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## RESEARCH ARTICLE

# Reversible mutations in gliding motility and virulence genes: A flexible and efficient phage defence mechanism in *Flavobacterium psychrophilum*

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## Abstract

Flavobacteria are among the most important pathogens in freshwater salmonid aquaculture worldwide. Due to concerns regarding development of antibiotic resistance, phage therapy has been proposed as a solution to decrease pathogen load. However, application of phages is challenged by the development of phage resistance, and knowledge of the mechanisms and implications of phage resistance is therefore required. To study this, 27 phage-resistant isolates of *F. psychrophilum* were genome sequenced and characterized to identify genetic modifications and evaluate changes in phenotypic traits, including virulence against rainbow trout. Phage-resistant isolates showed reduction or loss of gliding motility, proteolytic activity, and adhesion to surfaces, and most isolates were completely non-virulent against rainbow trout fry. Genomic analysis revealed that most phage-resistant isolates had mutations in genes associated with gliding motility and virulence. Reversal of these mutations in a sub-set of isolates led to regained motility, proteolytic activity, virulence and phage susceptibility. Although costly, the fast generation of phage resistance driven by single, reversible mutations likely represents a flexible and efficient phage defence mechanism in *F. psychrophilum*. The results further suggest that phage administration in aquaculture systems to prevent *F. psychrophilum* outbreaks selects for non-virulent phage-resistant phenotypes.

## INTRODUCTION

## *Flavobacterium psychrophilum* and aquaculture

*Flavobacterium psychrophilum* is the etiological agent of bacterial cold water disease (BCWD) and is among the most important mortality factors in salmonid aquaculture worldwide (Gómez et al., 2013). Particularly in

the production of rainbow trout (*Oncorhynchus mykiss*) larvae and fry, this pathogen constitutes a major bottleneck as the resulting disease, rainbow trout fry syndrome (RTFS), causes substantial economic losses in the industry. Infections with *F. psychrophilum* result in skin erosion, necrotic lesions and anaemia, leading to juvenile fish mortality rates of 80%–90% over a few days, if left untreated (Barnes & Brown, 2011). A number of virulence factors, including biofilm formation and

secretion of adhesins, haemolysins and extracellular proteases, for example (Gómez et al., 2013), have been identified and shown to be globally distributed in *F. psychrophilum* populations (Castillo et al., 2016). Also, putative virulence genes associated with potential toxin production (Castillo et al., 2016), and genes involved in gliding motility (e.g. *gldD* and *gldG*) (Pérez-pascual et al., 2017), have been linked with virulence of *F. psychrophilum*.

No efficient vaccines for *F. psychrophilum* are currently available, especially as the larval and fry have undeveloped immune systems (Sundell et al., 2014). Antibiotics are therefore the primary treatment for BCWD and RTFS (Gómez et al., 2013). Antibiotic treatment is still efficient in controlling the pathogen (Ngo & Adams, 2018); however, increased concern about the emergence of antibiotic resistance in *F. psychrophilum* (Kum et al., 2008) has emphasized the need to develop alternative antimicrobial strategies for sustainable prevention and treatment of *F. psychrophilum* infections in rainbow trout.

The use of bacteriophages to control pathogens as alternatives to antibiotics in aquaculture has been explored for the past two decades with promising results, including research on phages infecting *Flavobacterium* pathogens, for example (Castillo et al., 2012; Stenholm et al., 2008; Sundell et al., 2020). Phages can reach the infected organs in rainbow trout fry through various delivery routes (Christiansen et al., 2014; Madsen et al., 2013), and can reduce mortality of rainbow trout fry by 50%–100% in challenge experiments with *F. psychrophilum* (Castillo et al., 2012; Sundell et al., 2020) and *F. columnare* (Laanto et al., 2015), thus emphasizing the potential of phage applications for reducing the infection pressure of *Flavobacteria* in larval rearing systems and fish fry.

The approach is, however, challenged by the rapid (hours) development of phage resistance in phage-exposed *Flavobacterium* populations (Christiansen et al., 2016). In *F. psychrophilum*, phage resistance has been associated with mutations in genes related to cell surface properties such as gliding motility, lipopolysaccharide synthesis and cell wall components. Likewise, mutations in gliding motility genes in *F. johnsoniae* strains were shown to be linked with resistance to bacteriophages, for example (Braun et al., 2005; McBride et al., 2003). In *F. columnare*, an active CRISPR-Cas region was shown to be involved in resistance against phages and to drive phage-host co-evolution over a longer time scale (years). However, indications of coupling between phage resistance and loss of gliding motility were also observed in *F. columnare* (Laanto et al., 2012, 2015).

Such potential fitness costs associated with mutational changes providing phage resistance in fish pathogens (Castillo et al., 2015; Rørbo et al., 2018) have been suggested as a selection mechanism for

alternative and more flexible phage defence mechanisms, such as cell aggregation, downregulation of phage receptor expression and extracellular proteolytic inactivation of phage particles, for example (Castillo et al., 2019; Kalatzis et al., 2018), that do not require permanent genomic changes in important genes. Consequently, the development of different strategies to overcome phage infection observed even among closely related fish pathogenic strains are likely key drivers of the different phage-host co-evolution and coexistence relations found in different strains and environments (Castillo et al., 2019).

In this study we examined phage-defence strategies in *F. psychrophilum* and their implications for host fitness, by analysing the genomic mechanisms of phage resistance and quantifying the phenotypic costs of phage resistance, including the effects on virulence. Furthermore, the stability of the phage-induced genomic and phenotypic modifications was examined during subsequent culturing in the absence of phages. The results revealed a dynamic and efficient phage-defence mechanism in *F. psychrophilum* not previously described. In addition to single nucleotide mutations, insertions and deletions, intragenomic rearrangements of small DNA sequences orchestrated by transposon elements were observed, generating a suite of specific genomic modifications, which prevented phage infection. These modifications targeted the Type 9 secretion system (T9SS) and other gliding motility-related genes, leading to loss of key functional properties, and a complete loss of virulence against rainbow trout in the phage-resistant clones. However, reversal of these mutations in phage-resistant isolates in the absence of phages led to regained motility, proteolytic activity, virulence and phage susceptibility, suggesting that the described resistance mechanism is a flexible strategy, adapted to the specific pathogenic lifestyle of *F. psychrophilum*.

## EXPERIMENTAL PROCEDURES

### *Flavobacterium psychrophilum* strains and phages

*Flavobacterium psychrophilum* strains and phages were isolated from rainbow trout aquaculture facilities in the Baltic Sea region that were experiencing RTFS outbreaks. Two virulent *F. psychrophilum* strains (Sundell et al., 2019) were used in this study, FPS-S6 isolated in Sweden in 2017 and 160401-1/5N isolated in Denmark in 2016. Both strains are serotype 2 and belong to clonal complex CC-ST10 (Sundell et al., 2019), the most common CC in RTFS outbreaks in recent years (Nilsen et al., 2014).

Lytic broad host range phages were used to control *F. psychrophilum* strains in order to evaluate their

TABLE 1 Lytic phages used in the current study

Phage	Source	Isolation host	Proliferation host	Genomic cluster	Country	Year
FPSV-D19	Water sample	FPS-D10	FPS-D10	II	Denmark	2017
FPSV-D22	Rainbow trout tissue	FPS-D5	FPS-S6	II	Denmark	2017
FPSV-S20	Water sample	FPS-S30	FPS-S6	II	Sweden	2017

potential to control bacterial pathogen populations and to obtain and characterize phage-resistant bacteria. Phage FPSV-D19 and FPSV-S20 were isolated from aquaculture water in Denmark and Sweden, respectively (Sundell et al., 2019). Phage FPSV-D22 was isolated from homogenized tissue of rainbow trout fry with RTFS in Denmark (Donati et al., 2021; Sundell et al., 2019). All phages were isolated in 2017 and belong to genomic cluster II (Table 1).

## Culture conditions

*Flavobacterium psychrophilum* strains were cultured on tryptone yeast extract salts (TYES) agar, consisting of 0.4% tryptone, 0.04% yeast extract, 0.05%  $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ , 0.05%  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$  and 1.1% agar (pH 7.2). For all tests, unless otherwise stated, cryopreserved ( $-80^\circ\text{C}$ ) stock cultures of each *F. psychrophilum* strain/isolate were cultured for 5 days on TYES agar at  $15^\circ\text{C}$ , followed by subcultivation in TYES broth for 48 h at  $15^\circ\text{C}$  under  $\sim 200$  rpm agitation.

For preparation of high-density bacteriophage stocks, 1 ml phage stock was added to 20 ml exponentially growing cultures ( $\text{OD}_{600\text{nm}} = 0.2$ ) of a suitable proliferation host and incubated at  $15^\circ\text{C}$  for 2–3 days under agitation. Lysed bacterial cultures were centrifuged ( $5000g$ , 10 min,  $4^\circ\text{C}$ ) and sterile filtered ( $0.22 \mu\text{m}$ ). Phage quantification was performed by spot plaque assay on TYES soft agar (0.4% agar) overlay (Mazzocco et al., 2009).

## Isolation of phage-resistant *F. psychrophilum* during phage exposure

In order to obtain phage-resistant isolates, phages and bacterial hosts were co-cultured in TYES broth at a multiplicity of infection between 1 and 10. Three experiments with different host + phage combinations representing a variety of efficient lytic phages and susceptible hosts were set up, Exp. 1: FPS-S6 + FPSV-D22, Exp. 2: FPS-S6 + FPSV-S20 and Exp. 3: 160401-1/5N + FPSV-D19 (Table 2). Phage-free cultures with only bacteria served as controls. Bacterial and phage abundance was monitored every 12–16 h for 4–5 days by measuring optical density ( $\text{OD}_{600\text{nm}}$ ) and plaque-forming units (PFU) respectively. For phage quantification, 1 ml culture was fixed in 0.2%

chloroform and centrifuged ( $7000g$ , 15 min,  $4^\circ\text{C}$ ). The supernatant was transferred to a clean tube and used for spot plaque assay (Mazzocco et al., 2009). PFUs were counted after 5 days of incubation at  $15^\circ\text{C}$ . When  $\text{OD}_{600\text{nm}}$  in phage + host cultures started to increase, sampling for phage-resistant colonies was initiated. Culture samples were serially diluted and plated on TYES agar plates. Single colonies were isolated and tested for resistance to the corresponding phage using the double-layer method (Stenholm et al., 2008).

## Genome sequencing

Wild types FPS-S6 and 160401-1/5N, control isolates from phage-free cultures FPS-S6-C1 and 160401-1/5N-C1, and 27 phage-resistant isolates (Table 2) were whole-genome sequenced. 100 bp paired-end sequencing was performed on the Illumina HiSeq platform (Institute of Molecular Medicine Finland). Wild types were also sequenced using PacBio technology (BGI, China) in order to obtain high-quality reference genomes. Prior to genome assembly, all Illumina reads were trimmed using Trimmomatic v. 0.36.0 (Bolger) to remove low-quality bases and adapter sequences.

For the wild types, a hybrid assembly with Illumina and PacBio reads was performed with Flye 2.1, followed by Illumina read error correction using Bowtie 2.3.5 (langmead), Samtools 1.9 (li) and Pilon 1.23 (Walker). FPS-S6 assembly resulted in a single  $\sim 2.86$  Mbp contig. 160401-1/5N assembly resulted in two contigs,  $\sim 2.83$  Mbp and  $\sim 2.9$  kbp. The small contig aligned perfectly to a  $\sim 7.5$  kbp repeat region in the large contig and was discarded from downstream analysis. Annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (Haft et al., 2017; Tatusova et al., 2016). No plasmid sequences were identified in the PacBio reads; therefore, a separate assembly using only Illumina reads was performed on SPAdes v. 3.12.0 (Bankevich). Plasmid sequences were identified by screening the SPAdes assembly for high coverage contigs and signature plasmid genes. Two plasmids were found in each wild type, 3360 and 2191 bp in length.

Alignment-based assemblies were performed for phage-resistant and control isolates using the wild-type hybrid assembly (chromosome) and plasmids as reference genome on Geneious software v. 11.1.5. In order to obtain control assemblies to sort out false-positive

**TABLE 2** List of phage-resistant isolates picked for phenotypic characterization, genome sequencing and screening for gliding motility recovery, and their experimental origin

Name of experiment	Host + phage combination	Isolate	Time of isolation (dpi)	Tested for motility recovery	Accession number
FPS-S6 Control	FPS-S6 No phage	FPS-S6-C1	10		CP095210
		FPS-S6-C2	5		N/A
Exp. 1	FPS-S6 + FPSV-D22	FPS-S6-R1	2		CP095213
		FPS-S6-R2	2	Yes	CP095216
		FPS-S6-R3	2	Yes	CP095217
		FPS-S6-R4	3		CP095222
		FPS-S6-R5	3		CP095223
		FPS-S6-R6	3		CP095228
		FPS-S6-R7	4		CP095686
		FPS-S6-R8	4		CP095691
		FPS-S6-R9	5		CP095694
Exp. 2	FPS-S6 + FPSV-S20	FPS-S6-R10	2		CP095697
		FPS-S6-R11	2		CP095700
		FPS-S6-R12	2		CP095703
		FPS-S6-R13	2	Yes	CP095704
		FPS-S6-R14	4	Yes	CP095709
		FPS-S6-R15	4		CP095712
		FPS-S6-R16	4		CP095715
		FPS-S6-R17	4		CP095718
		FPS-S6-R18	4		CP095721
160401-1/5N Control	160401-1/5N No phage	160401-1/5N-C1	10		CP095024
		160401-1/5N-C2	6		N/A
Exp. 3	160401-1/5N + FPSV-D19	160401-1/5N-R1	5	Yes	CP095027
		160401-1/5N-R2	4		CP095030
		160401-1/5N-R3	3		CP095033
		160401-1/5N-R4	3		CP095036
		160401-1/5N-R5	3		CP095039
		160401-1/5N-R6	3		CP095229
		160401-1/5N-R7	6	Yes	CP095199
		160401-1/5N-R8	6		CP095202
		160401-1/5N-R9	6		CP095207

Abbreviations: dpi, days post-inoculation; N/A, not applicable.

mutations, wild-type Illumina reads were assembled using this method as well. Mutations in phage resistant and control isolates were identified manually in read alignment files visualized in Geneious v. 11.1.5. If sequencing depth was  $<50\times$  or frequency of a putative

mutation in the reads was  $<90\%$ , the mutation was verified by PCR and Sanger sequencing (data not shown). Insertion of IS was also verified by PCR (data not shown). Genome assemblies were submitted to GenBank (Table 2).



## Phenotypic characterization

A total of 27 resistant isolates, nine from each host + phage combination, in addition to control isolates from phage-free cultures were selected for further characterization covering different time points (Table 2). Putative virulence factors and phage susceptibility of the isolates were characterized and quantified the same way as wild-type strains FPS-S6 and 160401-1/5N by Sundell et al. (2019). The bacterial cells of each broth culture were washed in fresh cooled TYES broth by centrifugation (5310g, 15 min, 4°C) and the OD was spectrophotometrically adjusted to 1.0 at 600 nm, corresponding to an *F. psychrophilum* concentration of approximately  $10^9$  CFU ml<sup>-1</sup>. The 48 h cultures were used for all phage-host combination experiments and phenotypic characterization studies unless otherwise noted. Each test was performed at least three independent times unless otherwise stated.

## Testing for cross-resistance

Phage resistance isolates were tested for cross-resistance development against cluster II phages FPSV-D22, FPSV-S20 and FPSV-D19 used in exp. 1, 2 and 3 respectively, in addition to phage FpV4 and FPSV-S8 representing genomic clusters I and IV respectively (Castillo & Middelboe, 2016).

EOP of all five phages was quantified on the collection of resistant bacteria, control isolates and wild types. Bacterial isolates were exposed to the same phage titers and the infectivity was measured by the drop plaque assay (Mazzocco et al., 2009). Plaques were counted after 5 days of incubation at 15°C. EOP was calculated as the relative infectivity of each phage compared to the highest titre obtained for that phage (EOP = 1) on any isolate in the collection.

## Quantification of gliding motility

The bacterial colony spreading, that is gliding motility quantification, was performed by spotting 5 µl triplicates of each *F. psychrophilum* isolate from a broth suspension containing  $10^9$  CFU ml<sup>-1</sup> on TYES agar (0.5% agar) plates supplemented with 0.1% baker's yeast (Sundell et al., 2019). The bacterial motility indicated by the colony diameter (mm) of each isolate was evaluated after 6 days of incubation at 15°C.

## Proteolytic activity

The ability of the *F. psychrophilum* isolates to hydrolyse casein, and gelatine was tested on TYES agar (1.5% agar) supplemented with (wt./vol.) skim milk

(5%) and gelatine (3%), respectively (Castillo et al., 2015). A 1 µl volume of a  $10^9$  CFU ml<sup>-1</sup> broth suspension of each isolate was spotted on the agar surface in triplicate, allowed to dry, and incubated at 15°C. Following incubation, a positive reaction was indicated by a clear zone in the surrounding turbid agar medium around the inoculum. To estimate the proteolytic activity of each isolate, the clear zone ratio (clearing zone/colony diameter) was determined after 7 (gelatinase) and 10 days (caseinase) of incubation.

## Adhesion to polystyrene

For study of the adhesion ability, the cryopreserved *F. psychrophilum* stocks were cultured for 5 days on TYES agar and recultivated another 3 days before inoculation of bacteria into sterile (autoclaved 121°C, 20 min) aquaculture tank water to an OD<sub>520nm</sub> of 1.0, corresponding to approximately  $10^9$  CFU ml<sup>-1</sup> (Sundell et al., 2020). Aliquots of 100 µl were added in triplicate to wells of a flat-bottomed 96-well polystyrene microtiter plate (Nunc) while sterile fresh water was used as a negative control. After static incubation at 15°C for 1 h, the contents were discarded. To remove non-adherent cells, the wells were washed three times with sterile 0.5% NaCl and air-dried. A 125 µl volume of a 0.1% CV solution was then added to each well and incubated at room temperature for 45 min. After discarding the contents, the plates were washed three times by submersion in a container of tap water and air-dried. Then, 150 µl of 96% ethanol was added to each well and incubated at room temperature for 15 min. A 100 µl volume of the solubilized CV was then transferred to a flat bottomed microtiter plate and the absorbance was quantified in a microplate reader (Victor2, Wallac) at 595 nm. Each isolate was examined in triplicate and the experiment was repeated three times.

## Virulence trials

The median lethal dose (LD<sub>50</sub>) of the selected *F. psychrophilum* phage-sensitive and phage-resistant isolates (18 of the 27 included) was estimated in rainbow trout fry (mean weight ~3 g) from the same cohort obtained from a commercial fish farm. Before start of the virulence experiments, fish were kept under laboratory conditions in tanks with a flow-through of dechlorinated tap water at about 13°C with continuous aeration and fed twice a day at 1% of body weight with commercial fish feed. Prior to the challenge trials, fish were fasted for 24 h and anaesthetized by immersion in a 0.05 g L<sup>-1</sup> bath solution of benzocaine just before marking of groups by fin clipping and injection with *F. psychrophilum* (Sundell et al., 2019).

For preparation of *F. psychrophilum* cells for virulence trials, optically adjusted TYES broth suspension containing washed *F. psychrophilum* cells at a concentration of  $10^9$  CFU ml<sup>-1</sup> was used to prepare serial 10-fold dilutions ( $10^8$ – $10^3$  CFU ml<sup>-1</sup>) of each isolate in 0.5% NaCl. A 50 µl volume of five serial dilutions was administered through intramuscular injection into five treatment groups consisting of seven fish each. Bacterial viability and concentration of each dilution were verified by colony counting on TYES agar after 7 days of incubation at 15°C. A control group consisting of seven fish was anaesthetized, marked and injected with 50 µl of a sterile 0.5% NaCl solution. After the injection procedure, each group of fish ( $n = 6$ ), that is 42 individuals in total per tested isolate, were transferred to 20 L test aquaria containing aerated dechlorinated tap water with a temperature of 13°C.

Mortality was recorded for 21 days during which water was renewed daily, and dead and moribund fish were removed as soon as observed. Moribund fish were euthanized by overdose of anaesthesia. To confirm Koch's postulate, tissue samples from the kidney and spleen of dead fish were streaked onto TYES agar plates and incubated at 15°C for 7 days. Bacteria forming yellow colonies on the agar plates were identified as *F. psychrophilum* by species-specific PCR. The *F. psychrophilum*-specific primers PSY1 and PSY2 (Toyama et al., 1994) were used for PCR amplification of a partial fragment (1089 bp) of the 16S rRNA gene followed by electrophoretic confirmation of the amplification product. When possible, the LD<sub>50</sub> for each tested isolate was calculated using the Reed–Muench method (Reed & Muench, 1938), which requires cumulative mortality to be above 50% in at least one of the treatment groups and below 50% in another. The virulence trials were conducted under project and personal licence for animal experiments.

## Screening for recovery of gliding motility

Six phage-resistant isolates showing no gliding motility, two from each phage + host combination, were tested for recovery of gliding motility when grown in the absence of phage (Table 2). Cultures were transferred with a single inoculation loop to three replicate TYES agar (0.5% agar) plates supplemented with 0.1% baker's yeast and one TYES agar (1.1% agar) plate for control. After 2–7 days of incubation at 15°C, the plates were inspected for signs of motility (colony spreading). One replicate per isolate was picked and transferred to fresh plates in triplicates + control for a total of 12 serial passages (50 days total incubation) or until motility was observed. When an isolate showed signs of motility, an additional transfer to fresh motility agar was performed to obtain a uniformly spreading colony, followed by

three rounds of purification by single colony isolation on TYES agar (1.1%).

Isolates with recovered motility (FPS-S6-R3M, 160401-1/5N-R1M) were characterized and tested for phage susceptibility, proteolytic activity and virulence against rainbow trout, together with their ancestral phage-resistant isolates (FPS-S6-R3, 160401-1/5N-R1) and phage susceptible control isolates (FPS-S6-C2, 160,401-1/5N-C2) as described above with a few exceptions. Plates for motility, caseinase and gelatinase activity were incubated for 7 days. The median lethal dose (LD<sub>50</sub>) was estimated in rainbow trout fry (mean weight ~5 g) from the same cohort. Five serial 10-fold dilutions per isolate were prepared and each was injected intramuscularly after anaesthetization into five treatment groups consisting of seven fish each.

## Statistics

To test the statistical significance of phenotypic differences between phage-resistant mutants and their wild-type ancestors, a *t*-test was used.

## RESULTS

### Isolation of phage-resistant colonies

A total of 27 phage-resistant *F. psychrophilum* colonies were isolated from the three selected phage/host combination experiments and further tested for phage susceptibility (Table 2).

In addition, two phage-susceptible isolates (FPS-S6-C2 and 160401-1/5N-C2) were isolated from each of the phage-free control cultures (Table 2). These 29 isolates documented in Table 2 were selected for further phenotypic and genomic characterization.

### Cross-resistance to other phages

Phage-resistant isolates and controls from phage-free cultures were exposed to a collection of five phages to test for development of resistance or reduced phage susceptibility. Three of the phages (FPSV-D22, FPSV-S20 and FPSV-D19) were used in the initial phage exposure experiments (Table 2), and belonged to the genomic cluster II [Table 1, (Castillo et al., 2021a, 2021b; Castillo & Middelboe, 2016)]. The two additional phages, FPSV-S8 and FPV4, represented the genomic clusters IV and I, respectively, which are genetically distant from cluster II phages and from each other [Table 1, (Castillo et al., 2021a, 2021b; Castillo & Middelboe, 2016)]. The control isolates FPS-S6-C2 and 160401-1/5N-C2, which had not been pre-exposed to

	Isolate	Phages				
		FPSV-D22	FPSV-S20	FPSV-D19	FPSV-S8	FpV4
Exp. 1	FPS-S6 WT	$5 \times 10^{-1}$	$7 \times 10^{-1}$		1	
	FPS-S6-C2	1	1		$7 \times 10^{-1}$	
	FPS-S6-R1					
	FPS-S6-R2					
	FPS-S6-R3					
	FPS-S6-R4					
	FPS-S6-R5					
	FPS-S6-R6					
	FPS-S6-R7					
	FPS-S6-R8					
Exp. 2	FPS-S6-R9				$7 \times 10^{-1}$	
	FPS-S6-R10					
	FPS-S6-R11					
	FPS-S6-R12					
	FPS-S6-R13					
	FPS-S6-R14					
	FPS-S6-R15					
	FPS-S6-R16					
	FPS-S6-R17					
	FPS-S6-R18					
Exp. 3	160401-1/5N WT	$3 \times 10^{-2}$	$2 \times 10^{-2}$	$3 \times 10^{-1}$	$6 \times 10^{-3}$	
	160401-1/5N-C2	$3 \times 10^{-2}$	$1 \times 10^{-2}$	$3 \times 10^{-1}$	$7 \times 10^{-3}$	
	160401-1/5N-R1					
	160401-1/5N-R2					
	160401-1/5N-R3					
	160401-1/5N-R4					
	160401-1/5N-R5					
	160401-1/5N-R6					
	160401-1/5N-R7					
	160401-1/5N-R8					
	160401-1/5N-R9					

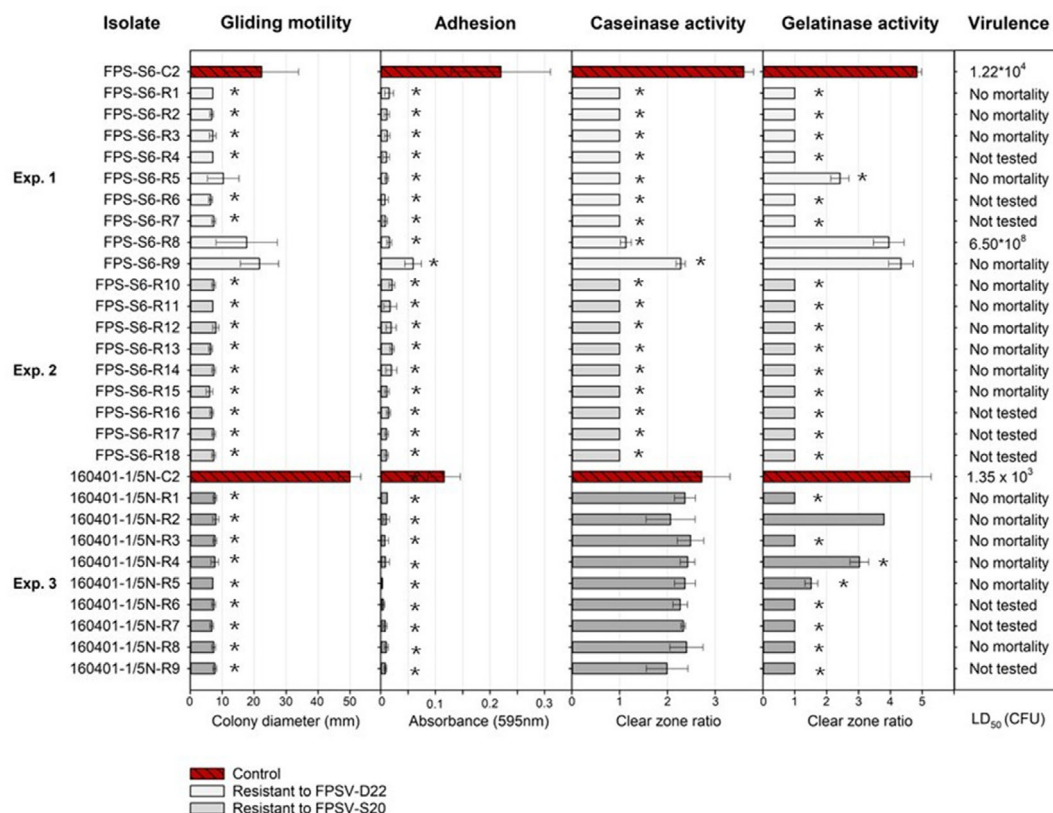
**FIGURE 1** Efficiency of plating of five lytic phages spanning different geographic locations, time of isolation, and genomic clusters on phage resistant isolates, phage susceptible control isolates, and their ancestral wild type. The designation R1–R18 indicates resistance to phage infection, whereas the C2 designation indicates isolates from phage-free control cultures. Black squares indicate clear plaques in the soft agar overlay, grey squares indicate turbid plaques and white squares indicate no infection. Numbers indicate efficiency of plating relative to the most susceptible strain/isolate. Shading of phage and isolate names indicate host-phage combinations in exp. 1, 2 and 3

phages were susceptible to the same phages as the wild types FPS-S6 and 160401-1/5N respectively and also demonstrated very similar efficiency of plating (EOP) (Figure 1).

Phage-resistant isolates from all three phage-host combinations showed complete resistance to all five phages tested in the EOP analysis (Figure 1) and a collection of 65 additional phages (data not shown). Only

isolate FPS-S6-R9 deviated from this pattern and remained susceptible to phages FPSV-S8 and FpV4. These two phages belong to different genetic groups than FPSV-D22, which FPS-S6-R9 was originally exposed to (Table 1). FPS-S6-R9 grew in aggregates when cultured in broth similar to controls and wild types, while no other resistant isolates formed aggregates in broth (data not shown).





**FIGURE 2** Phenotypic characterization of 27 phage-resistant isolates and control isolates from phage-free cultures. Gliding motility is shown as colony diameter (mm), adhesion is absorbance (595 nm) in a crystal violet (CV) assay. Caseinase and gelatinase activity is the clear zone ratio between colony diameter and clear zone diameter around the colony in protein supplemented agar. When no clear zone was observed, clear zone ratio = 1. Error bars are standard deviation of three replicates, and asterisk indicates a significant ( $p < 0.05$ ) difference from corresponding wild-type strain values. Virulence is shown as LD<sub>50</sub> (CFU) when applicable. Bar colour indicates isolate origin as shown in the figure legend

## Characterization of virulence factors and virulence

Phage-resistant isolates were phenotypically characterized with respect to selected putative virulence factors and antibiotic susceptibility. Furthermore, the median lethal dose (LD<sub>50</sub>) was used as a measure of virulence for 18 out of 27 isolates by performing rainbow trout fry infection trials (mean weight = 3 g). The results clearly demonstrated that there was a high cost associated with phage resistance, as gliding motility, adhesion capacity, proteolytic activity at different levels, and virulence against rainbow trout fry was negatively affected in phage-resistant isolates (Figure 2).

### Gliding motility

The gliding motility or colony spreading was impaired in most phage-resistant isolates (Figure 3).

Only isolates from exp. 1 (FPS-S6 + FPSV-D22) showed some motility, as three out of nine isolates, FPS-S6-R5, -R8 and -R9, still demonstrated colony spreading, although the motility of FPS-S6-R5 was

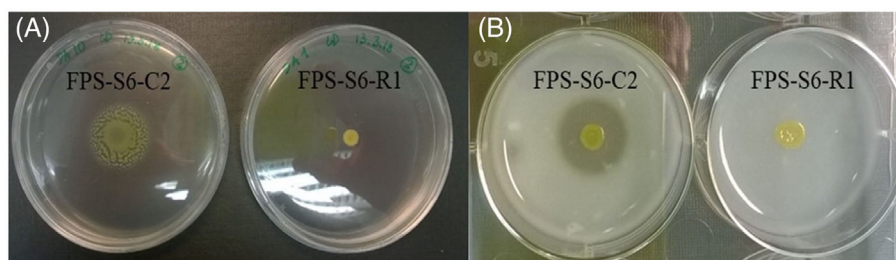
minimal (Figure 2). Thus, apart from strains FPS-S6-R5, -R8 and -R9, the resistant strains were non-motile and had a well-defined, rough, colony morphology [Figure 3(A)]. Resistant isolates derived from 160401-1/5N were all non-spreading in the gliding motility assay and had a significantly smaller colony diameter than the wild type ( $p < 10^{-4}$ ).

### Adhesion

All phage-resistant isolates had a significantly reduced ability to adhere to polystyrene surfaces compared to their respective phage-sensitive control ( $p < 0.05$ ) in the CV assay. Only isolate FPS-S6-R9 had maintained some adhesive properties and demonstrated a significantly higher absorbance than other phage-resistant isolates ( $p < 0.05$ ).

### Proteolytic activity

Most isolates derived from FPS-S6 had lost the ability to degrade extracellular casein and no clearing was



**FIGURE 3** Colony spreading and proteolytic activity. (A) Gliding motility/colony spreading assay with FPS-S6-C2 demonstrating colony spreading and phage-resistant FPS-S6-R1 showing no colony spreading. (B) Skim milk assay with the same isolates, demonstrating a clearing zone (casein degradation) around FPS-S6-C2 and no clearing in FPS-S6-R1

visible in the skim milk supplemented agar [clear zone ratio = 1, Figures 2 and 3(B)]. Extracellular casein degradation was only observed in isolates FPS-S6-R8 and FPS-S6-R9 but at a significantly lower level than in control isolate FPS-S6-C2 ( $p < 10^{-3}$ ). In contrast, all nine isolates derived from 160401-1/5N had maintained caseinolytic activity in the same range as the phage susceptible control 160401-1/5N-C2.

Gelatinase activity was similarly lost in many phage-resistant isolates (clear zone ratio = 1) although results were more variable. Isolates FPS-S6-R5, -R8 and -R9 digested gelatine but there was a reduced gelatinolytic activity in FPS-S6-R5 ( $p < 10^{-3}$ ) and FPS-S6-R8 ( $p < 0.05$ ) relative to FPS-S6-C2. Three isolates (160401-1/5N-R2, -R4, -R5) derived from 160401-1/5N still digested gelatine, but the clear zone ratio was significantly reduced in 160401-1/5N-R4 ( $p < 0.01$ ) and -R5 ( $p < 10^{-2}$ ) relative to the control isolate 160401-1/5N-C2.

## Virulence

In vivo fish experimental trials were performed with 18 of the 27 phage-resistant isolates to evaluate the median lethal dose ( $LD_{50}$ ).  $LD_{50}$  could not be determined for 17 of the isolates because no mortality was recorded in experimental fish despite intramuscular injection of bacteria (approximately  $10^7$  CFU fish $^{-1}$ ) (Figure 2). The calculated  $LD_{50}$  value for the phage-sensitive controls FPS-S6-C2 and 160401-1/5N-C2 was 12,200 CFU and 1350 CFU, respectively. Except from FPS-S6-R8, none of the phage-resistant isolates caused mortality or ulceration in experimentally infected rainbow trout fry. The virulence of the phage-resistant isolate FPS-S6-R8 was, however, severely attenuated with a  $>5 \times 10^4$ -fold increase in  $LD_{50}$  compared to that of control isolate FPS-S6-C2.

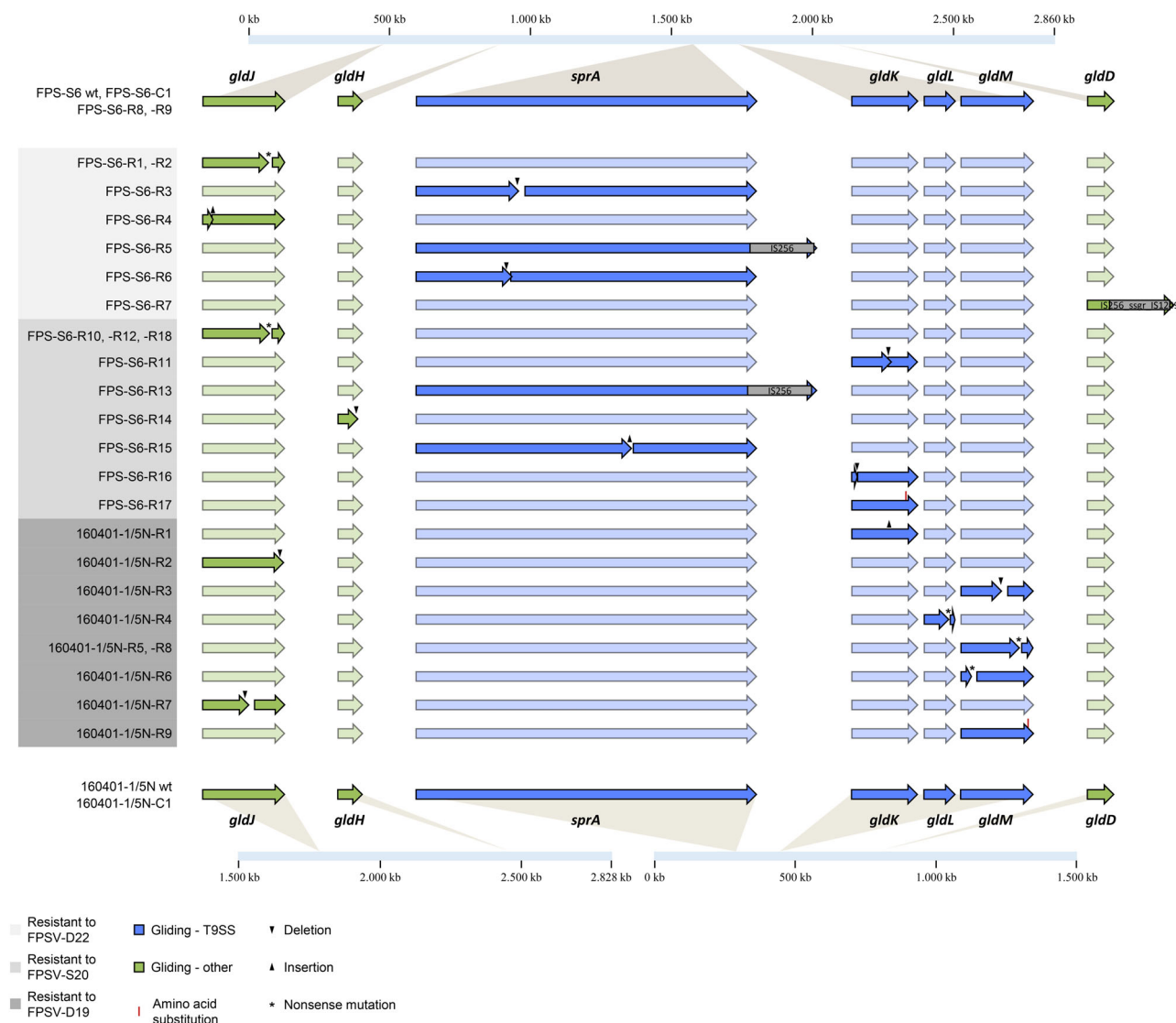
## Genomic characterization

A total of 42 mutations were identified among all 27 phage-resistant isolates (2.3 mutations on average),

and each isolate had between two and four mutations (Table S1). Around 40% of the mutations were found in genes related to the T9SS and other genes involved in gliding motility. Mutations were found in seven different genes involved in gliding motility (Figure 4). Fifteen isolates had mutations in genes encoding proteins that form the T9SS (four in *gldK*, five in *gldM*, one in *gldL*, five in *sprA*), while 10 isolates had mutations in other gliding motility-related genes (one in *gldD*, one in *gldH* and seven in *gldJ*). Most mutations were either non-sense mutations or frameshift mutations followed by a stop codon shortly after. Mutations resulting in amino acid deletions, insertions and substitutions were also present. Three isolates, FPS-S6-R5, -R7 and -R13, had motility genes that were disrupted by insertion sequences (ISs). In isolate FPS-S6-R7, an IS identified as IS256\_ssgr\_IS1249 had inserted in *gldD*. Interestingly, another IS (IS256) was inserted close to the end of *sprA* in both isolate FPS-S6-R5 and -R13, only 58 bp apart. Isolate FPS-S6-R5 demonstrated some degree of gliding motility and secretion indicating that the mutated *sprA* might still be somewhat functional.

Regardless of which gliding motility gene was mutated or which type of mutation was present in these genes, the bacteria were rendered non-spreading or had severely reduced gliding motility compared to the phage-sensitive control (Figures 3 and 4), based on the applied gliding motility assay.

Only isolates FPS-S6-R8 and -R9 did not have any mutations in genes related to gliding motility, and both of these isolates had maintained gliding motility similar to the control FPS-S6-C2. Gliding motility gene mutations were also coupled to reduced proteolytic activity, although the relationship was not straightforward. Isolates FPS-S6-R8 and -R9 showed a higher proteolytic activity than other phage-resistant isolates derived from FPS-S6. However, protein degradation was still decreased compared to the phage-sensitive control isolate FPS-S6-C2. Phage-resistant isolates derived from strain 160401-1/5N maintained the ability to degrade casein regardless of which mutation was present in the isolates. Proteolytic activity was not directly correlated to the specific gliding motility gene, which was mutated.



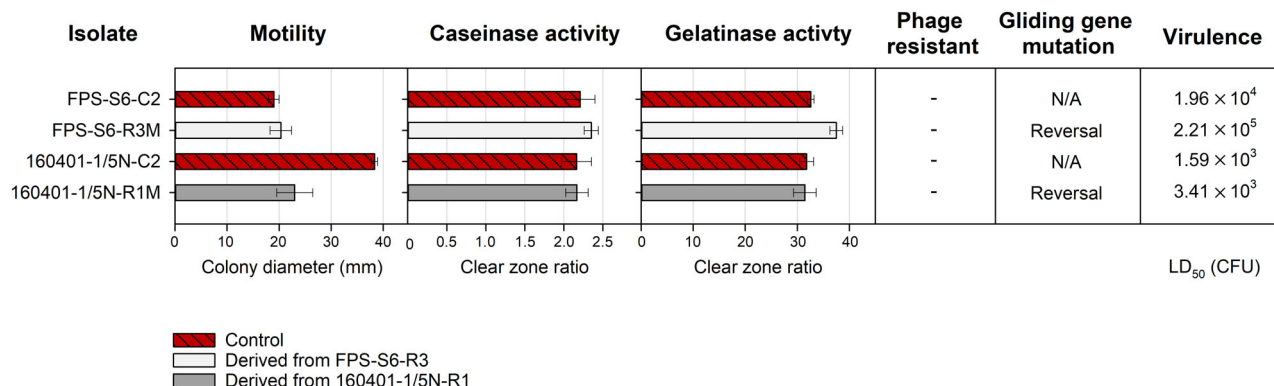
**FIGURE 4** T9SS and other gliding motility gene mutations in phage-resistant isolates. Top and bottom light blue lines represent the bacterial chromosome in strain FPS-S6 and 160401-1/5N respectively. Arrows indicate gene size and direction of transcription. Gene position in the chromosome is indicated by brown-grey triangles. Each row of arrows represents genes in the isolate/s indicated at the left. Mutated genes are highlighted by higher intensity colours. T9SS, type 9 secretion system

For instance, isolates 160401-1/5N-R5 and -R8 had identical mutations in *gldM* but while strain 160401-1/5N-R8 had lost gelatinase activity, 160401-1/5N-R5 did produce the enzyme (Figure 2).

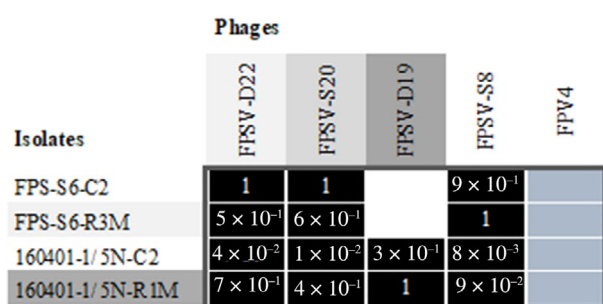
The remaining mutations present in phage-resistant isolates were mainly found in genes encoding cytoplasmic membrane proteins (Table S1). Some of these genes were also found to carry mutations in the phage-susceptible control isolates FPS-S6-C1 and 160401-1/5N-C1. These mutations are thus likely driven by culture conditions rather than phage predation. Fourteen isolates derived from FPS-S6 had mutations affecting a putative flippase (Table S1), assisting in membrane translocation of lipopolysaccharides. The mutation was either a 7 bp tandem repeat deletion immediately upstream of the gene or a frameshift

mutation caused by an A insertion in a polyA repeat. The nature of these mutations suggests that they could readily be reversed. The poly A repeat mutation was also found in FPS-S6-C1. Twelve isolates (four derived from FPS-S6 and eight from 160401-1/5N) had mutations in a FucP-like MFS transporter likely involved in sugar transport. A non-sense mutation in this gene was also found in 160401-1/5N-C1. Finally, a nucleoside symporter family gene (Table S1) contained mutations in six isolates derived from 160401-1/5N. The remaining mutations only occurred once and are listed in Supplementary Information S1.

Overall, the results show that phage-driven mutations under the experimental conditions used here clustered in gliding motility genes and T9SS genes, which affected the bacterial cell surface and hence likely phage



**FIGURE 5** Characterization of isolates with recovered traits in the absence of phage. Motility is shown as colony diameter (mm), caseinase and gelatinase activity is the clear zone ratio between colony diameter and clear zone diameter around the colony in protein supplemented agar. Error bars are standard deviation of three replicates. Virulence is shown as LD<sub>50</sub> (CFU). Bar colour indicates isolate origin as shown in the figure legend. '+': infection; '-': no infection; 'N/A': not applicable



**FIGURE 6** Efficiency of plating of five lytic phages on isolates with recovered traits and control isolates not pre-exposed to phage. Black squares indicate clear plaques in the soft agar overlay, grey squares indicate turbid plaques and white squares indicate no infection. Numbers indicate efficiency of plating relative to the most susceptible isolate. Shading of phage (black; clear plaques, grey: turbid plaques, white: no infection) and isolate names indicate host + phage combinations in Exp. 1, 2 and 3

adsorption. This corresponded well with the observation that phage-resistant bacteria exhibited dispersed growth, as indicated by turbid bacterial cultures, whereas the wild-type bacteria grew in aggregates. The only exception was FPS-S6-R9, which still formed aggregates but also retained gliding motility and susceptibility to some phages. Only one out of 68 mutations was silent (occurring in a transposase), and the most abundant point mutations in *F. psychrophilum* were G:C → T:A.

## Recovery of traits in the absence of phages

The stability of phage-induced changes in phage-resistant isolates was tested in a subset of isolates. Two phage-resistant isolates from each phage + host combination, six in total (Table 2), were tested for recovery of gliding motility. Regained

gliding motility was observed on motility-inducing agar plates in two out of six isolates after 10 (FPS-S6-R3M) and five (160401-1/5N-R1M) serial passages, whereas no gliding motility was observed in isolates FPS-S6-R2, -R4, -R13, or 160401-1/5N-R7 after 12 serial passages (~50 days of incubation in total) (Figure 5).

Colony spreading was fully restored in FPS-S6-R3M, whereas isolate 160401-1/5N-R1M only partly regained gliding motility, with colony spreading similar to the FPS-S6-C2 control, but still significantly lower colony than the control isolate 160401-1/5N-C2 ( $p < 10^{-2}$ ) (Figure 5). Recovered motility was accompanied by fully regained proteolytic activity and phage susceptibility (Figure 6).

FPS-S6-R3M had significantly increased gelatine degradation capabilities compared to FPS-S6-C2 ( $p < 10^{-2}$ ). Furthermore, FPS-S6-R3M and 160401-1/5N-R1M grew in aggregates in broth like the wild-type strains in contrast to the respective ancestral phage-resistant isolates FPS-S6-R3 and 160401-1/5N-R1 (data not shown). Genomic analysis revealed that the gliding gene mutations present in phage-resistant isolates FPS-S6-R3 (2 bp deletion in *sprA*) and 160401-1/5N-R1 (3 bp duplication in *gldK*) had been reversed in FPS-S6-R3M and 160401-1/5N-R1M leading to a functional gliding motility machinery.

In vivo fish experimental trials using intramuscular injection into rainbow trout (mean weight 5 g) were performed in order to test for virulence recovery. Gliding motile isolates FPS-S6-R3M and 160401-1/5N-R1M caused mortality (LD<sub>50</sub> =  $2.21 \times 10^5$  CFU and LD<sub>50</sub> =  $3.4 \times 10^3$  CFU respectively) although control isolates FPS-S6-C2 and 160401-1/5N-C2 were slightly more virulent (LD<sub>50</sub> =  $1.96 \times 10^4$  CFU and LD<sub>50</sub> =  $1.59 \times 10^3$  CFU respectively). LD<sub>50</sub> could not be determined for ancestral phage-resistant isolates FPS-S6-R3 and 160401-1/5N-R1 because few if any mortalities occurred.



## DISCUSSION

The strong selection for phage-resistant isolates in all three phage-host combinations confirmed a fast and efficient adaptation to phage predation in *F. psychrophilum*. Interestingly, resistance to the specific phages following exposure generally also resulted in cross-resistance to 65 different phages belonging to different genetic clusters with very little overlap in genomic composition (Castillo & Middelboe, 2016), suggesting that these highly different phages select for the same resistance mechanisms in *F. psychrophilum*. One exception from this, the resistant FPS-S6-R9, which maintained sensitivity to phages FPSV-S8 and FpV4, also deviated from the other resistant isolates in other ways, as discussed below.

In agreement with this host range pattern, the other phenotypic characterizations of phage-resistant isolates showed that most isolates behaved in a very similar manner irrespective of initial phage exposure. While phage-sensitive control isolates were virulent against rainbow trout in keeping with previous virulence trials (Sundell et al., 2019) (i.e. LD<sub>50</sub> values of 12,200 CFU and 1350 CFU for isolates FpS-S6-C2 and 160401-1/5N-C2, respectively), the complete loss of virulence in 94% of the tested phage-resistant isolates, and a  $5 \times 10^4$ -fold increase in LD<sub>50</sub> in the remaining isolate, clearly demonstrated the direct impact of phage resistance on host virulence properties. Overall, loss of gliding motility, reduced proteolytic activity (at different levels), reduced adhesion to polystyrene surfaces and lost or attenuated virulence against rainbow trout fry thus seems to be a general consequence of phage resistance in this pathogen.

By whole-genome sequencing of resistant isolates, we linked the phenotypic changes in phage-resistant isolates directly to mutations in specific genes related to gliding motility and T9SS. This is in agreement with previous studies in Flavobacteria, where phage resistance was associated to similar phenotypic changes and gliding motility gene mutations in *F. psychrophilum* (Castillo et al., 2015) and *F. columnare* (Kunttu et al., 2021; Laanto et al., 2012). The cross-resistance to other phage groups observed in phage-resistant isolates with gliding motility gene mutations also supports that a non-functional gliding motility machinery leads to broad phage resistance, as has previously been observed in *F. johnsoniae* (Chang et al., 1984). Similarly, artificial mutations constructed in gliding motility and T9SS genes resulted in loss of phage sensitivity (Hunnicuttt et al., 2002; Rhodes et al., 2011a; Rhodes et al., 2011b). Together, these results point to a specific group of target receptors for phage infection across a wide range of *Flavobacterium* phages and hosts. However, results by Castillo et al. (2015) showed that two out of three phage-resistant isolates with motility gene mutations could still be infected by some phages.

Phage-resistant isolates without gliding motility have been shown to have the same loss of gliding motility as genetically engineered *F. psychrophilum* with knockout mutations in gliding motility genes (Barbier et al., 2020; Pérez-Pascual et al., 2017). However, whereas both proteolytic and haemolytic activity was lost in the genetically engineered mutants, some phage-resistant isolates maintained the ability to degrade both casein and gelatine.

Proteolytic capabilities did not appear to correlate to a specific motility gene as mutations in, for example *gldJ* resulted in differing proteolytic profiles in isolate FPS-S6-R1 and 160401-1/5N-R2. Rather, these abilities appeared to be host-dependent, as all 160401-1/5N-derived isolates degraded casein while FPS-S6-derived isolates without gliding motility did not. Isolate 160401-1/5N-R2 was able to degrade gelatine unlike most other 160401-1/5N-derived isolates despite a five amino acid deletion close to the C-terminal of GldJ. Mutations in similar regions of *gldJ* in *F. johnsoniae* resulted in strains that secreted chitinase and motility adhesin SprB but demonstrated no colony spreading (Johnston & Abhishek Shrivastava, 2017).

Approximately 50% of all unique mutations among phage-resistant isolates were nucleotide substitutions. Around 70% of these were G:C → T:A, which indicates that many mutations were a result of unrepaired oxidative lesions in the bacterial host chromosome (Kino & Sugiyama, 2001). Interestingly, phage resistance was caused by IS insertions in three isolates, FPS-S6-R3, -R7 and -R13. IS elements have been demonstrated to be involved in the evolution of pathogens and symbionts as well as in antibiotic resistance development (Siguier et al., 2014). However, to our knowledge, ISs have only been shown to cause phage/virus resistance in Archaea (Deng et al., 2014), and have not previously been reported to function as a phage defence strategy in bacteria. It can be speculated that a stressful environment caused by high phage pressure has led to a reduced ability to repair oxidative lesions in chromosomal DNA and/or an altered IS expression in the bacterial host.

Altogether, both the current and previous data suggest that mutations in genes related to gliding motility and T9SS are selected for by phage exposure in both *F. psychrophilum* and *F. columnare* (Castillo et al., 2015; Laanto et al., 2012). However, no mutations were found in gliding motility-associated genes in the two phage-resistant isolates (FPS-S6-R8 and -R9), which still had gliding motility and secreted protease. Despite this, the phage-resistant isolates with gliding motility had severely attenuated or lost virulence against rainbow trout. This raises the question of whether gliding motility is required for virulence or if it is merely linked to virulence factors by a common secretion pathway. Although the current study provides no evidence to verify this, our data do indicate that there



are critical virulence factors that are not directly linked to gliding motility. The two motile phage-resistant isolates did have significantly reduced adhesion to polystyrene surfaces, which could contribute to or cause attenuated virulence against rainbow trout. Reduced adhesion was also reflected in cell-to-cell interactions as the resistant cells did not form aggregates in liquid cultures, whereas isolate FPS-S6-R9, which was significantly more adhesive than other phage-resistant isolates, did form cell aggregates similar to the wild type. Motile phage-resistant isolates FPS-S6-R8 and FPS-S6-R9 had no mutations linked to T9SS or known motility genes. The only mutation found in isolate FPS-S6-R8 was also present in phage susceptible control isolate FPS-S6-C1 and the underlying mechanism for phage resistance and altered phenotype could not be determined. Motile isolate FPS-S6-R9 had a non-sense mutation in MFS\_DtpA\_like transporter gene but whether or not this mutation is responsible for phage resistance remains to be verified.

The high mutation frequency in T9SS encoding genes among phage-resistant isolates indicates that this secretion system might serve as a receptor for a wide range of flavophages (Castillo et al., 2015; Kunttu et al., 2020). However, the T9SS is responsible for secreting dozens of molecules in *F. psychrophilum* (Barbier et al., 2020; Duchaud et al., 2007) and in other *Fibrobacteres-Chlorobi-Bacteroidetes* species (Veith et al., 2017), many of which are bound to the cell surface and are thus also potential phage receptors. Isolate FPS-S6-R9 had a functional T9SS and was susceptible to phages from genomic clusters I (FpV4) and IV (FPSV-S8), which suggests that phages from these clusters use different receptors than cluster II phage (e.g. FPSV-D22, to which the isolate developed resistance against). Consequently, while resistance to specific phages caused by mutations in the T9SS often confers resistance to phages from other genomic clusters, as mentioned above, our results indicate that the specific phage receptors may be different for different phage clusters. Alternatively, other phage defence mechanisms than cell surface modifications may have played a role for prevention of cluster II phage infections. No changes in CRISPR spacer content were observed in any of the isolates (data not shown), ruling out CRISPR-derived resistance under the specific experimental conditions. *Flavobacterium psychrophilum* has a number of very repetitive genes encoding proteins that are secreted through the T9SS, including putative adhesins likely involved in motility. In *F. johnsoniae*, motility adhesins SprB and RemA that are secreted from T9SS are thought to function as phage receptors (Nelson et al., 2008; Shrivastava et al., 2012). It is possible that for example tandem repeat number variation in these potential phage receptors was missed in our analysis due to short-read sequencing. Epigenetic modifications and/or altered

gene expression could also be possible explanations for phage resistance and attenuated virulence against rainbow trout in the two motile phage-resistant isolates FPS-S6-R8 and -R9. Overall, the observed coupling between phage resistance and mutations in T9SS and gliding motility genes in these phage-exposure studies supports the previous observation that large genetic variations in these specific genes among 35 *F. psychrophilum* isolates were strongly linked to phage susceptibility (Castillo et al., 2021b).

Although there is a substantial fitness cost associated with mutations in the motility machinery, it appears to be a widely used phage defence strategy among members of the *Flavobacterium* genus. This strategy requires a constant recruitment of the fitness impaired, phage-resistant mutants, and a possible explanation for the emergence of such mutants could be that the initial cost for the individual cell is small or absent due to the continuous production of common goods (e.g. T9SS-secreted proteases) by the surrounding bacterial population. When phage predation is high, the T9SS mutants have a competitive advantage due to phage resistance and ensure survival of a fraction of the population. When phage pressure is again alleviated, there would be a selection pressure for bacteria with a functional T9SS in order to colonize a suitable habitat (e.g. fish tissue) and gain access to nutrients. This would lead to a selection for reversal of T9SS mutations, and/or regrowth of a phage-susceptible subpopulation. The observed reversal of mutations in motility-associated genes (isolates FPS-S6-R3M and 160401-1RM) in the absence of phage exposure, leading to genotypes that were again identical to that of the respective wild-type strains, supports this scenario.

The presence of other mutations (e.g. an IS insertion in an MFS transporter in isolate FPS-S6-R3M) in the isolates with recovered traits emphasizes that motility gene mutations and their reversal were indeed causing the switches in phenotype between phage-resistance and -sensitivity. Two additional mutations (peptidase, outer membrane protein) had accumulated in FPS-S6-R3M compared to the ancestral FPS-S6-R3, and it cannot be ruled out that they also play a role in the recovery of phenotypic traits. The spontaneous T9SS mutations and mutation reversal might thus function as a switch for the bacteria to rapidly adjust to the surrounding environment, and thus a flexible strategy for a population to cope with changing phage infection pressures. The observation that IS elements contributed as drivers of the T9SS modifications adds a new mechanism for generating mutational changes in T9SS, as a specific host strategy to prevent phage infection. Spontaneous occurrence of non-motile variants as well as reversal back to a gliding phenotype is well known in other gliding *Flavobacteria* like *F. columnare* and *F. johnsoniae* (Chang et al., 1984;

Laanto et al., 2012), suggesting relatively frequent mutations in T9SS and motility genes.

While the response to phage exposure in *F. psychrophilum* is similar to the related fish pathogen *F. columnare*, it is in sharp contrast to phage–host interactions observed in the prevalent fish pathogen *Vibrio anguillarum* (Castillo et al., 2019). In *V. anguillarum*, mutational changes in the outer membrane protein K (OmpK) which serves as receptor for the broad host range phage KVP40 were very rare events and led to reduced virulence of the host. Instead, this species employed a suite of alternative defence mechanisms against this phage, including, receptor downregulation, biofilm formation, excretion of proteases that inactivate the phages and a putative abortive infection mechanism (Castillo et al., 2019). It was recently proposed that individual bacterial strains can rely on the pan-immune system (the entire array of phage defence mechanisms found among related bacteria) for phage protection, facilitated via horizontal gene transfer (Bernheim & Sorek, 2020). This way, bacterial hosts can survive predation by a wide diversity of phages, while avoiding the burden of carrying multiple, costly immune systems simultaneously. This hypothesis likely fits well to phage–host systems like *V. anguillarum* where horizontal gene transfer is frequent. However, *F. psychrophilum* has a relatively small and conserved genome across large geographical and temporal scales (Castillo et al., 2014; Castillo et al., 2021a, 2021b; Duchaud et al., 2018) with limited horizontal gene transfer. We speculate that, although costly, a rapid and broad phage resistance driven by single, reversible mutations might provide an effective short-term alternative.

From a phage therapy perspective, the emergence of phage-resistant bacteria is one of the major challenges to overcome. The results provided here show that phage administration to prevent or reduce RTFS is likely a low risk, as phage-resistant isolates have severely attenuated or lost virulence against rainbow trout. As our data indicate that phages from different genomic clusters use different host receptors, it is likely beneficial to use cocktails containing phage from different genomic clusters in order to reduce risk of escape mutants that still have a functional T9SS. Further studies on phage receptors and their importance on *F. psychrophilum* virulence would provide valuable information on how to produce phage cocktails that can effectively eliminate the pathogen while simultaneously minimizing the risk of virulent escape mutants.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## DATA AVAILABILITY STATEMENT

The DNA sequence data presented in this study are available in the NCBI repository (Accession numbers are given in the tables).

## ETHICS STATEMENT

All animal experiments were performed in Finland under the project (ESAVI/4225/04.10.07/2017) and 801 personal licence issued by the National Animal Experiment Board (Eläinkoelautakunta, ELLA).

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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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