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Original article

Propagation method for persistent high yield of diverse *Listeria* phages on permissive hosts at refrigeration temperatures

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Abstract

The efficient production of a high concentration of bacteriophage in large volumes has been a limiting factor in the exploration of the true potential of these organisms for biotechnology, agriculture and medicine. Traditional methods focus on generating small volumes of highly concentrated samples as the end product of extensive mechanical and osmotic processing. To function at an industrial scale mandates extensive investment in infrastructure and input materials not feasible for many smaller facilities. To address this, we developed a novel, scalable, generic method for producing significantly higher titer psychrophilic phage ($P < 2.0 \times 10^{-6}$), 2- to 4-fold faster than traditional methods. We generate renewable high yields from single source cultures by propagating phage under refrigeration conditions in which *Listeria*, *Yersinia* and their phages grow in equilibrium. Diverse *Yersinia* and *Listeria* phages tested yielded averages of 3.49×10^8 to 3.36×10^{12} PFU/ml/day compared to averages of 1.28×10^5 to 1.30×10^{10} PFU/ml/day by traditional methods. Host growth and death kinetics made this method ineffective for extended propagation of mesophilic phages.

Keywords: Biotechnology; Food safety; Bacteriophage; Phage replication; *Listeria monocytogenes*; *Yersinia enterocolitica*

1. Introduction

Bacteriophages are the most prevalent life form on earth by number, with an estimated 10^{31} virions, outnumbering prokaryotes by more than an order of magnitude and are the primary cause of bacterial death and turnover in natural environments [9]. These viruses are highly selective, infecting specific genera, species, strains or life stages of hosts such as pathogens, while leaving other bacteria unaffected. The diversity of phage covers the breadth of prokaryotic life, including species highly resistant to common antibiotics, and includes phage adaptations circumventing every known mechanism of host resistance [1,26]. High host specificity, bacterial susceptibility and potential for bioengineering for even more refined targeting and resilience to acquired resistance make phages particularly promising antimicrobials in contexts where antibiotics are ineffective or inappropriate [1,26]. Phage research offers promise for advancements both in historical applications of medical phage therapy against infection [12,17] and novel applications throughout biotechnology and agriculture [3,5,10].

Despite a long history of bacteriophage research and the importance of phage to global ecology, there are few truly standardized methods applicable for large-scale applied research [9]. A relatively small subset of phages receive extensive study and characterization, while the majority of phages remain underinvestigated, due in part to the difficulty in transferring methodology for large-scale experiments [4,11,22,23,26]. Protocols based on the well-studied phages tend to require non-trivial optimization when applied to new or understudied bacteriophages.

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As bacteriophages and their prokaryotic hosts comprise two of the most structurally and enzymatically diverse classes of life, the effects of specific phage-to-phage and host-to-host biophysical differences become increasingly influential and complicate the efficiency and efficacy of multi-day protocols dependent on purification through fine-tuned differences in physical properties [4,6,11,22,23,25,26]. Even when focusing only on the most common morphological form, the order *Caudovirales* [tailed, icosahedral, double-stranded DNA bacteriophages], significant differences exist above the species level in structure, size, morphology and genome sequence [4]. The smallest long-tailed *Caudovirales* identified, *Rhodococcus* phage RRH1, has a 43 nm diameter capsid, 14.2 kb genome and 81 nm tail [18]. The largest, *Pseudomonas* phage 201phi2-1, has a 130 nm diameter capsid, 316.7 kb genome and 195 nm sheathed tail, plus extensive fibers on the baseplate and capsid [21]. Short-tailed *Caudovirales* have similar capsid diameters, with tails of less than 17 nm. This is more than a 4-fold range in capsid diameter, 10-fold tail length range and 22-fold genome size range. Fibrous and globular appendages occur in variable number and size, attached to capsids, tail collars, tail tips and baseplates differing between phage strains [11]. Thus, diverse phages have distinctly different mass, surface charges, diffusion kinetics, specific gravities, solubilities and aggregation properties, which are the fundamental determining factors governing all forms of differential purification.

Sufficient supplies of concentrated bacteriophage suspension are essential to applied bacteriophage studies. Traditional propagation involves growing large volumes of a permissive host to stationary phase in liquid broth, spiking in the phage of interest at the mid-log phase of growth [29]. This culture is then treated by multi-step sterilization, purification, concentration and precipitation methods over several days, yielding final volumes several orders of magnitude below the input volume [25]. While this yield is sufficient for small-scale experiments, substantial resources and time are required to generate the amounts of phage necessary for a single moderate scale experiment. The large scales facilities necessary to efficiently generate the large quantities of phage necessary for longer-term experiments such as clinical applications are usually available only at the industrial level, where motivations and incentives tend to focus on short-range marketable products rather than foundational investigations for discovery or understanding which lead to long-term progress.

Thus, alternatives requiring less investment of time, materials and facilities should dramatically benefit phage research fields. To this end, using *Listeria* phage as a model, we developed a method of phage propagation yielding a continuous supply of high titer phage. Our approach uses resources and a scale of facilities accessible to the average microbiology researcher. Further, our methodology is intrinsically scalable to yield large volumes with, at most, linear increases in input resources and equipment requirements.

2. Materials and methods

2.1. Establishment of bacterial cultures

The bacterial and viral strains used in this study are listed in Table 1, along with source locations. Five-ml aliquots of sterile brain heart infusion [BHI] broth were pipetted into three 15 ml screw cap, sterile falcon tubes, respectively inoculated with a sterile loop of glycerol stocked monoculture of *Listeria monocytogenes* ATCC 19115 serotype 4b, *L. monocytogenes* LI 0529 serotype 1/2a and *L. monocytogenes* 08-5578 serotype 1/2a. These three tubes were incubated for 48 h at 30 °C to generate stationary phase starter cultures precursory to the phage propagation [5]. *Yersinia*

Table 1
Bacterial and viral strains used.

Taxon	Strain	Source
Bacteria: Gram-positive, psychrophilic	*Listeria monocytogenes* 08-5578 (serotype 1/2a) [Canadian 2008 outbreak] [8]	The National Microbiology Laboratory, Canadian Science Center for Human and Animal Health, Winnipeg, Manitoba
Bacteria: Gram-positive, psychrophilic	*Listeria monocytogenes* ATCC 19115 (serotype 4b)	University of Guelph Laboratory Services Division, Guelph, ON, Canada
Bacteria: Gram-positive, psychrophilic	*Listeria monocytogenes* Li0529 (serotype 1/2a)	University of Guelph Laboratory Services Division, Guelph, ON, Canada
Bacteria: Gram-negative, mesophilic	*Salmonella typhimurium* WG49	University of Guelph, Food Science Department, Guelph, ON, Canada
Bacteria: Gram-negative, psychrophilic	*Yersinia enterocolitis* 6471/76-c [14]	University of Guelph Laboratory Services Division, Guelph, ON, Canada
Virus, Bacteriophage: Myoviridae	*Listeria* phage A511 [13]	The Institute of Food, Nutrition and Health, ETH, Zurich, Switzerland
Virus, Bacteriophage: Siphoviridae	*Listeria* phage A118 [16]	The Institute of Food, Nutrition and Health, ETH, Zurich, Switzerland
Virus, Bacteriophage: Siphoviridae	*Listeria* phage 2389 [19]	Université Laval, Félix d'Hérelle Reference Center for Bacterial Viruses, Quebec City, QC, Canada
Virus, Bacteriophage: Myoviridae	*Salmonella* phage SF-1 [28]	University of Guelph, Food Science Department, Guelph, ON, Canada
Virus, Bacteriophage: Podoviridae	*Yersinia* phage vB_YenP_AP5 [14]	University of Guelph Laboratory Services Division, Guelph, ON, Canada