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Diversity and Geographical Distribution of *Flavobacterium psychrophilum* Isolates and Their Phages: Patterns of Susceptibility to Phage Infection and Phage Host Range

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Abstract *Flavobacterium psychrophilum* is an important fish pathogen worldwide that causes cold water disease (CWD) or rainbow trout fry syndrome (RTFS). Phage therapy has been suggested as an alternative method for the control of this pathogen in aquaculture. However, effective use of bacteriophages in disease control requires detailed knowledge about the diversity and dynamics of host susceptibility to phage infection. For this reason, we examined the genetic diversity of 49 *F. psychrophilum* strains isolated in three different areas (Chile, Denmark, and USA) through direct genome restriction enzyme analysis (DGREA) and their susceptibility to 33 bacteriophages isolated in Chile and Denmark, thus covering large geographical (>12,000 km) and temporal (>60 years) scales of isolation. An additional 40 phage-resistant isolates obtained from culture experiments after exposure to specific phages were examined for changes in phage susceptibility against the 33 phages. The *F. psychrophilum* and phage populations isolated from Chile and Denmark clustered into geographically distinct groups with respect to DGREA profile and host range, respectively. However, cross infection between Chilean phage isolates and Danish host isolates and vice versa was observed. Development of resistance to certain bacteriophages led to susceptibility to other phages suggesting that "enhanced infection" is potentially an important cost of resistance in *F. psychrophilum*, possibly contributing to the observed coexistence of phage-sensitive *F. psychrophilum* strains and lytic phages across local and global scales. Overall, our results showed that despite the identification of local communities of phages and hosts, some key properties determining phage infection patterns seem to be globally distributed.

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Introduction

Flavobacterium psychrophilum is a fish pathogen with a global distribution, causing the septicemic diseases "cold water disease" (CWD) or "rainbow trout fry syndrome" (RTFS) in freshwater aquaculture [1, 2]. The infection spreads to all the organs and results in high rates of juvenile mortality, increased susceptibility to other infections [3–5]. The consequences are high costs of treatment with antibiotics and significant economic implications for salmonid aquaculture worldwide [3, 4]. Treatment with oxolinic acid (OXA), sulfadiazine (S), and amoxicillin (AMX) are required to reduce mortality, however increased microbial resistance to these approved drugs have been observed in *F. psychrophilum* [6, 7]. A specific vaccine is currently at an early stage of development [8], however, this is targeting larger fish and is not expected to be applicable in the treatment of fish fry (<5 g) as the fish require a well-developed immune system for the vaccine to be efficient.

For these reasons, *F. psychrophilum*-specific bacteriophages (or phages) may be attractive therapeutic agents for controlling pathogenic bacterial infections of fish fry. Several phages have been reported for fish pathogenic bacteria such as *Aeromonas salmonicida* [9], *Aeromonas hydrophila* [10],

Edwardsiella tarda [11], *Yersinia ruckeri* [12], *Lactococcus garvieae* [13], and *Pseudomonas plecoglossicida* [14]. Also for *F. psychrophilum*, a number of studies have shown the ability of phages to control the pathogen at in vitro conditions [15, 16] and to reduce fish mortality in phage protection assays [17].

However, while phage infection can provide an efficient control of individual *F. psychrophilum* strains under controlled laboratory conditions, the use of phages to control *F. psychrophilum* under natural conditions is challenged by the fact the pathogen community is composed of numerous co-occurring strains with large differences in the sensitivity to the variety of potentially applied *F. psychrophilum* phages [15]. It is well documented that part of this diversity in the pathogen community is in fact driven by antagonistic co-evolution of phages and hosts, and that phage-resistant strains rapidly replace sensitive strains when the population is exposed to strong selective pressure by infectious phages [18–21]. Several different mechanisms of resistance or immunity have been described for well-studied bacteria-phage systems: alteration of host surface receptors [22], phase variation [23], restriction-modification systems [24], immunity by clustered regulatory interspaced short palindromic repeats (CRISPR) [25], and by the presence of prophages in bacterial genomes [26]. Recently, a specific prophage (phage 6H) has been found to be widely distributed in *F. psychrophilum* communities worldwide, influencing the phage susceptibility patterns in these communities [27].

Despite its global occurrence and pathogenic implications, little is known about the diversity and dynamics of *F. psychrophilum* and *F. psychrophilum*-specific phages with respect to phage sensitivity and host range, respectively, across large spatial scales, or about the mechanisms that regulate phage susceptibility in the host community. Thus, a detailed characterization of phage host community composition, interactions and acquisition of resistance is necessary for evaluating the potential of using phages to control *F. psychrophilum*. In this study, we therefore examined the genetic diversity of 49 environmental *F. psychrophilum* isolates from different geographical locations (Chile, Denmark, and USA) as well as the infectivity of 33 phages against this collection of strains. In addition, we explored the phage infectivity against 40 phage-resistant strains derived from sensitive wild-type strains in laboratory experiments. Our results demonstrate that local phage and host communities grouped in geographically distinct clusters according to host range and restriction analyses, respectively. However, some host range properties were distributed across the investigated geographic areas and thus a fraction of the Danish phage isolates were infective against Chilean pathogens and vice versa. Interestingly, development of phage resistance against certain phages lead to increased susceptibility to other phages in various *F. psychrophilum* groups, and in some cases this shift in sensitivity was associated with the loss of the specific prophage 6H.

Materials and Methods

Bacterial Strains, Bacteriophages, and Growth Conditions

Nineteen *F. psychrophilum* strains isolated from Chile [17, 27], 27 strains isolated in Denmark [15] and 3 strains isolated in USA were employed in this study (Table 1, Online resource). The Chilean *F. psychrophilum* strains were isolated from ten trout or salmon aquaculture farms, while Danish *F. psychrophilum* strains were isolated from eight different trout farms as well as two locations downstream from the farms.

In addition to the environmental isolates a number of phage resistant strains were used which were derived from selected sensitive hosts after exposure to phages (see details below). Moreover one strain (950106-1/1^{c}) was included in the analysis which had been cured for the presence of the prophage 6H in the genome after induction of the wild-type strain (950106-1/1) [27], hence 950106-1/1^{c} has lost the 6H prophage from the genome.

The 33 bacteriophages used in this study were previously isolated from fish farms in Chile and Denmark (Table 2, online resource; [15, 17]). For preparation of high-density bacteriophage stocks, the bacteriophages were eluted from agar plates with confluent lyses by adding 5 mL of Buffer SM (50 mM Tris-Cl, pH 7.5, 99 mM NaCl, 8 mM $MgSO_4$) and subsequent purification by centrifugation and filtration [15]. Bacteria were grown in liquid TYES-B medium (0.4 % tryptone, 0.04 % yeast extract, 0.05 % $CaCl_2$ and 0.05 % $MgSO_4$) and incubation was performed at 15 °C for 48–72 h with agitation.

Marker 16S rRNA Alleles

For primary identification of *F. psychrophilum* isolates, bacterial DNA was extracted from liquid cultures of 1 mL in TYES-B using the Wizard Genomic DNA Purification kit (Promega) and used for PCR assay detection of the specific CSF 259-93 and ATCC 49418^{T} alleles (Table 3, online resource). PCR cycling conditions included 10 min denaturation step at 95 °C, followed by 30 amplification cycles with each cycle consisting of denaturation at 95 °C for 1 min, annealing at 61 °C for 1 min, extension at 72 °C for 1 min and a final extension step at 72 ° C for 10 min [28]. Products are clearly distinguishable (298 and 600 bp).

Application DGREA to *F. psychrophilum*

Direct genome restriction enzyme analysis (DGREA) provides a fingerprint of the DNA fragments obtained after restriction analysis and was performed as described previously to *F. psychrophilum* [17]. Briefly, each reaction mixture consisted of 8 μg DNA digested with 10 U of *xhoI*

endonuclease (Promega) for 2 h at 37 °C, and treated with proteinase K (0.020 μg/μl) (QIAGEN) for 1 h at 37 °C. Nine microliters of each digestion were electrophoresed in 8 % nondenaturing polyacrylamide gels for 3.5 h at 100 V. The bands on the gel (only fragments between sizes of 500 bp and 2500, the size range well resolved in this gel) were visualized by silver staining, as described previously [29]. For construction of the dendrogram, bands with similar and different migration were distinguished and employed in a similarity matrix, calculated using the Nei and Li coefficient [30]. This matrix was finally used to obtain the dendrogram applying WPGM in Treecon [31].

Selection and Purification of Phage-Resistant *F. psychrophilum* Strains

A collection of 16 phage-resistant strains derived from Danish wild-type strain 950106-1/1 were isolated after exposure to the phages FpV4, FpV9, FpV21 or a cocktail of 11 phages in infection experiments (Christiansen et al., unpublished results) (Table 4, online resource). Similarly, 24 phage-resistant strains derived from the Chilean strains MH1, MH2, T23, T26, VQ79, BV7, BV8, A2, P2 and the American strain ATTC 49418^T, after infection with one, two or a combination of several phages isolated in Chile (Table 3, online resource). For the isolation of resistant strains, the sensitive wild-type strains were grown to exponential phase (OD525=0.1–0.2) and incubated for 2 h with phages at a multiplicity of infection (m.o.i) of 100 to ensure that all bacteria were infected with at least one bacteriophage. Subsequently, 0.1 mL aliquots were diluted and inoculated onto TYES plates (1.1 % agar) embedded with a total of 10^{11} phages and incubated at 15 °C for 6–7 days to allow slowly growing colonies to appear. This procedure was repeated three times and finally one clone was selected from each incubation and kept at −80 °C in TYES-B with 15 % glycerol.

Bacteriophage Host Range Test and Efficiency of Plating

The host range of the collection of bacteriophages was determined by spotting 10 μL of bacteriophage concentrate on top of a TYES-A plate (1.1 % agar) freshly prepared with 4 mL top agar (0.4 % agar) inoculated with 0.3 mL of investigated strain (OD525=0.4-0.5) [14]. The plaques were examined for cell lysis after 3–5 days. Since the reaction of the spot test can vary according to the growth condition of the host strain, these spot tests were performed three times with independent host cultures. An unweighted-pair group method using average linkages (UPGMA) tree was constructed using the software Treecom [31], where the sensitivity/no sensitivity matrix was converted to pairwise distances using the Dice similarity coefficient.

Efficiency of plating was determined exposing the strains to the same phage titer and infectivity was quantified by small drop plaque assay [32]. Plaque forming units (PFU) were examined after 3–5 days. Each experiment was performed three independent times.

Screening for Prophage 6H ORFs in *F. psychrophilum* Strains

In order to screen for the presence of the prophage 6H in the collection of *F. psychrophilum* strains, the entire collection was analyzed for the presence of four open-reading frames found in the prophage genome. Bacterial DNA was extracted as is indicated above. The open-reading frames (ORFs) coding for integrase, tail protein and two hypothetical proteins from phage 6H were PCR amplified using Pure taq™ ready-to-go™ PCR beads (GE Healthcare) and the primers described in supplementary information (Table 3, online resource). PCR was performed using approximately 10 ng of total bacterial DNA per reaction tube. The thermal program consisted of 10 min at 96 °C, 30 cycles of 1 min of denaturation at 96 °C, 1 min of annealing at 58 °C, and 1 min of extension at 72 °C, followed by 10 min at 72 °C. PCR products were subjected to agarose gel electrophoresis (1 %, 100 V, 45 min) and stained with GelRed™ (Invitrogen).

Determination of Phage Kinetic Parameters

One-step growth experiments to determine life cycle characteristics (latency time, burst size, and adsorption rate) of bacteriophage FpV15 during infection of *F. psychrophilum* strains 950106-1/1, V3-5, V3-16 and 950106-1/1^C (cured of a 6H-type prophage) were performed according to Stenholm et al. [15]. Phage adsorption rate (K) was calculated from the decrease in unadsorbed phages over time, according to the following equation:

$$K = {}^{2.3}\Big/_{(B)t} \times \log(p_0/p)$$

Where B=concentration of bacteria (cells per milliliter), p_0=PFU at time zero, p=PFU in supernatant (i.e. phages not adsorbed) at time t (min). The adsorption rate (K) is the velocity constant (milliliters per minute) [33]. For latency times and burst sizes, samples for PFU were collected every hour for 12 h and quantified by the small spot plaque assay [32].

Results

Bacterial Strains

A previous study has identified two variable regions in the 16S rRNA gene, that can be used to distinguish CSF 259-93 and ATCC 49418^T type strains [28]. Therefore, as a first approach to discriminate the isolated bacteria, analysis of the

distribution 16S rRNA alleles was carried out for all the 49 *F. psychrophilum* strains isolated from Chile, Denmark, and USA. The results showed that 94 % of the strains were type CSF 259-93. The only exceptions from this were the Chilean isolates BV7 and T23 which were type ATCC 49418^{T} (Table 1, online resource).

Genetic Diversity of *F. psychrophilum* and Susceptibility to Phage Infection

DGREA patterns of 49 *F. psychrophilum* strains showed a relatively good separation, displaying among seven to ten fragments of sizes ranging from 3,000 to 500 bp. The strains could be clustered into three different groups basically in accordance with geographic origin, designated I, II, and III (Fig. 1). Group I contained 93 % of the strains isolated in Denmark and the American isolate NCIMB 1947^{T}. Group II contained all the strains isolated in Chile, except CSF 295-93 and ATCC 49718^{T} isolated in USA. Finally, group III contained two Danish strains (001026-1/35C and 001026-1/38B). These main groups were further divided in 11 subgroups (A to K) based on the similarities in DGREA.

The host range of 33 phages from Chile and Denmark were examined for collection of 49 *F. psychrophilum* strains (Fig. 2). Over all, the total phage collection was able to lyse 40 out of the 49 (82 %) strains investigated, but with large differences in host ranges according to their geographical origin. Host range of the 11 isolates from Chile was narrow, infecting 16 out of 49 (33 %) of the *F. psychrophilum* strains. Five Chilean isolates (1H, 6H, 2P, 23T, and 2A) were infective to strains isolated outside Chile (Denmark and/or USA), while five phages (P1–P5) had extremely narrow host ranges and could only infect 1–2 of the Chilean strain (Fig. 2). Bacteriophages P1–P4 were isolated in the same geographical site and have identical host range, and are probably identical phages. In contrast to this, the Danish phage collection [15] was able to lyse 77 % of the strains: 13 out of 19 strains isolated in Chile, 25 of 27 isolated in Denmark and 1 of 3 isolated in USA (CSF 259-93). For example, phages FpV3, FpV4, FpV5, and FpV6 had identical host ranges infecting almost all the *F. psychrophilum* strains isolated in Chile although turbid plaques were observed, with the exception of the strains VQ50 and PL1R2 where clear plaques were formed (Fig. 2). To compare the genetic characteristics (DGREA) with bacterial susceptibility to phage infection, the latter was analyzed by UPGMA and converted to a dendogram based in the matrix of sensitivity/no sensitivity. According to the analysis, 25 different patterns of sensitivity were observed and

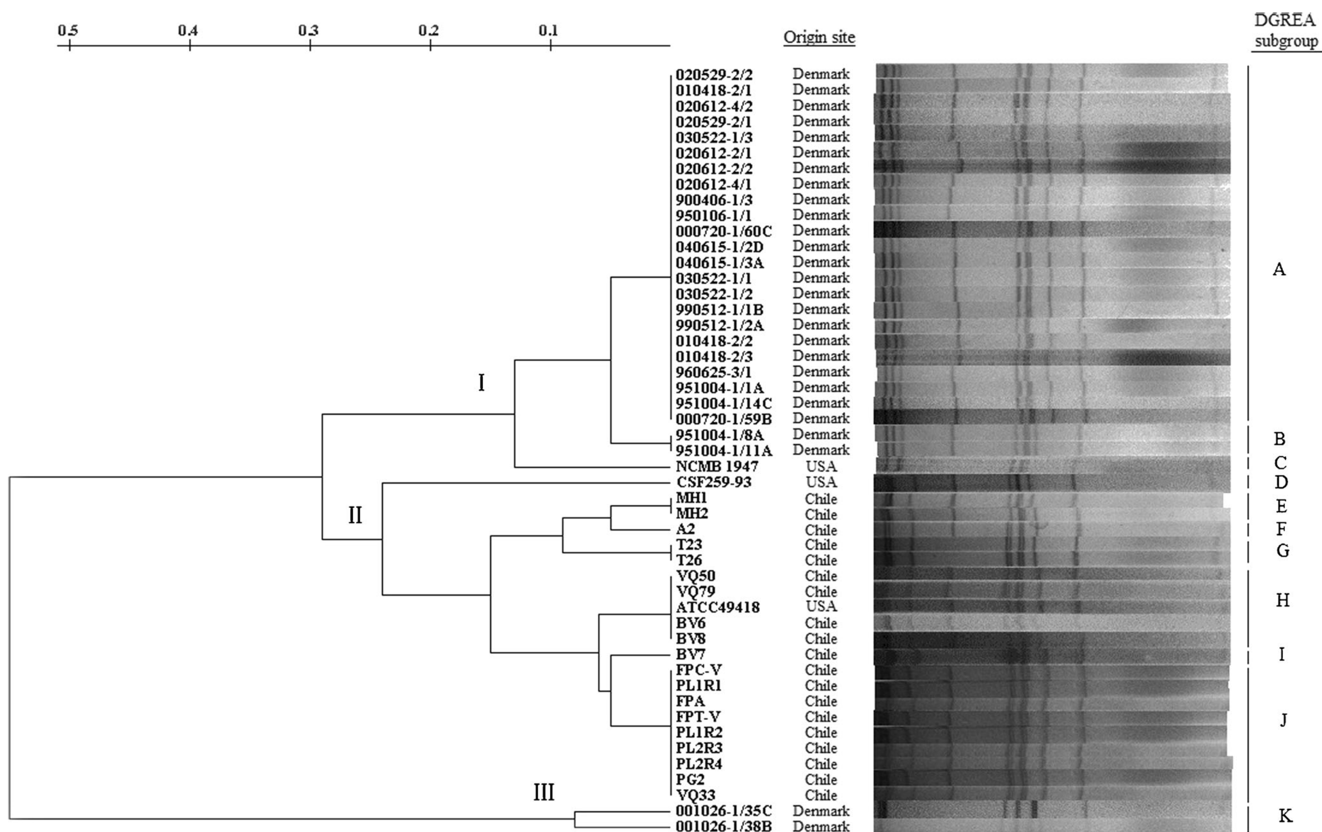

Fig. 1 Direct genome restriction enzyme analysis (DGREA) with *xhoI* and corresponding dendrogram by dissimilarity for 49 *F. psychrophilum* strains used in this study. Gels show observed pattern for each strain. DGREA subgroups are indicated with a *specific letter* (*A–K*) *on the right*. The *scale* corresponds to the fraction of dissimilar bands

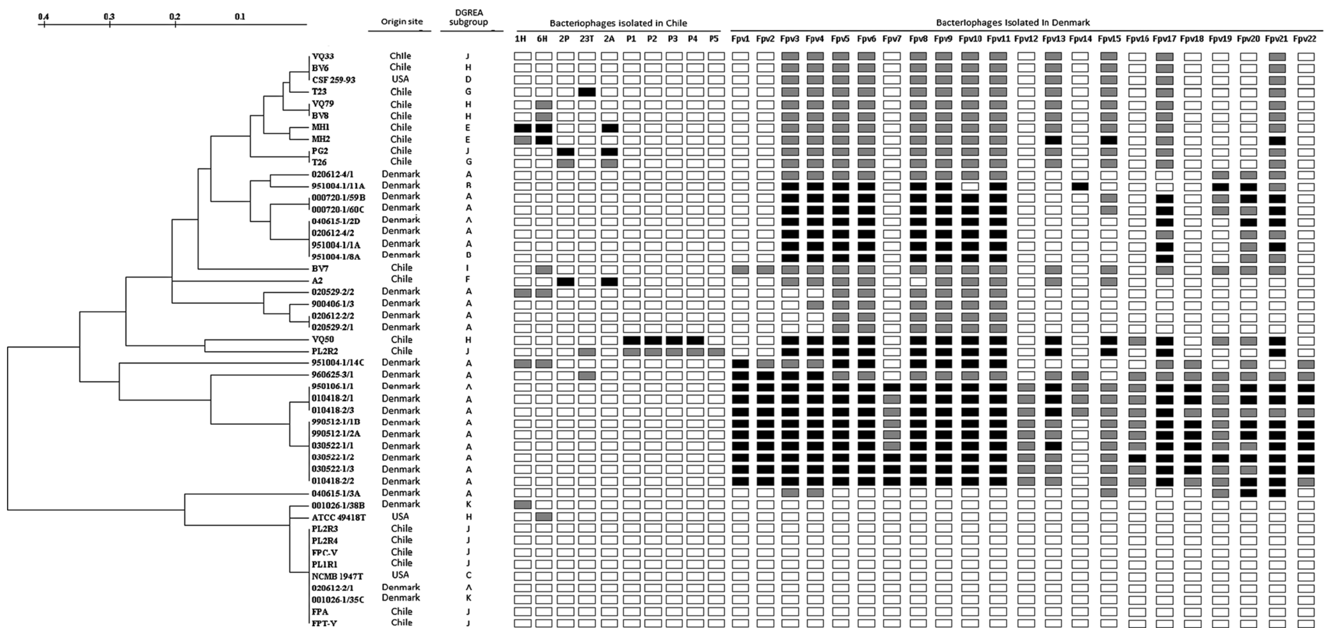

Fig. 2 Host range of bacteriophages against the collection of 49 *F. psychrophilum* strains isolated from different localities. Strains were grouped based on their susceptibility to phages infection using the unweighted-pair group method. Infectivity is categorized as: *white* "no plaques observed", *gray* "turbid zone", *black* "clear zone". DGREA subgroups and origin of the strains are inserted to facilitate comparison

compared with the DGREA subgroups. The results showed that there was no clear overall relation between DGREA subgroups and susceptibility patterns of the strains. However, specific associations could be observed; strains VQ79 and BV8, and strains MH1 and MH2 with similar susceptibility patterns also belonged to the same DGREA subgroups (H and E, respectively). Likewise, almost all strains which could not be infected by any of the bacteriophages belonged to DGREA subgroup J (Fig. 2).

Diversity and Phage Susceptibility Properties of Phage-Resistant Isolates

Effects of the resistance acquisition against specific phages on the sensitivity to other phages were examined for phage resistant isolates derived from the environmental *F. psychrophilum* strains 950106-1/1, MH1, MH2, T23, T26, A2, PG2 VQ79, BV7, BV8 and ATCC 49418^{T} (Table 4, online resource). From these 11 environmental strains 40 phage-resistant clones were randomly selected following exposure to specific phages (Table 4, online resource). For example, MR162 was derived from MH1 cells that had been challenged for resistance to bacteriophages 1H, 6H, and 2A. Each of 40 phage-resistant strains showed a unique sensitivity pattern to phage infection that differed from its respective ancestral sensitive strain (Figs. 3 and 4). In general, phage-resistant strains that were isolated after exposure to specific phages had evolved resistance patterns far beyond the resistance against the phages they had been exposed to, and thus developed cross-resistance to other bacteriophages (Figs. 3 and 4). For example, complete cross-resistance was obtained for phage-resistant strains V1-17, V2-23, V3-15, V4-14, and V4-24 derived from Danish strain 950106-1/1 when was exposed to 1, 3 or 11 different phages (Fig. 3). In the same way, phage-resistant strains derived from Chilean strains MH2, T23, A2, and PG2 showed cross-resistance to bacteriophages isolated in Denmark (Fig. 4). However, in some cases Chilean phage-resistant strains retained sensitivity to Danish bacteriophages (e.g., phage-resistant strains derived from MH1, T26, VQ79, BV7, and BV8).

Interestingly, acquisition of resistance against certain phages resulted in the loss of resistance to other phages in both bacterial groups. For example, the ancestral Danish strain 950106-1/1 was not infected by any of the phages isolated in Chile; however, the phage-resistant strains V3-4 and V3-5 derived from 950106-1/1 had become sensitive to 9 of the Chilean phages, forming clear plaques in spot assay (Fig. 2). Likewise, the Danish phages FpV15 and FpV16 produced turbid plaques in the 950106-1/1 strain, whereas clear plaques were observed for these phages when infecting the phage-resistant strains V3-4, V3-5, and V3-16 (Fig. 2). Also resistant strains derived from environmental Chilean strains showed increased sensitivity to new phages to which they were previously resistant: For example, the resistant strain MR1, MR12, MR62, and MR162 derived from ancestral strains MH1 or MH2 had increased sensitivity to the Danish phages FpV13 and FpV15 and the resistant isolate B7R6 had developed sensitivity to the Chilean phages 23T and 2A, which were unable to infect the ancestral strain BV7, from which it was derived (Fig. 4).

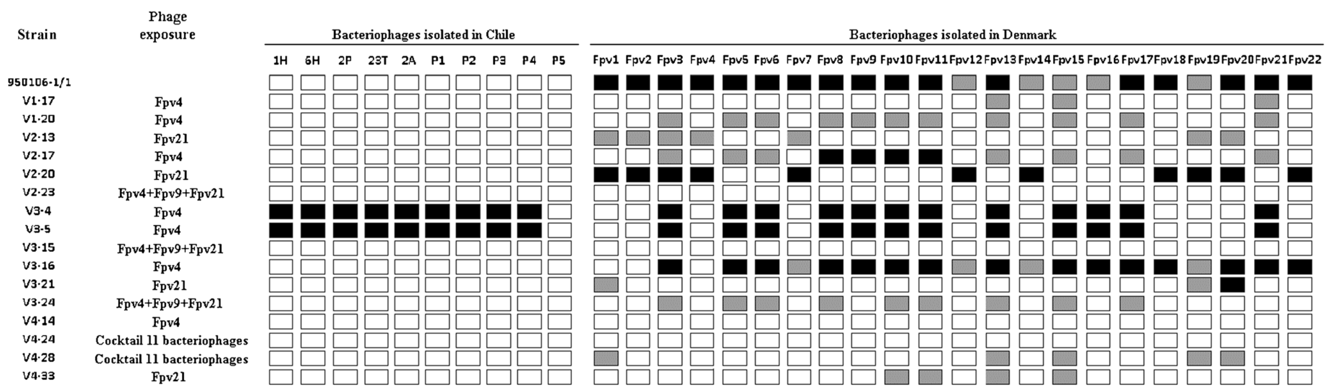

Fig. 3 Host range of bacteriophages against the collection of 16 phage-resistant strains derived from *F. psychrophilum* strain 950106-1/1. Infectivity is categorized as: *white* "no plaques observed", *gray* "turbid zone", *black* "clear zone". Host range against the ancestral Danish strain 950106-1/1 is added to facilitate comparison

In order to elucidate possible mechanisms that provided sensitivity in otherwise resistant isolates, the relationship between phage sensitivity and presence of the prophage 6H was examined by amplification of phage 6H ORFs. The results showed that all the phage-resistant strains which had gained sensitivity to new phages were negative in amplification of prophage 6H genes (Fig. 1a, online resource) in contrast to their ancestral strains, and hence had lost the prophage. However, this feature was not exclusive of these strains, as some phage-resistant strains which maintained resistance against other phages had also lost the prophage 6H genes (data not shown).

To examine in more detail the possible role of prophage 6H for phage sensitivity and life cycle properties, in the small group of Danish phage-resistant strains which gained sensitivity, one-step experiments with the phage FpV15 was carried out for the ancestral strain 950106-1/1, the phage-resistant

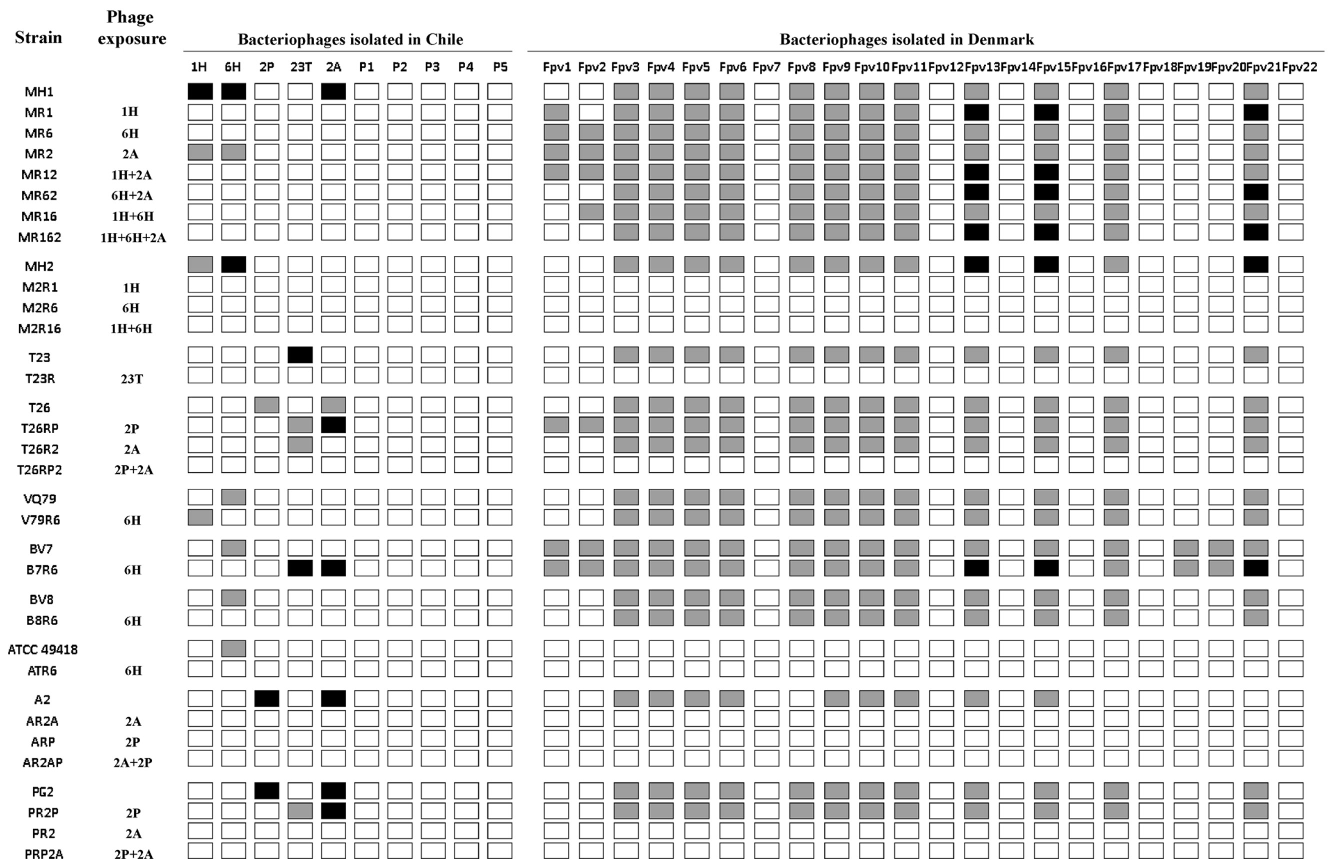

Fig. 4 Host range of bacteriophages against the collection of 24 phage-resistant strains derived from *F. psychrophilum* strains isolated in Chile and USA. Infectivity is categorized as: *white* "no plaques observed", *gray* "turbid zone", *black* "clear zone". Host ranges against the ancestral Chilean strains (MH1, MH2, T23, T26, VQ79, BV7, BV8, A2, and PG2) and USA (ATCC 49418^{T}) are added to facilitate comparison

strains V3-5, V3-16, and phage-cured strain 950106-1/1^C (cured for prophage 6H). The results showed a similar adsorption constant for phage FpV15 in all the strains ranging from $1.1–2\times10^{-10}$ min ml^{-1}, proposing that changes in adsorption to the bacteria was not the explanation for the increased susceptibility to FpV15 in the phage resistant V3-5 and V3-16 and cured strain 950106-1/1^C (Table 1). Interestingly, however, a 13–20-fold increase in burst size and a 2-h decrease in the latency time were observed in the phage-resistant and cured strains compared with the ancestral strain. In addition, a $>10^3$-fold increase in the efficiency of plating was observed for the phage-resistant strains relative to the ancestral strain (Table 1). Consequently, in addition to the loss of the prophage 6H from the genome, the phage-cured strain and the resistant strains also shared key new properties of phage sensitivity and life cycle properties compared with the common ancestral strain 950106-1/1. For the Chilean resistant isolates, on the other hand, we did not find a similar relationship between the loss of prophage 6H in the genome and the gain of new sensitivity properties (Figure 1b, online resource).

Discussion

Diversity of *F. psychrophilum* by DGREA

Based on a variety of molecular typing methods, *F. psychrophilum* have been shown to belong to a highly homogenous clonal complex when compared across geographical scales ranging from local geographic areas [4, 34], to differences between countries [35, 36], and even across four continents [2]. The present analysis of *F. psychrophilum* strains isolated from Chile and Denmark supported that the global *F. psychrophilum* community is genetically homogenous. Only relatively small variations between geographically distant communities were observed by the DGREA analysis (i.e. ~70 % similarity in DGREA profiles between Chilean and Danish isolates), and the group of Chilean strains (Cluster II) showed higher similarity to the main cluster of the Danish strains (Cluster I) than to small cluster of deviating Danish isolates (Cluster III). This suggested that the diversity at the local scale may be equally high or higher than at the global scale. However, the discrimination of two geographically distinct populations by DGREA (Fig. 1), suggested the presence of local clonal complexes in the *F. psychrophilum* communities.

The highly clonal population revealed for Danish *F. psychrophilum* strains (93 % strains belonged to DGREA subgroup A and all the strains were 16S RNA type CSF 259-93) could be explained by the host-specific association between certain types *F. psychrophilum* isolates and their fish species [2, 37] (Table 1, online resource), as most of the isolates originated from the same fish species. However, this clonal feature of the Danish *F. psychrophilum* strains based on DGREA did not correspond to the distinct groups formed by the patterns of phage susceptibility observed for each bacterial isolate (Fig. 2). In general, susceptibility to phage infection did not correlate with DGREA subgroups, indicating that DGREA classification does not reflect sensitivity to phage infection. This large diversity with respect to phage susceptibility supports previous suggestions that phages drive diversification of *F. psychrophilum* on a local scale, thus explaining the large local diversity in phage susceptibility observed for a number of aquatic bacteria including *F. psychrophilum* [15], and *Cellulophaga baltica*, also belonging to the *Flavobacteriaceae* group [38].

The 19 *F. psychrophilum* strains isolated in Chile showed a larger DGREA-based diversity and were distributed in 6 different DGREA subgroups (E to J). This differentiation is consistent with the variety of fish species used for isolation of the Chilean strains (Atlantic salmon, Coho salmon, and Rainbow trout), and thus supporting previous suggestions that clonal complexes of *F. psychrophilum* are associated with particular fish species rather than geographical location [2].

In Chile, rainbow trout and salmon eggs have been imported mainly from Europe (Ireland, Denmark, Scotland, Sweden and Norway) and USA [39]. Several fingerprinting studies have shown genetic homogeneity among *F. psychrophilum* strains from these locations based on multilocus sequence typing (MLST), ribotyping and plasmid profiling, amplified polymorphic DNA (RAPD) and 16S rRNA, suggesting that international trade of brood fish and fish eggs has promoted a worldwide introduction of *F. psychrophilum* [36, 37, 40].

Table 1 Kinetic parameters and efficiency of plating (EOP) for bacteriophage FpV15 on four bacterial strains

Strain	Adsorption constant (min ml^{-1})	Burst size (phages/cell)	Latency time (h)	EOP[a]
950106-1/1	$1.9\times10^{-10}\pm5.6\times10^{-8}$	9±1	5±0.1	1
950106-1/1^C	$2\times10^{-10}\pm3\times10^{-8}$	195±10	3±0.03	ND
V3-5	$1.1\times10^{-10}\pm8.7\times10^{-8}$	125±20	3±0.2	$>3\times10^3$
V3-16	$1.7\times10^{-10}\pm5.6\times10^{-8}$	183±9	3±0.4	1.5×10^3

ND not done

[a] Strains were exposed to the same FpV15 concentration. Efficiency of plating is expressed in relative PFU, where concentration of FpV15 in the strain 950106-1/1 is considered to be 1

Interestingly, our results showed little support to this hypothesis (Fig. 1) as the community analyses rather suggested that geographic separation may have facilitated local diversification and thus differentiation between the *F. psychrophilum* strains from Denmark and Chile.

Host Range and Dynamics of Phage Resistance

This study represents a host range analysis of *F. psychrophilum* bacteriophages and their hosts covering different communities isolated across large geographical (>12,000 km) and different temporal scales (>60 years). The geographical differences in the host range of *F. psychrophilum* phages suggest that the two environments harbor distinct phage communities with different properties, related to the characteristics of the host communities. The clonal population characteristic of the *F. psychrophilum* strains (Fig. 1) from Denmark may have selected for a bacteriophage community with a host range that covered the majority of hosts, whereas the much higher genetic variation characterizing the bacterial population isolated in Chile may have selected for a community of more strain-specific phages. Obviously, variability in the host range of phages is also dependent of the genetic composition of the phage community, however, little is known about the genetic basis for phage host range properties. Interestingly, despite their relatively similar host range, the Danish phage isolates represent a range in genome size from 8 to 90 kb, and thus most likely high genetic diversity. On the other hand, *P. aeruginosa*-specific bacteriophages belonging to the ΦKMV group, which showed a high level of sequence identity (83–97 %) among them, showed large host range variations (from 5 to 58 %), which were associated with a few specific mutations in fiber tail protein [41]. Obviously, more knowledge about genetic properties in both phages and hosts determining susceptibility and host range properties is needed to understand the complex network of interactions between *F. psychrophilum*-phage systems. Moreover, future studies need to address potential effects of *F. psychrophilum*-specific bacteriophages on the beneficial natural microbiota of the fish.

In all isolate-based studies of phage diversity, the results are biased by the choice of host strains used for isolation, as each individual strain will only target a subset of the infective phages present in the sample. Although a similar phage isolation procedure was applied in both the investigated environments, using a collection of different host strains to isolate phages from a variety of different fish farms, we therefore do not know to what extent the isolated phages are representative for the local phage community. Consequently, we cannot exclude that the host populations used in Chile and Denmark may have selected for phages with predominantly narrow and broad host ranges, respectively.

Some phage-resistant strains (22 out of 40) derived from environmental *F. psychrophilum* isolates (22/40) after exposure to specific phages also obtained cross-resistance to other phages. For example, the phage-resistant strains V4-14 (derived from strain 950106-1/1 and resistant to phage FpV4) and all the strains derived from MH2 (resistant to phages 1H, 6H, or both) were also resistant to the 22 Danish phages (Figs. 3 and 4). Similarly, resistance in *E. coli*-phage systems has shown that host mutations on certain receptors confer resistance to different bacteriophages [20, 42]. In the same way, our results suggest that some *F. psychrophilum* phages could use the same receptors, and that these receptors were present in isolates obtained in both Chile and Denmark.

Interestingly, development of resistance to certain bacteriophages led to sensitivity to other phages (Figs. 3 and 4). Such antagonistic pleiotropy has been reported previously for phage-resistant strains from *Synechococcus* [43] and *Prochlorococcus* [44], and our results thus demonstrate that this cost of resistance is also a potentially important mechanism in heterotrophic bacteria, possibly contributing to the observed co-existence of phage-sensitive *F. psychrophilum* strains and lytic phages across local and global scales.

The relationship between the acquired enhanced susceptibility to other phages in some phage-resistant strains and the loss of the prophage 6H from the genome (Table 1) suggested that the prophage played a role in the *F. psychrophilum* susceptibility pattern. Both the phage-cured strain (strain 950106-$1/1^c$) in which the prophage had been chemically induced and permanently lost, and in strains V3-5 and V3-16 in which the prophage was lost after exposure and development of resistance to phage FpV-4, had gained sensitivity to phage FpV-15 with very similar life cycle characteristics. We suggest therefore that the enhanced infection in these strains were associated with loss of the prophage and that the prophage therefore provided resistance or reduced sensitivity to FpV-15 by a superinfection exclusion (Sie) mechanism [45]. The prophage 6H genome contains several open-reading frames encoding putative membrane proteins [27]. Possibly, the prophage 6H therefore encodes a membrane protein which blocks the translocation of FpV-15 phage DNA into the cell, thus preventing infection by this phage, as observed for T-even-like phages in *E. coli* [46]. It is important to point out, however, that the loss of 6H-type prophage was a common feature among the phage-resistant strains derived from ancestral 950106-1/1 (data not shown), including those which maintained the resistance to phage infection. The susceptibility pattern of a given host is therefore the combined effect of resistance-providing mutations and loss of resistance by other mechanisms.

Overall, our results showed highly dynamic changes in the gain and loss of resistance in the global *F. psychrophilum* community in response to phage exposure, and despite the identification of local communities of phages and hosts, some key properties determining phage infection patterns seem to be universally distributed.

Implications for Phage Therapy in *F. psychrophilum*

Effective application of bacteriophages in the treatment of bacterial diseases requires a detailed characterization of phage-bacteria interactions [47]. Although, two separated genetic groups were obtained for *F. psychrophilum* strains isolated in Chile and Denmark using DGREA methodology, positive cross-reaction between Danish phages and Chilean host and vice versa was observed even in some cases when phage-resistant strains were isolated (Figs. 3 and 4). In phage therapy context, this is interesting as the global distribution of phenotypic traits related with phage susceptibility suggest that phage combinations (phage cocktail) used for treatment of CWD and RFTS may not be limited to local environments or specific fish farms but possibly have global scale applications. In this context, our understanding of the application of bacteriophages to control *F. psychrophilum* could improve by the identification of phage receptors and phage-resistance barries. Further knowledge on phage-resistance mechanisms in *F. psychrophilum* is therefore needed.

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