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Diversity of *Flavobacterium psychrophilum* and the potential use of its phages for protection against bacterial cold water disease in salmonids

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Abstract

Flavobacterium psychrophilum causes rainbow trout fry syndrome (RTFS) and cold water disease (CWD) in salmonid aquaculture. We report characterization of *F. psychrophilum* strains and their bacteriophages isolated in Chilean salmonid aquaculture. Results suggest that under laboratory conditions phages can decrease mortality of salmonids from infection by their *F. psychrophilum* host strain. Twelve *F. psychrophilum* isolates were characterized, with DNA restriction patterns showing low diversity between strains despite their being obtained from different salmonid production sites and from different tissues. We isolated 15 bacteriophages able to infect some of the *F. psychrophilum* isolates and characterized six of them in detail. DNA genome sizes were close to 50 Kbp and corresponded to the *Siphoviridae* and *Podoviridae* families. One isolate, 6H, probably contains lipids as an essential virion component, based on its chloroform sensitivity and low buoyant density in CsCl. Each phage isolate rarely infected *F. psychrophilum* strains other than the strain used for its enrichment and isolation. Some bacteriophages could decrease mortality from intraperitoneal injection of its host strain when added together with the bacteria in a ratio of 10 plaque-forming units per colony-forming unit. While we recognize the artificial laboratory conditions used for these protection assays, this work is the first to demonstrate that phages might be able protect salmonids from RTFS or CWD.

Keywords: bacterial cold water disease, *Flavobacterium psychrophilum*, phages salmonids, protection.

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Introduction

Flavobacterium psychrophilum (syn. *Cytophaga psychrophila*, *Flexibacter psychrophilus*) is the worldwide causative agent of bacterial cold water disease (CWD) and rainbow trout fry syndrome (RTFS) in juvenile salmonids (Nematollahi, Decostere, Pasmans & Haesebrouck 2003). Bacteria classified within the *F. psychrophilum* species form a closely related group. Among seven isolates obtained from France and the United States, the genomic DNA relatedness was above 90% (Bernardet & Kerouault 1989). A larger and more recent study using multilocus sequence typing (MLST) including 50 isolates originating from 10 host fish species and four continents confirmed this particularly low level of diversity (Nicolas, Mondot, Achaz, Bouchenot, Bernardet & Duchaud 2008). In addition, 20 isolates from Chile have proved to constitute a very homogeneous group by phenotypic and genetic properties (Valdebenito & Avendaño-Herrera 2009). Nucleotide diversity between pairs of sequences at 11 protein-coding loci of the core genome amounted to fewer than four differences per kilobase, on average. The MLST website based on seven highly informative loci is available at http://www.pasteur.fr/recherche/genopole/PF8/mlst/Flavopsy.html. The analysis also showed the existence of several clonal complexes with worldwide geographic distribution but marked asso-

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ciation with particular fish species. The complete genome of the *F. psychrophilum* virulent strain JIPO2/86 (ATCC 49511) (Duchaud, Boussaha, Loux, Bernardet, Michel, Kerouault, Mondot, Nicolas, Bossy, Caron, Bessiéres, Gibrat, Claverol, Dumetz, Le Hénaff & Benmansour 2007) consists of a 2 861 988-bp circular chromosome with 2432 predicted protein-coding genes. Among these predicted proteins, stress response mediators, gliding motility proteins, adhesins and many putative secreted proteases are probably involved in colonization, invasion and destruction of the host tissues.

Despite the substantial economic losses that *F. psychrophilum* causes in freshwater aquaculture, a specific vaccine is currently unavailable (Plant, Lapatra & Cain 2009) and antibiotics are the only treatment (Nematollahi *et al.* 2003). With the appearance of antibiotic resistance (Izumi, Ouchi, Kuge, Arai, Mito, Fujii, Aranishi & Shimizu 2007; Hesami, Parkman, MacInnes, Gray, Gyles & Lumsden 2010) and the adverse environmental effects of using antibiotics in aquaculture, alternative methods of treatment are needed. Phage therapy is a potential alternative approach for controlling pathogenic bacteria in aquaculture, and *F. psychrophilum* phages have already been isolated and characterized with this aim. Reports on the effects of bacteriophages against numerous pathogenic *F. psychrophilum* have provided the foundation for future exploration of the potential of phages in the treatment for RTFS and CWD. Among these, 22 *F. psychrophilum* phages from Danish rainbow trout farms were isolated and characterized into three major classes according to their genome sizes. They showed highly variable patterns of infectivity among *F. psychrophilum* strains, some with broad-host-range and strong lytic potential (Stenholm, Dalsgaard & Middelboe 2008). In another study, five bacteriophages that infect *F. psychrophilum* were isolated from pond water collected from Japanese ayu farms. The five phages isolated were classified as members of the *Myoviridae*, *Podoviridae* and *Siphoviridae* families and had highly variable patterns of infectivity for different *F. psychrophilum* isolates. They were stable in ayu farm conditions, and some proved to be efficient for the reduction in bacterial growth in laboratory culture conditions (Kim, Gomez, Nakai & Park 2010).

The different sensitivities of the pathogenic *F. psychrophilum* strains complicate the application of phages in control efforts unless either a wide-host-range phage is identified or a mixture of phages can be obtained that can infect most of the *F. psychrophilum* isolates. To explore further the potential application of phage therapy, we isolated and characterized *F. psychrophilum* strains and phages from aquaculture sites in Chile and measured their host range on *F. psychrophilum* strains from Chile and Denmark and their potential protection after experimental infection. This study is a step further towards the application of phages in controlling *F. psychrophilum* and consequently their potential application as a treatment for RTFS and CWD in aquaculture.

Materials and methods

Strains and growth media

Twelve *F. psychrophilum* strains isolated from different aquaculture sites in Chile were used in this study. Strains were isolated by culturing homogenized tissue from kidney, spleen or skin ulcers on TYES agar (Holt, Rohovec & Fryer 1993; Valdebenito & Avendaño-Herrera 2009). Besides the isolates obtained in Chile reference strains CSF 259-93 and ATCC 49418^T, and three Danish strains 951004-1/11, 950106-1/1 and 020612-4/1 (Stenholm *et al.* 2008) were also included in this study (Table 1). Bacteriophages were isolated from bacteriophage enrichment cultures obtained by the incubation of the homogenized tissue suspension or water sample (Van Twest & Kropinski 2010) with the *F. psychrophilum* isolate indicated in Table 2. The cell-free suspension was prepared after homogenization of the tissue and subsequent centrifugation at 5000 ***g*** for 10 min. The bacterial cells in the phage-enriched culture were pelleted by centrifugation at 5000 ***g*** for 10 min, and the remaining bacteria were removed by filtration (0.22 μm) (Bastias, Higuera, Sierralta & Espejo 2010). Phages in the filtrate were detected by plating 100 μL using the standard method for a double-layer agar plaque assay (Adams 1959). One plaque was selected from each positive sample and re-plated twice to ensure clonal phage stocks. Phages FpV4 and FpV9 were isolated in Denmark (Stenholm *et al.* 2008). Growth media consisted of tryptone 0.4%, yeast extract 0.04%, $CaCl_2$ 0.05% and $MgSO_4$ 0.05%, and incubation was performed at 15 °C (modification of Holt *et al.* 1993).

Table 1 Properties of *Flavobacterium psychrophilum* isolates employed in this study

Isolates	Site origin	Fish	Tissue isolation	CSF 259-93 marker[a]	ATCC 49418 marker[a]
VQ33	Bío Bío	Symptomatic trout		+	–
VQ50	Araucanía	Symptomatic trout		+	–
VQ79	Los Lagos	Asymptomatic Atlantic salmon		+	–
MH1	M Harvest Ensenada Los Lagos	Symptomatic Atlantic salmon	Skin	+	–
MH2	M Harvest Ensenada Los Lagos	Symptomatic Atlantic salmon	Skin	+	–
BV8	Concepción/Nehuentué	Atlantic salmon	Kidney	+	–
BV6	Lago Llanquihue	Trout	Gills	+	–
BV7	Concepción/Nehuentué	Atlantic salmon	Gills	+	+
A2	Salmonera antártica/Los Angeles	Trout	Skin	+	–
T23	TRUSAL, Peñaflor	Trout	Spleen	+	+
T26	TRUSAL, Peñaflor	Trout	Kidney	+	–
PG2	Pesquera del Golfo, X región	Trout	Kidney	+	–
CSF 259-93	Idaho	Rainbow trout	Spleen	+	–
ATCC 49418^T	Washington	Coho salmon	Kidney	+	+
951004-1/11[b]	Denmark fish farm	Trout	Spleen	+	–
950106-1/1[b]	Denmark fish farm	Trout	Spleen	+	–
020612-4/1[b]	Denmark fish farm	Trout	Spleen	+	–

[a]Marker corresponds to16S rRNA alleles described by Ramsrud *et al.* (2007).
[b]Data and detailed origin of these strains are in Stenholm *et al.* (2008).

Table 2 Properties of bacteriophages employed in this study

Bacteriophages	Enrichment *Flavobacterium psychrophilum* host isolates	Class	DNA size (Kbp)[a]	Density CsCl (g mL^{-1})
1H	MH1	*Siphoviridae*	49 (46)	1.38
6H	MH1	*Siphoviridae*	51 (48)	1.32
9H	MH1	*Siphoviridae*	50 (46)	1.38
2P	PG2	*Siphoviridae*	51 (50)	1.40
23T	T23	*Siphoviridae*	50 (49)	1.41
2A	A2	*Podoviridae*	52 (47)	1.40
FpV4[b]	900406-1/3 950106-1/1 951004-1/11	*Podoviridae*	90	NA
FpV9[b]	900406-1/3 950106-1/1 951004-1/11	*Siphoviridae*	48	NA

NA, not available.
[a]Size of the DNA was calculated by their migration in 0.4% agarose. Numbers between parentheses indicate the size calculated by adding the sizes of restriction fragments, as indicated in Materials and methods.
[b]Three isolates were used for enrichment of these phages (Stenholm *et al.* 2008).

Characterization of *F. psychrophilum* strains and their phages

Flavobacterium psychrophilum strains were characterized by their phenotypes including flexirubin-type pigment, congo red test as described by Bernardet & Kerouault (1989), API 20 NE (Biomerieux) and by the presence of 16S rRNA alleles (Ramsrud, LaFrentz, LaFrentz, Cain & Call 2007). Selected isolates were also grouped by direct genome restriction fragment analysis (DGREA). The procedure originally described for *Vibrio parahaemolyticus* (Fuenzalida, Hernandez, Toro, Rioseco, Romero & Espejo 2006) was employed except that 0.5 μL of the *Xho*1 (Promega) restriction enzyme was used in 1.5 μL buffer, incubated for 2 h.

Bacteriophage isolates were partially purified as indicated previously. Phages in the filtrate were centrifuged at 100 000 ***g*** for 1 h; subsequently, the pellet was resuspended in 1 mL of SM buffer (50 mM Tris–HCl, pH 7.5, 99 mM NaCl, 8 mM

$MgSO_4$) and centrifuged over a 1.5–1.2 g mL^{-1} CsCl gradient for 24 h at 100 000 ***g*** in a swinging bucket ultracentrifuge rotor (Zabala, Garcia & Espejo 2009). The phages were obtained from visible bands subsequently shown to contain more than 90% of the plaque-forming units (PFUs). The density of the fraction containing the phages was calculated by weighing 100 μL of the fraction.

Electron microscopy samples dialysed against SM buffer were stained with 2% uranyl acetate on grids with carbon-stabilized formvar and observed using a Phillips CM 100 transmission electron microscope. Phage DNA was extracted after incubation of the partially purified phage preparations with DNase (2 μg mL^{-1}) and RNase (100 μg mL^{-1}) for 1 h at 37 °C. These samples were subsequently treated with 500 μg mL^{-1} proteinase K for 1 h at 65 °C. Sodium dodecyl sulphate (SDS) was added to a final concentration of 0.5% (wt/vol). After incubation at 65 °C for 1 h, the solution was extracted twice with phenol–chloroform. Finally, the DNA was precipitated by addition of 1/10 volume of 3 M sodium acetate (pH 5.0) and two volumes of absolute ethanol at −20 °C. After the pellet was washed with 70% ethanol, it was dissolved in TE buffer (0.01 M Tris, 0.001 M EDTA, pH 7.5) (Bastias *et al.* 2010).

DNA size was estimated by electrophoresis in 0.4% agarose using a high-molecular-weight standard and also by adding the molecular weights of the restriction fragments obtained by digestion with the restriction enzyme *Hae*III (Promega) according to the manufacturer's instructions. The fragments were subsequently separated by electrophoresis on a 7.5% polyacrylamide gel for 2 h at 70 V and their size calculated using the GeneRuler 1-kb DNA ladder (Fermentas) as reference. Chloroform treatment was performed by mixing one volume of the phage suspension with 0.02 volumes of chloroform, followed by strong agitation for 1 min and subsequent determination of PFUs in treated and untreated samples.

Host ranges of the isolated bacteriophages were determined by spotting 10 μL of 1/10 serial dilutions of bacteriophage on top of a TYES-A plate freshly prepared with 4 mL of top agar inoculated with 0.3 mL of a culture of the strain to be tested (A_{525} = 0.24–0.5) (Stenholm *et al.* 2008). PFUs were counted on the appropriate dilution spot with the help of a magnifying glass.

Challenges and phage protection assays

Pathogenicity of four *F. psychrophilum* strains (MH1, T23, A2 and PG2) was tested by intraperitoneal injection of 10^8 bacteria per fish as described by Madsen & Dalsgaard (1999) except (i) that they were infected with bacteria suspended in 100 μL of growth medium, (ii) *Salmo salar* L. or *Oncorhynchus mykiss* (Walbaum) of 15–30 g were employed and (iii) water temperature was 15 °C, and the challenges lasted 15 days. The bacteria were grown in Cytophaga medium as reported by (Hadidi, Glenney, Welch, Silverstein & Wiens 2008). To test phage protection, phages resuspended in Cytophaga medium were added to the bacterial suspension immediately before injection to obtain a multiplicity of infection (MOI) of 10 PFU per CFU.

Phage protection was assessed by comparison of mortality after challenge with bacteria alone vs. bacteria plus phage. Fish were inoculated with 10^8 colony-forming units (CFUs) of *F. psychrophilum* strain MH1 alone or with the same number of bacteria containing 10^9 PFU of either phage 1H or 6H. *Flavobacterium psychrophilum* infection as the cause of death was confirmed by isolation of the bacteria from spleen or kidney of the dead or moribund fish. These animals showed dark pigmentation of the skin, enlarged spleen and haemorrhages at the anus, often accompanied by oedema and skin injury adjacent to the site of injection. The significance of the differences found was calculated using the χ^2 test with a significance level of 5%.

Challenges and phage protection were performed in 80-L tanks with separate water circulation and aeration. Tanks were disinfected before each experiment. Two-thirds of the water was changed every 2 days. The origin, number and weight of the fish used each challenge experiment are described in Table 3.

Results

Diversity among *F. psychrophilum* isolates obtained in Chile

Twelve isolates obtained in Chile and representing a large diversity according to farm, date of isolation and preliminary identification were selected for characterization (Table 1). In addition, two well-characterized *F. psychrophilum* strains, CSF 259-93 isolated in Idaho, USA (Nicolas *et al.* 2008) and

Table 3 Percentage mortality 15 days after injection of salmonids with *Flavobacterium psychrophilum* strain MH1 and *F. psychrophilum* strain MH1 with bacteriophages

Experimental conditions			% Mortality after injection with			
Experiment	Fish species	No. fish/ weight (g)	Cytophaga medium	Strain MH1	Strain MH1 plus phage 1H	Strain MH1 plus phage 6H
1	*Salmo salar*	11/20	0	45	18 (60)	ND
2	*Oncorhynchus mykiss*	15/15	7	47	ND	20 (67)
3	*O. mykiss*	15/15	7	80	67 (16)	47 (41)
4	*S. salar*	15/10	0	13	0 (100)	6 (50)

ND, not done.
(): Percentage mortality reduction in the presence of the phage.

ATCC-type strain 49418^T (Bernardet & Kerouault 1989), and three strains obtained in Denmark (Stenholm *et al.* 2008) were included for worldwide comparison of diversity. These isolates were also characterized by DGREA, a restriction enzyme analysis using total bacterial DNA, which permits direct genome restriction enzyme analysis by conventional polyacrylamide gel electrophoresis, adapted to *F. psychrophilum* (Fig. 1a). DGREA has been previously used for comparison of *V. parahaemolyticus* (Fuenzalida *et al.* 2006) and *Vibrio vulnificus* (Gonzalez-Escalona, Whitney, Jaykus & Depaola 2007) strains. In accordance with the low diversity observed between isolates of the current study species, the DGREA patterns between *F. psychrophilum* isolates were quite similar, an observation very different from that obtained for isolates of the two *Vibrio* species. In spite of the overall similarity in pattern, however, small differences in the electrophoretic migration of a few fragments allowed distinction of nine clusters among the 12 strains tested (Fig. 1b). As previously

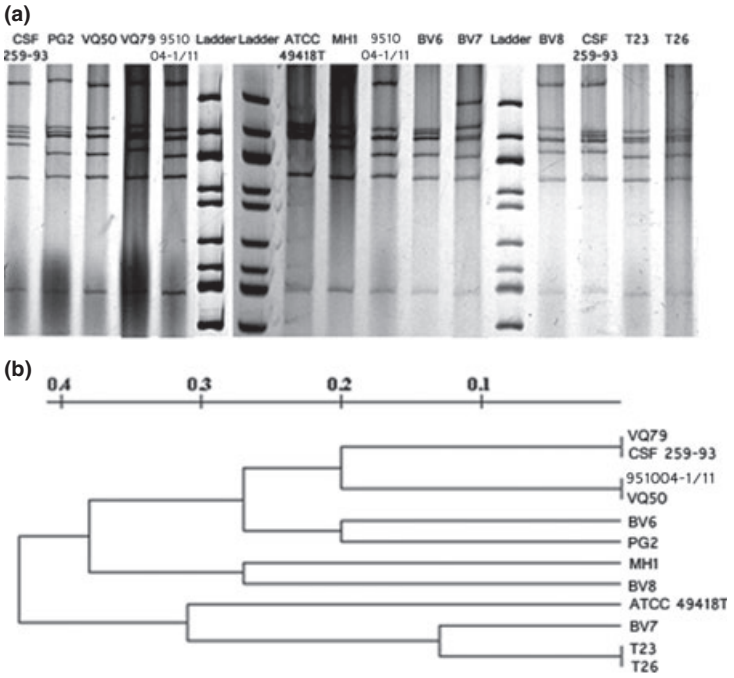

Figure 1 Direct genome restriction enzyme analysis with XhoI of *Flavobacterium psychrophilum* isolates obtained from symptomatic fish and dendrogram illustrating the clusters of the patterns by dissimilarity. The dendrogram was obtained applying WPGM using Treecom to a distance matrix calculated using the Nei and Li coefficient and the matrix was finally used to obtain the dendrogram applying weighed pair group method (WPGM) in Treecom. The scale corresponds to the fraction of dissimilar bands.

observed by MLST (Nicolas *et al.* 2008), some isolates from very different locations showed identical DGREA patterns. Strains VQ50 and VQ79, for example, both isolated in Chile, had DGREA patterns identical to strains 951004-1/11 and CSF 259-93, isolated in Denmark and Idaho, USA, respectively.

Flavobacterium psychrophilum bacteriophages

Fifteen bacteriophage isolates were obtained after enrichment of phages in tissue extracts of *F. psychrophilum*–infected fish in single bacterial isolate cultures. Six isolates selected because they produced easily distinguishable plaques in the enrichment host were characterized (Table 2). Figure 2 shows electron micrographs of the six phages. Notably, isolate 6H with a buoyant density in CsCl of 1.32 g mL^{-1} was also chloroform-sensitive and thus most likely contains a lipid membrane like the PM2 *Corticoviridae* and PRD1 *Tectiviridae* families (Bamford & Bamford 2006). Inactivation was higher than 99.9% after chloroform treatment. All isolates contained genomes of about 50 Kbp.

Host ranges of the six phage isolates from Chile and two phage isolates from Denmark (Stenholm *et al.* 2008) were determined for the 12 *F. psychrophilum* isolates obtained in Chile and for the five isolates from the USA and Denmark (Table 4). In general, phage isolates only rarely infected bacteria isolated from a different farm. When such infection did occur (phages 6H, 2P, 2A), PFUs were turbid and in smaller numbers than in the strain used for the original enrichment. The Danish phage isolates FpV4 and FpV9 infected both Danish bacterial strains and the Chilean strain VQ50. Interestingly, Danish strain 951004/11A (MM04) and the Chilean strain VQ50 appeared identical by DGREA.

Pathogenicity of *F. psychrophilum* isolates and phage protection

The pathogenicity of four strains (MH1, T23, A2 and PG2) was tested by intraperitoneal injection of 10^8 bacteria per fish (15–30 g weight). All four *F. psychrophilum* strains caused mortality from 40% to 100% within 10 days after injection (results not shown). Strain MH1 was tested in more detail in both salmon and trout (Table 3). For salmon, mortality after 15 days was 45% and 13%, respectively, in the absence of phages. In two parallel challenge experiments with trout, the mortality was 47% and 80%, respectively. Controls injected with sterile medium showed 0% mortality except in two experiments with 7% (Table 3).

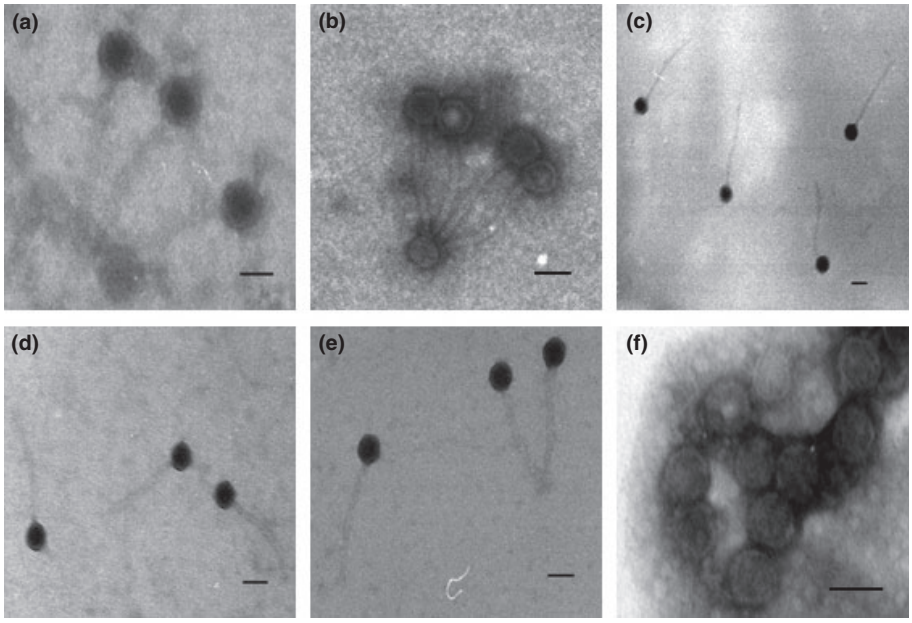

Figure 2 Uranyl acetate negative staining observation of *Flavobacterium psychrophilum* bacteriophages using electron microscopy. A, 1H; B, 6H; C, 9H; D, 2P; E, 23T; F, 2A (bar = 50 nm).

Table 4 Host range of the bacteriophages against the different *Flavobacterium psychrophilum* strains; numbers indicate PFU observed in the indicated strain

	Bacteriophage							
Bacterial strains	1H	6H	9H	2P	2A	23T	FpV4	FpV9
MH1	$1*10^{7}$ [a]	$1*10^{8}$ [a]	$1*10^{8}$ [a]	< 10	$3*10^{6}$	< 10	< 10	< 10
MH2	$2*10^{6}$	$1*10^{7}$ [b]	$1*10^{8}$	< 10	< 10	< 10	< 10	< 10
PG2	< 10	< 10	< 10	$3*10^{8}$ [a]	$3*10^{7}$	< 10	< 10	< 10
A2	< 10	< 10	< 10	$2*10^{8}$	$7*10^{7}$ [a]	< 10	< 10	< 10
T23	< 10	< 10	< 10	< 10	< 10	$6*10^{7}$ [a]	< 10	< 10
T26	< 10	< 10	< 10	$1*10^{5}$ [b]	$1*10^{2}$ [b]	< 10	< 10	< 10
951004-1/11	< 10	< 10	< 10	< 10	< 10	< 10	$1*10^{8}$	$3*10^{7}$
950106-1/1	< 10	< 10	< 10	< 10	< 10	< 10	$4*10^{7}$	$1*10^{8}$
020612-4/1	< 10	< 10	< 10	< 10	< 10	< 10	$1*10^{7}$	$3*10^{7}$
VQ33	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
VQ50	< 10	< 10	< 10	< 10	< 10	< 10	$1*10^{7}$	$1*10^{7}$
VQ79	< 10	$1*10^{5}$ [b]	< 10	< 10	< 10	< 10	< 10	< 10
BV6	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
BV7	< 10	$1*10^{5}$ [b]	< 10	< 10	< 10	< 10	< 10	< 10
BV8	< 10	$1*10^{5}$ [b]	< 10	< 10	< 10	< 10	< 10	< 10
ATCC49418^{T}	< 10	$1*10^{5}$ [b]	< 10	< 10	< 10	< 10	< 10	< 10
CSF 259-93	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10

PFU, plaque-forming units.
[a]Bacterial isolate used for enrichment.
[b]Titre estimated because no clear plaques were observed.
Gray: titre in heterologous host.

Phage protection was assessed by a comparison of mortality after challenge with bacteria alone vs. bacteria plus phage. In every experiment, mortality decreased when phages were present (Table 3). The χ^2 tests to assess the significance of these findings indicated values of 0.01 for the decreased mortality with the addition of 1H and 0.02 with the addition of 6H.

Discussion

This study adds to the number of phages reported for *F. psychrophilum* and confirms the idea that phages are widespread in aquaculture sites. Only *Siphoviridae* and *Podoviridae* were observed, and the six isolates studied in detail contained a genome of about 50 Kbp. Phage 6H stands out because of its sensitivity to chloroform and low buoyant density in CsCl, which strongly suggest that lipids form an essential component of its virion, as with PM2 (Espejo & Canelo 1968) and PRD1 (Olsen, Siak & Gray 1974). Phage 6H may represent a new and unique group of lipid-containing phages because its genome is much larger than those in the lipid-containing phages previously described. Phages 1H and 9H have identical restriction patterns (not shown) and probably correspond to the same phage.

Successful application of bacteriophages in the treatment for bacterial diseases requires a group of broad-host-range phages to cover a possible wide spectrum of potential pathogenic host strains associated with a disease outbreak. Unfortunately, for our phage therapy goal, phage isolates tested in this study only rarely infected *F. psychrophilum* strains independently isolated from the strain used for their enrichment. On those occasions, plaques were turbid and several orders of magnitude smaller than in the enrichment strain. Preliminary characterization of resistance suggests that this outcome arises from the presence of a large fraction of resistant host cells even when the population has been recently cloned. The absence of broader-host-range phages will require the use of a phage mix with a large number of phages to attain effective treatment for the different *F. psychrophilum* strains present in diverse aquaculture sites. However, broad-host-range mutants may be discovered by testing a larger number of phages. On the other hand, it is worth noting that some phages can infect, although poorly, strains isolated from disparate geographical regions and conditions; for example, phage 6H isolated in Chile could infect the ATCC 49418^{T} strain isolated in the USA (Bernardet & Kerouault 1989) or phages FpV4 and FpV9 isolated in Denmark could infect the VQ50

strain isolated in Chile. However, the low infectivity efficiency in the 'heterologous' strain could be a significant handicap in therapy. This handicap could be overcome with a better understanding of the causes of resistance or partial sensitivity.

Characterization of the *F. psychrophilum* isolates obtained in Chile confirms the low diversity observed within this species (Bernardet & Kerouault 1989; Nicolas *et al.* 2008). Differences between isolates were, however, observed by DGREA, although all isolates showed a very similar pattern with only small differences in the migration of a few fragments. We have no simple explanation for the conservation of the restriction pattern, even though restriction segments varied in size (although only slightly). The different sensitivity of the strains to different phages also suggested some diversity among the bacterial isolates. Most of the *F. psychrophilum* strains could kill juveniles of both salmon and trout after intraperitoneal injection of 10^8 cells. The repeatability of these challenges allowed exploration of the potential protection of bacteriophages against bacterial infection, in spite of the high variation observed between experiments. However, it seems difficult to obtain better repeatability unless more uniform fry populations can be obtained. In preliminary experiments to explore the effectiveness of phage therapy, we chose a simple test that would favour a potential protection by the phage, even though the procedure could not be used to treat *F. psychrophilum* infections in practice. Under these conditions, when the phage was added together with the bacteria in a ratio of 10:1, bacteriophages could significantly decrease mortality. Bearing in mind the artificial laboratory conditions employed, this outcome is the first observation that phages may protect against RTFS or CWD. However, although each of the reported experiments was performed once and does not firmly demonstrate phage protection, they could serve as a starting point for developing effective protection using infection methods more like those occurring in nature.

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