RESEARCH LETTER

Genomic structure of bacteriophage 6H and its distribution as prophage in *Flavobacterium psychrophilum* strains

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Abstract

Flavobacterium psychrophilum is currently one of the most devastating fish pathogens worldwide causing considerable economic losses in salmonid aquaculture. Recently, attention has been drawn to the use of phages for controlling *F. psychrophilum*, and phages infecting the pathogen have been isolated. Here, we present the genome sequence of *F. psychrophilum* bacteriophage 6H and its distribution as prophage in *F. psychrophilum* isolates. The DNA sequence revealed a genome of 46 978 bp containing 63 predicted ORFs, of which 13% was assigned a putative function, including an integrase. Sequence analysis showed > 80% amino acid similarity to a specific region found in the virulent *F. psychrophilum* strain JIP02/86 (ATCC 49511), suggesting that a prophage similar to phage 6H was present in this strain. Screening for a collection of 49 *F. psychrophilum* strains isolated in Chile, Denmark, and USA for the presence of four phage 6H genes (integrase, tail tape protein and two hypothetical proteins) by PCR showed the presence of these prophage genes in 80% of the isolates. In conclusion, we hypothesize that bacteriophage 6H belongs to an abundant group of temperate phages which has lysogenized a large fraction of the global *F. psychrophilum* community.

Introduction

Flavobacterium psychrophilum is a Gram-negative bacterium, belonging to the family *Flavobacteriaceae* (*Bacteroidetes*), and an important fish pathogen in salmonid aquaculture worldwide. The pathogen is the causative agent of bacterial cold water disease and rainbow trout fry syndrome (RTFS), and responsible for substantial economic losses in the aquaculture industry (Nematollahi *et al.*, 2003). The complete genome sequence of a virulent *F. psychrophilum* strain (JIP02/86) contained 2432 genes, including mediators of stress response, motility proteins, adhesins, and proteases that possibly participate in the invasion, colonization, adhesion, and host tissue destruction (Duchaud *et al.*, 2007). *Flavobacterium psychrophilum* is globally distributed with an apparently low level of diversity and high recombination rate based on nucleotide polymorphism among 11 protein-coding loci (Nicolas *et al.*, 2008), suggesting high species homogeneity on a global scale. Despite its global distribution and severe pathogenic implications, little is still known about the genomic diversity, and the factors controlling the genetic recombination and virulence of *F. psychrophilum* (Högfors-Rönnholm & Wiklund, 2010).

The past decade's increased sequencing of bacterial genomes has revealed the presence of integrated viral genomes (prophages) in most of the analyzed genomes. Prophage DNA sequences can constitute up to 10–20% of bacterial genomes and are major contributors to the genomic differences between individual bacterial species (Casjens, 2003). Prophages play an important role in horizontal gene exchange within bacterial populations, promoting genetic recombination and influencing the properties and evolution of bacteria (Brüssow *et al.*, 2004). The interactions between temperate phages and their bacterial hosts have shown to affect bacterial fitness by a number of mechanisms: Modification of anchor points for genomic rearrangements (Canchaya *et al.*, 2003a), induction of cell surface alterations (Nnalue *et al.*, 1990), production of toxins (Waldor & Mekalanos, 1996), and protection against lytic phage infection (Riipinen *et al.*, 2007) are all documented effects of infections by

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temperate phages. Prophages therefore play important roles in microbial performance and evolution.

A few studies have documented the presence of a particular prophage in multiple isolates of the same bacterial species, using PCR and DNA hybridization, for example *Paenibacillus polymyxa* (Santos *et al.*, 2002), *Salmonella enterica* serovar *Enteritidis* (Agron *et al.*, 2001), and *Streptococcus pneumoniae* (Ramirez *et al.*, 1999). These reports concluded that the presence of prophage sequences in the bacterial genome had profound effects on host cell pathogenicity and population fitness.

Prophages have been described for several pathogens such as *Salmonella enterica* (Hanna *et al.*, 2012), *Staphylococcus aureus* (Pantůcek *et al.*, 2004), *Clostridium difficile* (Shan *et al.*, 2012), but information about the geographical distribution and frequency of specific prophages within a specific bacterial population is currently not available. Here, we describe the genomic sequence of bacteriophage 6H, which infects the fish pathogen *F. psychrophilum*. Further, by analyzing a collection of 49 *F. psychrophilum* strains, we show that the phage 6H is widely distributed as prophage in the host genome of strains originating from geographically distant localities in Denmark, Chile and the USA.

Materials and methods

Bacterial strains and culture conditions

In this study, 49 *F. psychrophilum* strains were used, including 12 strains isolated in Chile (Castillo *et al.*, 2012), 27 strains isolated in Denmark (Stenholm *et al.*, 2008), and three stains obtained from culture collections originating from the USA (ATCC 49418T, NCMB 1947T, and the virulent strain CSF 259-93; Supporting Information, Table S1). In addition, six new isolates from Chile were identified as *F. psychrophilum* by species-specific PCR with DNA primers against a sequence of the 16 rRNA gen (Wiklund *et al.*, 2000) and included in the collection. All isolates were originally isolated from trout and salmon aquaculture farms. The strains were stored at −80 °C in tryptone-yeast extract-salts broth (TYES-B) with 15% glycerol and grown in TYES-B medium at 15 °C (Stenholm *et al.*, 2008).

Phage lysate concentration

Isolation and preliminary characterization of the free bacteriophage 6H has been described previously (Castillo *et al.*, 2012). For the subsequent DNA extraction and characterization, bacteriophage 6H was proliferated and concentrated according to the previously described protocol (Castillo *et al.*, 2012). Briefly, *F. psychrophilum* strain MH1 was used as host for phage proliferation and grown in TYES-B amended with the phage. Following 3-day incubation, the phage-enriched culture was pelleted by centrifugation at 5000 ***g*** for 10 min, and the remaining bacteria removed by filtration (0.22 μm). Phages in the filtrate were centrifuged (100 000 ***g*** for 1 h), and the pellet subsequently resuspended in 1 mL of SM Buffer (50 mM Tris–HCl, pH 7.5, 99 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin).

Extraction of phage and bacterial DNA

Bacterial DNA was extracted from cultures grown in TYES-B ($OD_{600\ nm} = 1.0$) using the Wizard Genomic DNA Purification Kit (Promega) following the manufacturer's standard protocol. Phage DNA was extracted as previously described (Castillo *et al.*, 2012).

6H genome sequencing and bioinformatic analysis

Sequencing of the prophage 6H genome was performed using the TOPO shotgun Subcloning Kit (Invitrogen) as is described by the manufacturer. 480 clones were sequenced in Macrogen (Seul, Korea) using the Sanger method and applying M13 forward and reverse universal primers. 21 contigs were obtained and the total sites determined corresponded to sixfold coverage of the genome. Sequence assembly and scanning for potential ORFs and G + C content were performed using the program Geneious (Drummond *et al.*, 2010, http://www.geneious.com), and tRNAscan-SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE/) was used for tRNAs. Deduced ORF sequences were compared with known proteins using standard protein–protein BLASTP (July 2013, nonredundant proteins database) at the National Centre for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/) (Altschul *et al.*, 1990).

Primer design and prophage 6H screening in *F. psychrophilum* strains

To examine the presence of 6H prophage sequences in the genomes of the collection of *F. psychrophilum* strains (Table S1), specific primer sets were developed for amplification of specific phage genes in host genomes based on the 6H genome sequences, using the program Geneious (Drummond *et al.*, 2010). The primers were designed to target the phage genes Integrase (ORF1), Tail tape protein (ORF 4), and two arbitrary assigned hypothetical proteins, HP1 (ORF 17) and HP2 (ORF 33; Table S2). PCR products for tail tape protein and HP1 were sequenced by Macrogen (Seul, Korea). For gene amplification, a PCR mixture (10 pmol of each primer, 200 μM of each dNTPs, 1.5 U Taq DNA polymerase, 1x PCR buffer, and 1 ng of

the template DNA) in a total reaction volume of 20 μL was run in the thermocycler: 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. The PCR products were analyzed on agarose gels (1%, 100 V, 1 h) and stained with GelRed nucleic acid gel stain (Biotium). A 100-bp DNA ladder (Omega Bio-Tek) was used as a molecular size marker.

Curing *F. psychrophilum* strain 950106-1/1 for prophage 6H

To investigate whether the presence of the prophage 6H provided resistance against superinfection by the phage 6H, a strain was produced which was cured of prophage 6H. The phage-curing experiment was performed with the prophage 6H-containing *F. psychrophilum*, strain 950106-1/1 using an UV induction protocol modified from the study by Georghiou *et al.* (1981). Briefly, the bacterial culture was grown to a cell density of 5–6 × 10^8 cells mL^{-1}, centrifuged (5000 ***g*** for 10 min, 4 °C), and resuspended in 1 mL of 1% NaCl. Angelicin was then added at a final concentration of 0.1 mM, and the cells incubated in the dark for 20 min at 15 °C. The cells were transferred to a petri dish and exposed to 30 min UV (310-nm wavelength 6-W lamp) irradiation of 5000 μJ m^{-2} and 2500 μJ m^{-2}, respectively. The cells were subsequently collected by centrifugation as described above, washed with 1% NaCl, and irradiated once more with 2500 μJ m^{-2} of UV for 30 min. Serial dilutions were then plated on TYES agar plates and incubated at 15 °C for selection of specific colonies. These were purified and screened for the presence of 6H prophage by PCR amplification of the four 6H ORFs as described above. One cured strain was isolated (950106-1/1^c), and the susceptibility of this strain and its ancestral lysogenized strain 950106-1/1 to phage 6H infection was tested using spot assay (Stenholm *et al.*, 2008).

Nucleotide sequence accession numbers

The complete genome sequence of bacteriophage 6H is available in the GenBank database (Accession number KC959568). The GenBank accession number of the *F. psychrophilum* JIP02/86 genome used in this study is AM398681.

Results

Characterization bacteriophage 6H

The genome of bacteriophage 6H was a double-strand DNA molecule of 46978 nucleotides with a G + C content of 31.6%, which is 1% lower than the G + C content of *F. psychrophilum* genomic DNA (Duchaud *et al.*, 2007). tRNA genes were not found. 92% of the genome enclosed 63 ORFs, 87% of which encoded hypothetical proteins (Fig. 1; Table S3). The phage genome did not show amino acid similarity to any other sequenced phage. However, amino acid sequences showed over 80% similarity to a region in the genome of *F. psychrophilum* virulent strain JIP02/86 (Duchaud *et al.*, 2007) (See below). No other similar prophages were found in the genome of this strain.

The regions comprised in ORF 1 and 4 encoded a putative integrase and tail protein probably involved in

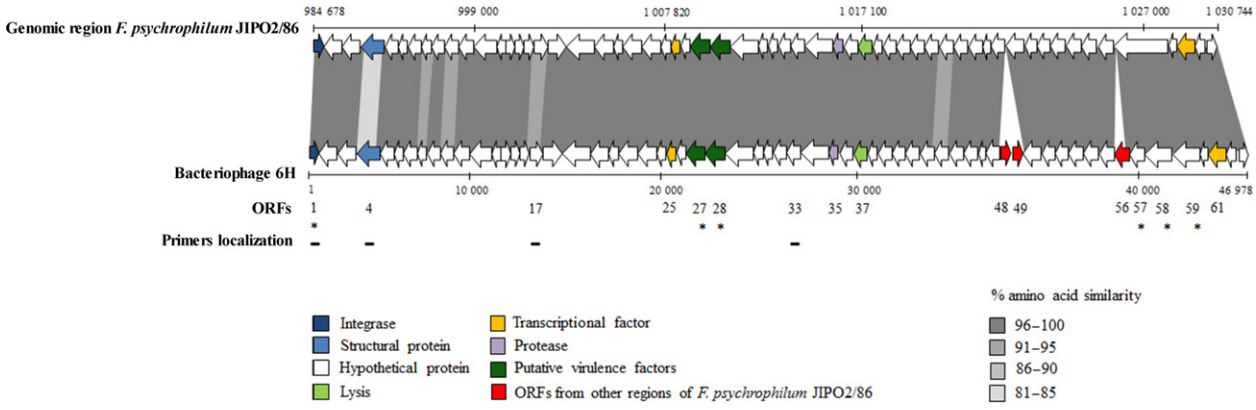

Fig. 1. Graphic representation of annotated map of the 46 978-bp bacteriophage 6H genome and its relation to the genomic region of *Flavobacterium psychrophilum* JIP02/86. The colors were assigned according to the possible role of each ORF as is shown in the figure. Amino acid similarity between the genes of the phage and bacterium is shown as gradients of grey color. The black bar represents the location of the primers, and proteins marked with '*' are discussed in the text. The complete genome sequence of bacteriophage 6H is available in the GenBank database (Accession number KC959568).

lysogeny and morphogenesis, respectively. ORF 25 encoded an XRE family of transcriptional regulators, which are known to be involved in a wide range of gene regulating processes, including restriction and modification systems, bacteriophage transcription control, plasmid copying, and stress response (Santos *et al.*, 2009). Possible virulence factors were identified in ORF 61, which is a probable transcriptional regulator of the LuxR family, and in ORFs 27 and 28, which possessed similarities to IbrA and IbrB described in *Escherichia coli* (31 and 37% amino acid similarity respectively, Table S3; accession number for the region of the IbrAB island is AF460182) (Sandt & Hill, 2000). However, whether these proteins are expressed and involved as virulence factors remains an open question. The ORFs 35 and 37 encoded a serine protease and a cell wall hydrolase, respectively, of which the latter can operate as an endolysin. Finally, ORFs 57–59 are predicted to have some similarity to DNA primase (29%, 39%, and 36% amino acid identity to *E. coli*, respectively, Table S3); however, these ORFs are represented as a single gene in the genome of *F. psychrophilum* strain JIP02/86. Interestingly, the phage genome contained three ORFs found in other regions of *F. psychrophilum* strain JIP02/86 genome: ORF 48 encoding a cytochrome c oxidase subunit I, ORF 49 encoding hypothetical protein, and finally ORF 56 encoding di-trans, poly-cys-decaprenylcystransferase.

Detection of prophage 6H genes in *F. psychophilum* strains

As mentioned above, a region of the virulent *F. psychrophilum* strain JIP02/86 genome showed > 80% amino acid similarity to phage 6H sequence (Duchaud *et al.*, 2007) (Fig. 1). To investigate the occurrence of this region in the full collection of 49 *F. psychrophilum* strains isolated in Denmark, Chile, and the USA (Table S1), we used phage-specific primers to target four 6H phage genes [the putative integrase (ORF1), tail tape protein (ORF 4), and two hypothetical proteins (ORF 17 and ORF 34, respectively)] in the individual isolates. In total, 39 of the 49 strains showed amplicons for at least two of the four genes tested, with some variability in the incidence of the specific genes (Fig. 2, Table 1). Seven (37%) of the Chilean strains, 11 (41%) of the Danish strains, and one (33%) of the USA strains showed positive identification of all four genes (e.g. strains MH2, 950106-1/1, Fig. 2), whereas none of the four genes were amplified in 3, 7, and 1 of the Chilean, Danish, and US strains, respectively (e.g. strains VQ50, 00720-1/59B, Fig. 2). A total of 20 strains were positive for 2 or 3 of the targeted genes (e.g. 010418-2/1, Fig. 2, Table 1).

A sequence analysis of the PCR products targeting phage 6H tail tape protein (ORF4) and hypothetical protein 1 (ORF17) obtained from *F. psychrophilum* strain 950106-1/1 isolated in Denmark and nine strains isolated in Chile, confirmed that the host-derived sequences were identical to the corresponding sequences in the bacteriophage 6H (data not shown).

Prophage 6H-mediated immunity

To determine whether the prophage 6H confers immunity against bacteriophage 6H, the susceptibility to phage 6H was tested in the Danish strain 950106-1/1 (positive amplification of all 4 ORFs), and the derived strain 950106-1/1^{c} which was cured of prophage 6H (negative amplification of all 4 ORFs). The results showed that both strains were resistant to phage 6H infection (data not shown).

Discussion

The genomic sequence of bacteriophage 6H showed 84% of the encoded proteins to be hypothetical proteins (Fig. 1; Table S3). Among the small group of ORFs with

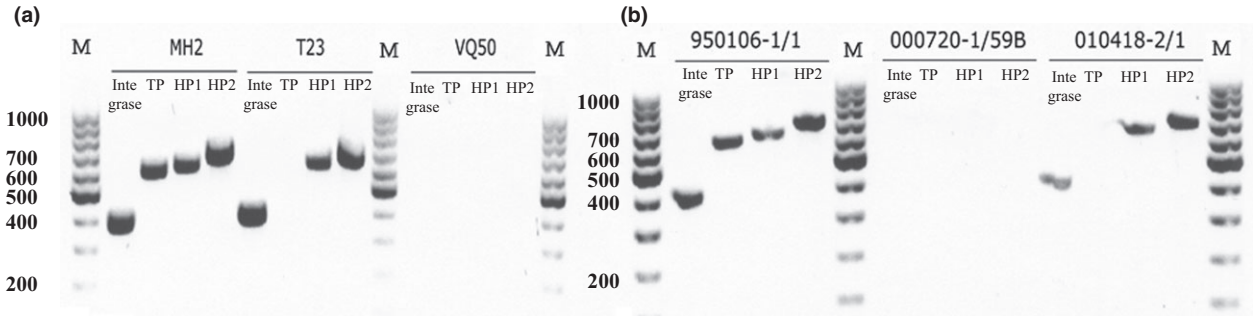

Fig. 2. Amplification profiles of integrase, tail tape protein (TP), hypothetical protein 1 (HP1), and hypothetical protein 2 (HP2) of the bacteriophage 6H and their distribution in selected *Flavobacterium psychrophilum* strains. (a) PCR amplification profiles for *F. psychrophilum* strains isolated in Chile. (b) PCR amplification profiles for *F. psychophilum* strains isolated in Denmark. M: molecular size marker 100 bp.

Table 1. Results of amplification by PCR of ORFs phage 6H in the *Flavobacterium psychrophilum* strains

Strain	Origin	Amplification profile of prophage 6H genes			
		Integrase[a]	HP1[a]	HP2[a]	TP[a]
MH1	Chile	–	+	+	+
MH2	Chile	+	+	+	+
PG2	Chile	+	+	+	+
A2	Chile	+	+	–	+
T23	Chile	+	+	+	–
T26	Chile	+	+	+	+
BV6	Chile	+	+	+	+
BV7	Chile	+	+	+	+
BV8	Chile	+	+	+	+
VQ33	Chile	+	+	+	+
VQ50	Chile	–	–	–	–
VQ79	Chile	+	+	+	–
FPA	Chile	–	–	–	–
FPT-V	Chile	+	+	+	–
FPC-V	Chile	+	+	+	–
PL1R1	Chile	+	+	+	–
PL1R2	Chile	+	+	+	–
PL2R3	Chile	+	+	+	–
PL2R4	Chile	+	+	+	–
900406-1/3	Denmark	+	+	+	–
950106-1/1	Denmark	+	+	+	+
040615-1/2D	Denmark	+	+	+	–
040615-1/3A	Denmark	+	+	+	–
030522-1/1	Denmark	+	+	+	+
030522-1/2	Denmark	+	+	+	+
030522-1/3	Denmark	+	+	+	+
020612-2/1	Denmark	+	+	+	–
020612-2/2	Denmark	+	+	+	+
020612-4/1	Denmark	+	+	+	+
020612-4/2	Denmark	+	–	+	–
020529-2/1	Denmark	–	–	–	–
020529-2/2	Denmark	–	–	–	–
010418-2/1	Denmark	+	+	+	–
010418-2/2	Denmark	+	+	+	–
010418-2/3	Denmark	+	+	+	–
990512-1/1B	Denmark	+	+	+	–
990512-1/2A	Denmark	+	+	+	+
960625-3/1	Denmark	+	+	+	+
951004-1/1A	Denmark	–	–	–	–
951004-1/8A	Denmark	–	–	–	–
951004-1/11A	Denmark	+	+	+	–
951004-1/14C	Denmark	+	+	+	+
001026-1/35C	Denmark	+	+	+	+
001026-1/38B	Denmark	+	+	+	+
000720-1/59B	Denmark	–	–	–	–
000720-1/60C	Denmark	–	–	–	–
NCIMB 1947	USA	+	+	+	+
ATCC 49418	USA	–	–	–	–
CSF 259-93	USA	–	–	–	–

Integrase[a], HP1[a], HP2[a], and TP[a] = Integrase (ORF1); HP1 [hypothetical protein 1 (ORF 17)]; HP2 [hypothetical protein 2 (ORF 33)]; and TP [tail protein (ORF 4)], respectively.

'+' positive PCR amplification; '–' negative PCR amplification.

predictable function, a putative integrase was annotated (ORF1; Fig. 1), inferring that bacteriophage 6H is a temperate phage. Although this is not direct evidence for its capacity to integrate into the host's chromosome, the presence of this ORF and the similarity of the complete genome to a region of *F. psychrophilum* strain JIPO2/86 supported this suggestion. In addition, putative virulence factors were identified: ORF 61 is a probable transcriptional regulator in the LuxR family, which is a group of proteins involved in bacterial propagation, virulence, and biofilm formation (Alonso-Hearn *et al.*, 2010). Its function is to bind to specific promoter sequences, thus controlling the transcriptional activity (Fuqua & Greenberg, 2002), as observed in the fish pathogen *Vibrio alginolyticus*, where ValR, a homolog of the LuxR protein, regulates flagellum biosynthesis, biofilm formation, and the expression of genes involved in colony phase variation (Chang *et al.*, 2010). ORFs 27 and 28 had similarity to the 'immunoglobulin-binding regulator' proteins IbrA and IbrB, previously described in *E. coli* (31 and 37% amino acid similarity, respectively) (Sandt *et al.*, 2002). These proteins regulate the expression of *eib* genes which are involved in the regulation of cell–cell interactions and possibly, host–pathogen interactions, because they exhibit resistance to serum and Ig-binding (Sandt & Hill, 2000). However, even though these putative virulence factors are present in the prophage 6H, it is not known to what extent they are expressed in the host.

Three ORFs (ORFs 48, 49, and 56) from different regions of the *F. psychrophilum* genome were present in the 6H genome (Fig. 1; Table S3). One of them (ORF 56) encodes a decaprenylcistransferase that participates in the biosynthesis of bacterial cell wall polysaccharide components such as peptidoglycan and lipopolysaccharide (Lujan *et al.*, 2012). This protein probably participates in the modification of cell surface components, thus possibly affecting host virulence or bacteriophage receptor sites. This result indicates a transfer of genetic information between the putative temperate phage 6H and its host and hence that phage 6H is involved in transduction and thus the evolution of *F. psychrophilum*. This is in accordance with observations from other pathogens, for example *Vibrio cholerae* where vibriophages have been shown to transfer antibiotic resistance from toxigenic strains to nontoxigenic strains (Choi *et al.*, 2010).

The close similarity (> 80%) in the phage amino acid sequence to a specific region in the full genome sequence of virulent *F. psychrophilum* strain JIP02/86 suggested that this or a closely related phage was present as a prophage in the genome. Using a PCR-based approach, the 6H phage genes were found to be present in 80% of the 49 *F. psychrophilum* strains isolated in three different geographic localities in Chile, Denmark, and the USA,

suggesting that bacteriophage 6H is a cosmopolitan temperate phage. This is supported by previous observations of the distribution of *V. parahaemolyticus*-specific phages, which demonstrated the occurrence of genetically highly similar phages (based on restriction patterns using direct genome restriction enzyme analysis) in distant geographic regions (Bastias *et al.*, 2010). Similarly, the *Pseudomonas aeruginosa*-specific bacteriophages ΦKMV and LKD16, isolated in Belgium and Russia, respectively, showed 90% DNA homology (Ceyssens *et al.*, 2006), suggesting large-scale spatial distribution of similar phages. Sequence analysis of the tail protein gene in phage 6H supports the hypothesis of cosmopolitan temperate phage, because the sequences of this phage gene showed 100% homology to the corresponding sequences obtained from 10 host strain genomes originating from both Denmark (950106-1/1) and Chile . The wide distribution of phage 6H genes in *F. psychrophilum* populations may reflect a high recombination rate among *F. psychrophilum* as previously suggested (Nicolas *et al.*, 2008) and/or a rapid and efficient dispersal of the species among aquaculture farms.

The trading of fish eggs may have played an important role in the transmission of this pathogen across a large spatial scale (Taylor, 2004). However, we cannot rule out the possibility of an early lysogenization by a bacteriophage type 6H to a common ancestor of *F. psychrophilum* strains and thus a vertical transmission and subsequent large-scale distribution of this phage sequence among the global *F. psychrophilum* community. Possibly, the absence of a selective pressure for maintaining the prophage could have resulted in the subsequent loss of 6H genes from the host genome, resulting in a so-called prophage remnant (Canchaya *et al.*, 2003b). Alternatively, the gene sequences in these positions are different from the 6H genes, indicating either the presence of a different prophage or be the result of recombination (Juhala *et al.*, 2000), rearrangements, or deletions (Lawrence *et al.*, 2001), which are common features in prophages. It should be noted, however, that a limitation of the PCR assay used for the detection of prophage 6H genes in host isolates is the potential risk of missing genes which may fail to amplify due to small changes in the primer binding sequence. This means that false-negative amplifications of genes are possible, and the screening for prophage genes among the host collection should therefore be considered to provide a conservative estimate of the occurrence of the prophage. Alternative methods such as DNA hybridization with prophage fragments which target larger DNA sequences could potentially provide more detailed information on the presence of 6H phage sequences in the host genome. However, a PCR-based approach has the advantage of allowing the development of a phage locus typing system in *F. psychrophilum* isolates.

The loss of the 6H prophage in the phage-cured strain 950106-1/1^{c} (derived from the prophage containing strain 950106-1/1) did not result in susceptibility to phage 6H in the cured strain, as the cured strain remained resistant to 6H infection. This suggested that the prophage did not provide immunity to 6H in strain 950106-1/1, implying that other resistance mechanisms, such as blocking phage DNA injection (McGrath *et al.*, 2002), alteration phage receptors (Perry *et al.*, 2009), or CRISPR generated phage inactivation (Bikard & Marraffini, 2012), could be important for 6H resistance in *F. psychrophilum*.

Previous characterization of the *F. psychrophilum* strains using different molecular techniques revealed a low diversity within this species (Nicolas *et al.*, 2008; Valdebenito & Avendaño-Herrera, 2009). However, the observed diversity in occurrence and large-scale distribution of genomic sequences derived from prophages in *F. psychrophilum* may represent a new approach for further discrimination of *F. psychrophilum* diversity and distribution, as previously performed for other pathogens (Romero *et al.*, 2009). Thus, while this study provides a first approximation for the potential application of prophage 6H for future molecular typing of *F. psychrophilum* based on specific prophage sequences, further information on *F. psychrophilum* prophages and their dynamics is required to develop this approach for *F. psychrophilum*.

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References

Agron PG, Walker RL, Kinde H, Sawyer SJ, Hayes DC, Wollard J & Andersen GL (2001) Identification by subtractive hybridization of sequences specific for *Salmonella enterica* serovar Enteritidis. *Appl Environ Microbiol* **67**: 4984–4991.

Alonso-Hearn M, Eckstein TM, Sommer S & Bermudez LE (2010) A *Mycobacterium avium* subsp. paratuberculosis LuxR regulates cell envelope and virulence. *Innate Immun* **16**: 235–247.

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403–410.

Bastias R, Higuera G, Sierralta W & Espejo R (2010) A new group of cosmopolitan bacteriophages induce a carrier state in the pandemic strain of *Vibrio parahaemolyticus*. *Environ Microbiol* **12**: 990–1000.

Bikard D & Marraffini LA (2012) Innate and adaptive immunity in bacteria: mechanisms of programmed genetic variation to fight bacteriophages. *Curr Opin Immunol* **24**: 15–20.

Brüssow H, Canchaya C & Hardt WD (2004) Phages and evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiol Mol Biol Rev* **68**: 560–602.

Canchaya C, Fournous G, Chibani-Chennoufi S, Dillmann ML & Brüssow H (2003a) Phage as agents of lateral gene transfer. *Curr Opin Microbiol* **6**: 417–424.

Canchaya C, Proux C, Fournous G, Bruttin A & Brüssow H (2003b) Prophage genomics. *Microbiol Mol Biol Rev* **67**: 238–276.

Casjens S (2003) Prophages and bacterial genomics: what have we learned so far? *Mol Microbiol* **49**: 277–300.

Castillo D, Higuera G, Villa M, Middelboe M, Dalsgaard I, Madsen L & Espejo RT (2012) Diversity of *Flavobacterium psychrophilum* and the potential use of its phages for protection against bacterial cold water disease in salmonids. *J Fish Dis* **35**: 193–201.

Ceyssens PJ, Lavigne R, Mattheus W, Chibeu A, Hertveldt K, Mast J, Robben J & Volckaert G (2006) Genomic analysis of *Pseudomonas aeruginosa* phages LKD16 and LKA1: establishment of the phiKMV subgroup within the T7 supergroup. *J Bacteriol* **188**: 6924–6931.

Chang C, Jing-Jing Z, Chun-Hua R & Chao-Qun H (2010) Deletion of valR, a homolog of *Vibrio harveyiś* luxR generates an intermediate colony phenotype between opaque/rugose and translucent/smooth in *Vibrio alginolyticus*. *Biofouling* **26**: 595–601.

Choi S, Dunams D & Jiang SC (2010) Transfer of cholera toxin genes from O1 to non-O1/O139 strains by vibriophages from California coastal waters. *J Appl Microbiol* **108**: 1015–1022.

Drummond AJ, Ashton B, Buxton S *et al.* (2010) *Geneious v5.3*. Available from http://www.geneious.com.

Duchaud E, Boussaha M, Loux V *et al.* (2007) Complete genome sequence of the fish pathogen *Flavobacterium psychrophilum*. *Nat Biotechnol* **25**: 763–769.

Fuqua C & Greenberg EP (2002) Listening in on bacteria: acyl-homoserine lactone signalling. *Nat Rev Mol Cell Biol* **3**: 685–695.

Georghiou D, Phua SH & Terzaghi E (1981) Curing of a lysogenic strain of *Streptococcus cremoris* and characterization of the temperate phage. *J Gen Microbiol* **122**: 295–303.

Hanna LF, Matthews TD, Dinsdale EA, Hasty D & Edwards RA (2012) Characterization of the ELPhiS prophage from *Salmonella enterica* serovar Enteritidis strain LK5. *Appl Environ Microbiol* **78**: 1785–1793.

Högfors-Rönnholm E & Wiklund T (2010) Phase variation in *Flavobacterium psychrophilum*: characterization of two distinct colony phenotypes. *Dis Aquat Organ* **90**: 43–53.

Juhala RJ, Ford ME, Duda RL, Youlton A, Hatfull GF & Hendrix RW (2000) Genomic sequences of bacteriophages HK97 and HK022: pervasive genetic mosaicism in the lambdoid bacteriophages. *J Mol Biol* **299**: 27–51.

Lawrence JG, Hendrix RW & Casjens S (2001) Where are the pseudogenes in bacterial genomes? *Trends Microbiol* **11**: 535–540.

Lujan DK, Stanziale JA, Mostafavi AZ, Sharma S & Troutman JM (2012) Chemoenzymatic synthesis of an isoprenoid phosphate tool for the analysis of complex bacterial oligosaccharide biosynthesis. *Carbohydr Res* **359**: 44–53.

McGrath S, Fitzgerald GF & van Sinderen D (2002) Identification and characterization of phage-resistance genes in temperate lactococcal bacteriophages. *Mol Microbiol* **43**: 509–520.

Nematollahi A, Decostere A, Pasmans F & Haesebrouck F (2003) *Flavobacterium psychrophilum* infections in salmonid fish. *J Fish Dis* **26**: 563–574.

Nicolas P, Mondot S, Achaz G, Bouchenot C, Bernardet JF & Duchaud E (2008) Population structure of the fish-pathogenic bacterium *Flavobacterium psychrophilum*. *Appl Environ Microbiol* **74**: 3702–3709.

Nnalue NA, Newton S & Stocker BA (1990) Lysogenization of *Salmonella choleraesuis* by phage 14 increases average length of O-antigen chains, serum resistance and intraperitoneal mouse virulence. *Microb Pathog* **8**: 393–402.

Pantůcek R, Doskar J, Růzicková V, Kaspárek P, Orácová E, Kvardová V & Rosypal S (2004) Identification of bacteriophage types and their carriage in *Staphylococcus aureus*. *Arch Virol* **149**: 1689–1703.

Perry LL, SanMiguel P, Minocha U, Terekhov AI, Shroyer ML, Farris LA, Bright N, Reuhs BL & Applegate BM (2009) Sequence analysis of *Escherichia coli* O157:H7 bacteriophage PhiV10 and identification of a phage-encoded immunity protein that modifies the O157 antigen. *FEMS Microbiol Lett* **292**: 182–186.

Ramirez M, Severina E & Tomasz A (1999) A high incidence of prophage carriage among natural isolates of *Streptococcus pneumonia*. *J Bacteriol* **181**: 3618–3625.

Riipinen KA, Räisänen L & Alatossava T (2007) Integration of the group c phage JCL1032 of *Lactobacillus delbrueckii* subsp. lactis and complex phage resistance of the host. *J Appl Microbiol* **103**: 2465–2475.

Romero P, García E & Mitchell TJ (2009) Development of a prophage typing system and analysis of prophage carriage in *Streptococcus pneumonia*. *Appl Environ Microbiol* **75**: 1642–1649.

Sandt CH & Hill CW (2000) Four different genes responsible for nonimmune immunoglobulin-binding activities within a single strain of *Escherichia coli*. *Infect Immun* **68**: 2205–2214.

Sandt CH, Hopper JE & Hill CW (2002) Activation of prophage eib genes for immunoglobulin-binding proteins by genes from the IbrAB genetic island of *Escherichia coli* ECOR-9. *J Bacteriol* **184**: 3640–3648.

Santos SC, Rodrigues Coelho MR & Seldin L (2002) Evaluation of the diversity of *Paenibacillus polymyxa* strains by using the DNA of bacteriophage IPy1 as a probe in hybridization experiments. *Lett Appl Microbiol* **35**: 52–56.

Santos CL, Tavares F, Thioulouse J & Normand P (2009) A phylogenomic analysis of bacterial helix-turn-helix transcription factors. *FEMS Microbiol Rev* **33**: 411–429.

Shan J, Patel KV, Hickenbotham PT, Nale JY, Hargreaves KR & Clokie MR (2012) Prophage carriage and diversity within clinically relevant strains of *Clostridium difficile*. *Appl Environ Microbiol* **78**: 6027–6034.

Stenholm A, Dalsgaard I & Middelboe M (2008) Isolation and characterization of bacteriophages infecting the fish pathogen *Flavobacterium psychrophilum*. *Appl Environ Microbiol* **74**: 4070–4078.

Taylor PW (2004) Detection of *Flavobacterium psychrophilum* in eggs and sexual fluids of Pacific salmonids by a polymerase chain reaction assay: implications for vertical transmission of bacterial cold water disease. *J Aquat Anim Health* **16**: 104–108.

Valdebenito S & Avendaño-Herrera R (2009) Phenotypic, serological and genetic characterization of *Flavobacterium psychrophilum* strains isolated from salmonids in Chile. *J Fish Dis* **32**: 321–333.

Waldor MK & Mekalanos JJ (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**: 1910–1914.

Wiklund T, Madsen L, Bruun MS & Dalsgaard I (2000) Detection of *Flavobacterium psychrophilum* from fish tissue and water samples by PCR amplification. *J Appl Microbiol* **88**: 299–307.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. *Flavobacterium psychrophilum* strains used in this study.

Table S2. Primers for PCR amplification of ORFs bacteriophage 6H.

Table S3. ORFs of the bacteriophage 6H genome and BLASTP protein hits.