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Toothpicks, logic, and next-generation sequencing: systematic investigation of bacteriophage-host interactions☆

Aisylu Shaidullina and Alexander Harms

Bacteriophages are abundant and diverse predators that drive community dynamics in many ecosystems and hold great potential for biotechnology and as therapeutics for bacterial infections. Previous research has largely explored phage-host interactions one-by-one, which limited our ability to observe phenotypic patterns, to uncover their genetic basis, and to unravel the underlying molecular mechanisms. However, the famous 'toothpicks and logic' were recently joined by large-scale sequencing of phage genomes and bacterial genome-wide screens that enable us to systematically investigate phage-host interactions. In this article, we highlight recent breakthroughs from the molecular basis of phage host range and receptor recognition over new insights into bacterial immunity to the serendipitous discovery of a new bacterial surface glycan. Future work will enable the understanding, prediction, and engineering of more complicated phage traits for new applications and extend the scope of these studies from simple test tube experiments to natural communities of phages and hosts.

Address
Biozentrum, University of Basel, Basel, Switzerland

Corresponding author: Alexander Harms (alexander.harms@unibas.ch)

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Introduction

Bacteriophages (or short 'phages') are the viruses infecting bacteria and top predators of the microbial world [1,2]. The ubiquity, abundance, and diversity of phages make them a major driving force of community dynamics in all ecosystems from the human microbiome to the open ocean with implications for human health and biotechnology [2–4]. The so-called tailed phages (*Caudovirales*) are most frequently isolated and studied in the laboratory, comprising three major types of virion morphology, including myoviruses (contractile tail), siphoviruses (long, flexible tail), and podoviruses (short, stubby tail) [1,2]. Phages can have two different lifestyles being either virulent — that is, always killing their host through direct replication — or temperate with the alternative of integrating into the host genome as a prophage to form a so-called lysogen [1,2]. Their accessibility as model systems made phages major workhorses of the first golden age of molecular biology around the middle of the last century [5], and they have in parallel been used for decades as potent therapeutics to treat bacterial infections [6].

However, our knowledge about the molecular basis of phage biology and, consequently, its applications, is limited by the historical focus on a small number of model phages infecting few hosts such as *Escherichia coli* or *Bacillus subtilis*. Nevertheless, even for these phages, many "data and experiments remain largely disorganized, anecdotal, and poorly cross-comparable" [7••]. In this article, we therefore argue that a more systematic approach to study phage-host interactions holds great potential to reveal patterns in the data, which might indicate molecular mechanisms and their underlying genetic basis that would otherwise be inaccessible (Figure 1). Such insight could hand us the keys to unlock the prediction of relevant phage properties from viral genomes, a more rational selection of phages for therapy, and countless new options for phage engineering to overcome the limitations of natural viruses [7••–9].

T-phages and the 'phage treaty' initiate the golden era of molecular biology

While there is no single starting point of systematic research on phage-host interactions, we feel that the 'phage treaty' announced by Max Delbrück in 1944 is a

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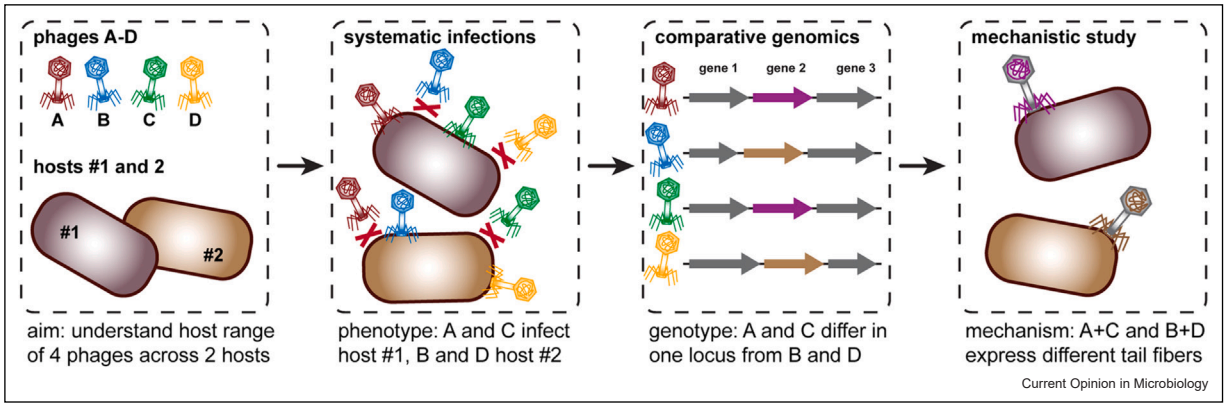
Figure 1



Schematic overview of how systematic approaches reveal new phage biology. Starting with two exemplary host strains (#1 and #2) and four exemplary phages (A–D), this illustration shows how systematic approaches rather than an investigation one-by-one can uncover the molecular basis of phage host range. Briefly, the combination of an observed pattern in phenotypic data (second from left) with a genomic analysis (third from left) indicates a genetic basis of the observed host range. Mechanistic studies (right) later uncover that the genetic differences cause the expression of different tail fiber variants that cause the observed host range differences.

key turning point [10]. With the explicit aim of building a deep body of interconnected results, Delbrück suggested that researchers should work with only one bacterial host strain (*E coli* B) and seven phages T1–T7. These had been chosen from previous work based on their diversity (as estimated by plaque morphology) and following practical considerations such as well-countable plaques. The T-phages as such were first described by Demerec and Fano in 1945, who used them to study phage cross-resistance based on the manual analysis of hundreds of *E. coli* mutants [11]. This work and others initiated a golden era of molecular biology from the 1950 to 1970s in which the T-phages and few others such as lambda or P1 were studied at unparalleled depth to unravel fundamental molecular principles of life using not much more than 'toothpicks and logic' [1,2,12]. These studies are still a major reference point for phage research today and have developed many critical tools of modern molecular genetics from restriction–ligation cloning over Cre–*loxP* recombination to different ways of controlling ectopic gene expression [1,2].

Techniques and tools to systematically investigate phage-host interactions

These tools enabled researchers to study the molecular mechanisms underlying the development and cell biology of complex organisms such as *Drosophila*, mice, or humans, which resulted in a greatly reduced interest in phage biology until the new millennium. Besides the renaissance of phage therapy to counter the escalating crisis of antibiotic resistance [6], the re-emergence of phage research in the early 2000s was largely driven by the high throughput and comparably low cost of newly available next-generation sequencing technology [2,13]. When applied to environmental metagenomes, these techniques revealed the vast abundance and diversity of phages across ecosystems, which triggered a new wave of research on their biology [2–5]. Subsequently, the further development of these high-throughput sequencing technologies has enabled new approaches to explore the bacterial genetics of phage sensitivity or resistance. Simple genome-wide loss-of-function screens in model organisms such as *E. coli* are either based on targeted gene knockouts or gene disruptions by a transposon and have enabled researchers to systematically identify host factors that are required for phage infections [14–16]. This approach is inherently limited by gene essentiality, though this issue can be overcome by clustered regularly interspaced short palindromic repeats (CRISPRi)-Seq, that is, by replacing permanent knockouts with the inducible transcriptional repression of genes by a catalytically inactive CRISPR–Cas9 system [17,18••]. An orthogonal approach to explore phage-host interactions is the quantification of phage infectivity upon overexpression of host genes ('Dub-Seq') [19••].

While these techniques are very informative about the host side of phage infections, they can only indirectly probe the viral side, and phage genetics so far lacks the throughput to develop analogous approaches. Instead, key insights are primarily gained from the genomic and phenotypic comparison of different bacteriophages. We recently described an assorted 'BASEL collection' of ca. 80 *E coli* K-12 phages as an expanded and rationally composed set of models for systematic experiments analogous to the original concept of the T-phages [20••]. Notably, these phages cover all known major groups of virulent, tailed phages infecting *E. coli* K-12. Despite the incredible diversity of bacteriophages, such coverage is possible because most phages isolated on a certain host

belong to a limited number of abundant taxa while outliers are rare [3,21–23]. The diversity and intensive characterization of BASEL phages make them a powerful tool analogous to the Escherichia coli reference (ECOR) collection of *E. coli* strains that are commonly used to explore the diversity of this species [24].

Systematic investigation of bacteriophage host receptors

Host-centered genetic screens of phage infections are generally based on bacterial survival after phage challenge. This feature limits the depth of investigation because an inhibition of phage infection downstream of genome injection would usually still result in host cell death. These screens therefore primarily reveal the identity and upstream signaling of phage receptors on the bacterial cell surface, but these are of major interest as key determinants of phage-host range and spontaneous bacterial resistance [25,26]. Consequently, genome-wide screens on phage-host interactions are commonly benchmarked by confirming the known surface receptors of classical *E. coli* model phages and their signaling networks [14–16,18••,19••,25]. These approaches can then be applied to any other phages and have, for example, been used to determine the lipopolysaccharide (LPS) core as the terminal surface receptor of the understudied yet environmentally abundant myoviruses of the *Vequintavirinae* subfamily that infect enterobacteria [14,20••,27•].

In other cases, open-minded genetic screens have revealed unprecedented biology that had been hidden in plain sight. A landmark study by Mutalik and colleagues revealed that phage N4 infection depends on the bacterial second messenger cyclic di-GMP (c-di-GMP) that normally controls sessile behavior and biofilm formation [19••]. Genetic perturbations that would decrease c-di-GMP levels totally abolished N4 infection as previously only observed for knockouts of the suspected receptor components *nfrA* and *nfrB*. Follow-up work later showed that the actual surface receptor of phage N4 is a previously unknown enterobacterial surface glycan now called N4 glycan receptor (NGR) that is exported by the NfrAB machinery and controlled by c-di-GMP signaling [28•,29].

Beyond individual results, all these studies on phage receptors of *E. coli* and related enterobacteria have confirmed and extended the pattern that siphoviruses virtually always target porins on cell surface, while podoviruses target glycans and myoviruses can target either of these [30]. As an example, the large siphoviruses related to T5 (family *Demerecviridae*) were long known to express receptor-binding proteins that are noncovalently loaded onto the tip of the phage tail, often targeting BtuB but occasionally also other porins such as FhuA or FepA [19••,20••,27•,31]. Based on a systematic comparison of experimentally determined receptor specificity and phage genomes, we recently discovered an analogous mechanism shared by different groups of small siphoviruses that recognize a larger set of porins [20••]. Notably, a considerable number of these phages specifically target LptD, the essential LPS export channel of Gram-negatives, which had previously been overlooked as a phage receptor because it is essential for bacterial viability [20••]. We also confirmed previous notion that the large myoviruses of *Tevenvirinae* do not only target porins as cell surface receptors but that some also require parts of the LPS core for infectivity [19••,20••]. This behavior correlated with an alternative allele of the short-tail fibers that mediate the final, irreversible adsorption of the virus to the cell surface, suggesting that there are at least two different ways of how T-even phages arrange this final, committed step of host recognition [20••].

Systematic investigation of host cell barriers and bacteriophage host range

The different layers of external barriers to phage infection on the cell surface are intimately connected to phage-receptor recognition. Intriguingly, several genetic screens on *E. coli* phage infections showed that any intervention inducing the Rcs signaling pathway — known to counter cell envelope stress by activating colanic acid biosynthesis — caused broad phage resistance, likely because this exopolysaccharide blocks access to phage receptors on the cell surface [15•,18••,19••]. While mucoidy caused by colanic acid secretion had been known before to cause phage resistance in *E. coli* [32], the strength and breadth of this phenomenon had not been anticipated.

However, the most important surface barrier of Gram-negative bacteria is the O-antigen formed by long glycan chains at the distal end of the LPS in their outer membrane. This glycan has been lost by the commonly used *E. coli* K-12 and B strains during laboratory domestication, but is ubiquitously expressed among natural isolates where it forms a barrier that shields cell surface receptors from phage recognition. Consequently, the vast majority of phages in the BASEL collection lose the ability to infect their *E. coli* K-12 host when O-antigen expression is genetically restored, likely because they lack specific tail fibers to recognize the O16-type O-antigen expressed by this strain [20••]. One important exception are phage N4 and its relatives as well as the *Vequintavirinae* that can both bind the NGR glycan and thereby likely bypass the O-antigen barrier on their way to the cell surface [20••,28•].

However, O-antigen glycans can also serve as an essential phage receptor themselves [33]. In *Salmonella* and *E. coli* strains expressing O-antigen, many tested podoviruses such as P22, distant relatives of N4, and T7-like

phages (*Autographiviridae*) depended on O-antigen recognition for infectivity [27•,31,33]. This result is a strong example for the value of studying hosts beyond the standard laboratory strains of *E. coli* to explore features of phage-host interactions that are not accessible in these model organisms. Work on more distantly related bacteria such as *Pseudomonas aeruginosa* revealed even more relevant differences in phage-host recognition. While enterobacterial phages often bind porins on the cell surface, *P. aeruginosa* phages near-exclusively target either the LPS or the type-IV pili that are critical for virulence and lifestyle of this organism, possibly because the cell surface is commonly shielded by exopolysaccharides [34,35].

The access to powerful genomic analyses in the age of next-generation sequencing has recently enabled researchers to broadly characterize large numbers of phage isolates sampled for a given host such as *E. coli* K-12 or *P. aeruginosa* [23,36], often combined with a host range analysis across a panel of strains from the same species and related organisms [22••,37]. These studies highlighted interesting differences in host range between different phage groups. While phages infecting staphylococci often exhibited a cross-species host range (likely by targeting conserved components of the Gram-positive cell wall and/or due to prevalent polyvalency), phages infecting *E. coli* were limited to a few strains of this organism [22••,37]. However, *E. coli* phages targeting the NGR as cell surface receptor — N4-like podoviruses of the *Schitoviridae* and myoviruses of the *Vequintavirinae* — exhibited very broad host recognition, possibly aided by the wide conservation of this glycan that could be exploited as a path to bypass the O-antigen barrier [14,20••,28•].

Systematic investigation of bacterial immunity to phage infection

Besides host recognition and extracellular barriers, bacteriophage-host range is determined by intracellular mechanisms of bacterial immunity that have recently become a major focus of phage research [38]. An early landmark study by Bondy-Denomy et al. systematically explored the cross-resistance of lysogens of thirty temperate phages of *P. aeruginosa* to each other's virions [39••]. Briefly, the authors found that almost all lysogens were resistant to at least one other phage due to prophage-encoded defenses ranging from receptor inactivation (by manipulation of O-antigen or type-IV pili) to intracellular interference. This work was highly influential because many of these phages continue to be studied in the field and because several lysogens were later shown to have impaired CRISPR–Cas functionality, an observation that directly resulted in the discovery of the first anti-CRISPR (Acr) proteins in their prophages [40].

The discovery of new bacterial immunity systems typically begins with their computational prediction in 'defense islands' of bacterial genomes or hypervariable loci of prophages [41,42]. After confirming functionality of a new immunity system, the key to unraveling its molecular mechanisms are often spontaneously immunity-resistant phage mutants. Besides altering a direct trigger of the new immunity system, these 'escape mutants' can obtain resistance in different ways that are all informative about the molecular basis of immunity [42,43]. As an example, two variants of the DarTG defense system specifically targeted the T4 relative RB69 or siphoviruses T5 and SECphi18, respectively [43]. While T5 failed to evolve resistance to DarTG, the other phages became resistant either by activation of an anti-immunity protein (RB69) or by mutating DNA polymerase to overcome the inhibition of DNA replication by DarTG (SECphi18) [43]. Owing to these specific associations, it is advantageous to employ a wide diversity of phages when characterizing new immunity systems.

When testing the BASEL phages for this purpose, we confirmed previous notion that classical restriction-modification (RM) systems are a potent first line of bacterial defense against foreign DNA. As expected, diverse RM systems strongly inhibited almost all tested phages, except those with covalent DNA modifications such as the T-even phages and some groups of small siphoviruses [20••,44]. In parallel, we also explored other immunity systems that were more elusive. While the RexAB and PifA systems only inhibited each one or two phages very specifically, others had a much broader target spectrum and revealed exciting patterns due to the assorted composition of the BASEL collection. As an example, we confirmed that Tin — known to target the DNA replication of T-even phages — specifically inhibited all representatives of this group but no other phage. Conversely, Fun — whose molecular mechanism is completely unknown — potently inhibited all phages with large genomes, including all tested myoviruses of *Tevenvirinae* and *Vequintavirinae*, as well as large siphoviruses of *Demerecviridae* [20••]. A promising path toward understanding Fun would therefore be to systematically isolate and characterize escape mutants from phages of these different groups as a key to unlock its molecular mechanism.

Applications and future directions of systematic phage research

In this review, we highlighted how recent systematic studies of phage-host interactions have advanced our understanding of their molecular mechanisms in unprecedented ways. While technological breakthroughs such as next-generation sequencing have been crucial for these studies, their core ideas are more an innovative

extension of the 'toothpicks and logic' approach that has driven phage biology already through its last century of successes [12].

A major strength of systematic studies is that they highlight patterns in the behavior of phages, for example, within a given taxonomic group or across taxonomic groups, that can be leveraged to unravel the genetic basis of these phenotypes by comparative genomics. Notably, the described features of porin recognition by *E. coli* siphoviruses or the LPS core binding of T-even phages had only a small number of different states, so that the BASEL collection was diverse enough to capture and understand them [20••]. Consequently, this knowledge can now be used to predict the receptor specificity of newly isolated phages and throughout multiple related genomes in the databases [20••]. More complicated features such as the specificity of phage tail fibers for the around two hundred different types of *E. coli* O-antigen or sensitivity/resistance to known immunity systems will require a considerably larger data set for reliable predictions. However, there is a great clinical and technological interest in predicting and, consequently, engineering these features and others in the next generations of 'designer phages' for medical applications, agriculture, and other purposes [7••–9]. Another application of this knowledge is the construction of bacterial strains that are more refractory to phage infection, for example, to guard industrial processes against failure due to phage contamination [45].

A systematic perspective on the ecology of phage-host interactions

While we have highlighted the clinical and biotechnological implications of systematic studies on phage diversity, this knowledge is also crucial for understanding the ecology and evolution of phages in natural ecosystems. As an example, the global dominance of a finite number of phage groups for a given host is generally understood as an indication that these groups each have different biological features that enable them to partition the ecosystem into different niches [3,21,46]. While the molecular basis of these proposed 'functional types' has remained largely elusive [46], systematic comparative studies on different groups of phages would be an efficient way to approach this question in the laboratory. As an example, the systematic phenotyping of the BASEL phages has revealed striking differences between phage groups regarding their host range or strategies to deal with host immunity, which are likely part of these divergent ecological profiles [20••]. This question will eventually need to be studied closer to natural environments to capture the complexity of phage-host interactions in real life [47], for example, like it has been pioneered for different *Vibrio* species and their phages in two recent landmark studies [48•,49•].

Next frontiers and limitations of current approaches

This review article has necessarily inherited the focus of recent studies on tailed phages infecting Gram-negative model organisms (mostly *E. coli*). It would therefore be interesting to see future work systematically exploring the biology of tailless phages such as the environmentally abundant microviruses and inoviruses with similar approaches [50,51], for example, by building upon previous work studying superinfection exclusion of diverse microviruses [52]. These studies could also reveal interesting new aspects of host biology as pioneered by a recent preprint on diverse single-gene lysins of these small phages that exploit any imaginable aspect of bacterial cell wall biosynthesis to lyse their host, a phenomenon with obvious potential to inspire new antibiotic drugs [53]. Another promising avenue would be to extend this research to Gram-positives such as *B. subtilis* or *Staphylococcus aureus* that — owing to their different cell surface properties — are targeted differently by bacteriophages and could thus reveal new facets of biology, for example, in cell wall recognition [30,31].

Conflict of interest statement

The authors declare no conflicts of interest.

Data availability

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

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