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Genomic characterization of a novel virulent phage infecting the *Aeromonas hydrophila* isolated from rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The virulent bacteriophage MJG that specifically infects *Aeromonas hydrophila* was isolated from a water sample from a river in Harbin, China. The genome of phage MJG was a double-stranded linear DNA with 45,057 bp, possessing 50.11% GC content. No virulence or resistance genes were found in the phage genome. Morphological observation, genomic characterization, and phylogenetic analysis indicated that MJG was closely related to phages belonging to the genus *Sp6virus* in the *Podoviridae* family. This phage is a novel member within *Sp6virus* that could infect and lyse *A. hydrophila*. This study could serve as a genomic reference of *A. hydrophila* phages and provide a potential agent for phage therapy.

A. hydrophila belongs to family *Aeromonadaceae*. It is a Gram-negative bacterium that is broadly distributed in global aquatic environments. *A. hydrophila* is an opportunistic pathogen of various animals, including fish (Dong et al., 2018). Motile *Aeromonas* septicemia (MAS) caused by *A. hydrophila* reportedly causes high mortality rates and confer considerable economic losses to the aquaculture industry (Zhang et al., 2015). Antibiotics are commonly used to control MAS in fish; however, excessive administration of antibiotics has led to the development of antibiotic-resistant *A. hydrophila* strains (Lee and Wendy, 2017; Vivekanandhan et al., 2002). This phenomenon may not only cause failure of antimicrobial therapy but also raise safety concerns on fish products (Chang et al., 2015).

Phages are bacterial viruses that infect and kill bacteria by using a different mechanism compared with antibiotics and are suitable for controlling antibiotic-resistant bacteria. Given its high specificity, a phage will not affect the natural flora (Lin et al., 2017). Additionally, when ayu fish (*Plecoglossus altivelis*) were administrated orally with phage-impregnated feed, the bacterial cells quickly disappeared in fish; bacterial growth in freshwater was low, thereby protecting the fish from infection (Park et al., 2000). Rainbow trout (*Oncorhynchus mykiss*) could be protected from *Flavobacterium columnare* infection by a single addition of phage to the flow-through tank system (Laanto et al., 2015). Therefore, immunization *via* a phage through oral delivery or immersion might provide a labor-saving control method for the MAS caused by *A. hydrophila*, especially the antibiotic-resistant strains.

Several studies have confirmed the protective abilities of phages against *A. hydrophila* infection in fish. When unfiltered fish pond water was treated with phages, 99% of *A. hydrophila* in water was reduced within 8 h (Hsu et al., 2000). Two *A. hydrophila* phages could inhibit the growth of the *Aeromonas* spp. under laboratory conditions and provide significant protection of up to 100% to striped catfish (*Pangasianodon hypophthalmus*) against pathogenic strain infections (Le et al., 2013). Immediate injection with a single administration of either phage (pAh1-C or pAh6-C, morphologically classified as *Myoviridae*) into cyprinid loaches (*Misgurnus anguillicaudatus*) increased survival rates against *A. hydrophila* infection (Jun et al., 2013). Given that phages are highly specific to a single bacterial species or even only one bacterial strain, the sources of phage should be abundant to ensure the therapeutic efficacy against bacterial infection in practice. However, only three *A. hydrophila* phage strains have been isolated from seawater samples in China thus far (Shen et al., 2012; Yuan et al., 2018).

The bacterial strain used in the present study as a host bacterium (*A. hydrophila* 2016-76) was isolated from the spleens of infected rainbow trout in an organized fish farm of China. The bacterial strain has been identified by 16 s rDNA sequencing and biochemical test as *A. hydrophila*. The strain could cause approximately 60% death of rainbow trout

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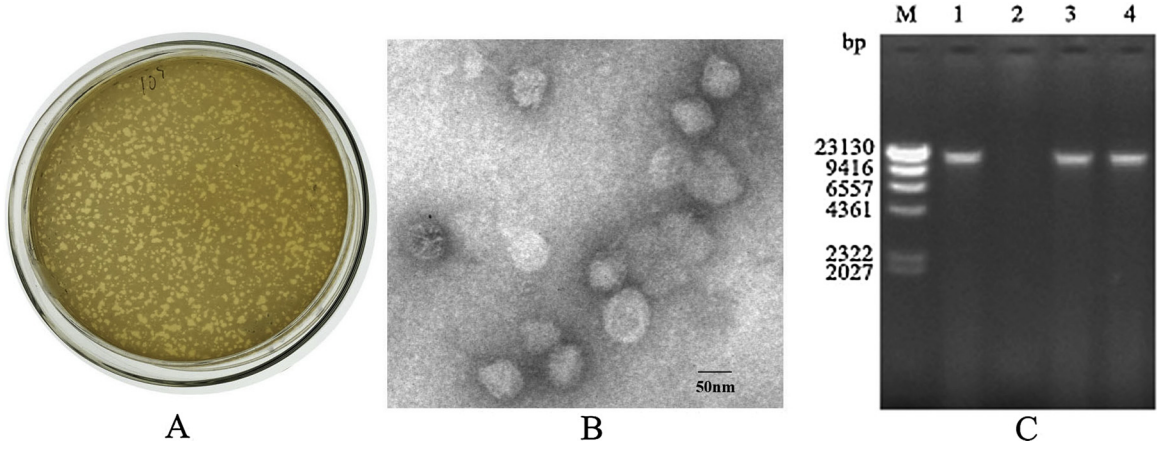

Fig. 1. Isolation, morphology, and genomic determination of bacteriophage MJG. (A) The formed plaques of phage MJG using *A. hydrophila* 2016–76 as a host strain; (B) Transmission electron micrograph of phage MJG; (C) The nucleic acids of phage MJG were untreated (lane 1), digested with DNase I (lane 2), RNaseA (lane 3), and mung bean nuclease (lane 4), following determination by using 0.7% agarose gel electrophoresis. Note: Agarose gel electrophoresis was performed to identify the type of the MJG genome and not to confirm the exact size of the genome.

via intraperitoneal injection at a dose of 10^8 cfu/fish. *A. hydrophila* 2016-76 has been kept at the China Center for Type Culture Collection (CCTCC M2019532).

Phage MJG was successfully isolated from a water sample from a river in Harbin, China by using *A. hydrophila* 2016-76 in accordance with a previously described method (Ross et al., 2016) (Fig. 1A). The phages were proliferated, tested, and purified through the routine process (Mirzaei and Nilsson, 2015). The phage morphology was determined using transmission electron microscopy (HITACHI H-7650) at 80 kV after 2% potassium phosphotungstate negative staining (Ackermann, 2012). The MJG morphology is characterized by a head with a diameter of approximately 50 nm and a possible short tail that could be spotted to the head (Fig. 1B) similar to other *Podoviridae* family phages (de Leeuw et al., 2017). MJG was stable after treatment with chloroform. One-step growth curve indicated that MJG had a burst size of 69 PFU (plaque forming unit) per infected cells. MJG could not lyse *Escherichia coli*, *Edwardsiella ictaluri*, *Yersinia ruckeri*, and *Pseudomonas*. In *Aeromonas* species, no plaques could be observed when phage MJG was cultured with *A. salmonicida* subsp. *masoucida*, *A. salmonicida* subsp. *achromogenes*, *A. sobria*, or *A. caviae*, respectively. Therefore, MJG could be specific to *A. hydrophila*.

Phage nucleic acids were extracted with a kit from Norgen Biotek Inc. under the manual protocol to further characterize the genome of phage MJG. The purified nucleic acids were digested with DNase I, RNaseA, and mung bean nuclease, followed by determination *via* 0.7% agarose gel electrophoresis. The nucleic acids could be only degraded by DNase I but not by RNaseA and mung bean nuclease (Fig. 1C). Therefore, the genomic nucleic acids of phage MJG were double-stranded DNA. Whole genome sequencing was performed using Illumina MiSeq platform based on the constructed library with different inserted segments (Illumina Inc., USA).

The clean reads were obtained after eliminating adaptor sequences and low-quality reads from raw reads. The high-quality reads were assembled using SPAdes v3.9.0 (Bankevich et al., 2012). Finally, the genome of MJG is double-stranded DNA of 45,057 bp, with 50.11% GC content (Supplementary S1). The sequences of MJG genome was BLASTN searched against the known phage genomes in NCBI. Significant alignments were observed as *Pseudomonas* phage Njord (NCBI: MH113812.1), with 57.0% identity. Additionally, the organization and arrangement of MJG genome were similar to those of *Pseudomonas* phage Njord (Fig. 2A and B). However, the query coverage was below 10%. Therefore, the newly isolated phage MJG could be novel.

The virus genome termini were determined on the basis of high frequency sequences in the high throughput sequencing result (Li et al., 2014). The ratio of the highest frequency/average frequency was 37, that of the forward top 1 frequency/forward top 2 frequency was 5, and that of the reverse top 1 frequency/reverse top 2 frequency was 7. According to the established criterion to distinguish the type of termini (Li et al., 2014), MJG is a linear genome. The genomes of SP6-like phages have direct terminal repeat (DTR) at the end. Toward the end of the infection cycle, the terminase of SP6-like phage would recognize its own DTR and cut the DNA to form the mature chromosome during packaging (Garneau et al., 2017). However, the similar DTR was not found at the end of the virion DNA of MJG by using PhageTerm software. As such, the phage MJG might utilize an alternative packaging mechanism.

The open reading frames (ORFs) were predicted using GeneMarkS (Besemer et al., 2001). The results showed that the MJG genome contains 45 ORFs (Supplementary S2), with total length of 41,793 bp and 92.8% gene encoding sequences. No sRNA, tRNA, or rRNA genes were found in the genome. Also, no antibiotic resistance gene, phage-coded virulence gene, or bacterial virulence gene was detected in the genome of phage. According to in silico analysis, phage MJG is safe a potential agent against *A. hydrophila* infection. The potential functions of ORFs were annotated with BLSASTP. Only 17 ORFs for MJG were predicted to have known functions and can be classified as replication, structural proteins, scaffolding and resembling proteins, and lysis proteins (Supplementary S3). Aside from the abovementioned genes with known functions, 10 unique genes with unknown function were only found in MJG genomes (Fig. 2A).

The amino acid sequences of ORFs were analyzed using the conserved domain database and Pfam database, respectively (Petrovski et al., 2011), to further identify the conserved motifs and determine the protein family allocations. Among the above 17 genes with known functions, 8 genes are involved in bacteriophage DNA replication, modification, and repair. The predicted hydrolase/topoisomerase-primase (MJG_9) may load on at the origin of replication and unwind DNA or maintain DNA replication to prevent the DNA double helix from supercoiling. DNA polymerase (MJG_13) would make DNA in the 5′ to 3′ direction. The generated gaps during replication could be sealed by DNA ligase (MJG_24). Deoxyribonucleoside kinase (MJG_22) was only found in phage MJG but not in other phages that infected *Aeromonas* bacteria. The deoxyribonucleoside kinase of bacteriophage T5 is essential for the rapid synthesis of DNA in large amounts (Mikoulinskaia et al., 2003). Thus, the DNA synthesis mechanism of phage MJG might be different from that of previous *Aeromonas* phages. Additionally, MJG_6 encodes a DNA-directed RNA polymerase, which catalyzes the transcription of phage DNA into RNA.

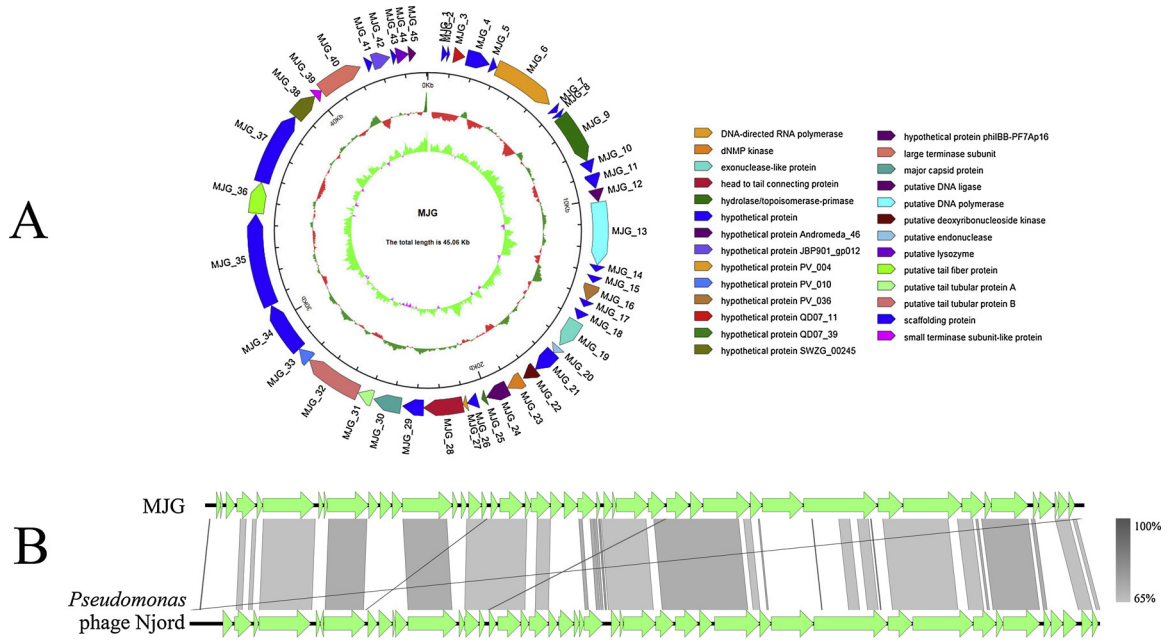

Fig. 2. Organization and arrangement of bacteriophage MJG. (A) Genomic map of phage MJG, plotted with Circos software; (B) Genomic comparison of phage MJG and the reference Pseudomonas phage Njord (Genbank No.MH113812.1), constructed with EasyFig. Arrows represent ORFs. The level of identity is indicated by the gray shading.

The virion of phage MJG consists of genomic DNA, capsid protein (MJG_30), head-to-tail connecting protein (MJG_28), scaffolding protein (MJG_29), two tubular proteins (MJG_31 and 32), and tail fiber protein (MJG_36). Before a mature virion forms, the terminase plays an important role during the packing of a genome into the phage head (Rao and Feiss, 2008). The small terminase (MJG_39) might determine the binding specificity of phage DNA, whereas the large terminase (MJG_40) might mediate the cleavage of the genomic DNA. However, no proteins with any identity to known or putative terminase of *Aeromonas* phages have been found, thereby suggesting that phage MJG utilizes an alternative DNA packing mechanism.

Several bacteriophages recognize and bind to the host receptors by tail fiber proteins (Le et al., 2013). MJG_36 was predicted to encode a tail fiber protein in the phage MJG. The tail fiber protein consists of 423 amino acids, matching over its N-terminal 157-amino-acid sequence in the Phage T7 tail fiber protein. Two protein binding regions of the N-terminal domain (Methionine 1-Arginine 4 and Proline 31) were predicted within the MJG tail fiber protein and may mediate the interaction of fiber and virion-like phage T7 (Dobbins et al., 2004). The C-terminal domain of bacteriophage tail fibers serves a function in cell receptor binding and may be responsible for specific recognition (Garcia-Doval and van Raaij, 2012). The C-terminal domain of phage MJG tail fiber protein did not share identities with that of any other *Aeromonas* phages but showed 66.5%–72.6% identities with *Pseudomonas* phage Achelous, Nerthus, Njord, Alpheus, and Uligo. However, the binding mechanisms of these *Pseudomonas* phages were still unknown. Additionally, a single point mutation of a putative tail fiber protein caused an altered host specificity of phage (Le et al., 2013). Therefore, the recognition and binding to the *A. hydrophila* receptor of phage MJG, which uses a unique tail fiber protein that is quite different from known *Aeromonas* phages, should be further analyzed.

When bacteriophages come to the end of the reproductive cycle, they can utilize lysins to degrade the cell wall of the infected bacteria and release their progeny. Additionally, phage lytic enzymes could lyse the bacteria exogenously, with lysis spectrum broader than that of its parent phage. Thus, lysins have been widely developed as efficient antibacterial agents for Gram-positive pathogens in various animal models (Fischetti, 2018). Phage lysins usually consist of endolysin and holin. However, only one putative lysozyme gene (MJG_44) was identified within the MJG genome, and it had no similarity with any known phage lysozyme genes. Thus, the lysozyme encoded by MJG_44 might be sufficient for lysis. In silico analysis with SignalP 4.1 server revealed that the MJG_44 protein sequence possesses a putative signal sequence and a cleavage site (Alanine 19-Histidine 20) in the N-terminus (Nielsen, 2017). Therefore, MJG lysozyme should be secreted in a membrane-tethered and released after the cleavage of the signal peptidase (Xu et al., 2004). Phage lysins were effective for Gram-negative bacteria pretreatment with an outer-membrane permeabilizer (Helander and Mattila-Sandholm, 2000). Consistently, a novel lysin named PlyF307 was proved to efficiently kill Acinetobacter baumannii in a mouse model (Lood et al., 2015). Therefore, whether MJG lysozyme possesses the intrinsic antimicrobial activity against *Aeromonas hydrophila* should be confirmed. A catalytic domain was found in the N-terminus of MJG lysozyme by using the catalytic site identification web server (https://catsid.llnl.gov/catsid/). Truncated lysins only containing the catalytic domains can increase protein solubility and provide comparable lytic activity with parental lysins (Kong and Ryu, 2015). The antimicrobial ability of MJG lysozyme might be enhanced by peptic digestion and subsequent tryptic digestion (Mine et al., 2004) or fusion with cationic peptides at the N or C terminus (Briers et al., 2014). Therefore, the natural or engineered lysozyme might be another potential use of phage MJG against *A. hydrophila* infection.

MEGA version 10.0.5 was used for phylogenetic analysis based on the amino acid sequences of the major capsid protein and putative tail tubular protein A to classify phage MJG (Fig. 3). The results clearly showed that MJG had a high homologous relationship with *Pseudomonas* phages but form a distinct clade different from other phages within the *Sp6virus* genus of the *Podoviridae* family. Based on the available genome data in GenBank, *A. hydrophila* phages can be divided into *Podoviridae* (25AhydR2PP, Ahp1 and CF7), *Siphoviridae* (like AhSzw-1 and AhSzq-1), and *Myoviridae* (CC2, Aeh1, and Ah1). In contrast to previous *A. hydrophila* phages Ahp1 and CF7 that belong to the unclassified *Autographivirinae* (Wang et al., 2016), phage MJG is classified into the *Sp6virus* in the *Podoviridae* family whose novel *Sp6virus* members can infect and lyse *A. hydrophila*. Compared with the three other Chinese *A. hydrophila* marine phages AhSzw-1, AhSzq-1 m, and

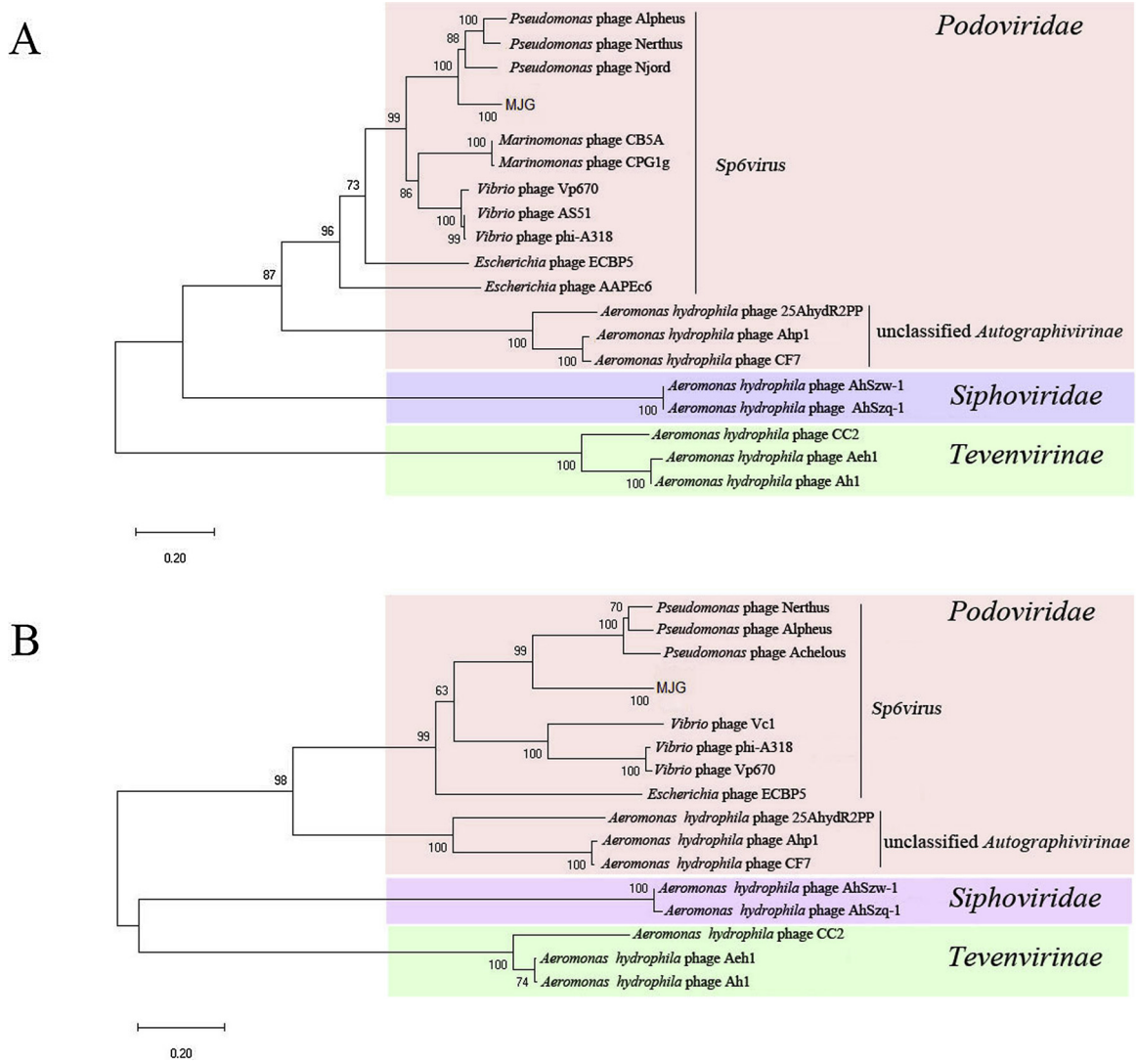

Fig. 3. Phylogenetic analysis of the selected phages within *Podoviridae* and *A. hydrophila* phages based on their major capsid proteins (A) and tail tubular proteins (B). The phylogenetic trees were constructed using the neighbor-joining method with 1000 bootstrap replicates in MEGA version 10.0.5 software.

CC2 (Shen et al., 2012; Yuan et al., 2018), phage MJG has been isolated from freshwater sample and is phylogenetically different from the marine relatives.

A novel *A. hydrophila* bacteriophage, MJG, was isolated in this study. Although phage MJG could infect and lyse *A. hydrophila*, genomic characterization and phylogenetic analysis strongly suggested that phage MJG is closely associated with several *Pseudomonas* phages within *Sp6virus*. Thus, further investigation on the recognition and lysis mechanism of phage MJG could provide novel insights into the phage evolution and ecology. The phage MJG and its encoded lysozyme may contribute to the development of alternative antimicrobial approaches for controlling *A. hydrophila* infection.

Nucleotide sequence accession numbers

The complete genome sequence of phage MJG has been deposited in the GenBank database under the accession numbers MK455769.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197764.

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