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Comparative genomic analysis of *Lactococcus garvieae* phage WP-2, a new member of *Picovirinae* subfamily of *Podoviridae*

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ABSTRACT

To date, a few numbers of bacteriophages that infect *Lactococcus garvieae* have been identified, but their complete genome sequences have not yet been investigated. For the first time, herein, the complete DNA sequence of a new phage of *L. garvieae* (phage WP-2) is reported and analyzed. The morphological characteristics indicated that the phage had a small isometric head along with a short and non-contractile tail, suggesting that WP-2 belongs to the family *Podoviridae*. Bioinformatic analysis revealed that phage WP-2 can be classified as a new member of *Ahjdlikevirus* in the *Picovirinae* subfamily because it had a small dsDNA of 18,899 bp with 24 open reading frames and a protein-primed DNA polymerase. The phage nucleotide sequence and predicted protein products have been identified to share very limited evidence of homology with complete genome and proteome of other phages. To our knowledge, this is the first *Ahjdlikevirus* bacteriophage which can infect a member of the *Lactococcus* genus.

1. Introduction

Lactococcal and streptococcal infections of fish are considered as economically important diseases caused by the members of the *Streptococcaceae* family. *Lactococcus garvieae*, *Lactococcus piscium*, *Streptococcus iniae*, *Streptococcus agalactiae*, *Streptococcus parauberis* and *Vagococcus salmoninarum* are the main fish pathogens of this group affecting a variety of wild and farmed finfish such as rainbow trout, yellow tail and tilapia (Toranzo et al., 2005; Vendrell et al., 2006). The diseases (lactococcosis and streptococcosis) caused by these bacteria are similar and can be characterized by exophthalmia, meningoencephalitis and hemorrhagic septicemia. Among them, *L. garvieae* is one of the most serious aquatic pathogens in rainbow trout farms of Iran (Sharifiyazdi et al., 2010; Soltani et al., 2008). The elevated rates of mortality (more than 50%), a decrease in the growing rates of fish, and unpleasant appearance of the infected fish are the reasons that make lactococcosis one of the devastating problems in aquacultures (Chang et al., 2002; Sharifiyazdi et al., 2010). *L. garvieae* is not only a fish pathogen, but also considered as a potential zoonotic agent capable of causing disease in humans and different animals (Aguado-Urda et al., 2011; Devriese et al., 1999; Villani et al., 2001).

Bacteriophages or phages are viruses that replicate within bacterial cells following the injection of their genomes. They are the most abundant and widely distributed biological entities on earth (Bergh et al., 1989; Whitman et al., 1998). Based on their life cycle, phages are found to have different potential applications including phage therapy, cancer therapy, biosensor technology and recombinant DNA technology (An et al., 2013; Nakai, 2010). According to the last report of the International Committee on Taxonomy of Viruses (ICTV, 2013 release), the *Podoviridae* family of phages has been divided into two subfamilies. Among them, the members of *Picovirinae* possess unique features including the existence of a small dsDNA as their genomes, similar gene number and arrangement, and a DNA polymerase type B that is also found in the *Tectiviridae* family of phages. To date, only two genera (the *Ahjdlikevirus* and the *Phi29likevirus*) have been characterized for this subfamily. Furthermore, several unassigned phages, which are not related to these genera but share similar properties with them, have been included in the *Picovirinae* subfamily (http://ictvonline.org/virusTaxonomy.asp).

Up until now, a few numbers of phages that are specific for *L. garvieae* and other streptococcal fish pathogens have been identified (Ghasemi et al., 2014; Park et al., 1997; Wright et al., 2013). However, their complete genomic DNA sequences and proteomic analyses have not yet been reported. In this study, a new phage belonging to the *Picovirinae*

Abbreviations: DNA, deoxyribonucleic acid; dsDNA, double stranded DNA; ssDNA, single stranded DNA; ICTV, International Committee on Taxonomy of Viruses; NCBI, National Center for Biotechnology Information; BHI, Brain Heart Infusion; PEG, polyethylene glycol; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; ORF, open reading frame; UPGMA, unweighted pair group method with arithmetic mean; MUSCLE, multiple sequence comparison by log-expectation; CHAP, cysteine, histidine-dependent amidohydrolases/peptidases.

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subfamily was isolated from a rainbow trout farm and its genome was completely sequenced. The genome and the proteome of *L. garvieae* phage WP-2 were analyzed and compared with all sequences deposited to the GenBank database (NCBI).

2. Materials and methods

2.1. Bacterial host strains and phage isolation

The bacterial host strains used in this study were *L. garvieae* strains RT-IRI (accession number in NCBI: KF849269), SEM-IRI (accession number in NCBI: KF849270) and SI-IRI (accession number in NCBI: KF849271) which were isolated from diseased rainbow trout of different farms in Iran. The phage specific for these bacteria was isolated from water samples obtained from a rainbow trout farm that presented an outbreak of lactococcosis. Briefly, the phage was isolated by adding 50 mL of the water into 50 mL of 2× Brain Heart Infusion (BHI) broth inoculated with 1 mL of an overnight culture of *L. garvieae*. After a 24 h incubation at 30 °C with gentle shaking, the culture was centrifuged at 8000 *g* for 10 min at 4 °C using a micro 17TR centrifuge (A1.5S-24 rotor, Hanil Science, South Korea), and then the supernatant was passed through a 0.22-μm cellulose acetate filter (Sartorius, South Korea). The enriched phage sample was examined to detect the presence of phage by plaque assay using a double agar overlay. An individual plaque was picked and eluted with SM buffer (50 mL of 1 M Tris–HCl pH 7.5, 5.8 g of NaCl, and 2 g of $MgSO_4 \cdot 6H_2O$ in 1 L of distilled water), and then re-isolated three times to ensure the purity of the phage isolates (Sambrook and Russell, 2001).

2.2. Phage purification

The enriched phage sample was concentrated by adding 10% polyethylene glycol (PEG 8000, Sigma, South Korea) and 1 M sodium chloride. After a 6 h incubation at 4 °C with gentle shaking, the mixture was centrifuged at 9000 *g* for 30 min at 4 °C (JA-14 rotor, Beckman Avanti J-E centrifuge, USA). The pellet was suspended in SM buffer, and then was purified using CsCl gradient ultracentrifugation in a Beckman Optima L-100K ultracentrifuge (SW 28 rotor, 35,000 *g*, 16 h, 4 °C). Finally, the phage band was extracted from the gradient and dialyzed against SM buffer (Sambrook and Russell, 2001).

2.3. Transmission electron microscopy (TEM)

Ten microliters of the CsCl-purified phage sample was put on a Formvar/carbon-coated grid (200-mesh copper grid, Ted Pella, USA) for 1 min. Then, excess sample was drained from the grid using filter paper. The phage particles were negatively stained with 10 μL drops of 2% (w/v) aqueous uranyl acetate (pH 4.5) for 1 min, and then excess dye was removed from the grid. The grid was examined in a JEM-2100 Electron Microscope (JEOL, Japan) operated at 100 kV (Ghasemi et al., 2014).

2.4. Phage genomic DNA isolation

The phage DNA was extracted by performing chemical lysis and phenol/chloroform/isoamyl alcohol (25:24:1) extraction method (Sambrook and Russell, 2001). To remove the bacterial DNA and RNA, DNase I (10 μg/mL) and RNase A (20 μg/mL) were added to the enriched phage filtrate and incubate at 37 °C for 15 min. Phage particles were concentrated by centrifugation at 28,000 *g* for 60 min at 4 °C using a micro 17TR centrifuge (Hanil Science, South Korea) with an A1.5S-24 rotor. Then, 600 μL lysis buffer (25 mM Tris–HCl, pH 8; 10 mM EDTA, pH 8; 10 mM NaCl; 1% SDS) and proteinase K (1 mg/mL) were added to the concentrated phage particles, and followed by incubation at 37 °C for 10 min and 60 °C for 10 min. After incubation, the DNA was extracted by adding an equal volume of phenol/chloroform/isoamyl alcohol solution (Bioneer, South Korea). The phage DNA was precipitated with iso-propanol and washed twice with ethanol, and then resuspended in sterile distilled water. The purified nucleic acid was stored at −20 °C.

2.5. Phage DNA fingerprinting

The phage DNA was used to investigate its restriction profiles produced by HindIII, AseI, Sau3aI, XbaI and DraI restriction enzymes (all from Promega, South Korea). Each endonuclease and corresponding enzyme buffer was added on the viral genome, according to the manufacturer's protocol. After incubation at 37 °C for 45 min, the products were examined by 1% agarose gel electrophoresis at 100 V for 30 min (Ghasemi et al., 2014).

2.6. Phage DNA sequencing and analysis

The whole genome of the isolated phage was sequenced at Macrogen company (South Korea) using a Roche 454 Genome Sequencer FLX system with an average of 7.5-fold sequencing coverage. Multiple sequences were assembled in one single sequence by the GS De Novo Assembler version 2.9.

Open reading frames (ORFs) were predicted by GeneMark.hmm for Prokaryotes, version 2.8 (http://opal.biology.gatech.edu/GeneMark/gmhmm2_prok.cgi) (Besemer and Borodovsky, 2005). The criteria for the characterization of each ORF were the presence of a start codon (ATG, GTG, or TTG), a stop codon (TAA, TGA, or TAG) and a minimum size of 50 amino acids. Translation of each ORF region to a protein sequence was performed by ExPASy translate tool (http://web.expasy.org/translate/). The amino acid sequences were then comparatively analyzed based on homology searches using the protein Basic Local Alignment Search Tool (BLASTP) at NCBI website (http://www.ncbi.nlm.nih.gov/blast/) (Altschul et al., 1997). Also, ExPASy compute pI/Mw tool (http://web.expasy.org/compute_pi/) was used to determine isoelectric pH and molecular weight of the predicted ORFs (Gasteiger et al., 2005). The search for tRNA-encoding region was performed with tRNAscan-SE (http://lowelab.ucsc.edu/tRNAscan-SE/) to determine whether the phage contains tRNA sequences or not (Lowe and Eddy, 1997).

2.7. Construction of phylogenetic tree

To determine the phylogenetic position of the isolated phage, two predicted ORFs were chosen to draw the UPGMA (unweighted pair group method with arithmetic mean) dendrograms. DNA polymerase and major capsid amino acid sequences of the investigated phage and other phages that belonged to *Podoviridae* and *Tectiviridae* were aligned in MEGA6 software utilizing MUSCLE (Tamura et al., 2013). Phylogenetic trees were then constructed using UPGMA method with two thousand bootstrap replications. DNA polymerase and capsid protein of *Microbacterium* phage vB_Mox-ISF9 were used as out-groups (Zamani et al., 2014).

3. Results and discussion

3.1. Isolation and characterization of phage WP-2

Since bacteriophages exist in close proximity to their host bacteria, we screened for a phage infecting three *L. garvieae* strains using water samples of a rainbow trout farm. Accordingly, a bacteriophage specific for these fish pathogenic bacterial strains was isolated and designated WP-2. The lactococcal phage WP-2 formed small plaques with an average diameter of 2.3 mm on the bacterial lawn.

To date, a wide variety of phages has been studied; however, most of phages as well as the lactococcal phages belonged to three families of bacteriophages including *Siphoviridae*, *Myoviridae* and *Podoviridae* (Deveau et al., 2006; Hanlon, 2007). Transmission electron microscopy