The Selection and Optimization of Phage Hosts

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Abstract

Most published methods for bacteriophage culture and purification have been developed around the needs of laboratory-scale production. As the use of phages as antibacterials continues to develop, phages infecting a diverse array of bacteria will need to be produced and purified economically at large scales. Historically, the hosts used for the propagation of phages infecting pathogenic bacteria have been selected largely out of necessity, but the choice of phage propagation hosts can influence the safety, economics, and scalability of phage cultures. This chapter will cover some of the basics of phage propagation and the means by which the choice of propagation host may improve culture yield, biological safety, and downstream purification processes.

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Introduction

The practice of using phages as antibacterials fell from favor in the West in the 1940s. Much of the historical and documented clinical use of phages is from the 1920s and 1930s, predating the advent of the FDA, phased clinical trials, or concepts of GMP manufacturing (see ▶ "Regulatory Aspects of the Therapeutic Use of Bacteriophages: Europe," and ▶ "Clinical Trials of Bacteriophage Therapeutics" chapters). As modern interest has increased in this field, many of the methods used for producing phage preparations today are not much different from those used 80 years ago: phages are propagated on their hosts in liquid medium, and the phages are separated from live cells and cell debris by a combination of centrifugation and filtration. There are no doubt many improvements or perhaps even entirely new technologies that could be deployed to make phage production more efficient, less costly, and less time-consuming.

For the production of phage to be sold commercially, product identity is a consideration that must be kept in mind throughout the development and production process. Simply put, product identity is the composition of the final product, and this is expected to be consistent from the initial product batches submitted for regulatory approval throughout the market lifetime of the product. The developer of a phage product initially has considerable control over product identity, but once this has been approved, alterations to product identity may require submission of additional data and re-approval by a regulator. A strong emphasis is placed on standard operating procedures with the goal of producing high batch-to-batch consistency. This includes not only consistent active titers of the phage(s) in the product but also consistent buffer composition and impurity profiles. This chapter will briefly review some of the considerations associated with the initial step of the phage production process: the propagation of phages on a live bacterial host, focusing on the characteristics of the host itself.

Phage Propagation

As viruses, phages can only increase their numbers by infection of viable host cells. Synthetic biology approaches to phage production that rely on cell-free transcription/translation are under study (Rustad et al. 2018; Shin et al. 2012), but these systems are currently limited to *E. coli*-based expression, and it is not clear if this approach can compete economically with culture-based phage production at the scales required. In many ways, the propagation of phage by traditional culture approaches can be considered as an extension of the propagation of their hosts. Rapidly growing, mid-log bacterial cultures have been the mainstay of phage replication and host physiology experiments, and cells in this state are considered to be the optimal hosts for phage production. For many phages, cells in stationary phase do not support lytic growth, as evidenced by the common observation of the halting of phage plaque growth as bacterial lawns mature and enter stationary phase (see "▶ Detection of Bacteriophages: Statistical Aspects of Plaque Assay" chapter). In

well-studied phages such as T4, stationary phase cells in liquid culture have been shown to support initial infection but not intracellular replication (Bryan et al. 2016; Kutter et al. 1994; Schrader et al. 1997). As cell growth slows, the ability to support phage replication also becomes diminished (Hadas et al. 1997). However, some phages, such as coliphage T7 and *Pseudomonas* phages UT1 and ACQ, are able to replicate in stationary phase cells with limited efficiency (Schrader et al. 1997). A recent study using phage T4 showed that the cell growth cycle affects the phage burst size, with newly divided, smaller cells producing smaller bursts than larger cells just prior to division (Storms et al. 2014).

The isolation of naturally occurring phages from the environment for therapeutic purposes is usually conducted using clinically relevant bacterial strains (see ▶ "Isolation of Bacteriophages" chapter). Since phages isolated from the wild are often cultured on whatever host was used for their initial isolation, in most cases this will mean propagation on pathogenic, clinical bacterial isolates. In some cases, such as the personalized medicine, "*sur mesure*" approach to clinical phage therapy (Pirnay et al. 2011), this initial host may be the most suitable choice for phage propagation due to considerations of time, phage host range and the amount of material ultimately required. In other commercial phage applications, however, the goal is highly repeatable, mass-production of one or a few carefully selected phages over an extended period of time. Given the investment in such phages, the selection of the propagation host for a phage product should be given some consideration.

Surrogate Hosts

The use of a surrogate host for phage propagation is often overlooked but can be highly desirable if such a host is available. In this context, a surrogate host refers to a member of a non-target and typically non-pathogenic bacterial species that will support phage replication as well as better than the phage's original pathogenic host. This approach can be very useful in a number of aspects, including increasing worker safety, reducing the burden of biological containment issues during phage production, and streamlining downstream purification processes. Additionally, if the target bacterium is highly fastidious or difficult to culture, a less-fastidious, rapidly growing surrogate host can greatly simplify phage production processes and increase final yields.

Bacillus anthracis is the causative agent of anthrax in humans and animals and is among the most significant potential biological weapon agents (Mark and Gerald 1999). The use of phages to treat or prevent *B. anthracis* infections has been explored by several groups; however the production of large quantities of *B. anthracis* phage could pose a significant biological hazard related to large-volume cultures of this select agent. The *Bacillus* phage Bc431v3 was reported to infect multiple strains within the *Bacillus cereus* group, including *B. anthracis*, *Bacillus thuringiensis*, *and Bacillus megaterium,* in addition to *B. cereus* itself (El-Arabi et al. 2013). This suggests the possibility of propagating a phage active against *B. anthracis,* which is a Risk Group 3 organism requiring close containment,

on a non-pathogenic surrogate such as *B. megaterium*. Similarly, the broad host-range *Listeria* phage P100, which has biological control activity against the foodborne pathogen *L. monocytogenes,* can be propagated on the closely related non-pathogenic host *L. innocua* (Carlton et al. 2005).

Surrogate hosts may be desirable not only to avoid culturing large volumes of pathogenic bacteria. Phages may be developed as antibacterials against slow-growing or fastidious organisms that are difficult to culture even at the laboratory scale, and large-scale fermentations for the purposes of phage propagation would pose significant barriers. Using such surrogate systems, phages active against fastidious or slow-growing organisms may be cultured and titered on a rapidly growing, easy-to-handle surrogate organism. Perhaps the most famous slow-growing human pathogen is *Mycobacterium tuberculosis,* which is not highly fastidious but is slow-growing with a doubling time of approximately 24 h under normal conditions (James et al. 2000). In contrast, the common environmental saprophytic relative of *M. tuberculosis, M. smegmatis,* grows under similar conditions as *M. tuberculosis* but has a generation time of approximately 3–4 h (Smeulders et al. 1999). *M. smegmatis* phages of Clusters A2, A3, G, and K are able to form plaques on *M. tuberculosis* at varying efficiencies (Jacobs-Sera et al. 2012), suggesting that phages for use against *M. tuberculosis* could be propagated and enumerated on *M. smegmatis.* Screening of a library of *M. smegmatis* phages also revealed that several were active against *Mycobacterium abscessus,* a more rapidly growing human pathogenic mycobacterial species (Dedrick et al. 2019).

The phytopathogenic bacterium *Xylella fastidiosa* is a slow-growing, fastidious organism that requires complex media to form plaques (Summer et al. 2010) and has a doubling time of 0.5–1.5 days, taking weeks to form colonies on solid medium (Hopkins 1989). Phages infecting *X. fastidiosa* can also be propagated on certain strains of *Xanthomonas* spp., which grow rapidly on standard, simple culture media and support robust phage replication (Ahern et al. 2014). The use of *Xanthomonas* surrogate hosts for the propagation of *Xylella* phages could allow for more efficient propagation of these phages for the prevention or treatment of plant disease caused by this pathogen.

Care should be taken with the use of surrogates, however, as serial propagation of a phage on an alternative host strain can inadvertently select for phages that are better adapted to the surrogate than the therapeutic target (see ▶ "Isolation of Bacteriophages" chapter). In commercial production, it is common to produce a set of master phage stocks from which batches of validated, single-use phage seed stocks are made. These single-use stocks are used to inoculate each phage production batch and are then discarded. This practice minimizes the number of serial passages (and the accompanying risks of genetic drift or cross-contamination) between the original phage isolate and the final product. It is advisable to generate master and seed phage stocks on the original target strain where practicable, although this could add significant process cost and complexity. In any case, the activity of the phage against the therapeutic target strain(s) should be assessed following production on the surrogate host to determine if propagation on the surrogate has resulted in changes to phage virulence.

Prophages

As the field matures, the criteria for product purity may become more stringent. The issue of low levels of contaminating temperate phages in therapeutic phage preparations has been noted as a quality control point for phage production (Merabishvili et al. 2009). These temperate phages may be present when a therapeutic, virulent phage is propagated on a bacterial host that contains one or more resident prophages (see ▶ "Temperate Phages, Prophages, and Lysogeny" chapter). Even when uninduced, temperate phage can spontaneously enter the lytic cycle at low frequency during bacterial growth and can often be detected in the culture supernatants of lysogenic strains (Nanda et al. 2015). DNA sequences associated with temperate phages have been identified in commercial phage cocktails (McCallin et al. 2013; Villarroel et al. 2017).

The presence of temperate phages in virulent phage preparations raises two potential risks. First, some temperate phages carry virulence determinants or other genes that can confer greater fitness on the lysogen, and these genes may be introduced into a new strain of the pathogen when the therapeutic phage is applied. Second, temperate phages can be capable of generalized or specialized transduction, and so the contaminating temperate phage could potentially transfer DNA from the phage propagation host into a new pathogen strain during treatment or move DNA between bacteria in the patient or the treatment environment after application. In addition to these general properties of temperate phages, some temperate phages may act as "helper" phages in the mobilization of mobile pathogenicity islands such as SaPIs in *S. aureus* (Lindsay et al. 1998; Novick and Subedi 2007). In this case, the temperate phage promotes the efficient transfer of mobile DNA elements among bacterial strains.

The detection of prophages resident in bacterial strains is not always a straightforward affair. Induction of resident prophages via the bacterial SOS response following exposure of the host to DNA damaging agents (e.g., UV light or low concentrations of mitomycin C) is known to induce many temperate phages (Nanda et al. 2015). Some phages, however, such as the canonical coliphage P2, are not inducible in this manner (Christie and Calendar 2016); thus the absence of SOS-induced lysis cannot be taken as a guarantee that a given bacterial strain is free of lysogens. Detection of spontaneously induced temperate phages in culture supernatants by plaque assay is also a possibility, as long as a suitable plating host which is known to be sensitive to the resident phage(s) is available; temperate phages will not form plaques on their own lysogens due to superinfection immunity. Whole genome sequencing can be used to identify prophages residing in bacterial genomes, and a number of bioinformatic tools are now available for this purpose (Akhter et al. 2012; Arndt et al. 2016; de Sousa et al. 2018; Lima-Mendez et al. 2008). However, given the diversity of phages and the fact that even a single mutation could be enough to inactivate a prophage, the viability of a potential prophage element in a bacterial genome cannot always be directly inferred bioinformatically, and this approach can lead to false-positive identification of viable prophages. Defective prophage remnants may be present which have similarity to known prophages

but which are otherwise unable to produce infectious virions that might appear in a therapeutic phage preparation.

Deletion of part of the prophage, such as the genes that control induction and excision of the lysogen, may not be sufficient since the remainder of the prophage could be induced *in trans* by a related phage and could also still carry virulence determinants. The most conservative option would be to simply delete the entire prophage element. Targeted genome reduction, including deletion of prophage elements, has been conducted in commercially important bacterial strains to increase yields and streamline production (Ara et al. 2007; Heider and Wendisch 2015; Posfai et al. 2006). A method for targeted curing of desired prophage elements using counter-selectable markers has been described in *Streptococcus pyogenes* (Euler et al. 2016). Recently, CRISPR-based technology has been shown to have utility in deletion of unwanted genetic elements in *Lactococcus lactis* by expression of Cas9 with guide RNAs that target genes, mobile elements or prophages (van der Els et al. 2018).

The potential risks associated with temperate phage contamination, however, should be balanced against other factors, such as the fact that the temperate phage are likely to be outnumbered by 10,000:1 or more by the desired virulent phage in any given preparation, and that the number of temperate phage virions introduced during phage treatment is likely to be small compared to the number of temperate phages already resident in a patient's natural microbial flora. The frequency and mechanisms of gene transfer in natural systems are not well understood, and any risk associated with phage treatment in this regard can only be truly estimated in the context of the natural horizontal gene transfer processes already occurring in the system receiving phage treatment. Thus, while methods exist to detect and remove resident prophages in phage propagation strains, it is not clear if the risks associated with their presence are will always be great enough to justify the time and effort required to refactor phage propagation hosts.

Reducing Host Toxicity

If a phage is to be propagated on a pathogenic host strain, this strain could be rendered avirulent (or at least less virulent) by deletion of major detectable pathogenicity determinants and toxins. This approach could provide greater biological safety during production and processing and also potentially streamline the downstream phage purification process. In many cases, host virulence factors are not required for phage infection or replication, and their deletion does not have an appreciable effect on bacterial fitness *in vitro*. *B. anthracis*, for example, is typically handled under biosafety level 3 conditions. The *B. anthracis* Sterne strain is lacking the virulence plasmid pXO2, which renders it unable to form capsule, and thus this strain is attenuated (Green et al. 1985). *B. anthracis* strains lacking plasmids pXO1 and pXO2 are able to produce neither capsule nor disease-associated toxins, and virulence of these strains is attenuated further (Pezard et al. 1991). Deletion of discrete pathogenicity islands, episomal elements or individual genes in a phage

propagation host may be accomplished by any number of recombinant DNA technologies.

Endotoxin

A potential concern for any preparation containing phage of a Gram-negative host is the presence of bacterial lipopolysaccharide (LPS), also called endotoxin. Lipopolysaccharide is a lipid-modified carbohydrate that makes up the majority of the outer leaflet of the outer membrane in Gram-negative cells (Raetz and Whitfield 2002). It is comprised of three major parts: the highly conserved lipid A, the moderately conserved carbohydrate core, and the highly diverse *O*-antigen. The hydrophobic lipid A domain is what anchors the other hydrophilic, carbohydrate domains to the outer membrane and is responsible for most of the toxic effects of LPS in mammalian hosts (Raetz and Whitfield 2002). Lipopolysaccharide was long thought to be essential for cell viability in Gram-negative bacteria (Zhang et al. 2013); however LPS-deficient mutants of certain species such as *Acinetobacter baumannii* (Moffatt et al. 2010) and *Neisseria meningitidis* (Bos and Tommassen 2005) have shown that it can be dispensable in some organisms. If a phage propagation host (surrogate or otherwise) is able to tolerate LPS deletion, this may be a viable pathway to simplifying the downstream phage purification process, as phage produced on such strains will lack endotoxin in the crude lysate. The viability of this approach is dependent on the phage host's ability to live without endotoxin and on the cell surface receptors used by the phage. If the phage uses a part of the host LPS as its receptor, then obviously cells lacking LPS will be unable to support phage replication. Phages that use outer membrane proteins as their receptors may be able to use LPS-deficient strains as hosts; in the case of *N. meningitidis*, loss of LPS production does not appear to have significant effects on the production of integral outer membrane proteins (Steeghs et al. 2001).

Bacterial strains with modified LPS have also been explored to simplify downstream processing demands in the production of biologicals, and these approaches may also be applicable to phage production. An *E. coli* strain expressing the lipid A precursor lipid IV_A is able to support robust protein expression (Mamat et al. 2015), suggesting that it may also be able to support phage replication, although this has never been examined to this author's knowledge. Lipid IV_A appears in the outer leaflet of the outer membrane but does not induce the TLR4-mediated inflammatory response in humans that is associated with intact lipid A, and lipid IV_A is in fact an antagonist of this response (Muroi and Tanamoto 2006; Park et al. 2009). The use of such strains has been suggested as a means of producing recombinant therapeutic proteins (Mamat et al. 2015) and membrane vesicles (Watkins et al. 2017). Quality control monitoring for products based on this approach may be difficult, however, as lipid IV_A will still produce endotoxin signal in the standard *Limulus* amebocyte assay (Mamat et al. 2015) and in mice (Muroi and Tanamoto 2006). Thus, it may be technically challenging to quantify contaminating endotoxin or conduct murine preclinical studies using phage produced with lipid IV_A-expressing cells.

Conclusion

Phage therapy is receiving increasing attention as a potential antibacterial strategy, particularly for the treatment of drug-resistant infections. With multiple Phase I and Phase II clinical trials underway, there is demand for industrial-scale production of phages infecting a potentially broad swath of human pathogens. In the case of a mass-produced, defined phage cocktail, investment in selecting and optimizing the phage production host may provide a cost benefit by facilitating more rapid phage production and/or simplified downstream processing requirements. As the field continues to move forward, undoubtedly new technologies will emerge to increase the efficiency of phage production and purification at scale.

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