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Unlocking the next generation of phage therapy: the key is in the receptors

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Phage therapy, the clinical use of viruses that kill bacteria, is a promising strategy in the fight against antimicrobial resistance. Before administration, phages undergo a careful examination of their safety and interactions with target bacteria. This characterization seldom includes identifying the receptor on the bacterial surface involved in phage adsorption. In this perspective article, we propose that understanding the function and location of these phage receptors can open the door to improved and innovative ways to use phage therapy. With knowledge of phage receptors, we can design intelligent phage cocktails, discover new phage-derived antimicrobials, and steer the evolution of phage-resistance towards clinically exploitable phenotypes. In an effort to jump-start this initiative, we recommend priority groups of hosts and phages. Finally, we review modern approaches for the identification of phage receptors, including molecular platforms for high-throughput mutagenesis, synthetic biology, and machine learning.

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Introduction

Just over a century ago, acquiring a severe bacterial infection was a death sentence. Without antibiotics, even minor injuries and commonplace infections would frequently lead to major complications, including sepsis. Antibiotics have revolutionized healthcare and have saved millions of lives. Unfortunately, the current rise of antibiotic resistance jeopardizes this success story and threatens to usher in a postantibiotic era. The fight against this crisis has to be approached from many angles, but the consensus dictates that a keystone should be the research and development of new therapeutic strategies against bacterial infections. One of these strategies is phage therapy [1••].

Phages are the natural predators of bacteria, and lytic phages can quickly kill their bacterial hosts. This bactericidal activity is the basis for phage therapy, the clinical administration of phages to patients suffering from bacterial infections. Phage therapy was discovered and first used before antibiotics, enjoying a peak in scientific research and societal interest and use during the 1920s and 30s. Patients with infections ranging from typhoid to conjunctivitis, mastitis to cholera, all benefited from the first uses of phage therapy [2]. However, the popularity of phages was short lived. Eclipsed by the arrival of antibiotics with their wide spectrum of action and ease for large-scale production, the field of phage therapy was largely dismissed. Nowadays, with the emergent threat of antibiotic resistance, phage therapy is in the spotlight again. And, because of our improved understanding of phage biology, genetics, and interactions with bacterial and human cells, we are better prepared to take advantage of its boons [1••].

The first step in the lifecycle of a phage is the adsorption to its bacterial host. It consists of an initial contact and reversible binding to the host, followed by the irreversible *lock-and-key* recognition and attachment of the phage's receptor binding protein to the receptor on the bacterial surface [3,4]. The nature of this interaction grants phages their high host specificity, and successful phage adsorption then primes the virus for ejection of its genome into the bacterial cell. Previous reviews have delved into the diversity of bacterial structures that can serve as phage receptors, with many including tables that nicely classify them [3–6,7•]. Simply put, phages can target any structural element of the bacterial surface. Some common examples include elements of the Gram-positive cell wall, such as peptidoglycan and teichoic acids, glycolipid moieties of the Gram-negative outer membrane, polysaccharide components of bacterial capsules, or a wide array of integral membrane proteins and appendages, such as pili and flagella. Such is the importance of the bacterial surface receptors that we can safely say that without them there is simply no phage infection [8].

This perspective piece focuses on phage receptors and the attention we should pay to them in the context of phage therapy. The choice of phage for therapeutic use is not made lightly. Preclinical characterization of phages

requires answers to questions regarding their effectiveness, stability, and their safety [9]. The phage's genome is analyzed to rule out the presence of toxin, antibiotic resistance, or integrase genes; its morphology and lifecycle variablessuch as latency time and burst sizeare assessed; its antibacterial activity and host range are measured. However, for many of these phages, one conspicuous gap is seen in this evaluation: the identification of the receptor they use. Our aim here is to promote that the determination of phage receptors is a desirable step in the characterization of all phages used therapeutically. To achieve this, first, we discuss the reasons why this is not already a common practice; second, we explore the potential benefits to be obtained with this endeavor; and, finally, we propose some strategies to fuel this initiative. We believe there is a next generation of phage therapy in the making, and that phage receptors are a key to unlock it.

We do not routinely identify the bacterial receptor used by a phage. Why not?

Decisions in policy-making, entrepreneurship, and, of course, clinical practice are consistently guided, among other things, by risk-benefit and cost-benefit analyses [10]. Let us assume, then, that whether or not to identify the bacterial receptor of all phages expected to be used therapeutically, is a decision worth making. We will begin our analysis by assessing the potential risks, costs, and obstacles associated with this practice.

The main stakeholders for phage therapy are perhaps the patients that stand to benefit from it and the medical teams providing their care. Currently, access to phage therapy for a given patient is extremely low, with one of the main pathways being extended-access (compassionate use) programs [1••]. In this context, we denote patients with severe, often life-threatening infections, with documented antibiotic failure and no other conventional treatments available [11]. For these patients, delays as short as a few days in the availability of phage therapy can have a detrimental impact on their clinical outcomes. Phage isolation and characterization need to be streamlined, and any additional test performed with the viruses should ideally not cause an unreasonable delay in administration. Unfortunately, characterization of phage receptors, at least currently, is not a quick process. Previous descriptions of phage receptors [4,7•] have primarily come from research settings where time constraints were not necessarily an issue. In order to be clinically applicable, phage receptor identification needs to be achievable in a clinically relevant timeframe, as for patients receiving phage therapy, time is of the essence.

A second group of stakeholders include the scientists and biotechnology experts in charge of sourcing and preparing phages for therapy. They are already aware of the paradoxical problem: in spite of being the most abundant organisms on Earth, the number of phages currently available for therapeutic use is quite small [12]. As a result, turning to environmental sources to hunt for specific phages against strains of pathogenic bacteria is a necessary but seemingly never-ending task. Likewise, identification of the phage receptors in the bacterial hosts is a daunting mission poised to take time, consume resources, require specialized infrastructure, and demand expertise in many fields such as bioinformatics, genetics, bacteriology, and phage biologyto name a few. Furthermore, the diversity of phages is mirrored and driven by the diversity of their hosts. As many techniques used in microbiology require some degree of tailoring to specific microorganisms, it is unlikely the 'one-size fits all' approach to identify phage receptors will work. Thus far, it would be reasonable to say that there are solid reasons to refrain from phage receptor analysis as a standard procedure. This is where a breakdown of what we stand to gain from this practice is needed.

Why would it be a good idea?

Some of the greatest advantages of phage therapy are its diversity and versatility. We have already alluded to the practically infinite number of phages in nature. There are also numerous ways of designing and administering phage-based treatments to patients, including conventional phage therapy, the use of phage cocktails and phage-derived proteins, or combinational treatment with antibiotics and the immune system [1••]. We believe that all of these approaches to phage therapy can be improved by leveraging an expanded knowledge of phage receptors.

Optimization of phage cocktails

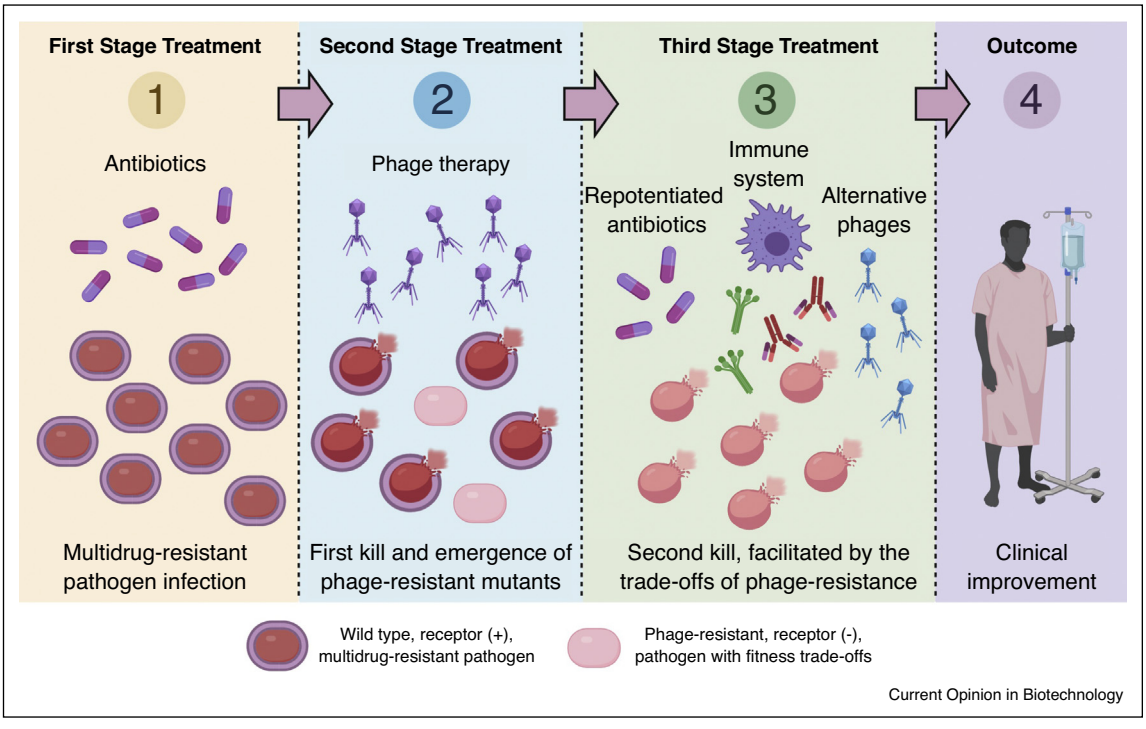
The use of a single phage against a bacterial pathogen is ill-advised in the clinical setting. The phage–host match performed in the laboratory may not correlate with the lytic activity of the phage *in vivo*. Furthermore, bacteria can, and frequently do, evolve phage resistance rather quickly [13]. Phage cocktails attempt to solve these issues by combining phages targeting a single strain, multiple strains of a single species, or even multiple species. However, the more phages in a cocktail, the less predictable its pharmacokinetics, pharmacodynamics, and safety, and the more complex its production [14]. Cocktail design does not involve the random mix of phages, and cocktail optimization aims to make rational, evidence-based decisions for the selection or exclusion of phages in a cocktail [15]. We can leverage the knowledge of bacterial receptors used by phages as a criterium to optimize cocktails. We can, for example, prioritize combinations of phages that target different receptors in the same host, reducing both the likelihood and speed of phage resistance, and limiting potential antagonism between the phages, as demonstrated by the following examples. Yang *et al.* [16•] designed a cocktail against *Pseudomonas aeruginosa* containing a phage targeting the O-antigen, and a second

targeting a truncated version of the O-antigen, which the pathogen produced upon becoming resistant to the initial O-antigen targeting phage. Tanji *et al.* [17] combined phages targeting two different receptors and managed to delay the emergence of phage-resistance in *Escherichia coli* O157:H7 by almost a day, compared to single-phage treatments. Gu *et al.* [18] also provided experimental validation of this principle by designing a cocktail against *Klebsiella pneumoniae* with three phages targeting three different receptors. An alternative approach, is to isolate phages that bind to more than one receptor or different conformations of the same receptor. For example, Takeuchi *et al.* [19] described how staphylococcal Twort-like phages can use teichoic acid in the cell wall as their receptor, regardless of whether or not the host has modified it via glycosylation, making them ideal components of phage cocktails. Using intelligent receptor-based design of phage cocktails, we can maximize their spectrum of action, while reducing the possibility of phage resistance.

Discovery of new phage-derived antimicrobials

For some phages, reaching their receptor located in the deeper levels of the cell envelope involves breaking through barriers, such as biofilms, capsule layers, or outer membrane lipopolysaccharides. For others, the binding process itself demands the enzymatic cleavage of their receptor [4]. In such cases, successful adsorption to their receptor demands surgical precision from phages, lest they compromise the bacterial viability before replication is complete [20]. The phage genome encodes a number of enzymatic families capable of executing these functions. These proteins can be located on the phages' tail fibers, tail spikes, or base plates, and many of them independently present antibacterial or antivirulence properties [20]. Depolymerases, for example, are used by phages to access receptors concealed by polysaccharides, orin cases where the polysaccharide chain is the receptor itselfto cleave it, thereby stabilizing the attachment [21]. Alternatively, and demonstrated by Born *et al.* [22] in the plant pathogen *Erwinia amylovora*, phage-derived proteins, including depolymerases, can be used to unmask deep phage receptors, priming the bacterial host for infection by phages it was previously resistant against. Although a detailed review of these proteins falls outside the scope of this perspective, we do propose that understanding a phage receptor, its location and structure, and what is needed from a phage to access and bind to it, can lead to the discovery or engineering of new phage-derived antimicrobials.

Figure 1



Clinical exploitation of receptor-mediated phage-resistance. A multidrug-resistant pathogen will typically withstand antibiotic treatment (first line of treatment). We can pursue the use of phage therapy (second line of treatment), with phages that have a known receptor that, if lost upon emergence of phage-resistance, will generate a trade-off. The lytic activity of the phages results in a first killing and the emergence of phage-resistant mutants. We can exploit the receptor-mediated trade-offs of phage-resistance using a third line of treatment that can include re-potentiated antibiotics, alternative phages, and the immune system. This will result in a second killing leading to an improved clinical outcome.

Understanding, predicting, and exploiting phage resistance

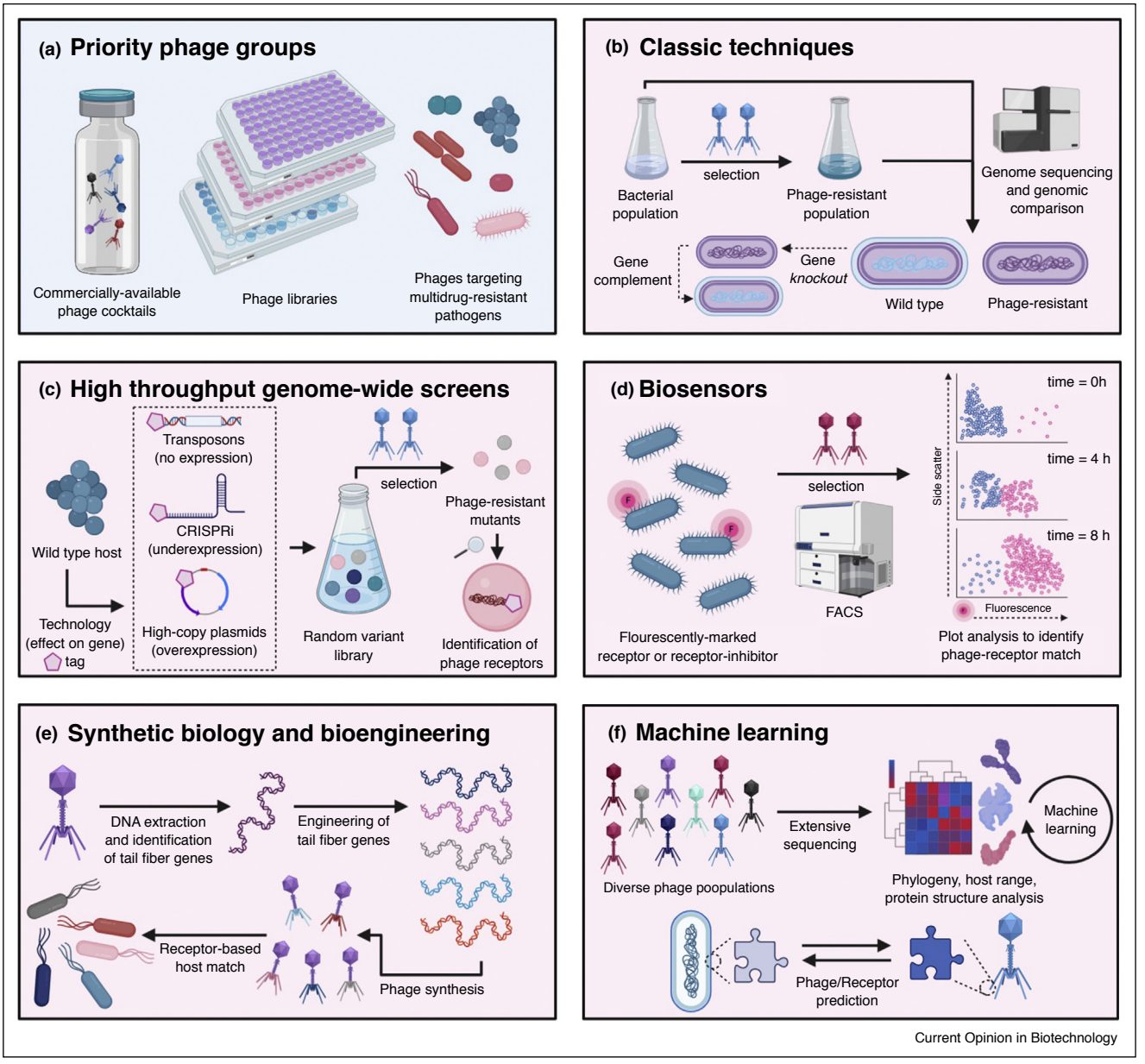
We have already referred to phage resistance and how optimization of phage cocktails can delay its emergence. However, it is naïve to expect that resistance can be completely avoided during the clinical use of phages. Bacteria have a wide array of mechanisms to develop resistance to phages, and perhaps the best understood and quickest to emerge involves the phage receptors [23]. Bacteria can mutate, mask, downregulate, or replace their phage receptors in order to inhibit phage adsorption. But, in doing so, they stand to lose the physiological role the receptor played, explaining the existence of so-called evolutionary *trade-offs* of phage resistance. Fortunately, this grants us the opportunity to use phages with known receptors to reverse-engineer or 'steer' [24•] bacterial evolution towards clinically exploitable phenotypes, as demonstrated by previous studies (Figure 1). Chan *et al.* [25] described how the receptor used by the *P. aeruginosa* phage OMKO1 is a membrane component of a drug efflux pump, and that phage resistance appeared with loss-of-function of this receptor; without the efflux pump, antibiotic sensitivity was restored in the previously multidrug-resistant strain. Filippov *et al.* [26] identified six receptors for eight phages in different sites of the inner and outer core of the lipopolysaccharide (LPS) of *Yersinia pestis*. They then observed how phage resistance emerged through mutations in different enzymes involved in the biosynthesis of LPS and discovered a direct correlation between the level of truncation of the LPS molecules and loss of virulence. In *Enterococcus faecalis*, Chatterjee *et al.* [27] described how the enterococcal polysaccharide antigen (Epa) was necessary for the adsorption of 19 different phages, and how phage-resistant mutants lacking Epa became vulnerable to antibiotics that target the cell wall, such as vancomycin. In another Gram-positive pathogen, *Staphylococcus aureus*, Capparelli *et al.* [28] identified that phage M^{Sa} used the *N*-acetylglucosamine of the cell wall as its receptor, and upon losing it, the phage-resistant mutants exhibited decreased growth and a dysregulation of gene expression. Gordillo Altamirano *et al.* [29] reported that *Acinetobacter baumannii* phages used the bacterial capsule as their phage receptors and that strains became resistant to phage adsorption by disruption of the capsule biosynthesis genes. After having lost their capsule, however, the strains were unable to form biofilms, lost their virulence in a murine model of bacteremia, and became vulnerable to three antimicrobial agents: the complement system, alternative phages, and antibiotics they used to resist. Alternatively, the clinical benefits of capsule loss could be elicited with the use of purified phage depolymerases [30,31]. Finally, Kim and Ryu [32] described phage SPC35, capable of infecting *E. coli* and *Salmonella enterica* using the membrane protein BtuB, which is involved in vitamin B12 uptake, as its receptor. Although resistance to this phage through receptor disruption would likely not reduce bacterial virulence [33], it could potentially be leveraged in the clinical setting through interventions that modulate the availability of this nutrient.

All the aforementioned studies agree that receptor-based steering of phage-resistance can open the door for innovative applications of phage therapy. Capparelli *et al.* [28], for example, proposed the use of their avirulent phage-resistant mutants as vaccines. Chatterjee *et al.* [27] suggested using phage therapy to prevent overgrowth of *E. faecalis* during antibiotic treatment of other infections, after observing that the Epa-deficient phage-resistant mutants could not colonize the intestine of mice with antibiotic-mediated dysbiosis. Chan *et al.* [25,34] translated their *in vitro* findings into the successful treatment of an aortic graft infection by *P. aeruginosa* using phage OMKO1 and ceftazidime, theoretically exploiting the receptor-driven phenomenon of antibiotic resensitization. Gordillo Altamirano *et al.* [29], envisioned a model of sequential therapy against *A. baumannii* that exploits the emergence of antimicrobial resensitization through loss of the bacterial capsule, which was used *a priori* in a clinical case study of sepsis by this pathogen [35]. Although these findings paint an exciting panorama, further research needs to address the questions of repeatability and pleiotropy in the evolution of receptor-mediated phage resistance [36•]. Nevertheless, if we have accomplished our goal of highlighting the importance of studying phage receptors, it is also our duty to delineate a pathway to start doing so efficaciously.

Where and how can we get started?

Insurmountable tasksor those that simply appear to becan often be deconstructed into smaller, more approachable ones. Understandably, the first step is sometimes the most difficult to take. With that in mind, we recommend three categories of phages where we, as a field, can begin the daunting task of identifying phage bacterial receptors (Figure 2a). First, we should characterize phage receptors for commercially available phage cocktails. While, traditionally, many of these cocktails were produced and distributed in eastern European countries, they are increasingly being produced and marketed in the West as well [37]. Some studies have looked into the safety of these cocktails, their activity against certain bacterial species, and even their metagenomic composition [38–40]. However, to the best of our knowledge, we have not acquired any insight about the receptors used by the phages in these cocktails. Second, we should identify bacterial receptors used by phages in readily available phage libraries. Established and emerging comprehensive libraries include PhageBank® from Adaptive Phage Therapeutics, Baylor College of Medicine's TAILΦR initiative, Texas A&M University's Center of Phage Technology, the Israeli Phage Bank, the Eliava Institute in Tbilisi, Georgia, among many others [37,41,42]. These phage libraries are ideal candidates to pre-emptively

Figure 2



Where and how to start characterizing phage receptors? (Where: blue background) **(a)** Phage groups to be prioritized include those in commercially available cocktails and libraries, and those targeting multidrug-resistant pathogens. (How: red background) **(b)** Classic techniques involve the genomic comparison of wild type and phage-resistant strains to identify genes and structures involved in phage-resistance. The action of these genes can be confirmed through knockout and complementation experiments. **(c)** High-throughput genome-wide screens using molecular technologies to test the effect of complete disruption, underexpression, or overexpression of genes in phage-resistance. Barcodes or 'tags' on these molecular probes facilitate the downstream recognition of the affected genes. **(d)** Biosensors use fluorescent tags on candidate receptor or receptor-inhibitor genes. After putting the bacterial population through phage-selection, the analysis of the fluorescence cell-sorting can confirm the function of the genes. **(e)** In the absence of phages from natural sources capable of targeting a given receptor, synthetic biology and bioengineering can allow us to modify or *de novo* produce phages or their tail-fibers specifically designed towards a receptor or host. **(f)** As our body of knowledge of phage receptors and phage tail fibers increases, we can feed data on phylogeny, host range, and protein structure into machine learning algorithms, allowing the accurate prediction of receptors used by a given phage.

characterize receptors, before compassionate usage or clinical trials. We encourage all phage researchers to begin this undertaking with their own phage collections. Third, we can largely focus this initiative on phages against the most dangerous pathogens faced in clinical practice. From the perspective of antimicrobial resistance, species with the ability to acquire resistance mechanisms and render antibiotic therapy useless are the top priority [43,44]. These include, of course, the *ESKAPE* group (*Enterococcus faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa*, and *Enterobacter* spp.) in healthcare-associated infections, as well as leading causes of community-acquired resistant infections such as *Mycobacterium tuberculosis* and *Neisseria gonorrhoeae.*

And, finally, we are left with the question of *how* to discover phage receptors in a resource-savvy and time-savvy way. Traditional approaches involve the cross-screening and genome sequencing of phage-resistant isolates (Figure 2b). If, for example, we observe that a phage-resistant isolate harbors loss-of-function mutations in an outer membrane protein, we can putatively identify it as the phage receptor in the wild type strain. Confirmation of the receptor may then be pursued with genetic knockout and complementation experiments, although this may not be required in all cases. As a disclaimer, most of the benefits associated with the identification of phage receptors could be attained with a broad approximation of their general location and/or function. For example, to optimize a phage cocktail it would be enough to know that a phage attaches to the capsule or the flagellum of its host, and not necessarily the exact polysaccharide chain or flagellin subunit, respectively. This 'proof of concept' approach may save considerable time and resources, but it could arguably fall short from convincingly tipping our risk- and cost-benefit analyses in favor of this venture.

Emerging technologies that enable high-throughput screening methods to reduce the time and resource burden associated with identification of phage receptors are welcome. The use of genomewide screens on bacterial culture collections, particularly the Keio and ASKA collections, have enabled the broad characterizations of genetic interactions between phage and bacterial host [45]. These genome-scale studies allow for comprehensive and unbiased identification of phage receptors and other resistance mechanisms [46–49]. Recently, Mutalik *et al.* [50••] expanded these approaches using three additional platforms to investigate the resistome of *E. coli* against 14 different phages (Figure 2c). In random barcode transposon site sequencing (RB-TnSeq), a barcoded transposon randomly inserts itself along the host's genome, leading to loss-of-function mutations at the insertion sites. For the CRISPRi (interference) method, nucleases are targeted towards specific mRNA sequences resulting in an underexpression of the genes. Finally, dual-barcoded shotgun expression library sequencing (Dub-seq) inserts random pieces of DNA into the host using shotgun cloning and a high-replication plasmid, resulting in the overexpression of genes. After putting the host through each of these platforms, the team was able to identify genes thatwhen either disrupted, underexpressed or overexpressed, respectivelygranted *E. coli* resistance against one or more of the 14 screened phages [50••]. The approach confirmed both known and unknown phage receptors, as well as identified novel genes involved in mechanisms of phage-resistance unrelated to phage adsorption. Olivenza *et al.* [51] proposed the methodology of epigenetic biosensors to detect phages and differentiate phage receptors. In this platform, candidate phage receptor genes, or their repressors, are fluorescently tagged. Next, flow cytometry was used to measure the changing levels of transcription of these genes as the bacterial population was exposed to various phages. The analysis of the fluorescence plots as the population becomes resistant to the phages, enables the confirmation of the role of the genes (Figure 2d). Once these libraries and platforms are created, they can be rapidly screened across multiple phages and conditions, massively increasing throughput and identification of putative phage receptors. However, many of these techniques still require optimization of the methods for each species, the generation of libraries is expensive, and ultimately, still relies on the availability of phages able to infect the host on which the libraries have been created. Regardless, these methods present a significant improvement in throughput and potentially cost over traditional homologous recombination approach for gene knockout and phage receptor identification.

Looking further ahead, we may be able to utilize recent advances in genomics and synthetic biology to predict and subsequently design phage–host recognition proteins (Figure 2e and f). Advances in sequencing and metagenomics have hugely increased the number of assembled phage genomes [52]. From these sequences we can identify the key genes associated with phage–host interactionsincluding tail fiber, tail spikes, base plate, or other host-recognition site proteinsand from that infer the proteins' structures. As our knowledge of phage receptors and their complementary phage host-recognition factors increases, we could use machine learning approaches to identify patterns, domains, and potentially three-dimensional structures that may allow us to predict the receptor and host-range of any given phage [53]. Using these predictions, and in the complete absence of a natural phage against a given bacterial species, we could utilize synthetic biology approaches to tailor-make phages that target specific receptors. These could include natural mechanisms, such as tropism-switching seen in *Bordetella pertussis* phages [54,55], along with promising research in

synthetic biology and targeted evolution of phage tail fiber proteins [56,57••]. Regardless of the approach taken, progress in new sequencing platforms, automation, and robotics is expected to significantly reduce the time, workload, and cost required to unveil phage receptors. And as every scientist knows, less time pipetting is more time writing.

Concluding remarks

Phage therapy by itself will not solve the antibiotic resistance crisis, but it is undoubtedly a promising therapeutic strategy in a field largely forgotten by the pharmaceutical industry. We believe that expanding our knowledge in a key component of phage–host interactions, the phage receptor, can open the door to improved and innovative applications of phage therapy. Identifying and understanding phage receptors allows us to improve the design of phage cocktails, accelerates the discovery of new phage-derived antimicrobials, facilitates the prediction and exploitation of phage-resistance, and enables synergistic combinations of phages with antibiotics and the immune system. We acknowledge that large-scale characterization of phage receptors is a demanding task. It is not an initiative that can be brought forward by just one part of the field, but should rather include phage biologists, biotechnology companies, clinicians, clinical trial managers, and other stakeholders. It seems challenging, but we are optimistic.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Fernando L Gordillo Altamirano: Conceptualization, Investigation, Visualization, Writing - original draft. **Jeremy J Barr:** Conceptualization, Investigation, Writing - review & editing, Supervision, Funding acquisition.

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