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Current Opinion in Microbiology

Review

Tapping the treasure trove of atypical phages

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With advancements in genomics technologies, a vast diversity of 'atypical' phages, that is, with single-stranded DNA or RNA genomes, are being uncovered from different ecosystems. Though these efforts have revealed the existence and prevalence of these nonmodel phages, computational approaches often fail to associate these phages with their specific bacterial host(s), while the lack of methods to isolate these phages has limited our ability to characterize infectivity pathways and new gene function. In this review, we call for the development of generalizable experimental methods to better capture this understudied viral diversity via isolation and study them through gene-level characterization and engineering. Establishing a diverse set of new 'atypical' phage model systems has the potential to provide many new biotechnologies, including potential uses of these atypical phages in halting the spread of antibiotic resistance and engineering of microbial communities for beneficial outcomes.

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Current Opinion in Microbiology 2024, **82**:102555

This review comes from a themed **Carol Gross Special Issue**

Edited by **Mark Buttner** and **Joan Mescas**

Available online 10 October 2024

https://doi.org/10.1016/j.mib.2024.102555

Introduction

The phageome — the community of viruses, or bacteriophages, that parasitize bacterial communities — has been known as a critical feature of microbiomes impacting their ecology, physiology, virulence, and nutrient cycling [1]. Isolation efforts over more than 100 years have shown that phages come in diverse morphologies, genome size, and genome composition and use different infection cycles. Most studies on bacteriophage abundance, diversity, genetic content, host specificity, and their impact on microbial communities have, however, focused on a handful of viral taxa, specifically within the double-strand DNA (dsDNA) phages [2]. This bias in isolating dsDNA phages compared with non-dsDNA phages (e.g. single-strand RNA [ssRNA] and single-strand DNA [ssDNA] phages) primarily comes from the widespread use of methods that are optimized for isolating highly abundant phages and also those that form a zone of clearance on a lawn of bacteria under standard laboratory conditions [3].

Despite their importance, our inability to culture most of the phage diversity present in any ecosystem using standard laboratory conditions has limited our true understanding of phage ecology and how phages influence microbial community dynamics [4]. Meanwhile, recent studies on metagenomes and metatranscriptomes demonstrated the true ubiquity and diversity of phages are largely underestimated [5,6]. In particular, non-dsDNA phages such as ssRNA and ssDNA phages are especially prominent in soil, ocean, human, and wastewater ecosystems [7–9]. Foundational studies on a handful of model non-dsDNA phages have already provided crucial insights into phage biology and have enabled development of a number of invaluable biotechnologies, including diagnostics, therapeutics, imaging, vaccines, phage display, and indispensable molecular biology reagents [10–12]. These applications stem from the unique characteristics and features of non-dsDNA phages (see below) and suggest more biological innovations and knowledge could be gained if more of these phages were available in cultures. Frustratingly however, metagenomic sequencing is often unable to associate these atypical phages with their target host bacterial species, identify infectivity pathways and modes of replication, or characterize mechanism of host lysis [2,7,13]. To fully leverage the potential of atypical phages, systematic methods will need to be developed for isolation, characterization, and engineering of more of these atypical phages that will fuel development of several next-generation biotechnologies, including potential uses in halting the spread of antibiotic resistance and engineering of microbial communities for beneficial outcomes.

In a clear example of such application potential, and owing to their small and simple genome, ssRNA and

ssDNA phages are viewed as a great platform for building completely synthetic viral particles or chimeric phages [13–15]. Early efforts demonstrated that by producing coat proteins without the entire ssRNA phage genome, virus-like capsids/compartments can be obtained via self-assembly of coat-protein monomers [13]. These virus-like particles (VLPs) have found a variety of applications, including diverse vaccine development programs around the world [13–15]. Metatranscriptomics-resolved ssRNA phage diversity offers an incredible opportunity to assemble new chimeric phages as well as build VLPs using sequences from uncultivated phages. Similarly, synthetic ssDNA phages could be constructed and engineered as vehicles for delivering payloads to specific target host(s) in a microbial community.

In this review, we focus specifically on leviviruses (a group of ssRNA phages) and inoviruses (a group of ssDNA phages). We provide an overview of current knowledge on their diversity and distribution, highlight current roadblocks in the establishment of larger culture collections for these phages, and describe how such expanded culture collections could reveal new critical information for these phages, especially regarding phage–host interaction mechanisms. Detailed reviews on these phages and other taxa are given elsewhere [13,16].

Key characteristics of leviviruses and inoviruses

The nonenveloped, positive-sense ssRNA phages of the *Leviviricetes* class (formerly *Leviviridae* family) have unique characteristics, such as the smallest phage genomes (~3–5 kb), and similar genetic content as some of the eukaryotic RNA viruses, including Severe acute respiratory syndrome coronavirus 2 (SARS-COV2) virus, that prompted their use as surrogate for establishing diagnostics of human enteric viral pathogens [2,17]. Levivirus genomes consist of three well-defined genes encoding capsid-forming coat protein, maturation protein, and replicase/RNA-dependent RNA polymerase (RdRp) protein, and a fourth gene spanning and embedded with two of the aforementioned gene regions encodes the lysis protein (Figure 1a,b). Though these RNA phages show clear plaques on a lawn of target *Escherichia coli* , they do not encode multiproteins involved in hosts lysis (e.g. holin, lysin, spanin) as in typical lytic dsDNA phages. Instead, ssRNA phages encode a single gene, encoding a protein that inhibits bacterial cell wall biogenesis without any PG-degrading activity [18,19]. Though the role of ssRNA phages on bacterial fitness, virulence, and evolution is unknown, decades of research on a group of canonical *E. coli* ssRNA phages, such as MS2, Qβ and R17, have enabled diverse biotechnological and biomedical applications [13].

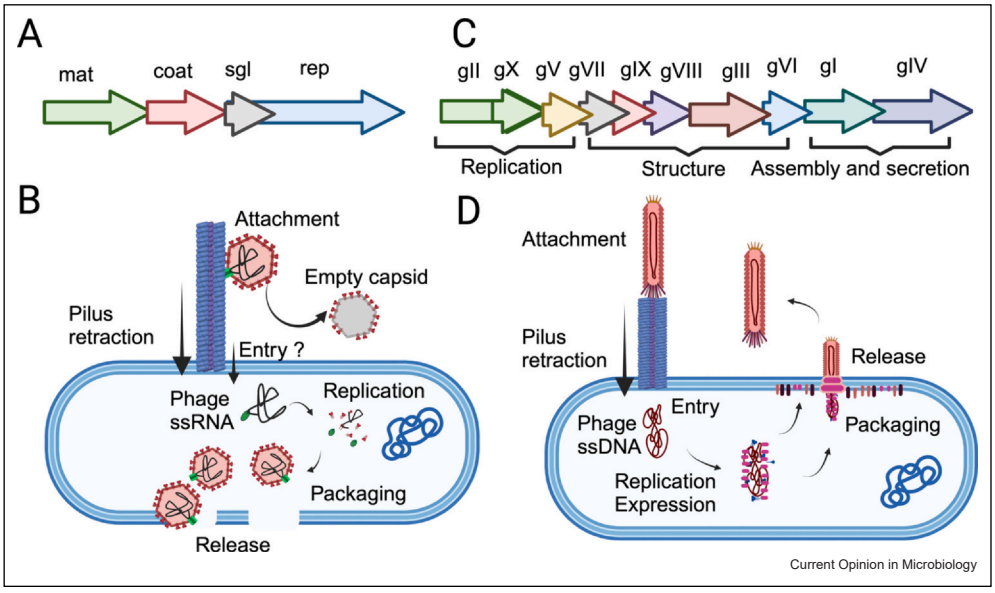
The inoviruses, or 'filamentous phages', are nonenveloped, rod-shaped phages, typically with circular ssDNA genomes belonging to the *Faserviricetes* class. Most of our understanding of these phages comes from highly similar *E. coli* Ff filamentous (F-pilus specific) phages members of the *Inoviridae* family, such as M13 and fd, even though they are unlikely to be representative of the entire diversity of filamentous phages [16,20,21]. These phages have genome size of about 8–10 kb and encode about 10–15 genes involved in morphogenesis and other structural functions (Figure 1c,d). One of the key characteristics of these phages is that they release their progeny through a phage-encoded transmembrane egress machinery without lysing their host cell (Figure 1d). Known examples of inoviruses show turbid plaques on the target bacterial lawn [22] and likely get often unnoticed during bacterial cultivation or phage isolation efforts in favor of clear plaque-forming phages. Decades of work has uncovered different phenotypic traits imparted by a few model filamentous phages on their target bacterial host [16,21,23]. For example, owing to their nonlytic activity, smaller genome size, and engineerability, Ff phages have served as the basis of critical biotechnological applications such as phage display [24].

In addition to their atypical genome types, sizes, and contents, cultivated leviviruses and inoviruses are also distinct from dsDNA phages in their mechanisms of host entry and host range. Well-studied models of both ssRNA and ssDNA phages recognize various types of retractile pili, such as type IV secretion system pilus (e.g. F-pilus encoded by F-plasmid) and the type IV pilus, a filamentous proteinaceous structure that extends from the cell surface of Gram-negative bacteria [25,26], generally encoded on mobile plasmids. Though pili have been observed in Gram-positive bacteria [27,28], there have been no reports on isolation of leviviruses while only a couple of reports on inoviruses [29] that may use pilus as a receptor. A detailed review on diversity, classification, assembly, and mechanisms of different pilus systems is available elsewhere [30–32], but the apparent reliance of most ssDNA and RNA phages on pili systems potentially encoded on mobile plasmids may lead to different phage–host dynamics compared with most dsDNA phages.

Metagenomic view of diversity and abundance of leviviruses and inoviruses

Most of the information regarding the diversity of leviviruses and inoviruses in nature comes from metagenomics, that is, shotgun sequencing of DNA or RNA extracted from a microbiome sample or from prophages identified in whole-genome shotgun sequencing of bacterial isolates. Integrated Microbial Genomes and microbiomes/Viral Resources (IMG/VR) [33] is an

Figure 1

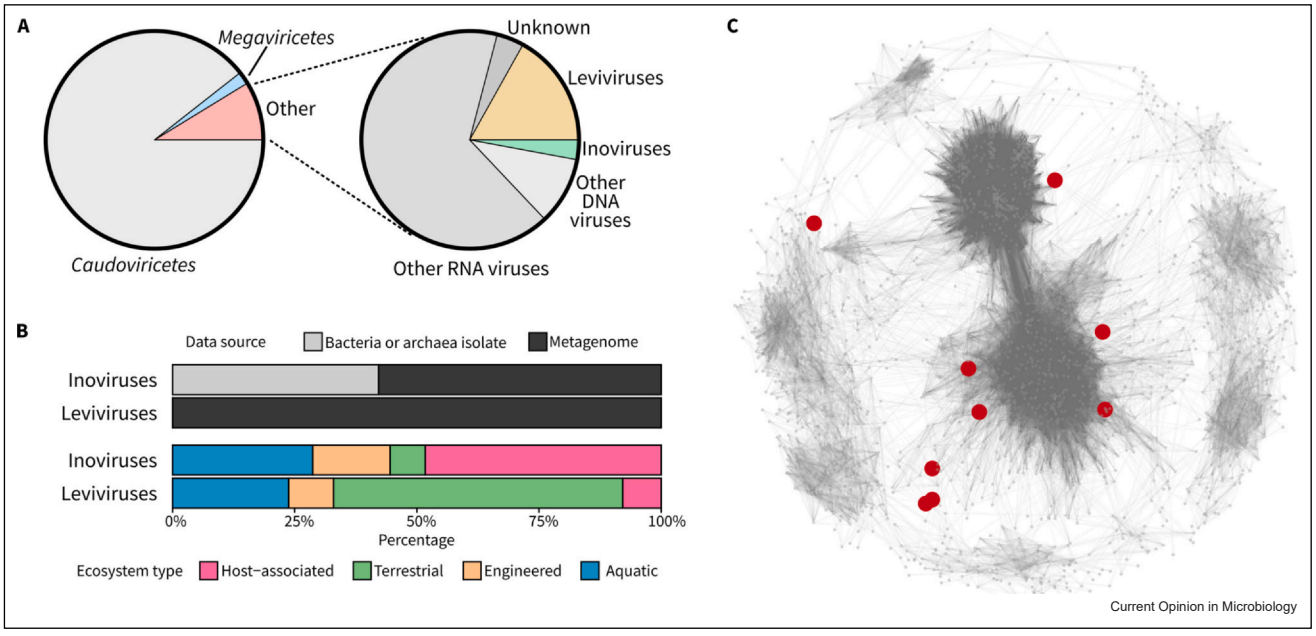


Infection pathways of model ssRNA and ssDNA phages. **(a)** Genes and genome organization of ssRNA phage. *mat* encodes the maturation protein responsible for adsorption to the receptor pilus, *coat* encodes the capsid protein, *sgl* encodes lysis protein, and *rep* encodes the replicase. **(b)** The ssRNA phage binds to the side of retractile pilus using the Mat (green) protein; the retraction of pilus forces phages to the cell surface; the next step is known as eclipse, in which capsid is released and force Mat-RNA genome into the host; At this phase of the infection cycle, the genomic RNA is sensitive to exogenous RNase. After the translation and RNA replication steps, mature virions are assembled, and the lysis protein of the ssRNA phage induces host lysis and releases the new viral progeny. **(c)** Genes and genome organization of filamentous ssDNA phage. Different genes are grouped by their functional role. (d) Filamentous phages bind to the tip of retractile pilus; pilus traction leads to phage disassembly and injection of ssDNA genome via unknown mechanism; Once the genome is inside the cell, expression processes produce structural components and produce nascent phages to release from the host cell without lysis.

extensive database of ~5 million genome and metagenome-derived viral sequences, which provides a unique opportunity to evaluate the distribution and abundance of leviviruses and inoviruses. It is important to note, however, that both inoviruses and leviviruses are expected to be under-represented in IMG/VR because of technical biases: the vast majority of metagenomic data set from which IMG/VR sequences are obtained use methods that select for dsDNA templates, for instance, because of the DNA extraction protocol chosen or the approach used for adapter ligation, excluding ssDNA (inoviruses) and RNA (leviviruses) genomes. Hence, the information provided here should be considered as a 'lower bound' and not representative of the entire diversity of inoviruses and leviviruses.

Based on the version 4 of IMG/VR, leviviruses and inoviruses represent a minority of all phages identified but still account for 82 176 (leviviruses) and 14 198 (inoviruses) genomes, several orders of magnitudes higher than the number of viral isolates available for each group (Figure 2a). About half of the inovirus sequences were obtained in whole-genome shotgun data sets of isolates, that is, identified as prophages when sequencing bacterial isolates (Figure 1b). This highlights how frequently inovirus prophages, like other prophages, can reside in bacterial populations without any noticeable phenotypic effect during cultivation [5]. This elevated ratio of prophage detection also probably reflects a technical bias, as integrated inovirus prophages will be dsDNA and more readily sequenced than the ssDNA-independent genome form. Meanwhile, the ssRNA leviviruses are exclusively detected in metatranscriptomes or in RNA viromes, that is, sequencing of RNA extracted from an environmental sample (Figure 1b). This is consistent with the fact that all cultivated leviviruses are exclusively virulent and do not enter into a lysogenic or chronic infection cycle, though there have been discussions on alternative phage lifestyles [34]. Finally, detection of inoviruses and leviviruses in metagenomes provides an opportunity to evaluate in which ecosystem these phages are most often detected in. Based on the current IMG/VR v4 data, inoviruses are primarily identified in host-associated ecosystems, while leviviruses are more often detected in soil ecosystems, suggesting these two phage groups have different host range and/or preferred ecological conditions. While more sampling is needed to confirm these trends, these initial results already provide useful information for follow-up studies, for example, which types of samples are best candidates for cultivation assays targeting specific groups of atypical phages.

Figure 2



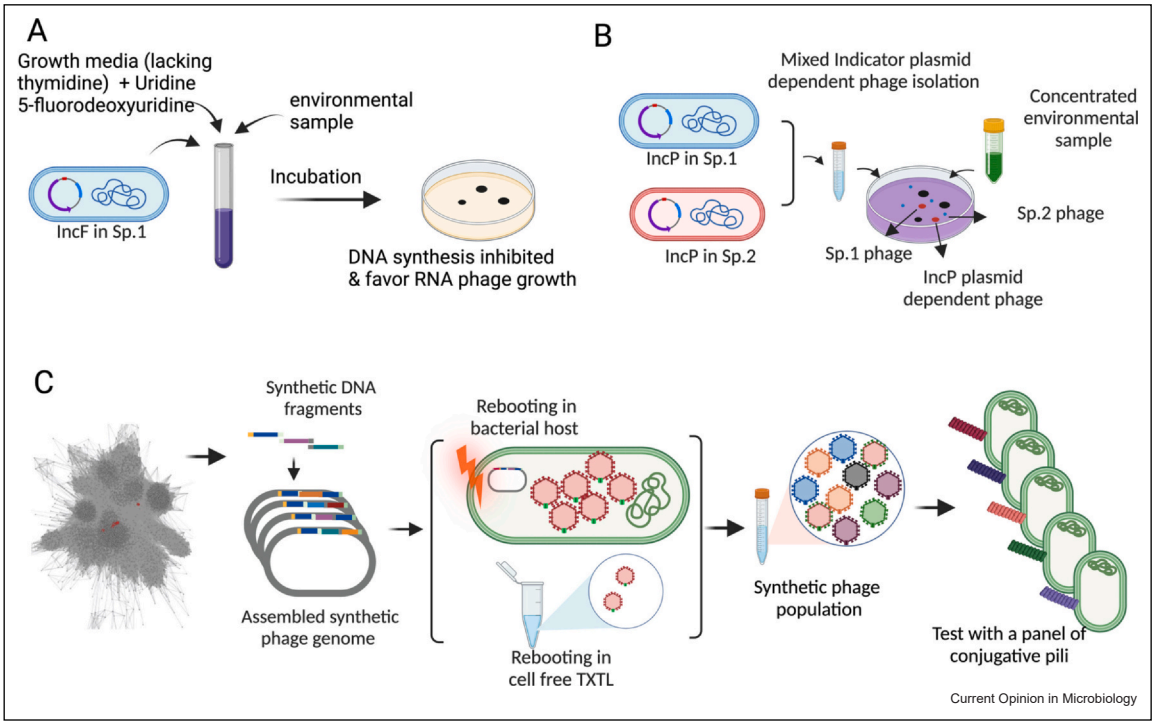
Overview of the prevalence, distribution, and diversity of some atypical phage groups. **(a)** Number of levivirus and inovirus sequences as part of the IMG/VR v4 database. **(b)** Source data information for inovirus and levivirus sequences in IMG/VR v4, including the type of data set (top), that is, sequencing of an isolate or a metagenome, and the ecosystem of origin (bottom). Ecosystem information is only displayed for detections in metagenomes. 'Aquatic' ecosystems include both saline and freshwater samples (n = 19 523 and n = 3123 for leviviruses and inoviruses, respectively), 'engineered' includes mostly bioreactors and wastewater samples (n = 7560 leviviruses and n = 1726 inoviruses), 'terrestrial' includes mostly soil and sediment samples (n = 48 592 leviviruses and n = 785 inoviruses), and 'host-associated' includes primarily human-associated microbiomes (n = 6481 leviviruses and n = 5258 inoviruses). **(c)** Similarity network of predicted *Leviviricetes* genomes [6]. Nodes in the network are individual nonredundant genomes, connected based on pairwise average amino acid identity. Genomes for which isolates are available are highlighted in red. For clarity, only the main connected component of the full network is presented here.

To further illustrate the diversity observed for these two groups of phages in metagenomes, we leveraged a recently collected data set of 10 795 high-quality nonredundant levivirus genomes [6] and performed all-versus-all comparison of predicted protein sequences to estimate the variability in this genome set. Strikingly, despite being classified in the same class (*Leviviricetes*) and sharing a conserved gene content with typically three genes coding for an RdRP, a coat protein, and a maturation protein, with an additional lysis gene more variable in sequence and location, most of the genomes in this set did not share any recognizable sequence similarity (i.e. ≥30% amino acid identity) across the three conserved proteins. Instead, when representing this set as a genome network where genomes displaying ≥50% amino acid identity for the three core proteins are connected (Figure 2c), levivirus genomes obtained from metagenomes show a remarkable diversity, much broader than the one currently represented by isolated genomes (highlighted with red circles). This sequence diversity is consistent with previous analysis of leviviruses detected in wastewater samples [2] and re-emphasizes the need to isolate and characterize model leviviruses that are more broadly representative of this phylogenetic diversity.

Isolation methods

In contrast to dsDNA phages, there has been no single accepted method developed that can be extended to different bacterial species for isolating diverse non-dsDNA phages from the environment [35]. The fact that all cultured leviviruses and inoviruses use retractable pilli as their primary receptors [11] provides a crucial avenue to develop a targeted method for their isolation. In agreement with this observation, protocols have been developed to use *E. coli* strains expressing F-pilus to isolate FRNA phages (ssRNA phages that bind to F-pilus) as indicators of fecal contamination in diverse environments [36]. Similar protocols have been developed to use *Salmonella* as an indicator strain for isolating FRNA phages [37]. Owing to their specificity for retractable pilli, mixed indicator strains (*Salmonella* and *Pseudomonas* sp. strains) carrying the same P-group plasmid (Figure 3a) were developed to isolate plasmid-dependent phages [38]. Application of environmental samples and identification of all phage plaques on such mixed indicator strains found these to be specific for the plasmid-encoded pilus. Phages

Figure 3



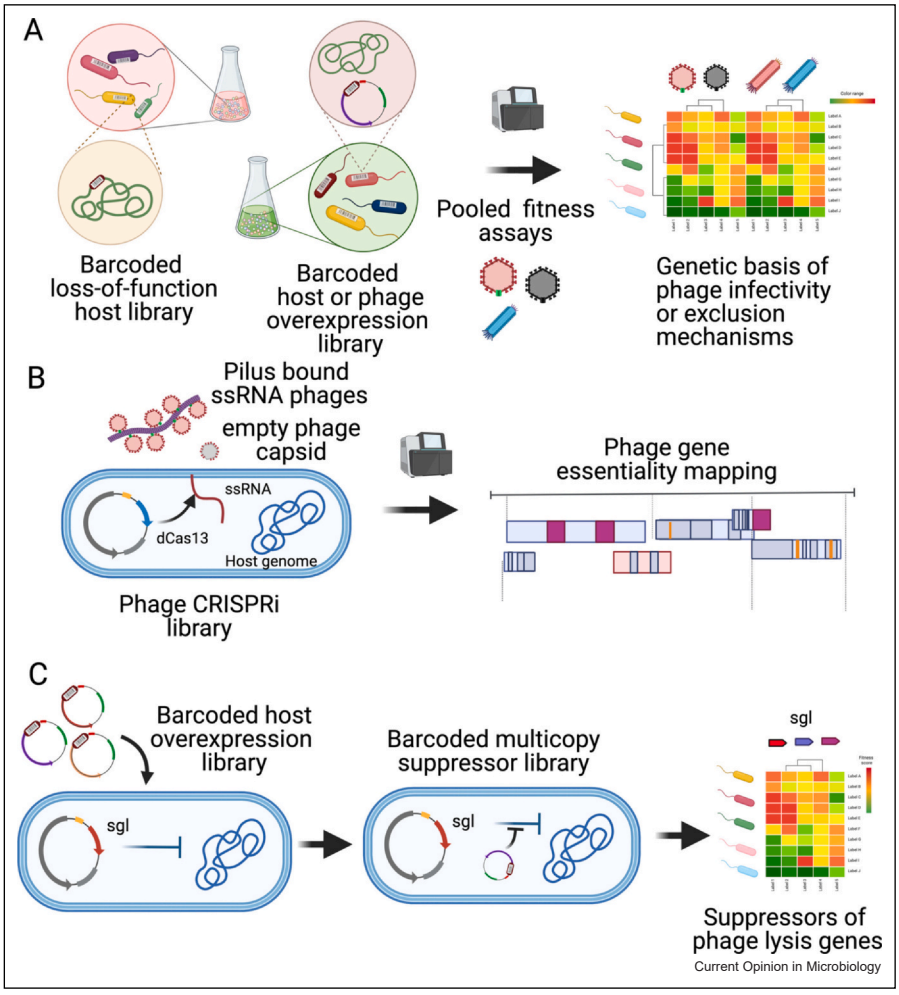
Approaches to isolate or assemble synthetic phages for characterization. **(a)** Media optimization approach for enriching ssRNA phages for diverse species. **(b)** Mixed indicator strain approach for isolating different plasmid-dependent phages for different target hosts. **(c)** Workflow for metagenomically resourced synthetic ssRNA and ssDNA phages using *in vivo* phage rebooting in a permissive host or use of *in vitro* TXTL systems. One approach is to use the pool of synthetic phages and subject them to a panel of strains expressing different conjugative pili.

that form clear plaques on mixed indicator strains have to be either specific for plasmid-encoded pilus or they need to be polyvalent dsDNA phages. As occurrence of such broad-host range dsDNA polyvalent phage is comparatively rare, the mixed strain isolation method facilitates enhanced detection and discovery of leviviruses and inoviruses. Recently, a revised method was reported that used Green fluorescent protein (GFP)-Red fluorescent protein (RFP) reporter combination in a mixed indicator strain assay, and authors were able to isolate a diverse group of plasmid-dependent phages [39]. This study isolated an impressive set of nontailed, lipid-containing dsDNA tectiviruses, ssDNA filamentous phages, and ssRNA phages, all dependent on plasmid-encoded pilus for infection. Compared with these above-mentioned methods, robust methods to isolate phages that bind to chromosomally expressed pilus in different species have been lacking. There are a number of reports on inducing genomically integrated filamentous phages using DNA damaging agents such as mitomycin-C [40,41]. These induced phages are often dependent on genomically encoded retractable pilus but yield turbid plaques and hence make it challenging to isolate individual plaques or increase the titer to prepare the genomic DNA for sequence identification.

Though we do not know if there are pilus-independent leviviruses and inoviruses in nature, the approaches adopted so far have been biased toward isolating pilus-specific phages and primarily focused on clear plaque formers on *E. coli* lawn. This means we must be missing phages that infect other hosts and probably use alternative receptors. These biased isolation approaches have further limited our ability to associate uncultured leviviruses and inoviruses to different hosts and different types of retractable pilus or to alternative receptors. Furthermore, we do not yet have data sets that help us to define the host range of these phages and if there is a cross-sensitivity between different pilus systems and infection patterns. Complications arising from conditionally repressed pilus expression, plasmid incompatibility (Inc) issues, and no general rules to associate a host with particular Inc plasmid and its sensitivity to a given phage have further constrained development of new approaches [42].

Despite these challenges, there are some approaches that may hold some promise for the development of future methods enabling the isolation of a broader diversity of leviviruses and inoviruses. For example, it may be possible to inhibit DNA synthesis/replication and

Figure 4



High-throughput methods to characterize phage–host interaction determinants and phage gene functions. **(a)** Barcoded loss-of-function methods such as RB-TnSeq or CRISPRi are used to identify phage receptors and regulators of receptors. Barcoded host fragment higher copy/overexpression methods such as Dub-seq enable characterization of genetic barriers for the phage infection cycle. The heatmap shows data representation from such genetic screens and indicates which host mutants survive phage selection conditions, uncovering which genes are crucial for phage injection pathways. **(b)** CRISPRi methods using different catalytically inactive Cas systems to systematically interrogate phage gene essentiality determinants. **(c)** Barcoded host-genome overexpression/increased copy number library used to characterize phage genome encoded single gene lysis. This high-throughput technique not only enables suppressor library screens but also opens up novel ways to assess gene function, for example, superinfection exclusion. The heatmap shows data representation from such a genetic screen to uncover which genes or genome fragments when overexpressed or present in high copy numbers, rescue the host toxicity associated with phage gene expression.

enhance RNA synthesis and favor the isolation of RNA phages. This can be achieved by adding DNA synthesis inhibitors such as 5-fluorodeoxyuridine that blocks thymidine synthase (Figure 3b) or by adding compounds that inhibit DNA replication or causes DNA damage (such as nalidixic acid and mitomycin-C, respectively) and thus favoring the reproduction of RNA phages [11,43]. Though this approach seems plausible, the toxicity associated with DNA synthesis/replication inhibitory compounds on the host growth, variability in host response, and poor phage reproduction may limit the scalability of this approach to different species. To favor isolation of ssRNA and ssDNA phages while constraining the enrichment of abundant dsDNA phages, it may be possible to evolve or engineer a host strain that is broadly resistant to diverse dsDNA phages in a particular environment [35]. Similarly, engineering the target host strain to overexpress extracellular polysaccharide such as colanic acid [44] or overproduce a rare capsule type might limit enrichment of dsDNA phages while favoring non-dsDNA phage isolations. In addition to such enrichment methods, we also need rapid methods to identify the genetic material in phage isolates. By supplementing RNase A into the top agar, it is possible to identify whether the phage is an ssRNA phage, for example, as RNase A will inhibit the ssRNA

phage infection cycle [45]. RNase degrades the genomic RNA during the infection process unlike other pilus-dependent phages where nucleases never have access to the genomic DNA. For detection and characterization of ssDNA phages in gut commensals, a recent study used a quantitative polymerase chain reaction (qPCR) and imaging workflow that can now be further developed for other species [29].

As the genome size of these phages is less than 10 kbps, it may also be possible to build synthetic ssRNA and ssDNA phages based on (meta)genomic data sets using synthetic DNA or PCR-amplified fragments if samples are available, and rebooting or directly express them in either highly competent bacterial host or by using cell-free transcription–translation (TXTL) system [46]. These synthetic phages then need to be screened against a panel of bacterial strains carrying different plasmid systems (incompatibility groups; Figure 3c). Finally, multiple displacement amplification on viral fraction (of pool samples) can help identify which inoviruses are present in a sample and could be followed by cesium chloride (CsCl) density gradient targeting filamentous particles layer for isolation.

Systematic characterization of phage infectivity cycle and phage gene function

Though a number of ssRNA and ssDNA phages have been known to bind conjugative pili for host recognition and entry, the knowledge on conditions that favor these interactions (e.g. ionic strength), and influence of co-infecting dsDNA phages and other biotic factors on phage ecology is limited [2,11,16]. Recently, structure-based studies have advanced our understanding of the mechanistic basis of phage–bacteria interactions for non-dsDNA phages, revealing intricate steps involved in phage infection cycle [47,48]. Most of our knowledge on the genetic basis of ssRNA and ssDNA phage infections, and bacterial resistance to them, are based on studies of only one to two *E. coli* phages [25,49]. These studies primarily isolated phage-resistant host mutants and characterized them using classical genetic approaches [50].

Recently, we reported high-throughput genetic technologies (RB-TnSeq, CRISPRi, and Dub-seq) that enable fast and effective genome-wide screens for discovering host genes, gene dosage barriers, or receptors crucial in phage infection and resistance [44]. Similarly, by using CRISPRi screen to knockdown different phage genes, it is possible to enumerate the genome-wide essentiality function of every gene on phage infection cycle in one single pot assay [51,52]. As shown recently, high-throughput screens can also be leveraged to gain mechanistic insights into the function of a gene (e.g. single gene lysis systems from ssRNA phages) across diverse phages [19]. Finally, after building a compendium of ssRNA and ssDNA phages associated with different bacterial species, it may be valuable to systematically map the pilus-phage interactions (Figure 4) and assay the infectivity modes under diverse abiotic conditions, such as a panel of divalent cations, chemical compounds, antibiotics, fertilizers, and in the presence of different dsDNA phages [53]. These studies could also be extended to higher order interaction studies such as assessing phage susceptibility in a microbial community setting and/or in presence of microeukaryotes. Systematically mapping the infectivity modes, synergies and constraints in laboratory models (plants, animals, and human cell lines), will enable phage ecology studies across diverse ecosystems.

Conclusions

The goal of this article was to spotlight the importance of looking beyond the tailed dsDNA phages, give an overview on the current knowledge on non-dsDNA phage diversity and distribution, and provide an assessment of current roadblocks in the establishment of larger culture collections for non–*E. coli* hosts. The leviviruses and inoviruses highlighted here represent only a subsample of the phage diversity known from metagenomic studies. While much less studied than their tailed dsDNA counterparts, ssRNA and ssDNA phages have a huge application space in gene therapy, vaccine development, and as a tool to rationally manipulate microbiomes. With alarming rise in antibiotic-resistant microbes, a trait primarily spread via conjugation elements, ssRNA phages are also considered as a powerful alternative to dsDNA phages in terms of therapeutic application because of their specificity to genomic/plasmid-encoded AMR-spreading pili elements. Here, we highlighted some of the challenges and opportunities to develop new rapid methods of ssDNA and ssRNA phage isolation, use metagenome sequencing to establish diverse culture collections for these phage groups, and gain a deeper understanding and engineering control of these new nonmodel ssDNA and ssRNA phages. Specifically, improving our understanding of the ecology, evolution, and (predicted) host interactions of these atypical phages will be critical to identify best candidates for different biotechnological applications and provide guidance for targeted isolation and thorough *in vitro* characterization of these candidates. Overall, given their broad diversity and unique characteristics, atypical phages such as inoviruses and leviviruses will undoubtedly play an important role next to tailed dsDNA phages in the emerging phage-based biotechnology toolkit.

Data Availability

Data will be made available on request.

Declaration of Competing Interest

None.

Acknowledgements

This material by BRaVE Phage Foundry at Lawrence Berkeley National Laboratory is based upon work supported by the U.S. Department of Energy, Office of Science, Office of Biological & Environmental Research under contract number DE-AC02-05CH11231. The work conducted by the U.S. Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, is supported by the Office of Science of the U.S. Department of Energy operated under Contract No. DE-AC02-05CH11231.

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