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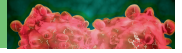
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## BRIEF REPORT

## ENVIRONMENTAL MICROBIOLOGY



# Determinants in the phage life cycle: The dynamic nature of ssDNA phage FLiP and host interactions under varying environmental conditions and growth phases

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## Abstract

The influence of environmental factors on the interactions between phages and bacteria, particularly single-stranded DNA (ssDNA) phages, has been largely unexplored. In this study, we used *Finnlakevirus* FLiP, the first known ssDNA phage species with a lipid membrane, as our model phage. We examined the infectivity of FLiP with three *Flavobacterium* host strains, B330, B167 and B114. We discovered that FLiP infection is contingent on the host strain and conditions such as temperature and bacterial growth phase. FLiP can infect its hosts across a wide temperature range, but optimal phage replication varies with each host. We uncovered some unique aspects of phage infectivity: FLiP has limited infectivity in liquid-suspended cells, but it improves when cells are surface-attached. Moreover, FLiP infects stationary phase B167 and B114 cells more rapidly and efficiently than exponentially growing cells, a pattern not observed with the B330 host. We also present the first experimental evidence of endolysin function in ssDNA phages. The activity of FLiP's lytic enzymes was found to be condition-dependent. Our findings underscore the importance of studying phage ecology in contexts that are relevant to the environment, as both the host and the surrounding conditions can significantly alter the outcome of phage–host interactions.

## INTRODUCTION

Like all other viruses, bacteriophages are obligate parasites and need host cells to reproduce. Phage–host interactions include all the host-dependent steps of the phage life cycle, such as adsorption to a host cell, genome entry, production of new phage particles, cell lysis and release of newly assembled phage particles into the environment (Stone et al., 2019). Furthermore, bacteria use different types of defence systems to avoid phage infections and phages respond by using anti-defence mechanisms, both of which are integral forms of phage–host interactions (Teklemariam et al., 2023). Phage research traditionally focuses on clinically important phage–bacterium interactions. The

life cycle details of environmental phages, especially non-dsDNA phages, are less well understood. Environmental phage isolates are also diverse and often considered difficult to work with as existing methods are optimized for more studied dsDNA phages. Secondly, phage research is often done under conditions that may not reflect the conditions of the environment of isolation. In nature bacteria rarely are in continuous maximal growth phase due to, for example, nutrient limitations and temperature fluctuations. Also, the presence of ecological interactions such as interspecific competition are not easily incorporated into all laboratory experiments.

Phage infections are important factors in all ecosystems as they have direct effects on food webs

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especially regulating bacterial prevalence. Both abiotic and biotic conditions like temperature, availability of nutrients and oxygen as well as microbial composition and density may influence bacterial growth and choice of phage life cycle strategies (Attrill et al., 2023; Brum et al., 2016; Bryan et al., 2016; Chevallereau et al., 2022; Clokie et al., 2011; Shan et al., 2014; Tokman et al., 2016; Woody & Cliver, 1995; Zhang et al., 2022). Because fluctuating environmental conditions and seasonality limit the possibility of exponential growth of bacteria in nature, it is advantageous for a bacteriophage to be able to infect hosts in different growth phases and conditions. However, most phages are efficient in infecting cells in the exponential growth phase of the host (Chibani-Chennoufi et al., 2004; Woods, 1976). Yet, as laboratory experiments are often implemented in optimized conditions, the variety of ecological interactions may not be easily detected (Bryan et al., 2016; Chevallereau et al., 2022; Koskella et al., 2022). It is also important to recognize that different steps of the phage infection may not be equally efficient, but rather depend on the available host and the surrounding conditions. Furthermore, inefficient phage infections are probably more common and thus more impactful in nature than previously assumed (Howard-Varona et al., 2017, 2018).

*Flavobacteriaceae* is a diverse family of bacteria within the phylum *Bacteroidetes* prevalent in both freshwater and marine habitats. They can constitute a significant portion of the microbial community depending on the biotic and abiotic factors (Kirchman, 2002). The ecological impact of *Flavobacteriaceae* is broad and well known, especially in aquatic environments, where they contribute to the degradation of organic matter, playing a crucial role in nutrient cycling and the decomposition of organic compounds (Bernardet et al., 2002; Kirchman, 2002). Some species are involved in the breakdown of algal blooms (Buchan et al., 2014), thus having a high impact on water quality. *Flavobacteriaceae* are also found in other environments like soil and sediments as well as animal hosts (Bernardet et al., 2002).

Until recent years, it seemed that the tailed dsDNA phages represent the dominant type of phages and that other morphologies, like non-tailed and lipid-containing phages as well as phages with other genome types, would be relatively rare having less prominent ecological impact. However, analyses of several different environments have shown the opposite (Chevallereau et al., 2022; Holmfeldt et al., 2013; Kauffman et al., 2018; Van Cauwenberghe et al., 2022; Zhan & Chen, 2019). According to microscopy and DNA-based methods (e.g., metagenomics) non-tailed phages, especially microviruses, seem to be abundant in many biomes (Brum et al., 2013; Hopkins et al., 2014; Kauffman et al., 2018; Kirchberger et al., 2022), suggesting the ecological importance of non-tailed phages has been underestimated. Most of the known *Flavobacteriaceae* infecting phages are tailed dsDNA phages.

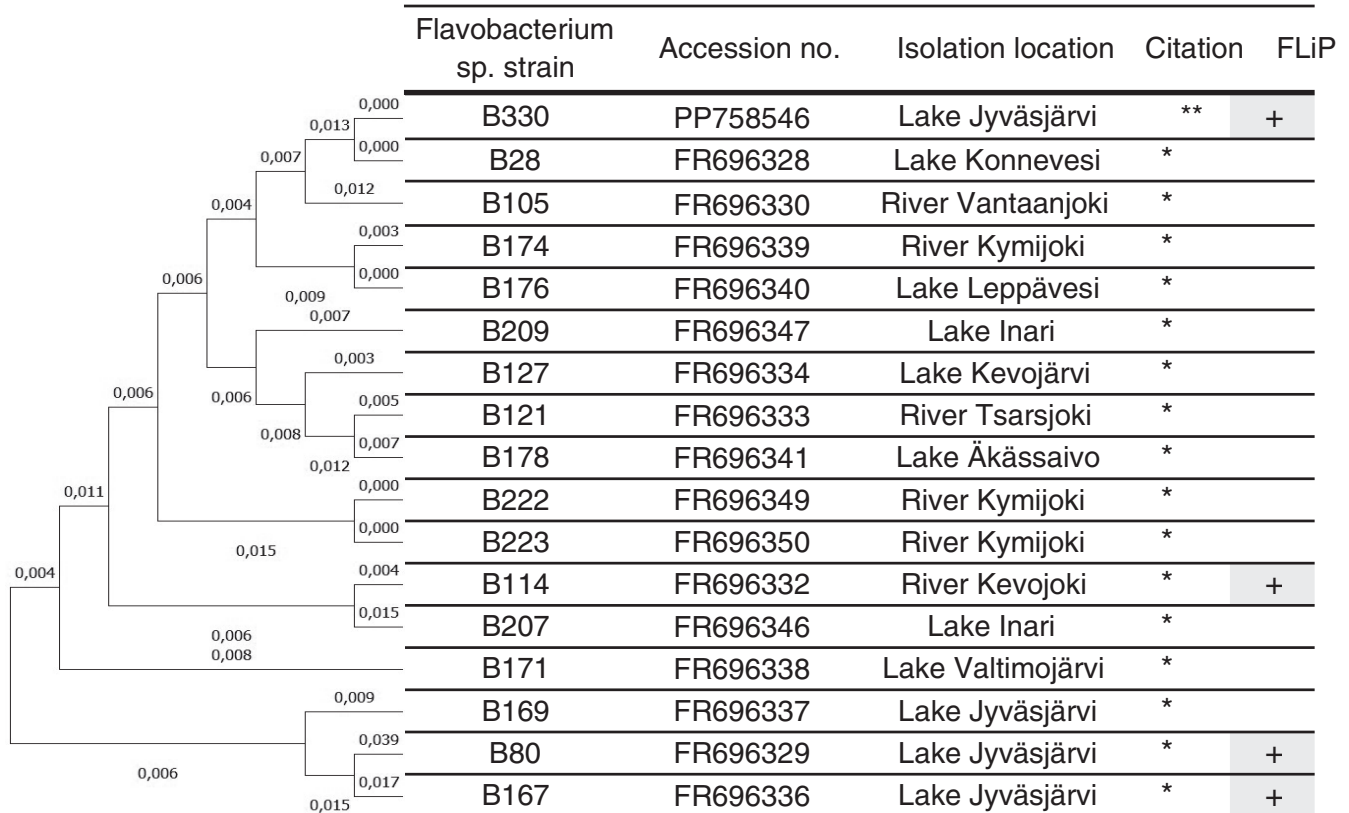
However, recent research has disclosed evidence of *Flavobacteriaceae*-infecting ssDNA phages, especially in aquatic environments (Bartlau et al., 2022; Holmfeldt et al., 2013; Kejzar et al., 2022). Yet the low number of non-tailed phage isolates, especially those with a ssDNA genome or lipid membrane, has led to a gap in understanding of the biology and host interactions of these phages. *Flavobacterium* infecting lipid-containing phage (FLiP), the only characterized species belonging to the virus family *Finnlakeviridae* (Consortium et al., 2020) has been isolated from the boreal lake Jyväsjärvi in Central Finland (Laanto et al., 2017). FLiP shares a unique combination of structural elements with only one other published phage phiCjT23 (Kejzar et al., 2022); most remarkably ssDNA genome and a lipid membrane inside an icosahedral, tailless capsid. The circular genome of FLiP is 9174 nt long and contains 16 putative ORFs, out of which five genes/gene products have been identified (Laanto et al., 2017). However, so far, the life cycle of FLiP and its possible relatives has remained enigmatic. Yet, it can be assumed that the wide temperature fluctuations in the boreal lake environment where FLiP originally was isolated influence its' replication. FLiP was isolated on 10th September 2010 from the surface water of Lake Jyväsjärvi, where water temperature ranges between 0°C in winter to 23°C in summer, at a depth of 1.5 m (PäijänneLTER, 2024; years 2013–2020). Water temperatures above 1.5 m may get even warmer due to direct sunlight onto the lake surface.

To understand the life cycle properties and host interactions in a lipid-containing ssDNA phage FLiP and its three hosts; *Flavobacterium* sp. strains B114, B167 (Laanto et al., 2011) and B330 (Laanto et al., 2017), we performed plate assays, adsorption experiments, long-term growth experiments, biofilm assays and zymogram analyses in different temperatures. We found that interactions with each host have different optimum temperatures. Additionally, FLiP infects stationary phase B167 and B114 cells more effectively than exponentially growing cells. Phage particle production with B330 starts faster, but FLiP replication in B167 is more efficient on a longer time scale. The attachment of host cells and FLiP to a surface enhances FLiP infection. Most remarkably, we provide the first experimental evidence of ssDNA-encoded endolysin lysis and its condition dependence.

## MATERIALS AND METHODS

### Phage and *Flavobacterium* hosts

ssDNA phage FLiP (Laanto et al., 2017) and *Flavobacterium* sp. strains B114, B167 and the isolation host of FLiP *Flavobacterium* sp. strain B330 (Figure 1), were used in this study. Also, a selection of other previously isolated *Flavobacterium* sp. strains was used to



**FIGURE 1** Bacterial strains used in this study and host range of FLiP. The same 780-nucleotide region from the beginning of the 16S rRNA gene sequences of bacterial strains was used to construct the phylogenetic tree. The evolutionary history of bacterial strains was inferred by using the Maximum Likelihood method and the Tamura-Nei model (Tamura et al., 2021; Tamura & Nei, 1993). + Sign in the grey background indicates infection at all tested temperatures: room temperature, 18°C and 8°C. FLiP did not infect any of the other bacterial strains at any of the tested temperatures. \*Laanto et al., 2011; \*\* this study.

determine the host range of FLiP using Shieh medium and double layer agar assay at three different temperatures: 8°C, 18°C and room temperature (RT). All bacterial strains were stored at –80°C with 20% glycerol.

Partial 16S rRNA gene sequences (780 nt from the beginning of the gene) of bacterial strains were used to construct a phylogenetic tree. The evolutionary history of bacterial strains was inferred by using the Maximum Likelihood method and the Tamura-Nei model (Tamura & Nei, 1993). The tree with the highest log likelihood (–2332.44) is shown (Figure 1). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value. Codon positions included were 1st + 2nd + 3rd + Noncoding. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

### Phage lysate preparation

High titre ( $10^{10}$ – $10^{11}$  PFU ml<sup>–1</sup>) FLiP lysates were produced by double layer plaque assay using either Shieh

(Song et al., 1988) or High-tryptone cytophaga (HTC) (Pate & De Jong, 1990) medium: 3 ml Shieh or HTC soft-agar (0.7% [wt/vol]) was tempered into 47°C, after which 100 µl of liquid culture of an overnight (21°C, 120 rpm) grown host bacterium and 100 µl of phage were mixed into it and the mixture was poured onto a plate with a layer of same but already solidified medium with agar (1% [wt/vol]). Plates were incubated for 1–2 days at room temperature, after which 5 ml of medium was applied on the plate and incubated for at least 5 h (8°C, shaking) to produce plate lysates. Lysate was collected and filtered (0.2 µm) and stored at 8°C.

For larger scale FLiP propagation, lake-water-based media (i.e., J-Shieh and J-HTC; where letter J refers to water from Lake Jyväsjärvi in Jyväskylä, Central Finland) were prepared in unfiltered lake water from lake Jyväsjärvi and autoclaved. J-Shieh-agar (250 ml, 2% [wt/vol]) was cast onto the bottom of a 2-litre Erlenmeyer flask, forming a ~2 cm thick layer. Before casting, J-Shieh-agar was tempered to 40–45°C, and 3 ml of overnight bacterial culture was added and mixed with it (Figure S1A). After solidification agar was pierced ~20 times with a pipette tip to further increase the available surface area for bacterial attachment. 500 ml of



Shieh medium and 3 ml of  $\sim 10^{10}$  PFU ml<sup>-1</sup> FLiP lysate were applied on top of the agar. The liquid phase of the culture was collected after 48–72 h of incubation (120 rpm, RT) and centrifuged (Sorvall SLA-3000 rotor, 10,800×g, 30 min, 4°C). The supernatant was filtered (0.2 µm) and stored at 8°C.

Drop titration was applied to determine lysate titres (Figure S2A). 3 ml soft-agar (0.7% [wt/vol]) medium was tempered into 47°C and 100 µl of liquid culture of a host bacterium was added and mixed to it and the mixture was poured onto a solidified medium-agar (1% [wt/vol]) plate (Ø 9 cm). 5 ml soft-agar and 170 µl bacterial culture were used for square plates (12.8 × 8.6 cm). A dilution series of phage lysates in 2 or 10 µl drops were pipetted on the plates. After incubating (2 days, RT) the PFU ml<sup>-1</sup> was determined.

## Purification of phage particles

FLiP-filtered stocks produced from plate lysates or collected from liquid cultures were precipitated using polyethylene glycol 6000 (PEG 6000) and purified for protein analyses as described by Laanto (Laanto et al., 2017). Rate zonal centrifugation in 5–20% (wt/vol) sucrose (in 20 mM KPO<sub>4</sub> buffer) and equilibrium centrifugation in 20–70% (wt/vol) sucrose (in 20 mM KPO<sub>4</sub> buffer) were used for purification of the phage.

## FLiP and host growth in different temperatures

In the first experiment temperatures 4, 7, 8, 9, 13, 14, 17, 19, 21, 25, 29, 30, 31, 33, 34 and 37°C were used to study temperature effect on FLiP plaque formation. Plates with drop-titrated dilution series of phage on top of bacterial hosts (*Flavobacterium* sp. B330, B167 or B114) mixed in soft agar were enclosed into a plastic container with water in a decanter flask to prevent the plates from drying out. The lid of the plastic container was closed lightly so that air ventilation was not completely prevented. Plates were incubated at each specific temperature until no further growth of bacterium and no changes in plaque number or morphology were observed (2–9 days). FLiP titre, plaque morphology, bacterial lawn growth and any impact on the size or morphology of the clear area caused by FLiP infection were recorded after incubating at each temperature.

Furthermore, we used similar approaches in three additional experiments for FLiP titre, plaque morphology and lysis area size at RT and 8°C. Each of these experiments was started with a single overnight bacterial culture of each strain. Plates to be incubated at RT and 8°C were plated at the same time to achieve the

same initial density of bacteria in top agar at both temperatures. In experiment A plating was done in two replicates, and a dsDNA phage, MaF61, infecting the *Flavobacterium* sp. strains B114, B167 and B330 was used as a control. 2 µl drops were used. In the experiments B and C, only FLiP-phage was used. The replicated experiments were otherwise similar but a drop size of 2 µl was used in experiment B and a drop size of 10 µl was used in experiment C. Plates were imaged with ChemiDoc MP Imaging system (Bio-Rad) using the application for Ethidium Bromide Gel (602/50, UV Trans) and Auto Optimal exposure. The data were analysed with One-way ANOVA using Tukey's post hoc comparisons and with a non-parametric Mann–Whitney *U* test (in GraphPad Prism 10) using the maximum values measured from lysis size measurements.

## Adsorption of FLiP to fresh and overnight-grown cells

FLiP adsorption at room temperature to both fresh cells in the exponential growth phase and to overnight cells in the stationary growth phase was experimented with using methods modified from Kropinski (Kropinski, 2009).

First, FLiP adsorption to fresh cells was tested. Bacterial host cultures (B330, B167 and B114) were grown overnight in HTC medium (RT, 120 rpm) after which they were subcultured ~1:3 in HTC to obtain fresh cultures. These fresh cultures were grown (RT, 120 rpm) for approximately 2.5 h to log phase and diluted to  $1.00 \times 10^8$  colony forming units (CFU) ml<sup>-1</sup>. Actual number of viable bacterial cells was recorded by plating. Cell densities of fresh cultures before infection were:  $2.5 \times 10^8$  CFU ml<sup>-1</sup> for B330;  $1.4 \times 10^8$  CFU ml<sup>-1</sup> for B167 and  $2.0 \times 10^8$  CFU ml<sup>-1</sup> for B114. Diluted cultures were incubated (120 rpm, 10 min, RT), after which they were divided into three replicates. Phage was added to each replicate culture in a final concentration of  $1.25 \times 10^4$  PFU ml<sup>-1</sup> and mixed. 50 µl samples were then taken every other minute for 10 min after infection while maintaining agitation between samplings. Samples were mixed with 950 µl ice-cold Shieh medium and centrifuged (4600×g, 3 min, 4°C) immediately. 100 µl of the supernatant was plated with B330 as described for double-layer plaque assay. After incubating (2 days, RT) the phage plaques were counted, and the titre of free phage was calculated in PFU ml<sup>-1</sup>.

Experiments for FLiP adsorption to overnight cells were done similarly but overnight cultures were not subcultured or diluted. Cell densities of overnight cultures before infection were  $2.0 \times 10^9$  CFU ml<sup>-1</sup> for B330;  $1.1 \times 10^9$  CFU ml<sup>-1</sup> for B167 and  $7.1 \times 10^8$  CFU ml<sup>-1</sup> for B114.



## Effect of host growth phase on FLiP plaque formation

The optimal host bacterial growth phase for FLiP plaque formation was determined by a double-layer plaque assay. *Flavobacterium* sp. host strains B330, B167 and B114 were subcultured 1:10 into Shieh medium and kept under shaking (120 rpm, RT). The same FLiP dilutions were plated with each host in time points 2, 4, 6 and 8 h after subculturing. An overnight-grown (19.5 h) bacterium culture was used as a control. After incubating the plates (2 days, RT) the plaque number was recorded.

## Imaging the interactions of FLiP and *Flavobacterium* sp. B330 cells

Thin section samples from the phage and bacteria were prepared from glutaraldehyde fixed samples. Overnight grown bacteria were inoculated to fresh Shieh-medium and grown (120 rpm, RT) to the density of  $1 \times 10^9$  CFU ml<sup>-1</sup> and infected with phage multiplicity of infection (MOI) = 10. Samples were taken at 5 and 180 min post-infection and fixed with 2.5% glutaraldehyde (in 0.1 M Sorenson's phosphate buffer, pH 7.4). After 2 h of fixation (RT), the samples were collected, washed with 100 µl of buffer and prepared for transmission electron microscopy (TEM) imaging as previously described (Bamford & Mindich 1980) at the University of Oulu, Biocenter Oulu Electron Microscopy Core Facility. The positively stained samples were examined with a JEOL JEM-1400HC microscope at 80 kV (University of Jyväskylä, Finland).

## FLiP growth in long-term liquid cultures containing agar

Because of the low increase in infective centres and lack of latent phase after the beginning of FLiP growth in liquid-based one-step experiments (Figures S3 and S4) cultures supplemented with solid J-Shieh-agar for bacterial attachment (Figure S1B) were used to monitor FLiP-host dynamics with *Flavobacterium* sp. B330, B167 and B114. As a preliminary experiment, FLiP attachment on agar surfaces was studied (Figure S5). The first longer-term experiment in liquid cultures containing agar lasted 26 h and the other 77 days.

The liquid cultures supplemented with solid J-Shieh agar were prepared as described above with some modifications: 2% J-Shieh agar was cast into Erlenmeyer flasks without bacterial addition. Overnight cultures of bacterial strains B330, B167 and B114 were adjusted to matching cell densities (approximately  $4.0 \times 10^8$  CFU ml<sup>-1</sup>) based on OD and mixed into J-Shieh applied on top of solidified J-Shieh-agar. FLiP

was used for infection so the titre was  $1.0 \times 10^6$  PFU ml<sup>-1</sup> at the beginning of the experiment and the MOI was ~0.4. Cultures were incubated by shaking (120 rpm) at room temperature (26-h experiment) or three different temperatures: RT, 17°C and 9°C (77-day experiment). The optical density of cultures (200 µl sample) in the 26-h experiment was measured using 595 nm wavelength (Multiskan FC Microplate Photometer and SkanIt Software for Microplate Readers) 10 times during the experiment. In both experiments, phage titre was monitored by taking samples. Samples were centrifuged (6000×g, 5 min, 4°C) and supernatant plated. In the 26-h experiment, samples were taken 13 times. In the 77-day experiment, samples were taken approximately once a week. Controls without bacterium were incubated in the same conditions to monitor phage decay in different temperatures without host bacterium.

Also, a separate experiment was conducted to monitor FLiP decay in Shieh and filtered lake water at room temperature. All samples were prepared in triplicates: 25 ml of Shieh medium or lake water from Jyväsjärvi with  $5 \times 10^6$  PFU ml<sup>-1</sup> phage was added into a 50 ml tube and lightly vortexed (1–2 s). Tubes were standing still between samplings and lightly vortexed (1–2 s) before samples were taken. The first sampling was done 1 h after starting the experiment and the next samplings once a week after that until no phage were left. PFU ml<sup>-1</sup> was determined on B330.

## Effect of FLiP on bacterial biofilm production

Biofilm formation capacity of *Flavobacterium* sp. B330, B167 and B114 were determined both with and without FLiP using microtiter biofilm assay as described by O'Toole (O'Toole, 2011). B330, B167 and B114 were grown overnight and adjusted to  $5 \times 10^8$  CFU ml<sup>-1</sup> using Shieh. Diluted bacterial culture was applied into wells of F96 Cert. Maxisorp nunc-immuno plate (Thermo Scientific) using 170 µl for phage treatment wells and 200 µl for control wells. FLiP (30 µl) was added to wells with a multiplicity of infection (MOI) 0.1 either directly after the bacteria or 24 h after the beginning of the experiment. The final liquid volume in each well was 200 µl, and all treatments were done in four replicates.

Plates were incubated at 18°C in plastic containers with water in a decanter flask to prevent samples from drying. A sample was taken from three replicates of each treatment 24 h after phage addition and a dilution series was pipetted on B330 containing soft agar to determine phage titre. To quantify the amount of biofilm formed, growth medium and unattached cells were discarded, and biofilm was stained incubating for 15 min in 0.1% crystal violet (CV) solution. The plate was



rinsed three times with water and dried in RT. CV was solubilized by incubating for 15 min in 30% acetic acid, moved to a new microtiter plate and the A595 was measured using Multiskan FC Microplate Photometer (Thermo Fisher) and SkanIt Software for Microplate Readers (Thermo scientific).

Biofilm data were analysed using 2-way ANOVA with multiple comparisons, and phage titre data by non-parametric Kruskal–Wallis ANOVA, with pairwise comparisons to bacteria-free control using the Dunn test. In both analyses, GraphPad Prism (version 10.0.3) was used.

## FLiP protein analysis in zymograms

To characterize the effect of temperature on phage lytic enzyme activities, zymogram gels were made by complementing 17% Tricine-SDS-PAGE gels with peptidoglycan isolated either from *Escherichia coli* (DH5 $\alpha$ ) or *Flavobacterium* sp. B330. Gels were run (100 V, 30 mA) at both room temperature (150 min) and 6°C (180 min) using  $\times 2$  purified (denatured by boiling, 5 min) FLiP samples as well as  $\times 2$  purified control phage samples (PRD1, PM2 and PhiCJT23) containing 30  $\mu$ g protein. Gels were rinsed with sterile water and incubated in sterile water (30 min, RT or 6°C). The gels were moved to renaturation buffer (25 mM KPO4 pH 7.4; 0.1% Triton X-100 [Sigma-Aldrich]) and incubated rocking (45 h, RT or 6°C). The gels were rinsed with sterile water and dyed (0.1% methylene blue; 0.01% KOH) rocking (1 h, RT or 6°C). Excess dye was removed by incubating rocking (1 h, RT) in sterile water.

To investigate if the activity of FLiP lytic enzyme is pH-dependent, experiments described above were repeated for FLiP and control phages at 6°C with three renaturation buffers with different pH (6.4, 7.4 and 8.0).

## RESULTS

### Host range of FLiP

FLiP has been isolated with *Flavobacterium* sp. B330 (Laanto et al., 2017). In host range experiments it also infected three other hosts: *Flavobacterium* sp. strains B80, B114 and B167 (Figure 1). Infection on B80 was weaker compared to other hosts (titre was about 1–2 orders of magnitude lower) and FLiP did not always infect B80 in a reproducible way, meaning that for unknown reasons sometimes plaques were not observed. Therefore, B80 was not included in further experiments. FLiP forms plaques of different sizes independent of the host bacterium, but the largest plaque sizes are typical on B114 while the smallest plaque sizes are observed on B330 (Figure 2A). FLiP plaques are usually clear with fuzzy edges on B330, B167 and

B114 bacterial lawns, but turbid plaques are occasionally observed especially on B167 and B114 hosts. Based on our surveys (data not shown) the turbidity of the plaques does not seem to be a heritable phenotype.

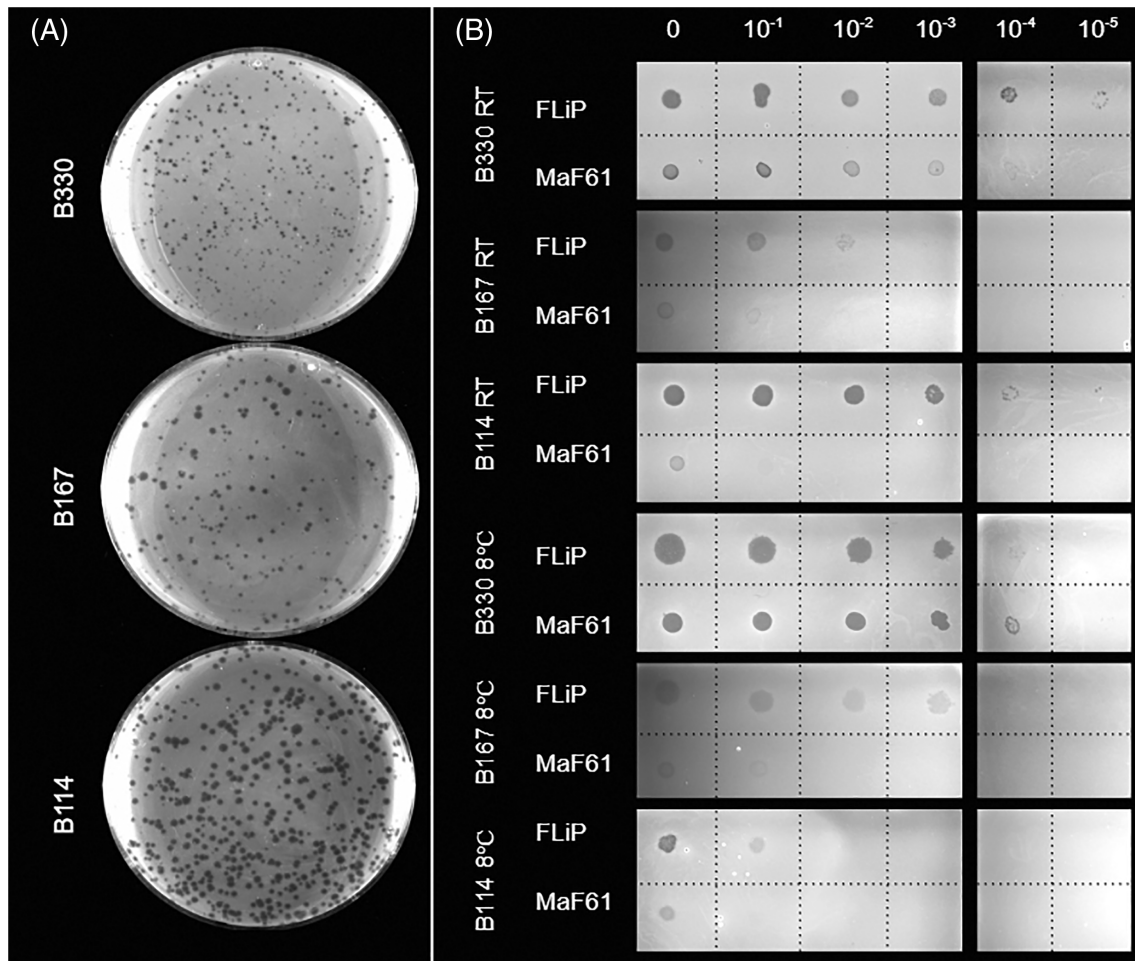
### Hosts of FLiP are psychrotolerant and FLiP is more lytic in cooler temperatures

In the first experiment, a large scale of temperatures was tested to analyse the range where FLiP and its hosts can grow and to detect possible temperature-dependent differences in FLiP infectivity. B330, B167 and B114 grew on Shieh-agar plates between 4°C and 33°C (Table 1), but an even bacterial lawn was formed only in the temperature range 7–25°C. For comparison bacterial growth in liquid at different temperatures is included in Figures S6 and S7. FLiP infected all its hosts from 4°C upwards as detected by a clear titration drop area. Clear FLiP plaques appeared on the B114 host only in temperatures between 13°C–21°C, at 4°C–30°C on B330 and 7°C–29°C on B167 (Table 1). The titre was high (at least  $1.55 \times 10^9$  PFU ml $^{-1}$ ) on all hosts at all temperatures allowing plaque formation (Table 1).

In the next temperature experiment, which was replicated three times, titration drop areas were measured, titres calculated and bacterial growth recorded at temperatures RT (~21°C) and 8°C (Table S1). In experiment A we compared the lysis area sizes of FLiP and the control phage MaF61(dsDNA phage) at room temperature and +8°C (Figure 2B). With B330, FLiP lysis area size was significantly larger at +8°C compared to RT (One-way ANOVA, Tukey's post hoc test  $p < 0.001$ ), whereas with MaF61 this effect was not observed (Figure 2B). Lysis areas were also significantly larger at +8°C with B167 (Mann–Whitney  $U$  test,  $p < 0.05$ ), whereas in B114 the effect was the opposite (Mann–Whitney  $U$  test,  $p < 0.05$ ). In experiment B (Figure S8B), FLiP lysis area sizes were larger at +8°C compared to RT in B330 and in B167 ( $p < 0.05$  for both).

FLiP titre on different hosts varied between experiments, but in all of them, better titre on B330 was achieved at RT, while titre on B167 was not much affected by temperature (Table S1). FLiP infection on B114 was weak and without individual plaques at 8°C in all the experiments. Plaques on B330 and B167 were more prone to spread out of the main lysis area at the colder temperature. This was not observed with MaF61 and lysis areas were always with sharp edges.

The difference between FLiP and the control phage MaF61 indicates that lysis area size at different temperatures is not only dependent on the growth rate of the bacterium but also phage characteristics are important determinants in lysis area formation.



**FIGURE 2** FLiP and MaF61 plaque and lysis area sizes and morphologies in double layer agar assay. (A) Plaque size and morphology of FLiP at room temperature on *Flavobacterium* sp. B114, B167 and B330 on Shieh agar. (B) Typical FLiP and MaF61 plaque and titration drop morphologies at room temperature and 8°C on *Flavobacterium* sp. B330, B167 and B114. One of the two replicated plates was imaged and presented in the figure. Undiluted phage lysate and a dilution series (2  $\mu$ l each) were pipetted on plates which were incubated for either 2 days at room temperature or 6 days at 8°C, after which no further growth of bacterium and no further change in phage infection was observed. Plates were imaged with ChemiDoc MP Imaging system (Bio-Rad). The figure is composed of separate parts of plate images: Solid lines indicate the edges of one part and dotted lines confine areas of each titration drop. RT, room temperature ( $\sim$ 21°C).

### FLiP adsorbs more efficiently to stationary phase cells than to exponentially growing hosts

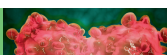
To see if adsorption is affected by the growth phase of the host, the adsorption of FLiP particles was followed in liquid in two experiments. In the first experiment, fresh cells in the exponential growth phase were used and in the second experiment, overnight-grown cells in the stationary growth phase were used.

In the adsorption assay with fresh cells, adsorption to B330 cells continued until the end of the experiment and maximal adsorption (66%) was reached at the time point of 10 min (Figure 3A). There was almost no adsorption to B167 (max. 5%) and weak adsorption to B114 (max. 18%) cells in the time scale of 10 min.

In the assay with overnight-grown cells FLiP adsorption was rapid and effective both to B330 and B114 cells (Figure 3B). When B330 cells were used, 57% of FLiP particles were already adsorbed during the first 2 min and maximal adsorption (63%) was reached in 6 min. In the B114 host, 52% of particles were adsorbed during the first 2 min and maximal adsorption (64%) was reached in 4 min. Adsorption to B167 cells was weaker (max. 19%) compared to the two other hosts.

The impact of the host cell growth phase on FLiP infection was observed on plates using a double-layer plaque assay. When B330 was used as a host in plaque assay, FLiP titre was similar independent of the growth phase of the host (Figure 3C). In contrast, B114 and B167 susceptibility to FLiP infection increased especially during the first 6 h after subculturing.





**TABLE 1** FLiP infectivity and titres on *Flavobacterium* sp. strains B330, B167 and B114 in the temperature range of 4°C–37°C.

Temperature (°C)	FLiP titre on B330 (PFU ml <sup>-1</sup> )	FLiP titre on B167 (PFU ml <sup>-1</sup> )	FLiP titre on B114 (PFU ml <sup>-1</sup> )	Recorded after (days)
4	1.55E+10	+	+	7
7	3.73E+10	5.55E+09	+	7
8	4.90E+10	9.70E+09	*	9
9	1.10E+10	–	+	3
13	2.00E+10	1.55E+10	8.18E+09	2
14	1.64E+10	9.00E+09	1.55E+09	2
17	7.50E+10	2.30E+10	6.10E+09	2
19	3.00E+10	1.00E+10	1.82E+10	2
21	7.00E+10	2.18E+10	1.50E+10	2
25	3.80E+10	2.80E+10	+	2
29	6.90E+09	2.18E+10	+	2
30	8.30E+09	*	–	2
31	+	*	–	2
33	+	+	+	2
34–37	–	–	–	9

Note: Recorded after (X) days indicate the number of days after which no further growth of bacterium and no further change in phage infection were observed.

+Infection without plaques: Bacterial cells lysed by the phage leading to a clear titration drop area. \*Inhibition of bacterial growth without infection: Bacterial cell growth is inhibited leading to a turbid titration drop area, where bacterial growth is lowered compared to the surrounding lawn. –No infection or inhibition: Titration drop area is covered with similar bacterial lawn than surroundings.

However, this result should be considered indicative as the experiment lacked replicates.

### FLiP interactions with cells of *Flavobacterium* sp. strain B330 under TEM

FLiP interactions with cells of *Flavobacterium* sp. strain B330 were also studied under TEM from thin sections. At 1 min p.i. FLiP particles were observed on the surface of cells (Figure 4A). While one-step growth experiments did not show a clear drop in bacterial CFU or very clear steps in FLiP propagation (Figures S3 and S4), from thin sections at 180 min p.i. we could see that most of the cells were intact but cells undergoing lysis with mature FLiP virions inside were also observed (Figure 4B). At 180 min p.i., only a small portion of the cells (~1%) were seen lysing (Figure 4C).

### Long-term cultivation experiments show that FLiP propagation is most effective with *Flavobacterium* sp. B167

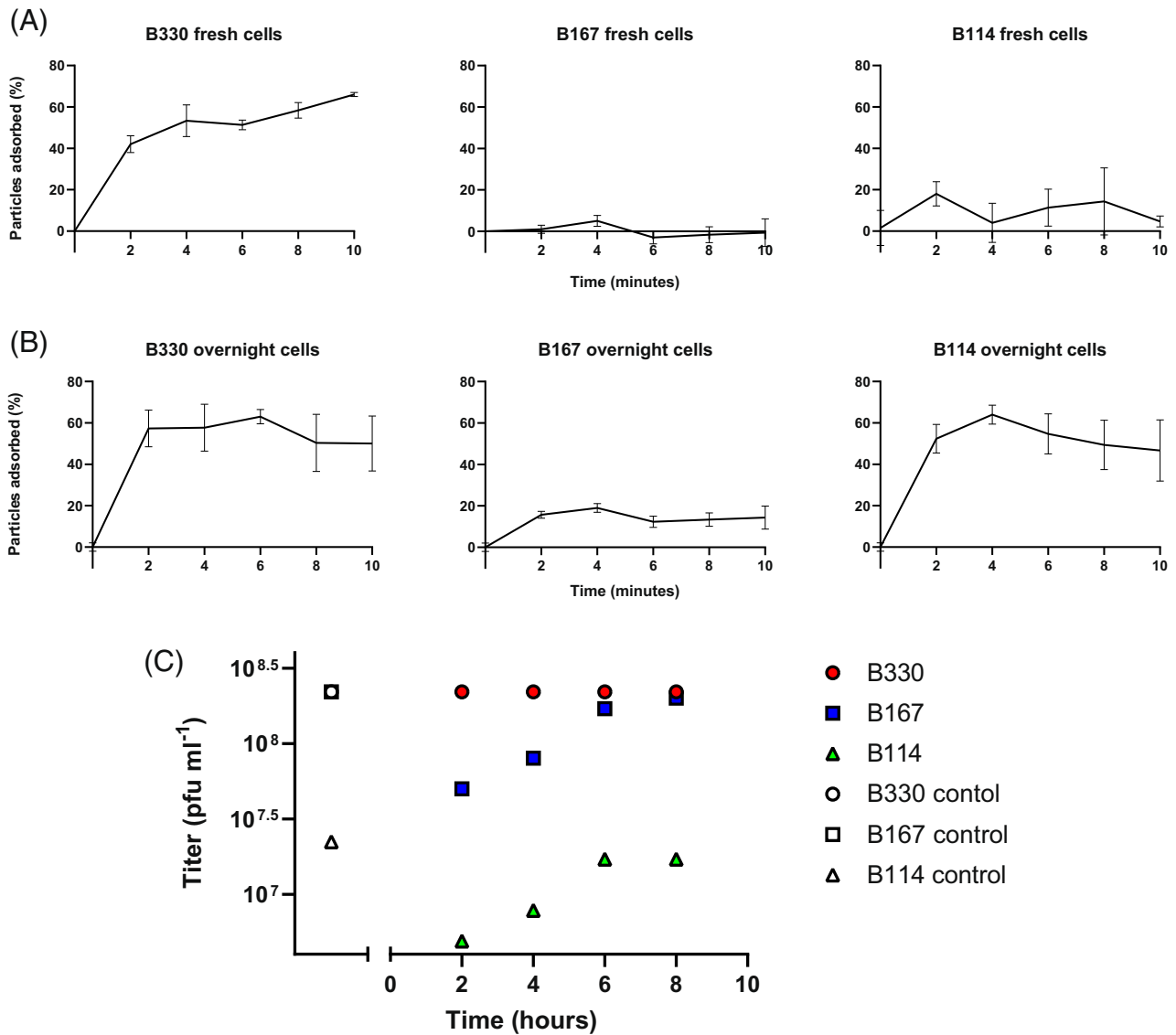
Since it seemed that FLiP–host interactions happen more slowly than phage–host interactions in most cases, we explored FLiP–host dynamics for an extended period of time. Longer-term growth experiments were performed in liquid cultures supplemented with agar surface for bacterial attachment (Figure S1B). To give plenty of time for cell attachment and several

infection cycles to occur, titres were followed for 26 h (Experiment 1) and 77 days (Experiment 2).

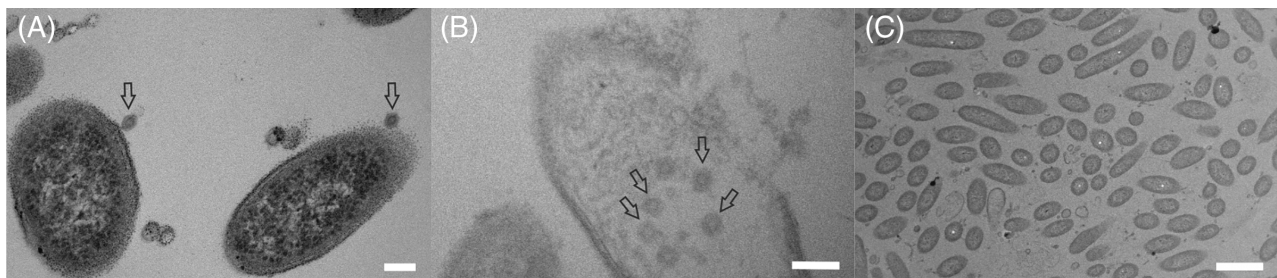
The initial titre of FLiP was  $1.0 \times 10^6$  PFU ml<sup>-1</sup> at the beginning of the first growth experiment (26 h). The highest titre ( $7.0 \times 10^{10}$  PFU ml<sup>-1</sup>) was achieved with B330 in 21–26 h, and FLiP replication with B167 was almost similar (Figure 5). Although the optical density of the B114 culture increased nearly as much as that of the B167 culture, FLiP replication in the B114 culture was weaker, resulting in a lower phage titre compared to the other two hosts.

The longest experiments (77 days) were done at three different temperatures: room temperature, 17°C and 9°C. FLiP titre increased rapidly at room temperature in B330 but then started to decrease (Figure 6A,B). When grown with B330, FLiP titre reached to maximum of  $\sim 3\text{--}4 \times 10^9$  PFU ml<sup>-1</sup> regardless of the temperature; within 1 day at room temperature (Figure 6A), 2 days at 17°C (Figure 6C) and 15 days at 9°C (Figure 6D,E).

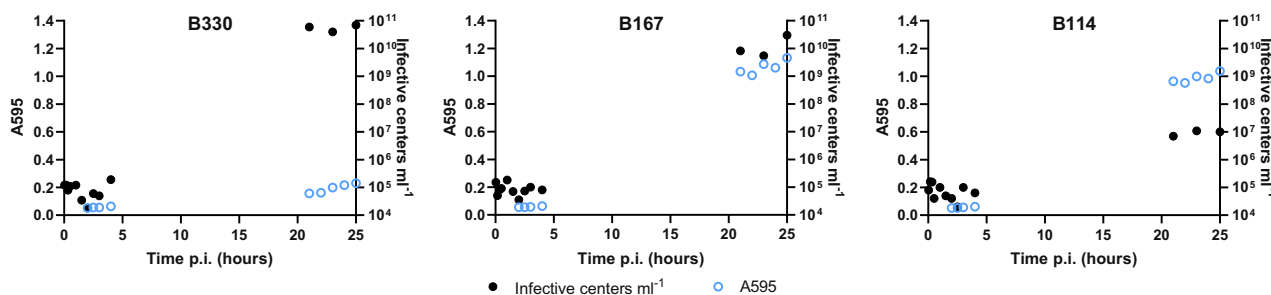
With B167, FLiP replication was slower compared to B330, but higher titres were observed, reaching  $\sim 3 \times 10^{11}$  PFU ml<sup>-1</sup> in 2 days at 17°C (Figure 6C). Maximal titres at both room temperature and at 9°C were  $\sim 2\text{--}3 \times 10^{10}$  PFU ml<sup>-1</sup> after 7 (Figure 6A) and 15 days (Figure 6E), respectively. FLiP replication with B114 was slower and titre rise was smaller compared to the other two hosts. At 17°C maximal titre of  $1.73 \times 10^8$  PFU ml<sup>-1</sup> was reached in 2 days (Figure 6C). However, the highest titre,  $1.64 \times 10^9$  PFU ml<sup>-1</sup>, with B114 was reached at 9°C, but it took 15 days



**FIGURE 3** Effect of host cell age on FLiP infection. Mean adsorption of three replicates ( $\pm$ SEM) of FLiP particles to (A) fresh and to (B) older host cells. As far as fresh cells were concerned, FLiP could only adsorb to B330 cells effectively. Adsorption was faster and more efficient to stationary phase cells of all strains. (C) Effect of host growth phase on FLiP propagation efficiency on plates. FLiP dilution was plated with the same batch of bacterial subculture every 2 h after the subculture. *Flavobacterium* strains B167 and B114 were more susceptible to FLiP 6 or more hours after subculture, whereas incubation did not influence *Flavobacterium* sp. B330.



**FIGURE 4** Thin sections of FLiP and cells of host *Flavobacterium* sp. B330 under TEM at (A) 1 min post-infection (p.i.) and in panels (B) and (C) 180 min p.i. Scale bar in (A) and (B) is 100 nm and 1  $\mu$ m in (C). Arrows indicate FLiP particles.



**FIGURE 5** Dynamics between FLiP and three *Flavobacterium* sp. hosts (B330, B167, B114) during 26 first hours in J-Shieh liquid cultures supplemented with agar. OD (blue, left axis) and infective centres (black, right axis) are presented.

to reach (Figure 6E). At room temperature, the titre remained at  $4.6 \times 10^7$  PFU ml<sup>-1</sup> with B114 (Figure 6A, B). A comparison of the highest titres achieved during these experiments is presented in Figure S2B.

At 9°C FLiP titre did not markedly drop during 77 days (Figure 6E), whereas at room temperature titre started to decrease and no infective particles were found after 30–50 days, depending on the host strain (Figure 6B). This result was confirmed in an additional experiment where FLiP particles were incubated at room temperature without host bacterium either in Shieh medium or in lake water. No infective particles were detected after the 50-day measuring point in lake water and the 70-day measuring point in Shieh (Figure S9).

### Effect of FLiP on bacterial biofilm formation

Next, we explored how FLiP influences bacterial biofilm formation, and on the other hand, if FLiP can infect bacteria that have already formed biofilm. In this experiment, FLiP was added to bacterial culture at the same time as the host, or after 1-day culture. In both cases, the biofilm was stained on the following day, and the FLiP titre was recorded. Both bacterial strain and phage addition significantly influenced biofilm formation ( $F_{5,36} = 549$ ,  $p < 0.001$  and  $F_{2,36} = 88.5$ ,  $p < 0.001$ , respectively) but there also was an interaction of these factors ( $F_{5,36} = 15.9$ ,  $p < 0.001$ ) indicating that the effect of phage was not similar in different hosts (Figure 7). Pairwise comparisons indicated significant differences in the bacterial ability to form biofilm, with B114 forming the highest amount of biofilm already after 1 day of culture. When comparing phage-free control cultures, the incubation time significantly increased biofilm formation in all bacterial hosts. However, the effect of FLiP or the timing of the infection was not uniform. FLiP did not have a significant impact on biofilm formation in B330, but in B114 FLiP reduced biofilm formation if added simultaneously with the bacterial host. In B167 FLiP was able to efficiently reduce biofilm

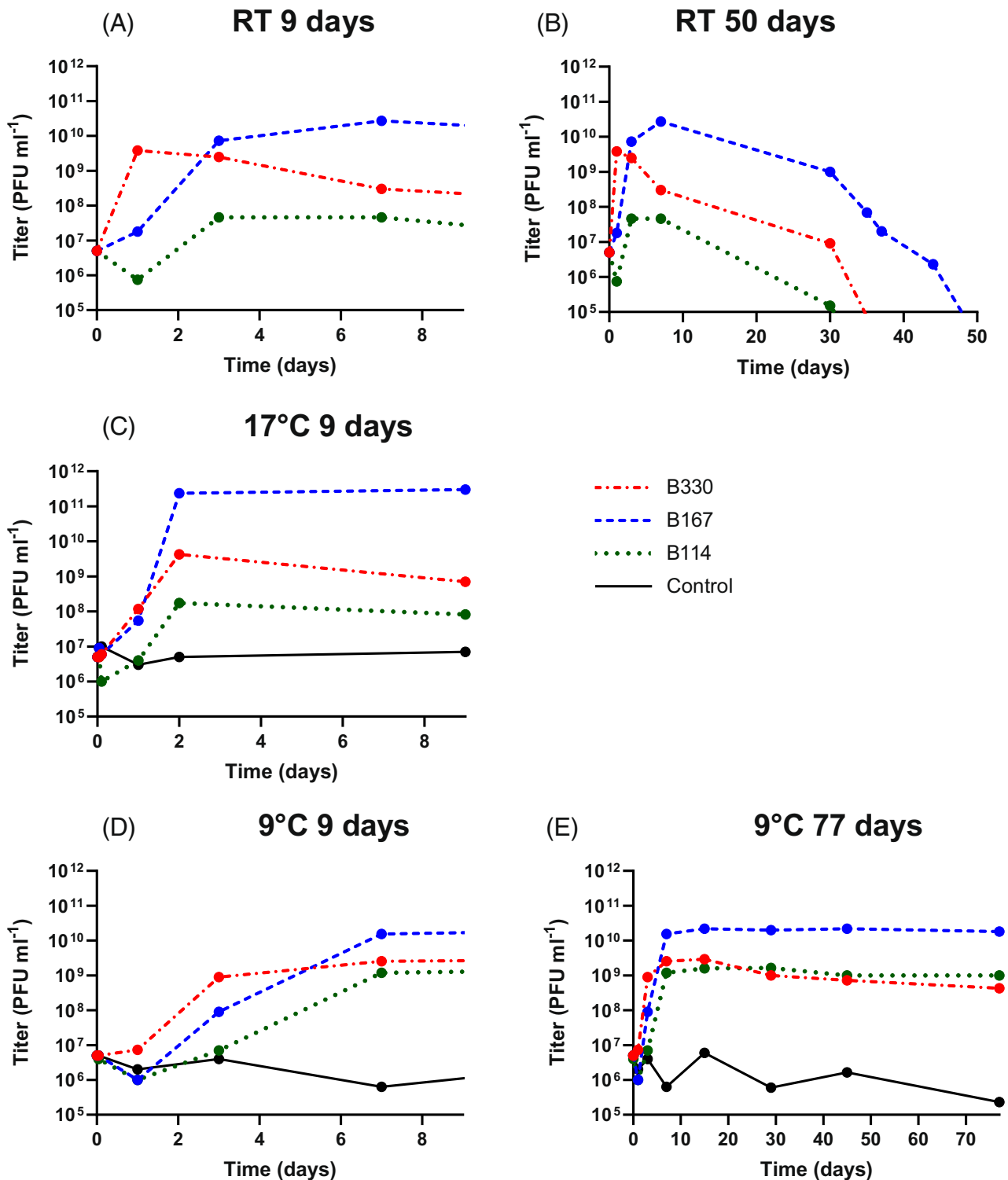
formation when applied to 1-day-old culture. However, it should be noted that the initial concentration of cells was slightly different between samples and controls.

FLiP replication occurred with B330 and B114 only when the phage was applied simultaneously with the bacterial host (Figure 7). Although the difference in no-bacterium control ( $9.8 \times 10^7$  PFU ml<sup>-1</sup>) was not statistically significant, FLiP titre increased to  $9.5 \times 10^9$  PFU ml<sup>-1</sup> in B330 and  $3 \times 10^9$  PFU ml<sup>-1</sup> in B114. With B167, phage titre increased to  $7.5 \times 10^{10}$  PFU ml<sup>-1</sup>, and in the treatment where phage was added 1 day after the bacterium the average titre was  $3 \times 10^9$  PFU ml<sup>-1</sup>.

### The activity of FLiP lytic enzymes is possibly temperature-dependent

Zymography suggested that two of the structural proteins of FLiP have lytic activities, and FLiP lytic enzymes fall into similar size ranges with those of PRD1. PRD1 lytic transglycosylase P7 (27.1 kDa) can be seen in all zymogram gels, but endolysin P15 (17.3 kDa) produced less visible clear zone (Rydman & Bamford, 2000, 2002) (Figures 8 and S10). FLiP gp14 with a calculated mass of 25.2 kDa is known to contain a conserved lytic transglycosylase domain based on sequence analysis (Laanto et al., 2017). A clear zone could indeed be seen approximately on the level of 25 kDa in most of the zymogram gels (Figures 8 and S10). Zymography done at both RT and at 6°C indicated that FLiP lytic transglycosylase can degrade B330 peptidoglycan in cold temperatures, but not always at room temperature (Figure 8A,C). Temperature dependence was not observed when peptidoglycan from *Escherichia coli* was used (Figure 8B,D), as *E. coli* peptidoglycan was degraded by FLiP lytic transglycosylase both at RT and at 6°C.

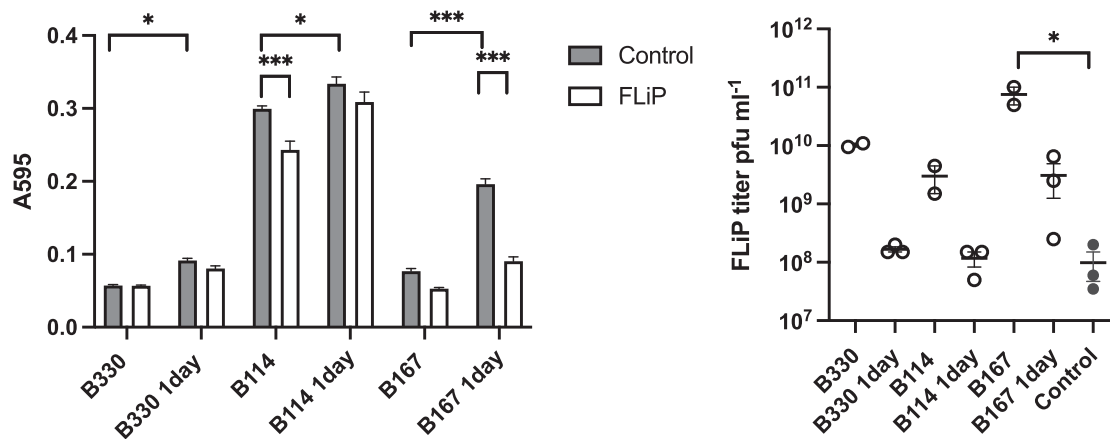
In addition to the transglycosylase enzyme, the FLiP sample produced another clear zone on the level of approximately 14 kDa in all zymogram gels. The zone size is very similar to that of lysozyme (Figures 8 and S10), which is known to be 14–15 kDa in mass. As compared to structural protein profiles of FLiP in SDS-



**FIGURE 6** Titres of FLiP grown with each host (B330, B167, B114) at 9°C, 17°C and room temperature in J-Shieh liquid cultures supplemented with 2% agar. Titre change at RT (A) 9 days and (B) 50 days. (C) Titre change at 17°C, 9 days. Titre change at 9°C (D) 9 days and (E) 77 days. RT, room temperature. Control indicates FLiP titre without bacterial host.

PAGE analysis (Laanto et al., 2017) the smaller lytic enzyme size suggests it to be gp7, which does not have functional annotation. This smaller lytic enzyme was active against both B330 and *E. coli* peptidoglycan

at both tested temperatures. Furthermore, both of the FLiP lytic enzymes degraded peptidoglycan extracted from B330 and *E. coli* in a pH range from 6.4 to 8 (Figure 8).



**FIGURE 7** FLiP–host interactions and biofilm formation in three *Flavobacterium* sp. hosts (B330, B114 and B167). (A) Effect of timing of FLiP addition on bacterial biofilm formation (mean  $\pm$  SEM). FLiP was added either at the same time with bacteria or after bacteria had been allowed to form biofilm for 1 day. Biofilm formation was measured 1 day after phage addition. (B) Effect of biofilm formation on phage titre (mean  $\pm$  SEM). Stars indicate statistical significance ( $p$  value) levels: \*0.05–0.01, \*\*0.01–0.001, and \*\*\*<0.001.

## DISCUSSION

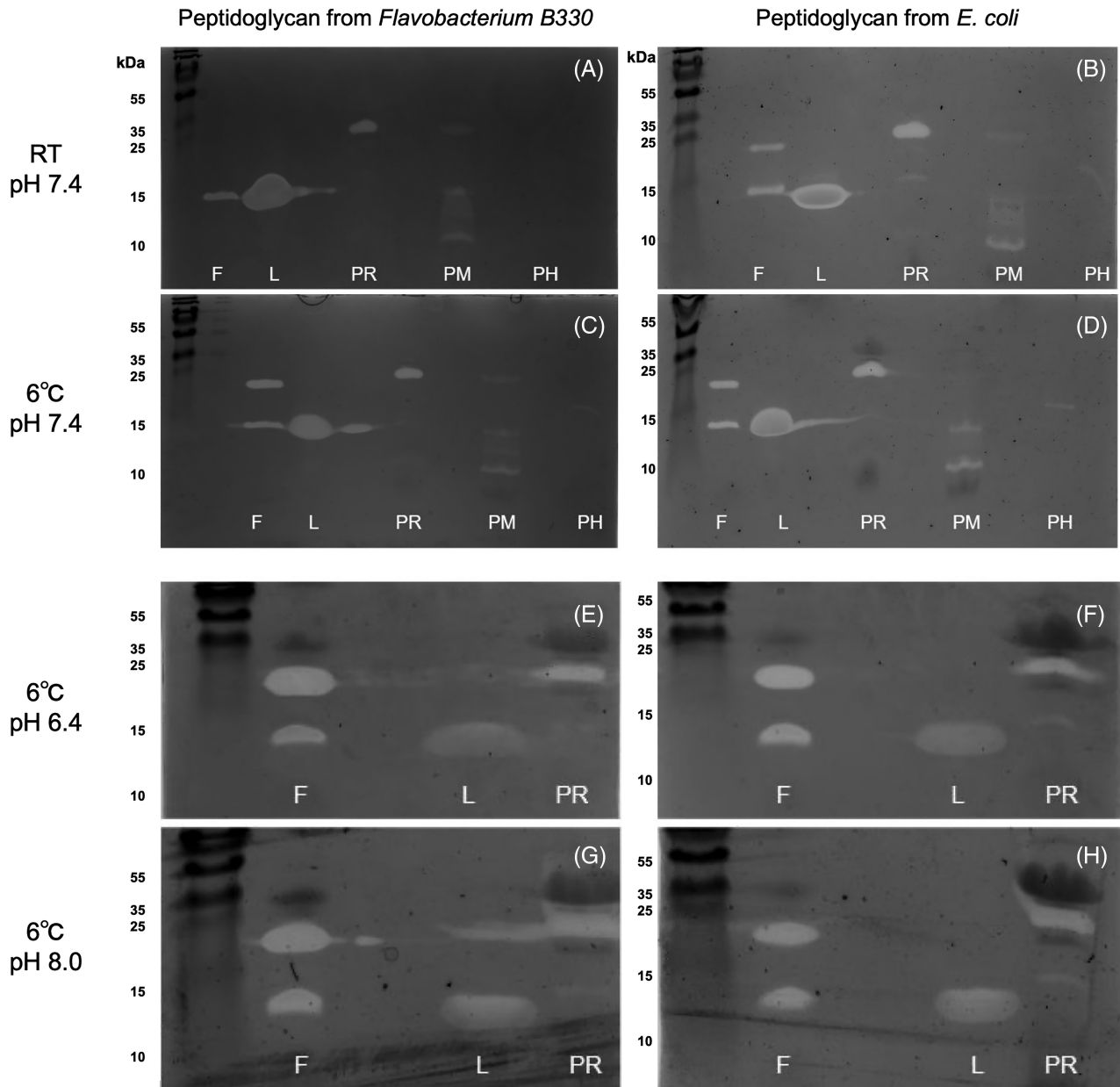
Historically, studies of bacteriophage life cycles and host interactions have been concentrated on a set of phage–bacteria combinations, which have been considered to serve as good model systems or are of clinical or economic importance. Especially tailed dsDNA phages, previously considered to be the dominant phage type in almost all ecosystems, have been studied more in-depth than other phage types. That has given a limited view of the entire diversity of phages and their life cycles. In addition, the dynamic nature of phage life cycles is not revealed if experiments are only carried out in optimized laboratory conditions (Attrill et al., 2023; Brum et al., 2016; Tokman et al., 2016). FLiP represents a minority in phage studies in at least three fundamental ways as it is tailless, lipid-containing and has a ssDNA genome. Although ssDNA viruses are important players in the aquatic ecosystem (Brum et al., 2013; Holmfeldt et al., 2013; Kauffman et al., 2018; Kirchberger et al., 2022; Kirchberger & Ochman, 2023; Van Cauwenberghe et al., 2022), the empirical studies concerning ssDNA phages have been overshadowed by studies done with dsDNA phages. Furthermore, FLiP infects flavobacteria, which form one of the most prevalent groups of environmental bacteria (Bartlau et al., 2022; Eiler & Bertilsson, 2004, 2007) indicating the possibility of a large ecological impact of FLiP and its relatives.

When new phage species or strains are characterized, one of the main aims is to investigate the host range of the phage. We wanted to go one step further and study if phage–host interactions are inherently different with different hosts as has been shown in other phage–host systems (Howard-Varona et al., 2017, 2018). We also aimed to reveal how phage infection is affected by variations in environmental conditions. An overarching theme in all experimental results is that

FLiP–host interactions are dependent on the host strain. FLiP can infect *Flavobacterium* sp. B330, the original isolation host, in a broad range of environmental conditions. Infections with B167 have more condition-dependent variability, and infections with B114 are generally weaker compared to others. *Flavobacterium* B330 and B167 have been isolated from Lake Jyväsjärvi in Central Finland, and strain B114 from a river in Northern Finland. Different isolation locations might be connected to bacterial adaptations to different environmental conditions. Since contact with these kinds of hosts is possible in real life, FLiP may benefit from different infection dynamics offered by hosts.

According to experiments done using either exponentially growing or stationary phase bacterial hosts, FLiP-infected older *Flavobacterium* sp. B114 and B167 cells in the stationary growth phase faster and more efficiently than fresh cells in the exponential growth phase (Figure 3). To infect stationary phase cells more efficiently than exponentially growing cells is a rare infection strategy, although more phages are probably capable of infecting stationary phase cells than generally assumed (Schrader et al., 1997; Woods, 1976). The vast majority of all known phages prefer exponentially growing cells (Bryan et al., 2016; Koskella et al., 2022), but context specificity of phage–bacterium interactions is rarely taken into account in laboratory experiments (Schrader et al., 1997) and full spectra of conditions in nature cannot be accurately reproduced in laboratory settings. The ability to infect encountered hosts in different growth phases could benefit FLiP as it could enhance phage survival and growth in natural environments, considering bacteria in nature are in different growth phases and the environmental conditions are rarely optimal for bacterial growth.

Most often, FLiP replication is weak in traditional liquid cultures and the OD of the liquid cultures do not drop during FLiP infection (Figures S3 and S4),



**FIGURE 8** Zymograms show the temperature dependence of FLiP lytic transglycosylase. Zymogram gels incubated at room temperature with (A) *Flavobacterium* sp. B330 peptidoglycan and (B) *E. coli* peptidoglycan. Zymogram gels incubated at 6°C with (C) B330 peptidoglycan and (D) *E. coli* peptidoglycan. The activity of lytic enzymes at different pH. Zymogram gels incubated at 6°C at pH 6.4 with (E) B330 peptidoglycan and (F) *E. coli* peptidoglycan. Zymogram gels incubated at 6°C at pH 8.0 with (G) B330 peptidoglycan and (H) *E. coli* peptidoglycan. F, FLiP; L, lysozyme; PH, PhiCJT23; PM, PM2; PR, PRD1; RT, room temperature.

meaning that only a few cells are lysed, which was also indicated by the EM images (Figure 4). The efficient FLiP infection seems to require the surface attachment of the host, as has been observed also with some other *Flavobacterium*-infecting phages (Almeida et al., 2019; Laanto et al., 2015) as well as in some other phage-host systems (Ulrich et al., 2022). So far described *Flavobacterium* infecting phages appear to be associated with Type 9 Secretion System (T9SS) (Castillo et al., 2021; Kunttu et al., 2021; Shrivastava et al., 2013). T9SS is associated with flavobacterial

gliding motility machinery, which is used in motility on a surface (Johnston et al., 2018; Shrivastava et al., 2013). Our data thus indicates that the FLiP receptor might be only expressed when the host cell is attaching or recently attached to a surface. However, it remains unclear if FLiP uses components of T9SS, or proteins translocated through that system, as its specific receptors. Therefore, experimental evidence is needed to verify the FLiP receptor(s).

Interestingly, the role of surfaces in the FLiP–host interaction is multilayered, extending beyond the host



cell gene expression. It has been estimated that most environmental bacteria live in biofilms (Adnan et al., 2010; Ansari et al., 2012; Watnick & Kolter, 2000; Yin et al., 2019). FLiP particles also adhered to the agar surface (Figure S5), as has been shown previously (Almeida et al., 2019). This benefits the phage by increasing the likelihood of encountering a suitable host, and by avoiding drifting in the environment. Our biofilm assays indicated that FLiP infections happen most efficiently in the very early phase of biofilm formation. However, also here the host matters, as FLiP seemed to be able to interact with *Flavobacterium* B167 cells in formed biofilm, and replicate (Figure 7). Together, these data suggest that surface interactions can be more important for aquatic phage-bacterium ecology than previously anticipated.

Enlarged plaque and titration drop size in cool temperature compared to room temperature could indicate either enhanced lytic enzyme activity (see below) or larger burst size caused by the slow growth of hosts and elongated time before lysis (Kannoly et al., 2023; Kim et al., 2018). In addition, FLiP particles maintained infective for several months at 9°C (Figure 6), suggesting the phage tolerates the cold winter months in boreal water bodies during the season when hosts are in a less active state. In contrast, phage decay was faster at room temperature (Figures 6 and S9), indicating that in nature phage needs to encounter a susceptible host to survive through high-temperature seasons. In conclusion, it may be advantageous for FLiP to maintain flexibility of infection strategies in different hosts to respond to the seasonal changes in the boreal environment, where water temperatures fluctuate considerably from frozen surface waters in winter to warm summers.

Zymogram assays revealed two structural proteins with lytic activity in FLiP virion resembling those of PRD1 (Figures 8 and S10). There are two different types of lytic proteins present in the structures of both species. FLiP lytic enzyme gp14 contains the same conserved lytic transglycosylase domain as PRD1 lytic enzyme P7, which are also similar in size (~25 and 27.1 kDa, respectively). The other FLiP protein with lytic activity, possibly gp7, does not have any sequence similarity in DNA or protein level to any known lytic enzymes. It would not be an efficient use of genomic space if these enzymes were not needed at some point in the phage life cycle. So far, known icosahedral, single-stranded nucleic acid phages rely on amurin-based single-protein lysis (Catalão et al., 2013; Grabowski et al., 2021; Székely & Breitbart, 2016). In recent studies (Roux et al., 2012; Van Cauwenberghes et al., 2022) putative endolysin sequences have been found in microvirus genomes. If gp7 is an endolysin, our study is the first to show experimental evidence of ssDNA phage-encoded endolysin.

Zymogram assays revealed that FLiP lytic enzymes have slightly different activities depending on

temperature (6°C or RT) and source of peptidoglycan (*E. coli* or *Flavobacterium* B330). At room temperature, gp14 was not active against B330, whereas at 6°C a signal in zymogram gel was observed (Figures 8 and S10). The other protein of FLiP with lytic activity, gp7, produced a signal in zymogram gels in all tested conditions, but the strength of the signal varied. If gp7 is an endolysin as hypothesized, it would mean that FLiP lysis is possible in a variety of conditions. In PRD1, lytic enzymes are more active in cold (Rydman & Bamford, 2000). Here, clear areas caused by FLiP lysis on plates were indeed larger in cold temperatures (Figure 2B), indicating higher activity of lysis protein in colder temperatures as compared to room temperature. This again suggests the role of gp7 as endolysin with the primary function to lyse cells, although the temperature connection was not seen in the zymogram gels.

## CONCLUSIONS

Environmental conditions in boreal lakes have strong seasonal variations, which affect all microbial interactions. When the aim is to evaluate the role of a certain phage in the microbial community, the experimental procedures should reflect the complexity of natural conditions. Using ssDNA phage FLiP as a model we show that phage infectivity is strongly condition-dependent. We conclude that it is necessary to further explore phage life cycles by systemically experimenting role of different environmental conditions (temperature, structure of environment, availability of nutrients, oxygen) as well as biotic factors like interspecies competition. This will help to estimate how the range of abiotic and biotic environmental conditions encountered during different seasons affect phage infections and ecology.

## AUTHOR CONTRIBUTIONS

**Kati Mäkelä:** Conceptualization; formal analysis; investigation; data curation; writing – original draft; writing – review and editing; visualization; funding acquisition; methodology; resources. **Elina Laanto:** Conceptualization; methodology; investigation; writing – review and editing; funding acquisition; visualization; supervision; resources. **Lotta-Riina Sundberg:** Conceptualization; methodology; resources; formal analysis; writing – review and editing; supervision; project administration; funding acquisition.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in JYX repository: <https://doi.org/10.17011/jyx/dataset/95450>.

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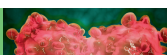
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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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