. This approach, following sequencing, in fact provided part of the basis for the P100 Generally Regarded as Safe designation as well as the FDA approval of LMP-102. Ongoing improvements in bioinformatics and other technologies suggest that the day may be approaching where it is phage characterization strategies, such as approaches to genome annotation, that will be subject to basic regulatory approval rather than individual phage isolates themselves. Therefore, we suggest that isolates that meet specific, stringent safety as well as efficacy criteria [13] might be given streamlined regulatory approval based on predefined, highly rigorous procedures rather than *de novo*, full-blown clinical safety and efficacy trials for all phage isolates proposed to be included in cocktails. We also imagine that the criteria that must be met to gain regulatory approval could differ as a function of proposed use, such as in terms of phage application strategy (*per os* vs topical vs parenteral) or the bacterium being treated (including being based on severity of infections or effectiveness of alternative available treatments). The chemical as well as physical nature of formulations, such as in terms of buffers, preservatives, osmolarity and pH, by contrast, would probably be required to remain within well-defined ranges, as subject to prior regulatory approval.

It is of interest that a number of these ideas are either in limited use for the influenza vaccine, such as in the case of live attenuation [66], or instead have been proposed for use. Cold-adapted and therefore nasal-limited and thus attenuated 'master' strains of influenza are literally mated with influenza strains against which a live-attenuated vaccine is sought [70]. Indeed, hybrid strains are also used to produce even inactivated influenza vaccines, as their ability to effectively replicate in embryonated eggs is important to vaccine production. This generation of modified viruses for vaccine production is similar to proposals for creating phage strains that are then modified in terms of which target bacteria they can impact (see [71] and above), though there can be limitations associated with that specific approach for phage cocktail development [28]. Bioinformatics is further suggested as a means of predicting the magnitude of immune responses to different gene variants, and a patient-centered, individual approach is also mentioned in terms of further influenza vaccine development [72]. In other words, improvement of virus-based pharmaceuticals within a streamlined regulatory framework is not without precedent [73,203].

Licensure of reformulated influenza vaccines in the USA requires a clinical trial to ensure the existence of a potential to generate adequate levels of protective antibody [74]. This requirement is logical as it represents an efficacy check prior to large-scale manufacturing. It can also be viewed as representing close monitoring of the early use of a given vaccine formulation, since individuals given successful vaccination will achieve an outcome (immunity) that is equivalent to subsequently vaccinated individuals. A similar approach undertaken with regard to new phage isolates, however, would have to involve some form of treatment of existing infections. We thus envisage an analogous licensure of reformulated phage products that involves limited testing with close monitoring of outcomes – the latter as an equivalent to Phase IV studies, which involves postmarketing assessment

of drug efficacy and safety. While healthy individuals represent the target population for most vaccine testing, it is a comparatively rarer bacterium-infected population that would need to be identified for equivalent phage testing.

Interesting parallels exist between phage therapy and its possible regulation with what are known as 'biopesticides', as well as microorganism-mediated biocontrol agents more generally [75]. As noted by the Environmental Protection Agency (EPA), "Biopesticides are usually inherently less toxic than conventional pesticides … [they] generally affect only the target pest and closely related organisms, in contrast to broad-spectrum, conventional pesticides … [and] often are effective in very small quantities and often decompose quickly" [204]. Potentially equivalent to phage therapy, biopesticide use is not necessarily equivalent to that of conventional, chemical pesticides, such that "users need to know a great deal about managing pests." In addition, with regard to biopesticide regulation, it is noted that: "Since biopesticides tend to pose fewer risks than conventional pesticides, EPA generally requires much less data to register a biopesticide than to register a conventional pesticide. In fact, new biopesticides are often registered in less than a year, compared with an average of more than 3 years for conventional pesticides. While biopesticides require less data and are registered in less time than conventional pesticides, EPA always conducts rigorous reviews to ensure that pesticides will not have adverse effects on human health or the environment. For EPA to be sure that a pesticide is safe, the agency requires that registrants submit a variety of data about the composition, toxicity, degradation and other characteristics of the pesticide" [204].

It is notable that phages can be employed within agricultural settings as antibacterial biopesticides [18]. Regulatory standards, however, are not equivalent between the EPA and the FDA, from region to region, nor necessarily between vaccines and antibacterial agents. Nonetheless, these alternative approaches to regulatory approval minimally indicate that frameworks for the regulation of bioactive substances are not monolithic (see [49]).

Strategies for continuous product development within a streamlined regulatory framework could be described as representing a quasipersonalized model of medicine. In this case, 'quasipersonalized' refers to populations or subpopulations of individuals existing at specific points in time or in specific locations, that is, where optimal treatment approaches may vary spatiotemporally. Here, either individual phages or phage cocktails would be 'personalized' to the current and also potentially regional characteristics of the etiology being treated, and this would be rather than strictly to pathogen characteristics as found in individual patients. If successfully developed, we envisage that this modifiable approach could serve as a means by which both the commercial and medical utility of phages may be aligned towards addressing the current need for diverse, safe and abundant antibacterial 'drugs' possessing novel mechanisms of action. Such an approach also basically represents the standard model of phage therapy development employed in the former Soviet Republic of Georgia, where phage therapy has enjoyed both historical and ongoing success in combating bacterial infections [8,9]. Within this framework, further personalization is also possible given a more labor-intensive per-patient matching between etiologies and specific phages or cocktails that also have been subject to the equivalent streamlined approval process – a strategy that is routinely practiced in Poland as well as episodically in Georgia. Alternatively, it has been suggested that "products for bacteriophage therapy deserve their own regulatory framework in Europe" [49]. Verbeken *et al.* also discuss many of the issues considered in this section [49].

Conclusion

The key advantage of naturally occurring antibacterial agents is the relative ease of their discovery, while the key advantage of bacteriophages in particular is their potential, once properly characterized, to negatively impact only their specific bacterial targets. These strengths of phages, abundance in combination with selective toxicity, must be balanced against their also typically narrow spectrum of activity (host range). One means by which this latter issue both can and has been addressed is through the combination of individual phage isolates into cocktails. This can result in both an increased potential for phage formulations to be used presumptively and an increased breadth of utility for individual formulations. The latter will probably be crucial to the commercial as well as clinical success of phage therapy in the context of western medicine, as it has been for phage therapy in the former Soviet Republic of Georgia.

The Achilles' heel of this strategy, as indeed for antibiotics generally, is the potential for bacteria to evolve resistance to antibacterial drugs, either in the course of use (as is the case of antibiotics) or as new bacterial strains become prominent within populations (as appears to often be the

case for phages). Current regulatory approaches to antibacterial agents, however, do not easily foster the rapid response by medical practice to what are literally emerging pathogens in terms of susceptibility to currently available treatments. In part, this situation is a consequence of an otherwise lack of surplus of diversely acting but, nonetheless, apparently inherently safe, naturally occurring antibacterial agents, as phage therapy seems to offer. The potential for phage therapy to robustly address the crisis of antibiotic resistance that is now seen among pathogenic bacteria is therefore dependent on both how and whether a regulatory model can be developed in the west that is capable of taking advantage of these remarkable phage properties.

Future perspective

Phages, in comparison with other antibacterial agents such as antibiotics, can display a greater diversity of mechanisms of action and, in many cases, can also be safer. The challenge for phage therapy is how to harness these positive attributes in light of existing regulatory practices as well as how phages might fit into the current economic models that underlie the distribution and use of antibacterial agents. Currently phage therapy appears to thrive particularly in regions where the regulatory climate is relatively friendly and the amount of money received by phage-product suppliers is nominal (e.g., as seen in Poland and the former Soviet Union). Our expectation is that phage therapy will show increasing promise for use as antibacterials to fight infections as mainstream western scientists and companies publish further, well-controlled phage therapy studies. This, in turn, could result in an increasing demand for phage therapy by both clinicians and the lay public, including in regions such as the USA where the regulatory climate is less conducive to its near-term implementation [73] and the primary economic models for drug development can be somewhat biased against antibacterial drugs [10,76].

Given these issues, phage treatment will probably attain a foothold in western medicine particularly where its use can prevent large medical costs. Therefore, over the next 5–10 years, we expect phage therapy to find its way into clinical practice towards the treatment of debilitating or life-threatening chronic bacterial infections that are otherwise resistant to available antibacterial drugs. Indeed, this approach is already happening within the EU in Poland. Over longer time frames, it may be that the dangers associated with disruption of the human microbiome owing to use of relatively broad-spectrum antibacterial agents [77] could result in a greater role for phage therapy as an alternative, narrow-spectrum antibacterial treatment. It is especially within this latter context that we see a role for prêt-à-porter phage cocktails that nonetheless are relatively modifiable rather than somewhat fixed in their formulation.

Executive summary

Phages & phage therapy basics

- Phages can be extremely abundant and their ability to kill target bacteria make them potentially useful candidates as antibacterial agents.
- Numerous phage therapy products have been developed against many bacterial types and in numerous systems (e.g., agriculture, food safety, veterinary medicine and, especially, for treatment of human patients).
- Products consist of single phages (monophage therapy) or, instead, a combination of phages (phage cocktails), and formulations may be custom generated for individual use, periodically modified (e.g., one or more times per year) or instead more or less fixed.

Polyphage therapy

- Many investigators have moved to design experimental phage therapy studies employing phage cocktails containing multiple phage types (polyphage therapy).
- Phage cocktails are intended to broaden the utility for phage formulations to treat specific bacterial diseases, and to prevent the development of phage-resistant bacterial mutants.
- Efforts towards cocktail optimization have been proposed, including various procedures that select for phages with enhanced or more beneficial lytic activities.

Integrating phages & phage cocktails into clinical medicine

- A brief overview of the strengths of phages as antibacterials is provided.
- Benefits of formulating phages into cocktails are discussed, in addition to costs that can be associated with basing cocktails on excessive phage numbers.
- Prêt-à-porter, sur-mesure and modifiable approaches are discussed, each in terms of its relation to more personalized approaches to phage-based medicine.
- Are 'modifiable' antibacterial strategies permissible in western medicine? There are regulatory challenges facing phage therapy, particularly in terms of a quasipersonalized, modifiable approach.

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