A perfect fit: Bacteriophage receptor-binding proteins for diagnostic and therapeutic applications

Review Article

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189957 - Engineered bacteriophages as antibiotic alternatives for treating catheter associated urinary tract infections (CAUTIs) (SNF)
Bacteriophages are the most abundant biological entity on earth, acting as the predators and evolutionary drivers of bacteria. Owing to their inherent ability to specifically infect and kill bacteria, phages and their encoded endolysins and receptor-binding proteins (RBPs) have enormous potential for development into precision antimicrobials for treatment of bacterial infections and microbial disbalances; or as biocontrol agents to tackle bacterial contaminations during various biotechnological processes. The extraordinary binding specificity of phages and RBPs can be exploited in various areas of bacterial diagnostics and monitoring, from food production to health care. We review and describe the distinctive features of phage RBPs, explain why they are attractive candidates for use as therapeutics and in diagnostics, discuss recent applications using RBPs, and finally provide our perspective on how synthetic technology and artificial intelligence-driven approaches will revolutionize how we use these tools in the future.

Introduction
Bacteriophages (phages) are environmentally ubiquitous viruses that solely infect bacteria, and often with a specific and limited host range. The majority of phages being explored for diagnostic [1] and therapeutic [2–6] applications are tailed dsDNA phages from the order Caudovirales that feature a tail organelle for translocation of their genomic DNA into the cytoplasm of a host bacterium [7–9]. Emanating from the tail are specialized receptor-binding proteins (RBPs) identified as tail fibers (TFs) or tailspikes (TSPs) that interact with specific ligands displayed on the surfaces of their bacterial hosts (Figure 1). As the first point of contact with bacteria, the binding range of phage RBPs is the primary determinant of their host range.

Similar to the evolution and adaptation of antimicrobial resistance mechanisms, competitive bacteria–phage coevolution has driven bacteria to develop a diverse array of intracellular antiphage defenses [10], with many still being discovered [11,12]. However, the more direct route to phage resistance for bacteria, as observed both clinically and experimentally, is to simply remove, modify, or block access to the surface-exposed phage receptors via phenotypic variation or spontaneous mutation. Naturally, phages have evolved effective counterstrategies to maintain their phenotypic diversity and infectivity [13,14], which has subsequently led to the evolution of a morphologically diverse repertoire of specialized RBPs and tail machineries optimized for infection of their target hosts [15]. In fact, the phage tail apparatus has been such an evolutionary success that phage tail-like complexes appear to be widespread in nature and adopted by bacteria for other purposes, from tailocins, for example, the Pseudomonas R- and F-type pyocins [16] to the type-VI secretion systems [17]; and the extracellular contractile injection systems involved in microbial competition across taxonomic domains, including effector injection into eukaryotic cells [18].

Structural features of receptor-binding proteins
RBPs can be classified as tail fibers (TF) or tailspikes (TSP). Morphologically, both types are composed of homotrimeric complexes, with TFs twisting their chains into long filaments that interact with proteinaceous and/or saccharidic receptors, whereas TSPs form more globular complexes featuring a central domain with enzymatic activity toward carbohydrate moieties on the bacterial surface [20].

Tail fibers
The three chains of a TF typically fold into an interweaving, β-helical ‘spine’, terminating with a distal tip domain of various shapes and sizes with exposed receptor-binding sites. For example, the distal segment of the phage T4 long tail fiber (gp37) [21] and the TF of...
Salmonella phage FO1a (gp75) (Figure 2a) both terminate with needle-like binding tips, whereas the TFs of phages T7 and T5 fold into more globular, ‘bullet-like’ heads [22,23] (Figure 2e,f). Alternatively, some TFs display adhesins or tail fiber assembly (Tfa) proteins at the distal ends that can confer receptor binding and/or act as chaperones to assist with TF maturation [24–27]. The attachment of adhesins to the tips of TFs is prevalent among the T4-like phages (excluding T4 itself). For example, attached to the end of the long tail fiber (LTF) of Salmonella phage S16 is a single adhesin protein (gp38) (Figure 2b). The C-terminus of the gp38 adhesin features ten polyglycine type-II (PGII) helices that fold into an unusual ‘PGII sandwich domain’ with exposed residues and β-turn loops that dictate receptor specificity [25]. The use of dual-function chaperones is best demonstrated by the Tfa protein of E. coli phage Mu (TfaMu), which functions first as a chaperone to assist with TF folding [27] and subsequently remains bound to the fiber tip and has been shown to interact with lipopolysaccharide (LPS) receptors [28] (Figure 2d). Distinct organizational similarities can be drawn between the receptor-binding tips of TFs and the complementarity-determining regions (CDRs) formed by the heavy and light variable chains of antibodies [25] (Figure 2g). In both cases, sequence variability within terminal loops and surface residues dictates the binding of specific bacterial receptors or antigens, respectively. Analogous to antibody engineering, the binding range of TFs can thus be altered using targeted or random mutagenesis within these receptor-binding sites to create new RBP or whole phages with variable binding ranges and affinities [29–31].

Tailspikes

While the N-termini of both TFs and TSPs function as specialized anchoring domains for attachment to the phage particle (typically to a baseplate structure at the distal end of the tail), the C-terminal domain(s) of TSPs differ significantly to TFs as they form globular structures with enzymatic activity that mediate binding and subsequent degradation or alteration of receptors [8,32,33]. In most cases, TSPs feature hydrolase, lyase, or esterase functions that act on sugar moieties such as...
A selection of structurally distinct tail fibers and tailspikes. All structures are shown as ribbon representations generated using a combination of structures solved by X-ray crystallography (blue line indicates each section and the corresponding PDB ID) and models predicted using AlphaFold-Multimer (identified by red lines) [19,42].

Representative TFs shown are (A) the T4-like TF of *Salmonella* FO1a gp75 that binds to *Salmonella* LPS (unpublished); (B) the LTF of phage S16 contains a receptor-binding adhesin attached to its distal tip (orange) (PDB ID: 6F45) [30]. The broad binding range of the S16 LTF was exploited in the development of an Enzyme-linked immunosorbent assay (ELISA)-like assay for *Salmonella* detection; (C) the Mu-like TF (shown in (D)) of *Burkholderia* phage E094 requires coexpression of Tfa proteins that assist with fiber folding and remain bound to the mature fiber tip (as shown in (D)). The E094 TF was recently developed into an agglutination assay for rapid detection of *B. pseudomallei* [24]; (D) the...
the teichoic acids for Gram-positive hosts or LPS and capsular polysaccharides (CPS) of Gram-negative hosts [34–39] (Figure 2 H–K). Many phages incorporate multiple TSPs in branched networks on their baseplates [35,40,41], such as the four TSPs used by Kutteriviridae phages CBA120 [35] (e.g. TSP2 and TSP3 shown in Figure J and K) and EP75 [41] with each TSP presenting activity against a specific E. coli or Salmonella O-antigen, another example of how phages have adapted to expand their host range.

**Exploiting receptor-binding proteins as diagnostic probes and antibacterial agents**

Owing to their high specificity toward their bacterial host receptors, RBPs are ideal candidates for development as bioprobes for labeling, detection, affinity-based immobilization, and separation of target bacteria. Furthermore, due to the ability of certain TSPs to enzymatically remove important components and virulence factors of bacteria cell walls (e.g. CPS), there is growing interest in leveraging TSPs as a new class of antivirulence tools. Below are examples of how the unique properties of RBPs have recently been exploited.

**Receptor-binding proteins as diagnostic bioprobes**

Table 1 provides an overview of RBP-based detection assays reported in the last three years. Among the most recent is the development of a latex bead agglutination assay using the TF of phage E094 as a bead-conjugated biobead for rapid detection of Burkholderia pseudomallei, the causative agent of melioidosis (Figure 2e). In a field evaluation using 300 patient isolates taken from different specimens (e.g. urine and blood), the assay presented a diagnostic accuracy of 90%, sensitivity of 98%, and specificity of 83% (the latter being improved through assay buffer modification) [24]. An alternative approach developed by Shi et al. leveraged the different interaction mechanisms of phage TFs and the antibiotic Polymyxin B to their respective cell wall targets to produce a dual-site detection assay for Pseudomonas aeruginosa. In brief, the TF of phage PaP1 TF was used as a highly specific biobead for bacterial immobilization before applying tracer Fluoreseinisothiocyanate (FITC)-conjugated Polymyxin B as a highly sensitive recognition element [45]. He et al. used the same recombinant PaP1 TF conjugated to magnetic beads to immobilize and separate P. aeruginosa from solution for downstream identification, an approach the authors demonstrated could also be combined with antimicrobial susceptibility testing [46]. The foodborne pathogens Yersinia enterocolitica and Y. pseudotuberculosis are notoriously hard to detect, especially with traditional culture-based methods. As such, Filik et al. used recombinant TFs from Yersinia phage YeO3–12, to develop an ELISA-based assay specific for the pathogenic Y. enterocolitica serotype O:3 [47]. For viable but nonculturable (VBNC) E. coli detection, Costa et al. combined magnetic enrichment with spectrophotometric approaches using the RBP of phage 285p as a biobead. The assay could detect VBNC E. coli cells in different clinical matrices (e.g. blood and urine), providing a clear advantage over conventional culture-based methods [48]. As a final example, a Salmonella enterica detection system using the LPS-binding Det7 TSP (Det7T) was developed. Here, TSPs were used as the sensing elements in a surface-plasmon resonance biosensor that could detect Salmonella down to concentrations of 5x10^4 CFU/mL within 20 min [49].

**Receptor-binding proteins as antibacterial agents**

Phages employ enzymatic TSPs to bind and degrade (or modify [34]) extracellular, saccharidic barriers such as the LPS, CPS, or components of biofilms as they navigate their tail apparatus toward the outer membranes of a target host cell. Together with endolysins, TSPs can also be referred to as ‘enzymbiotics’ [33,54]. A comprehensive review of the different depolymerase functions of TSPs can be found here.

**Klebsiella** are notorious producers of CPS barriers that facilitate tissue invasion and fend off attacks from the immune system, antibiotics, and phages [55]. Accordingly, the development of therapeutic agents that can inhibit or remove Klebsiella CPSs is of great clinical interest. Coevolution of Klebsiella phages with their capsule-protected bacterial counterparts has naturally led to an expansion in their use of TSPs featuring capsular depolymerase activity, with various phages carrying multiple antiCPS TSPs on their tails [56,57]. These TSPs have been developed by several groups to degrade the capsular types (e.g. K1 and K2) found in multidrug-resistant variants of K. pneumoniae [37,58,59]. AntiCPS TSPs can significantly reduce K. pneumoniae biofilms in vitro, and showed promising results in vivo against carbapenem-resistant K. pneumoniae infections in mice [58]. The uniform products of enzymatic degradation by
certain TSPs can also be exploited for vaccine production. For example, antiCPS TSPs were recently used to process *K. pneumoniae* K1 and K2 CPSs into oligosaccharide repeat units (complete with intact modifications), conjugated to carrier proteins, and subsequently applied as vaccines against *K. pneumoniae* in mouse models [60]. Similarly, TSPs have been developed as antivirulence agents against *Acinetobacter baumannii*, another CPS ‘specialist’ and significant cause of hospital-borne infections worldwide [61]. For example, the anticapsule TSP from *A. baumannii* phage PMK34 was used to remove the CPS barrier of *A. baumannii* cells in vitro and resensitize the pathogen to serum killing, enabling a greater than 5-log reduction of *A. baumannii* in human serum [62]. Finally, an array of capsule (K1, K5, and K30)-specific TSPs, for example, K1F (Figure 2i), have been used to resensitize *E. coli* to serum killing, however, with varying efficacy when tested in a mouse thigh infection model that was linked to the stability of the different recombinant TSPs [43].

**Receptor-binding protein fusion constructs as antibacterial agents**

‘Innolysins’ are hybrid enzybiotics that combine the enzymatic activity of endolysins with the binding specificity of RBPs [63]. As proof of concept, Zampara et al. fused the *E. coli* phage T5 endolysin and its tail fiber (Pb5) to produce an Innolysin-presenting antibacterial activity against *E. coli* and other species (e.g. *P. aeruginosa* and *Shigella sonnei*) when they had the same Pb5 receptor, FhuA, within their cell walls [63]. More recently, the Innolysin concept has expanded to develop Innolysins against *Campylobacter jejuni* [64].

**New approaches to identify and characterize receptor-binding proteins**

Even though the amino acid sequences of RBPs are highly diverse, there is a significant amount of domain conservation, allowing identification of putative RBPs (and their characterization as TFs or TSPs) by amino acid sequence and basic structure predictions. Novel approaches try to computationally predict phage host range from the various tail protein sequences [32]. However, experimental confirmation of RBPs and their bacterial targets is not a trivial task, especially when based on amino acid sequence alone. A few novel screening approaches have therefore been proposed to identify these proteins of interest. Gonzalez and Scharf developed a screen to identify the RBPs of flagellotropic *Acrobacterium* phage 7–7–1 [65], and Huss et al. used deep mutational screening to find receptor-binding sites of the T7 TF [66]. The amino acid sequences of known RBPs can also be used to predict a certain phage’s host range based on machine-learning algorithms [67]. With the advance of computational methods, we expect more such approaches to evolve and many more potential RBP functions to be identified.

**Understanding receptor-binding protein-ligand interactions: where do we go from here?**

The last few years has also seen incredible advancement in artificial intelligence-based modeling tools, such as AlphaFold [42], which are revolutionizing the field of structural biology by providing high-confidence models of proteins and their complexes within relatively short time-frames. It is therefore of no surprise that these tools are already being used to explore the structures and functions of interesting RBPs (Figure 1; Table 2) [62,68–70]. Nevertheless, despite these breakthroughs in protein modeling, combined with a growing number of RBP and phage tail structures solved empirically using X-ray crystallography and (cryo-)electron microscopy (Table 2), there remain a number of ‘blind spots’ in our understanding.

Principally, a deeper understanding of how the diverse array of RBPs interact on the atomic level with a similarly heterogeneous assembly of proteinaceous and/or saccharidic receptors is greatly desired. A number of high-resolution structures have been resolved for phage TSPs and their cell surface ligands, however, these structures typically reveal the interactions of only single saccharidic repeats (i.e. the products of TSP enzymatic degradation), and while these structures have provided important
atomic-level details regarding enzymatic process of TSPs, and identified unique ligand interaction sites, they do not reveal how intact, oligosaccharidic receptors entwine and interact across the complete surfaces of RBPs. Far less is known about the molecular interactions between TFs and their receptors, with the identification of binding sites limited to investigations with mutational studies and/or protein docking simulations (e.g. for studying phage T4 LTF and OmpC interactions [31,71]). Excitingly, the TF of phage T5, Pb5 (Figure 2e), was recently solved in complex with FhuA, providing the first high-resolution structure of a phage TF (T5 Pb5) interacting with a proteinaceous receptor (FhuA), an approach that could be expanded for modeling complex ligands into RBP-binding sites. However, predicting how protein structures change upon ligand binding, and how disordered receptor-binding sites could assume different folds during binding, remains a challenge [73]. Experimental determination and verification of RBP function is still needed, and macromolecular (baseplate and tail) cryo-EM studies to decipher activation mechanisms of phage tails are highly desirable. Importantly, more and more RBP-ligand interactions are being systematically dissected on an atomic level to reveal residues and regions that are critical for ligand binding and which are amenable to mutation in order to manipulate RBP-binding affinities and modification of binding ranges [29–31,35,37,66,68,74,75]. Such studies are essential for further development of RBPs as bioprobes or depolymerase tools and provide the necessary atomic-resolution ‘blueprints’ for host range programming of whole, engineered phage therapeutics.

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**Table 2**

Recent advancements in structure-function analysis of phage RBPs.

| Phage | Host organism | Main findings | Year | Reference |
|-------|---------------|---------------|------|-----------|
| S16   | *Salmonella*  | TF and attached adhesin structure | 2018 | [25] |
| TW1   | *P. phenolica* | Structure of the virus, focus on TSPs | 2018 | [76] |
| Det7  | *Salmonella*  | Time-resolved DNA ejection and crystal structure of TSP | 2019 | [74] |
| CBA120| *E. coli*     | Structure of ORF211, the specificity determinant TSP and structure of two other TSPs | 2019/2020 | [35,38] |
| A511  | *Listeria*    | Baseplate and tail structure | 2019 | [8] |
| Mu    | *E. coli*     | Tail fiber structure and folding | 2019 | [27] |
| PR722 | *E. coli, S. typhimurium, and so on* | High-resolution virus structure | 2019 | [77] |
| T4    | *E. coli*     | Interaction between T4 TFs and OmpC on an amino acid level. | 2019/2021 | [31,71] |
| Unnamed | *B. argentinensis* | TF and receptor-binding tip structure of an unknown prophage | 2020 | [78] |
| YeO3–12| *Y. enterocolitica* | Predicted structure of the gp17 TF and functional studies | 2020 | [79] |
| KP32  | *K. pneumoniae* | Crystal structure of gp38, a CPS depolymerase TSP | 2020 | [37] |
| GVE2  | *Geobacillus* | Crystal structure of its TSP combined with functional characterization | 2020 | [80] |
| V22   | *Alteromonas* | Recombinant proteins and functional studies of receptor-binding candidates | 2020 | [26] |
| 80x   | *S. aureus*   | Baseplate structure, atomic models of its RBPs | 2020 | [81] |
| SPP1  | *B. subtilis* | Molecular organization of the phage, structure, and assembly of TFs | 2021 | [82] |
| EP75 and EP355  | *E. coli* | Structural and functional characterization of two distinct RBP sets | 2021 | [41] |
| OE33PA and Vinitor 162 | *O. oeni* | AlphaFold predictions of different phage adhesion candidates | 2021 | [69] |
| T7    | *E. coli*     | Structure of the mature T7 phage in full and empty state with receptor bound | 2021 | [9] |
| T7    | *E. coli*     | Systematic dissection of the T7 receptor-binding tip using deep mutational scanning to identify individual residue functions | 2021 | [66] |
| G     | *Lysinibacillus* | Tail structure in contracted and uncontracted state | 2021 | [63] |
| Pam1  | *P. mucicola* | Structure and assembly pattern of phage, including tail spike protein | 2022 | [84] |
| PMK34  | *A. baumanii* | Prediction of capsule depolymerase structure and functional studies | 2022 | [62] |
| T5    | *E. coli*     | First high-resolution structure of a phage TF (T5 Pb5) interacting with a proteinaceous receptor (FhuA) | 2022 | [68,70] |
Conflict of interest statement
M.D. is a part-time employee of Micreos Pharmaceuticals. M.J.L. is a scientific advisor for Micreos Pharmaceuticals. J.K. declares no conflict of interest.

Data Availability
No data were used for the research described in the article.

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Supporting information
Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mib.2022.102240.

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An extensive bioinformatic analysis of the structural and genetic organization of depolymerase-containing RBP s in Klebsiella phages. Describes the modular composition and reuse of certain anchoring and branching domains that enables domain exchange between phages to rapidly shift capsular serotype specificity or to expand the spectrum, again, providing important information that could be used for engineering.

In this innovative study, the authors solve the problem of loss of immunogenicity of Klebsiella capsular polysaccharide in vaccine development by using two phage-derived depolymerases (TSPs), which yield an immunogenic conjugated Klebsiella pneumoniae vaccine. Mouse data is shown to support the effectiveness of the novel vaccine.

Groundbreaking study, which ‘transport s’ the concept of lysis-from-pherotype by deep mutational scanning. Elife 2021, 10:1-30. Systematically dissected the functional role of all residues within the receptor binding tip of the phage T7 TF (Figure 1). This approach built a functional map of the TF tip and identified individual residues important for host-specific interactions. Variants (1,560 in total) were identified for their different functional gains, such as modified host range or the ability to target phage resistant hosts. This approach combines studying sequence-function relationships with protein engineering and could be harnessed for engineering the host ranges of therapeutic phages.

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