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In Vitro Evolution of Specific Phages Infecting the Fish Pathogen *Flavobacterium psychrophilum*

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Abstract

**Background:** *Flavobacterium psychrophilum* is the causative agent of the bacterial cold-water disease and rainbow trout fry syndrome. Owing to the issues associated with increasing use of antibiotics to control the diseases, phage therapy has been proposed as an alternative method to control *Flavobacterium* infection within the industry.

**Materials and Methods:** We explored two simple and fast in vitro strategies for the isolation of evolved *F. psychrophilum* phages, using three well-characterized phages FpV4, FpV9, and FPSV-S20.

**Results:** During in vitro serial transfer experiments, 12 evolved phages were selected 72–96 h after phage exposure in the first or second week. Phenotype analysis showed improvement of host range and efficiency of plating and adsorption constants. Comparative genomic analysis of the evolved phages identified 13 independent point mutations causing amino acid changes mostly in hypothetical proteins.

**Conclusions:** These results confirmed the reliability and effectiveness of two strategies to isolate evolved *F. psychrophilum* phages, which may be used to expand phage–host range and target phage-resistant pathogens in phage therapy applications against *Flavobacterium* infections.

**Keywords:** phage evolution, phage mutations, phage–host range, *F. psychrophilum*

Introduction

*Flavobacterium psychrophilum* is a yellow-pigmented Gram-negative fish pathogenic bacterium with a global distribution in freshwater aquaculture, causing “bacterial cold-water disease” (BCWD) and “rainbow trout fry syndrome” (RTFS).1 The diseases result in high rates of juvenile mortality, increased susceptibility to other infections, high costs of treatment with antibiotics, and large economic losses in salmonid aquaculture worldwide.2–4 Treatment with antibiotics is essential to decrease mortality; however, increased microbial resistance to the licensed drugs has been documented in *F. psychrophilum*.5,6

In this context, alternative approaches to reduce the frequency of BCWD or RTFS are essential, and *F. psychrophilum*-specific bacteriophages have been proposed as attractive biological agents for controlling bacterial infections (phage therapy).7 Several phages with strong lytic capacity against *F. psychrophilum* have been reported, displaying diversity in morphology, genomic composition, kinetics and efficiency of infection, host range, and ability to control bacterial densities under laboratory conditions.8–10 Recently, the application of *F. psychrophilum* phages using different delivery methods (intraperitoneal injection, oral administration, and phages coated on feed pellets) in pathogen challenge trials with rainbow trout fry and eggs has shown variable degrees of protection, emphasizing the need for further optimization.11,12

One challenge with respect to the efficiency of phage therapy is the development of bacterial resistance to phage infection.13 Bacterial populations can evolve rapidly from a dominance of phage-sensitive clones to phage-resistant clones when exposed to the selection pressure from lytic phages.14,15 Studies based on comparative genomic analysis of phage-resistant and environmental *F. psychrophilum* strains have demonstrated that mutations in genes involved
in the type IX secretion system (T9SS), gliding motility, and hypothetical cell-surface-related proteins are linked to variability in phage infection patterns. Changes in these proteins are, therefore, a key factor in the development of phage resistance.

The selection for modified phages that are able to target the mutated receptors may, therefore, be a viable strategy to overcome the development of phage resistance and improve phage therapy efficiency. However, data supporting the potential of experimental evolution of phages with improved infection capacities are limited. According to conceptual models of arms race coevolution dynamics, phage genotypes can adapt to their evolving host, acquiring the ability to infect new hosts. In this scenario, experimental evolution studies would allow the selection for specific phage genotypes with expanded host ranges, and the ability to infect previously resistant strains.

This study presents and evaluates the efficacy of two experimental strategies that together provide an effective and robust method for the rapid isolation of evolved phages infecting the fish pathogen *F. psychrophilum*. Analysis of the mutation patterns that arise through serial incubations of phage-host systems elucidates the manner that specific genetic modifications bring significant improvements in phage-host range, thereby providing a better understanding of the genomic basis for phage infectivity in *F. psychrophilum*. This knowledge is important for developing successful phage therapy strategies to combat *Flavobacterium* infections in aquaculture facilities.

**Materials and Methods**

**Bacterial growth conditions and medium composition**

This study used 36 *F. psychrophilum* strains from rainbow trout fish farms in different geographic localities, covering a spatial scale of >2,000 km and a temporal scale of >22 years (Supplementary Table S1). The strains were stored at −80°C in tryptone yeast extract salts (TYES) broth (tryptone 0.4%, yeast extract 0.04%, CaCl$_2$·2H$_2$O 0.05% and MgSO$_4$·7H$_2$O 0.05%) with 15–20% glycerol. To culture the *F. psychrophilum* cells in the exponential growth phase (OD$_{600}$ nm = 0.3–0.4) and incubated at 15°C for ~30 min. A total of 4 mL of 43°C TYES top agar (TYES broth supplemented with 0.4% agar) was added, and the mixtures were poured onto TYES agar plates (TYES broth with 1.5% agar), which were immediately placed at 15°C. After incubation of the plates for 72–96 h, the presence of lytic bacteriophages in the form of plaques was detected.

**Preparation and purification of bacteriophage stocks**

A total of 100 µL of bacteriophage stocks (kept at −80°C with 20% glycerol) was mixed with 300 µL of *F. psychrophilum* cells in the exponential growth phase (OD$_{600}$ nm = 0.3–0.4) and incubated at 15°C for ~30 min. A total of 4 mL of 43°C TYES top agar (TYES broth supplemented with 0.4% agar) was added, and the mixtures were poured onto TYES agar plates (TYES broth with 1.5% agar), which were immediately placed at 15°C. After incubation of the plates for 72–96 h, the presence of lytic bacteriophages in the form of plaques was detected.

The bacteriophages were eluted by adding 5 mL of sodium chloride-magnesium sulphate buffer (50 mM Tris–HCl, pH 7.5, 99 mM NaCl, 8 mM MgSO$_4$, 0.01% gelatin) on top of the plate and incubated for 2 h with shaking (100 rpm), followed by chloroform fixation (10 µL/mL). Finally, supernatants were centrifuged (9,000 g, 10 min, 4°C) and transferred to a new tube. To determine phage concentrations, serial dilutions in SM buffer were used with the small drop plaque assay. Unless otherwise mentioned, the *F. psychrophilum* strains 950106-1/1 and FPS-S6 were always used for proliferation and quantification of phages.

For the purification of phage stocks, 250 mL of bacterial cultures (OD$_{600}$ nm = 0.3–0.4) were separately infected with the different phages and incubated for 72–96 h. The lysed bacterial cultures were centrifuged (9,000 g, 10 min, 4°C) and filtered through a 0.2-µm pore size sterile filter. Then, the phage stocks (>10$^9$ plaque-forming units [PFU]/mL) were concentrated by adding polyethylene glycol 8000 (PEG-8000) and sodium chloride (final concentration 10% w/v and 1 M, respectively), followed by incubation at 4°C for 24 h. Subsequently, phage solutions were centrifuged (10,000 g, 30 min, 4°C) and the phage pellet was resuspended in 2 mL of SM buffer.

**Experimental design and isolation of evolved bacteriophages**

Two strategies were conducted where selected *F. psychrophilum* strains, partially or fully resistant to phage infection by the selected phages, were coincubated with the phages FpV4, FpV9, and FPS-S20 to obtain evolved phages. The phage FpV4 belongs to genetic cluster I and phages FpV9 and FPSV-S20 belong to cluster II. The first experiment using a single phage stock approach (Fig. 1) was carried out as batch incubations of the *F. psychrophilum* strain FPS-F15 exposed to the phage FPS-S20, which is unable to infect the strain.

An individual colony of *F. psychrophilum* FPS-F15 was inoculated in 20 mL of TYES broth and incubated at 15°C with agitation at 150 rpm for 72 h. Subsequently, 1 mL of the exponentially growing culture (OD$_{600}$ nm = 0.6–0.7) was inoculated in three tubes containing 25 mL of TYES broth and incubated at 15°C with agitation at 200 rpm for 6 h.

Alongside, a single FPSV-S20 stock (10$^9$ PFU/mL) was produced as already indicated. An aliquot from the phage stock was added to bacterial cultures at a multiplicity of infection (MOI) of 10, in parallel with control cultures amended with the same volume of sterile SM buffer. After 4 days of incubation, 100-fold dilution of the infected bacterial cultures was done in TYES broth and reincubation for 72–96 h. After each passage, 500 µL of the original phage FPSV-S20 stock was added, corresponding to ~10$^7$ PFU/mL. The experiment was run for 2 weeks covering two to three transfers into fresh medium, with addition of new phages.

Subsamples for phages and bacteria were collected every day, and quantification was done by small spot assay and optical density, respectively. Aliquots from each day were plated onto a phage susceptible host strain (*F. psychrophilum* FPS-6) and the cocultured resistant strain (*F. psychrophilum* FPS-F15) to allow quantification of total phages and the quantification and isolation of potential mutant phages, respectively. In the case of plaque formation on plates with the coincubated host, 100 plaques were randomly selected before each transfer and were preserved as frozen stocks at −80°C.

The second strategy was performed with the same protocol already described but using multiple phage stocks to inoculate a different stock in each serial transfer. In these experiments, the *F. psychrophilum* strain 020612-2/1 was infected.
by phages FpV4 and FpV9 and the strain FPS-F15 with the evolved phage FPSV-S20A. For both strategies, all the experiments were done in triplicate.

**Lytic activity of evolved phages**

To quantify the lytic potential of evolved phage isolates, an *in vitro* lysis assay was performed in sterile flat 96-well plates using a FLUOstar OPTIMA plate reader (BMG, Labtech GmbH, Ortenberg, Germany). In brief, 12 wells were used for each condition. Wells were loaded with 200 µL of freshly prepared TYES broth and immediately inoculated with the *F. psychrophilum* strains 020612-2/1 and FPS-F15. The plate was incubated at 15°C with orbital shaking (100 rpm) until OD$_{595}$ nm = 0.05, and the bacterial cultures were then infected at MOI of 1 with the *F. psychrophilum* phages FpV4, FpV9, FPSV-S20, FpV4-1.1, FpV9-1.1, and FPSV-S20A and incubated at 15°C with orbital shaking (100 rpm) for 96 h.

Twelve wells without phages served as control of bacteria growth. The growth of each individual culture was monitored every 24 h for 96 h. For quantification of phage production, 5 µL of the phage-containing wells was serial diluted in SM buffer and 10 µL from each dilution was spotted at the lawn of *F. psychrophilum* strains 950106-1/1 or FPS-S6 and incubated at 15°C for 72–96 h.

**Host range analysis and efficiency of plating for evolved phages**

A total of 15 phages (3 wild type and 12 evolved phages) were tested for infectivity against the 36 *F. psychrophilum* strains. The host range of the isolated bacteriophages was determined by spotting 10 µL of the phage-containing wells was serial diluted in SM buffer and 10 µL from each dilution was spotted at the lawn of *F. psychrophilum* strains 950106-1/1 or FPS-S6 and incubated at 15°C for 72–96 h. In addition, the bacterial susceptibility to evolved *F. psychrophilum* phages was tested for the 36 *F. psychrophilum* strains by quantification of efficiency of plating (EOP) relative to the proliferation hosts (strains 950106-1/1 and FPS-S6) using small drop plaque assay after exposing the isolates to the same phage titer (10$^8$ PFU/mL). PFU were examined after 72 h of incubation. Each experiment was performed three independent times.

**Phage adsorption constants**

The adsorption constants of evolved and wild-type phages to *F. psychrophilum* strains were determined by adding these phages to 20 mL of exponentially growing cells (OD$_{600}$ nm = 0.5) at an MOI of 0.0001 and incubating the infected culture at 15°C with agitation (100 rpm). Samples were taken every 5 min for 30 min, centrifuged (5,000 g, 3 min), and diluted (1:10) in SM buffer with chloroform. The phage adsorption constant was calculated from the decrease in unadsorbed phages over time, according to the following equation: $K = 2.3/(B)r_t \times \log(p_0/p)$, where $B$ is the concentration of bacteria (cells/mL), $p_0$ is the number of PFU at time zero, $p$ is the number of PFU in supernatant (i.e., phages not adsorbed) at time $t$ (min), and $K$ is the velocity constant (mL/min).

**DNA extraction, sequencing, and bioinformatic analysis**

For DNA extraction, 250-mL phage stocks (>10$^9$ PFU/mL) were concentrated to 2 mL by PEG-8000 as is already described. Phage samples were treated with DNase I and RNase A (both a final concentration of 1 µg/mL) at 37°C for 1 h. After the incubation, enzymes were subjected to inactivation at 65°C for 10 min. Immediately, DNA extraction was performed according to Wizard Genomic DNA Purification Kit (Promega, Hilden, Germany). The amount of genomic DNA was measured using QuantiTm PicoGreen® quantification kit (Invitrogen, Waltham, MA).
The genomic DNA sequences of 12 evolved *F. psychrophilum* phages were obtained using Illumina HiSeq platform at FIMM Technology Centre (Finland) and BGI (China) with pair-end read sizes of 100 bp. Library construction, sequencing, and data pipelining were performed in accordance with manufacturer’s protocols. The Illumina data were mapped to the previously sequenced wild-type *F. psychrophilum* phages FpV4 (accession no.: KT876724), FpV9 (accession no.: KT876725), and FPSV-S20 (accession no.: MK764447), using Geneious software version 10.24

Total number of usable Illumina reads for evolved phages ranged from 0.9 to 13.6 million. Percentage of reads aligned to reference phage genomes ranged from 70.9% to 99.5%, generating coverages from 45,540× to 90,305× (Supplementary Table S2). Annotation of the genomic sequences was done by the NCBI Prokaryotic Genome Automatic Annotation Pipeline.25

Genome comparison among evolved phages and the reference ancestor phages was done using MAUVE v2.3.1 software.26 Mutations in DNA sequences were confirmed by direct inspection of the reads when present in >98% on this specific genome region.27 The effects of the mutations on DNA and amino acid sequences were analyzed by ClustalW algorithm version 2.0.64.28

Accession numbers

The GenBank accession numbers for the sequenced bacteriophages are FpV4 (KT876724), FpV9 (KT876725), FPSV-S20 (MK764447), FPSV-S20A (OK268422), FPSV-S20B (OK268423), FPSV-S20C (OK268424), FPSV-S20AI (OK268425), FPSV-S20A2 (OK268426), FPSV-S20A3 (OK268427), FpV4-1.1 (OK340820), FpV4-1.2 (OK340821), FpV4-1.3 (OK340822), FpV9-1.2 (OK340817), FpV9-2.1 (OK340819), and FpV9-1.3 (OK340818).

Results

Isolation and lytic potential of evolved *F. psychrophilum* phages

To isolate evolved *F. psychrophilum* phages, a strong phage selection pressure was imposed by subjecting them to...
a series of transfer experiments at two different conditions, both when added to the \textit{F. psychrophilum} strains 020612-2/1 and FPS-F15 (Fig. 1). These two strains displayed a variation in the susceptibility to phage infection, which was shown previously in the literature, with the strain 020512-2/1 being partially resistant to phages FpV4 and FpV9 and the strain FPS-F15, which is resistant to the phage FPSV-S20.\textsuperscript{17} In the first strategy, using \textit{F. psychrophilum} FPS-F15 and the phage FPSV-S20, the phage--host culture was supplemented with a single stock of phage FPSV-S20 during each transfer (Fig. 1A).

The growth of the host displayed the same dynamics of growth during the first week of the experiment in the presence of the phage as it did in the control without phage (Fig. 2A). However, during the second transfer, the host FPS-F15 showed a decrease in optical density 24 h post phage addition, and then returned to a bacterial density similar to the control culture in the third transfer (Fig. 2A). Attempts to isolate individual clear plaques in the second transfer revealed that all the purified phages from these plaques were able to infect the strain FPS-F15. Three specific clones were named FPSV-S20A, FPSV-S20B, and FPSV-S20C (Supplementary Table S2) and selected for further characterization.

In the second strategy, we added phages FpV4 and FpV9 from different independently produced stocks in each serial transfer to the strain 020612-2/1 to increase the probability of adding mutant phages to the culture (Fig. 1B). During the transfers with host 020612-2/1 and phage FpV4, the optical density of the infected cultures decreased 72 h post first phage addition and then remained at the same level of growth, relative to the phage-free control for the following week (Fig. 2B). Similar results were obtained using strain 020612-2/1 and phage FpV9, where a decrease in cell density, relative to the control, 72 h post second phage addition was observed (Fig. 2C).

The successive addition of the phage after the second week showed identical cell growth to the culture control (Fig. 2C). For both experiments, individual clear plaques were isolated on lawns of the cocultured host, and all the purified phage isolates were able to infect (clear zones) the previously unsusceptible strain 020612-2/1. Three specific phage clones derived from FpV4 were named FpV4-1.1, FpV4-1.2 and FpV4-1.3 and three clones derived from FpV9 were assigned as FpV9-1.1, FpV9-1.2, and FpV9-1.3 (Supplementary Table S2).

Finally, this second strategy was used for adding further selection pressure on phages with already improved infection

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Lytic capacity of evolved \textit{Flavobacterium psychrophilum} phages. (A–C) OD\textsubscript{595} of cultures of \textit{F. psychrophilum} (A, B) strain 020612-2/1, (C) strain FPS-F15 amended with wild type and evolved phages and control cultures without phages. Right plots represent abundance of phages as PFU/mL for each phage–host system. Error bars represent standard deviations from the mean based on triplicate cultures. OD, optical density.}
\end{figure}
abilities, using the previous isolated evolved phage FPSV-S20A and the strain FPS-F15 for isolation to select for phages with increased lytic abilities. The results showed a decrease in the optical density 48 h post first and third phage addition relative to the phage-free control (Fig. 2C). Purified phage stocks from this experiment were named FPSV-S20A1, FPSV-S20A2, and FPSV-S20A3 (Supplementary Table S2).

Infection experiments with the three representative evolved phages were performed in batch cultures at MOI of 1 (~ 10^8 PFU/mL). The results showed rapid propagation for evolved phages FpV4-1.1, FpV9-1.1, and FPSV-S20A during the first 24–48 h of incubation, relative to the wild-type phage–host systems (Fig. 3). In all the experiments, at 96 h post evolved phage addition, reductions in OD_{595} nm varied from 47% to 65% relative to levels for control cultures incubated with wild-type phages, suggesting that infection by evolved phages had a significant lytic control of host cell density (Fig. 3A–C).

For example, evolved phage FPSV-S20A reduced the host culture by 47% in comparison with both the free-phage culture and the FPSV-S20-enriched culture, and an 11-fold increase in phage abundance was observed at the end of the experiment (Fig. 3C).

**FIG. 4.** Host range of bacteriophages against the collection of 34 *Flavobacterium psychrophilum* strains. Infectivity is categorized as white “no inhibition observed,” gray “turbid inhibition zone,” and black “clear inhibition zone.” Results of EOP on each *F. psychrophilum* isolate are expressed in relative PFU, where the 10^8 PFU/mL were fixed as 1 in the proliferation hosts. Red and green squares represent wild-type and evolved phages, respectively. Top yellow squares highlight proliferation hosts. EOP, efficiency of plating.

**Table: Bacteriophages**

The host ranges of the 3 wild-type and the 12 evolved *F. psychrophilum* phages were examined using a collection of 34 *F. psychrophilum* strains. Overall, the evolved phages formed clear plaques on the isolation hosts (i.e., the previously nonsusceptible strains), whereas the wild-type phages could either not lyse or formed turbid zones with a very low EOP (Fig. 4). For example, the wild-type phage FpV4 was not able to infect the strains FPS-P1, FPS-P3, FPS-F15, P30-2B/9, and FPS-G1; whereas the evolved phages FpV4-1.1, FpV4-1.2, and FpV4-1.3 infected these strains with an increase in EOP of >10,000×, although turbid plaques were observed (Fig. 4).

Interestingly, clear zones and increased EOP were detected for these evolved phages on the strains 020612-2/1 and FPS-D10, which were resistant to FpV4 (i.e., no inhibition areas or plaques were found) (Fig. 4). Similarly, evolved phages from FpV9 infected the strains 001026-1/35C and M2/99, forming turbid plaques with an EOP increase of >9,000×. Specifically, evolved phage FpV4-2.1 formed clear plaques on the strains FPS-G6 and FPS-F36 with an increase of EOP.
>10\times(\text{Fig. 4}). Finally, evolved phages derived from FPSV-S20 infected the strains FPS-F15 and F482, producing clear and turbid inhibition zones, respectively (\text{Fig. 4}).

Clear plaques and an increase of EOP >10\times were also found for the evolved phages FPSV-S20A, FPSV-S20B, and FPSV-S20C in the strains 030522-2/1, 990512-1/2A, 010418-2/1, FPS-S9, and FPS-S10 (\text{Fig. 4}). In contrast, the second round of selection experiments using phage FPSV-S20A as the phage for selection, which generated the phages FPSV-S20A1, FPSV-A2, and FPSV-A3, did not result in any further improvement of host range or EOP (\text{Fig. 4}).

Overall, the evolution of phage FpV4 increased the phage–host range from 58.8\% to 73.5\% (FpV4-1.3) and 79.4\% (FpV-1.1 and FpV-1.2) of the tested bacterial host strains. For phage FpV9, the evolved phages infected the same number of host strains (70.5\%), as the ability to infect two new strains was associated with the loss of infectivity of two other strains (\text{Fig. 4}). The evolved derivatives of phage FPSV-D20 showed a slight increase in overall host range (67.6\% of the strain collection) relative to the wild-type phage (61.8\%).

\textbf{Sequencing of evolved F. psychrophilum phages with altered host range}

Genomic analysis revealed 12 independent mutations distributed along the genomes of all the evolved \textit{F. psychrophilum} phages. All of these were point mutations that caused amino acid changes in 10 open reading frames (ORFs) (\text{Fig. 5 and Supplementary Table S3}). The results showed that all the evolved phages from FpV4 contained a substitution of Asn525Ser in a hypothetical protein (FDG89_gp19) (\text{Fig. 5A}). Specifically, the evolved phages FpV4-1.2 and FpV4-1.3 harbored additional substitutions of Ala84Thr and Leu86Phe in a hypothetical protein (FDG89_gp34) and DNA polymerase (FDG89_gp62), respectively (\text{Fig. 5A}).

In contrast, evolved phages from FpV9 contained four independent nucleotide mutations distributed in two ORFs, of which only three caused amino acid changes (Supplementary Table S3). Evolved phage FpV9-1.2 had a substitution of Ala340Ser in a hypothetical protein (ALN97196.1), whereas FpV9-2.1 had one mutation of Ser464Pro in a different hypothetical protein (ALN97199.1). Also, the evolved phage FpV9-1.3 harbored a substitution of Phe567Leu in the same protein as FpV9-2.1 (\text{Fig. 5B}).

The genomic sequences of wild-type phage FPSV-S20 and the evolved phage FPSV-S20A showed a unique substitution of Tyr45Asp in a hypothetical protein (QCW20605.1), whereas evolved phages FPSV-S20B and FPSV-S20C harbored an identical substitution of His45Asp in a different hypothetical protein (QCW20624.1) (\text{Fig. 5C}). Finally, genomic comparison of FPSV-S20A1 and FPSV-S20A2 showed identical sequence to FPSV-S20A, from which they were derived, whereas the evolved phage FPSV-S20A3 showed the accumulation of three additional mutations (Asp384Asn, Val111Ile, and Asn449Lys in three hypothetical proteins (QCW20617.1, QCW20629.1, and QCW20630.1), respectively) (Supplementary Table S3 and \text{Fig. 5C}).

\textbf{Adsorption constant of evolved F. psychrophilum phages}

To examine in more detail the interaction of the wild-type and evolved \textit{F. psychrophilum} phages with different susceptible hosts, adsorption constants were determined for a selected group of evolved and wild-type phages. The results showed that all the evolved phages had increased adsorption rate to the isolation hosts in comparison with the wild-type phages. For example, the evolved phages FpV4-1.1, FpV4-1.3, and FpV9-2.1 had 26\times, 20\times, and 13\times increase in adsorption compared to the wild-type phages FpV4 and FpV9, respectively.
adsorption constant to the *F. psychrophilum* strain 020612-2/1, respectively, compared with the wild-type phage FpV4 and FpV9 (Fig. 6A, B).

Similarly, the evolved phage FPSV-S20A increased its adsorption constant $337 \times$ in the isolation host FPS-F15 in comparison with the wild-type phage (Fig. 6C). An increase $>10 \times$ in the adsorption rate was observed in the phage FpV4-1.1 when exposed to the *F. psychrophilum* strains FPS-D10 and FPS-G6 (Fig. 6A).

**Discussion**

In this study, we observed the *in vitro* evolution of *F. psychrophilum*-specific phages, using simple batch culture experiments. Within two to three serial transfers of phage stocks to nonsusceptible hosts, evolved phages were isolated with improved lytic abilities, host ranges, EOP, and adsorption constants (Figs. 3–4 and 6), which were associated with mutational changes in specific ORFs likely related to

![Discussion](https://example.com/figure6.png)

**FIG. 6.** Adsorption constants of wild-type and evolved *Flavobacterium psychrophilum* phages. (A) Adsorption constants of FpV4, FpV4-1.1, and FpV4-1.3 in the strains 020612-2/1, FPS-F15, FPS-D10, and FPS-G6. (B) Adsorption constants of FpV9, and FpV9-2.1 in the strains 020612-2/1, FPS-D10, and FPS-G6. (C) Adsorption constants of FPSV-S20, and FPSV-S20A in the strains FPS-S6 and FPS-F15.
phage–host interactions (Fig. 5). These findings are a key advance in the optimization of phage-based control of F. psychrophilum pathogens in aquaculture.

Emergence of bacterial resistance against phages is driven by several mechanisms to avoid viral infections. In phage therapy, a distinctive strategy to overcome phage-resistant bacterial strains is de novo isolation of phages from environmental sources; however, this can be a long and technically demanding process. In this study, we were able to isolate and characterize nine laboratory-evolved F. psychrophilum phages that could effectively infect strains that were susceptible to or inefficiently infected by the wild-type phages (Figs. 3 and 4).

Similar results have been obtained for phages infecting the pathogenic bacteria Listeria monocytogenes, Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa, where short rounds of phage–bacteria incubations allowed the isolation of phages with broader host ranges. Our findings showed that F. psychrophilum phages isolated from fish farm environments can be in vitro evolved to overcome phage-resistant strains and the experimental strategies presented here can specifically be used to improve the lytic potential and host range of phages (Figs. 1 and 3).

Both the tested strategies were equally successful in isolating genetic variants of F. psychrophilum phages with an expanded host range (Fig. 5), suggesting that a single round of phage stock production contained sufficient phage mutants to allow for selection of these in the phage exposure experiments. Thirteen independent genetic mutations were found in the 12 evolved phages, causing amino acid changes into hypothetical proteins in almost all cases (Supplementary Table S3). These substitutions are likely to be responsible for the expansion of host ranges and improved lytic abilities and adsorption constants (Figs. 3–4 and 6).

Earlier reports have established that gliding motility proteins and components of the T9SS are the putative receptors or coreceptors for F. psychrophilum phages. The increased adsorption constants of the evolved phages (Fig. 6) suggest that the mutations resulted in structural changes in the phages, which improved the interaction with these cell–surface proteins. Interestingly, the mutant proteins found in the evolved phages were not the main target of genetic diversity in wild-type F. psychrophilum phages isolated from fish farms sources.

The previous comparative genomic analysis of F. psychrophilum phage sequences showed that most of the genetic diversities among the environmental phage isolates were associated with annotated structural proteins. Thus, the results obtained in this study suggest the presence of additional ORFs in F. psychrophilum phages that are related to cell–surface interaction and host range, and thus important for phage adaptations to changing host communities.

Interestingly, these findings are in contrast to results found in phages infecting the fish pathogen Vibrio anguillarum, where the expansion of host ranges was highly dependent on the proliferation host and not related to genetic mutations in the phage genomic sequences. This indicated that non-mutational mechanisms encoded by the host, such as methylation of the phage DNA, were a major contributing factor for host range variation in vibrio phages.

Today, synthetic biology offers exciting new tools to build engineered phages through a variety of recombinating approaches and in vitro genome assembly. Thus, by these techniques natural phages can be engineered to new prototypes with defined properties, likely allowing to overcome phage-resistant phenotypes in infectious diseases. However, although the genetic modification of phages by evolution experiments is less specific than genetic engineering approaches, natural selection strategies may have advantages, particularly in the food production sector, where efficiency, safety, and therapeutic suitability of phages are extremely important, and where use of genetically engineered organisms may still raise concern.

In addition to the phage therapy implications, the demonstration of short-term phage evolution and the fast development of phage–host range variants also has implications for our understanding of phage–host coevolution in natural environments. For example, coevolution studies as described here, which focus on the molecular details of the occurrence and diversification of phage variant populations and their hosts, provide important information on the specific mechanisms that drive antagonistic coevolution between different phages and their respective hosts under certain conditions.

Overall, the in vitro serial transfer strategies presented in this study provide an effective and applicable method for the selection and isolation of evolved phages with specific lytic properties on a short time scale. In addition, we propose future efforts to investigate whether F. psychrophilum isolates appear less able to resist evolved phages and whether evolved phages are more able to adapt to counter mutations in their hosts. Thus, this study affords valuable information to optimize phage therapy approaches for the biocontrol of this fish pathogen in the aquaculture industry. Furthermore, we expect that the methods utilized here can be applied to explore a variety of evolutionary landscapes of different phage–host systems to potentially progress applications in phage therapy to prevent and overcome the emergence of phage-resistant strains.

Author Disclosure Statement

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Supplementary Material

Supplementary Table S1
Supplementary Table S2
Supplementary Table S3

References


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