VIROLOGICA SINICA 2015, 30 (1): 52-58
DOI 10.1007/s12250-014-3529-4

RESEARCH ARTICLE

Isolation and characterization of glacier VMY22, a novel lytic cold-active bacteriophage of *Bacillus cereus*

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As a unique ecological system with low temperature and low nutrient levels, glaciers are considered a "living fossil" for the research of evolution. In this work, a lytic cold-active bacteriophage designated VMY22 against *Bacillus cereus* MYB41-22 was isolated from Mingyong Glacier in China, and its characteristics were studied. Electron microscopy revealed that VMY22 has an icosahedral head (59.2 nm in length, 31.9 nm in width) and a tail (43.2 nm in length). Bacteriophage VMY22 was classified as a *Podoviridae* with an approximate genome size of 18 to 20 kb. A one-step growth curve revealed that the latent and the burst periods were 70 and 70 min, respectively, with an average burst size of 78 bacteriophage particles per infected cell. The pH and thermal stability of bacteriophage VMY22 were also investigated. The maximum stability of the bacteriophage was observed to be at pH 8.0 and it was comparatively stable at pH 5.0–9.0. As VMY22 is a cold-active bacteriophage with low production temperature, its characterization and the relationship between MYB41-22 and *Bacillus cereus* bacteriophage deserve further study.

KEYWORDS ***Bacillus cereus*; characterization; cold-active phage; lytic; *Podoviridae***

INTRODUCTION

Bacteriophages (phages) are viruses that infect and proliferate in bacteria. They play important roles in regulating the structure of microbial communities, metabolism and global biogeochemical cycles (Danovaro et al., 2008; Rohwer and Thurber, 2009; Suttle, 2005). Cold-active bacteriophages are capable of infection and reproduction at temperatures ≤ 4 °C (Rex et al., 2006). A number of cold-active bacteriophages have been isolated, including six phage isolates from Baltic Sea ice infecting *Shewanella* spp. and *Flavobacterium* spp. (Luhtanen et al., 2014; Sencilo et al., 2014). Forty phage-resistant isolates from 49 *Flavobacterium psychrophilum* strains were isolated in Chile, Denmark and the USA (Castillo et al., 2012; Jian et al., 2013; Stenholm et al., 2008). Bacteriophage 9A infecting *Colwellia psychrerythraea* strain 34H was isolated from an Arctic nepheloid layer (Colangelo-Lillis and Deming, 2013; Wells and Deming, 2006). Bacteriophages vB_EcoM-VR5, vB_EcoM-VR7 and vB_EcoM-VR20 showing an unusual low-temperature plating profile were isolated from Lithuanian aquatic environments (Kaliniene et al., 2010). Three different phage-host systems were isolated from Arctic sea ice and the ocean north-west of Svalbard (Borriss et al., 2003).

Bacillus species are aerobic, Gram-positive, spore-formers and rod-shaped. They are ubiquitous in aquatic environments, soil and dried foods. A number of specific *Bacillus* species are used in molecular production and food fermentation. Although *Bacillus* is a very diverse genus with more than 100 species, only the *Bacillus cereus* group of species is associated with non-opportunistic infection of mammals (Zwick et al., 2012). Cases of *B. cereus* associated with immunocompromised patients are increasing (Ceuppens et al., 2013).

Received: 30 October 2014, Accepted: 21 January 2015
Published online: 2 February 2015
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Although many *Bacillus* spp. phages have been isolated, *B. cereus* phages, especially cold-active *B. cereus* phages, have not received much attention, although some temperate phages of *B. cereus* have been characterized (Kong et al., 2012; Lee et al., 2012). Bacteriophages are divided into two categories, lytic and lysogenic. In the former case, bacterial cells are lyzed after replication of the virion in the lytic cycle. Thus, lytic phages have been used in the control of *B. cereus* in mashed potatoes (Lee et al., 2011). In contrast, host cells infected by lysogenic phages do not immediately lyze.

The isolation and characterization of cold-active bacteriophages from low temperature environments contributes to the understanding of cold-adaptation mechanisms and co-evolution of bacteriophages and their hosts. Glaciers are a unique ecosystem. Mingyong Glacier is located in the Meili Snow Mountains, part of the Hengduan Mountains in Yunnan Province, China, and shows high biodiversity (Tang and Zheng, 1990). In this study, a novel cold-active bacteriophage, VMY22, specific for *B. cereus* strain MYB41-22, was isolated from Mingyong Glacier and the biological features of this bacteriophage were characterized.

MATERIALS AND METHODS

Sampling and bacterial isolation

Culture medium: Peptone-Yeast power-Glucose-Vitamins (PYGV) liquid medium (pH 6.0) was prepared as described (Atlas, 2004). Fifteen grams of agar or 4 g of agarose were added to liquid medium to prepare solid PYGV medium or semi-solid PYGV medium, respectively.

Bacterial strain: Water samples were obtained from Mingyong Glacier (E98°81′18.4″, N28°47′33.0″, 4–6 °C, pH 5.4–6.0) located in Yunnan province, China. Samples were spread on PYGV solid plates and incubated for 2–3 d at 15 °C. Bacterial strains were isolated and purified by streaking on solid plates. Isolates were identified by 16S rRNA gene sequencing. PCR universal primers used in this study were as follows: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′), and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lin et al., 2011; Liu et al., 2006). The PCR product of 1.5 kb was ligated into pMD-18T vector (Takara, Dalian, China) and the ligation product was transformed into *Escherichia coli* DH5α competent cells. Positive clones were sequenced by Shanghai Sangon Biotech (Shanghai, China). The morphology of *B. cereus* strain MYB41-22 was investigated by scanning electron microscope.

A phylogenetic tree was constructed by neighbor-joining using Molecular Evolutionary Genetic Analysis software (MEGA 6.0) with 1000-fold bootstrap support. The 16S rRNA gene sequences of *Bacillus* strains and an outgroup of the type strain *E. coli* ATCC 11775^T were analyzed.

Bacteriophage isolation

B. cereus strain MYB41-22 was used as the host to isolate, propagate and characterize phage. Water samples (50 mL) collected from Mingyong Glacier were pre-incubated with host cells in order to enrich the phages. After incubation at 15 °C for 10 d, the culture was centrifuged (12000 × *g*, 15 min, 4 °C, Beckman Avanti J-25, USA) and 0.22 μm filters (Millipore Corp., Bedford) were used to filter the supernatant.

Phage was isolated by successive single-plaque isolation using the double-layer agar method (Adams, 1959). Bacteriophage stock (100 μL) was mixed with MYB41-22 host cell culture (200 μL; OD_{600} = 0.3–0.6) and incubated at 15 °C for 10 min, and then mixed with 4 mL of semi-solid PYGV medium and poured onto a PYGV solid plate (Shen et al., 2012). Plaques were enumerated after the plates had been incubated at 15 °C overnight.

Purification of phage particles

The method of single plaque isolation was used to purify phages (Xiang et al., 2005). All of the centrifugation was carried out at 4 °C. Cells were removed from culture containing 1 × 10^9 PFU/mL phage particles by centrifugation (12000 × *g*, 30 min, Beckman Avanti J-25). The supernatant was supplemented with DNase I and RNase A (Sigma-Aldrich, 1 μg/mL) and incubated for 30 min at 37 °C. 10% (w/v) PEG 8000 and 1 mol/L NaCl were added to the supernatant in order to precipitate phage particles. The phage particles were pelleted by centrifugation (11000 × *g*, 15 min) after incubation on ice overnight, then resuspended in Suspension Medium (SM) buffer (50 mmol/L Tris-HCl, 100 mmol/L NaCl, 10 mmol/L $MgSO_4$, 0.01% gelatin), followed by extraction with chloroform and centrifugation (11000 × *g*, 10 min). Solid CsCl (0.45 g/mL) was added to the supernatant which was then subjected to ultracentrifugation (280000 × *g*, 24 h, SW41 rotor, Beckman LE-80K, USA). The band of phage was collected and dialyzed against SM buffer without gelatin.

Electron microscopy

Phage morphology was examined by transmission electron microscope (TEM). Approximately 1×10^9 PFU/mL phage was dropped into 200 mesh copper grids and stained with 2% (w/v) uranyl acetate. Morphology analysis was conducted with a JEM-1230 TEM at 80 kV (JEOL, Tokyo, Japan).

Determination of optimal multiplicity of infection (MOI)

MOI was defined as the ratio of phage particles to potential bacterial host cells (Lu et al., 2003). MYB41-22 host cells (3×10^8 CFU/mL) were infected with VMY22 at different ratios (0.001, 0.01, 0.1, 1, 10 and 100 PFU/CFU). The mixture was centrifuged (11000 × *g*, 10 min) to remove the pellet after incubation for 4 h at 15 °C. A 0.22 μm filter was used to filter the supernatant and the phage titer was determined.

Adsorption experiments

Phage adsorption experiments were carried out as follows: MYB41-22 host cells (3×10^9 CFU/mL) in PYGV were infected with phage at an MOI of 0.1 and incubated at 15 °C. Aliquots of 100 μL were taken 0, 2, 4, 6, 8, 10, 15 and 20 min after infection, diluted in 0.9 mL cold PYGV, and centrifuged (12000 × *g*, 5 min, 15 °C). The supernatants containing the unabsorbed phages were then titrated using the double-layer agar method.

One-step growth curve of cold-active phage

Fresh MYB41-22 host cells (1 mL, 3×10^9 CFU/mL) were mixed with phage at an MOI of 0.1 and adsorbed for 30 min at 15 °C (Haq et al., 2012). The mixture was centrifuged (13000 × *g*, 30 s, 15 °C), and the pellets were suspended in 5 mL of fresh medium before shaking at 15 °C. Samples were taken at 5-min intervals and the phage titer was measured by the double-layer agar method. Plates were incubated at 15 °C overnight to allow the detection of plaques. Assays were repeated three times.

Thermolability of cold-active phage

To examine the thermolability of the cold-active phage, VMY22 (2×10^9 PFU/mL) was incubated at different temperatures (40, 50, 60 and 70 °C) for 1 h. The survival rates of samples were determined at 10 min intervals.

The pH sensitivity of cold-active phage

In order to keep the pH stable, 10 μL phage stock (1×10^9 PFU/mL) was mixed with 990 μL PYGV broth medium, and incubated at room temperature for 1 h (Haq et al., 2012). The pH values of the mixture were adjusted from 3.0 to 11.0 with different buffers, including 50 mmol/L citrate buffer (pH 3.0–5.0), phosphate buffer (pH 6.0–8.0), Tris-HCl buffer (pH 9.0) and carbonate buffer (pH 10.0–11.0). The survival rates of samples were determined.

Sensitivity to organic solvent and detergents

The sensitivity of phage to organic solvents and detergents was studied. Phages were treated with 15% chloroform for 10 min, 1 mg/mL proteinase K for 2 h at 37 °C, Triton X-100 (0.3% w/v) for 10 min and SDS (0.3% w/v) for 30 min at room temperature, respectively. The surviving phage number was determined. The plaques were examined after incubation at 15 °C overnight.

Extraction of phage DNA and restriction endonuclease digestion

Twenty millimolar EDTA, 10% SDS and 50 μg/mL proteinase K were added to a CsCl-purified phage suspension and incubated at 56 °C for 3 h. Phenol/chloroform and ethanol were used to extract and precipitate DNA. The air-dried DNA pellet was dissolved in TE buffer (10 mmol/L Tris-HCl, 5 mmol/L EDTA, pH 8.0) and digested with *Eco*R I, *Bam*H I, *Hin*d III and *Pst* I (TaKaRa, Dalian, China), respectively.

Protein analysis

CsCl-purified phage particles mixed with loading buffer were boiled for 5 min, and then subjected to 12% SDS-PAGE. Coomassie brilliant blue R-250 (Bio-Rad, USA) was used to stain the protein bands.

RESULTS

Isolation and morphology

The nucleotide sequence of 16S rRNA gene of strain MYB41-22 has been deposited in the NCBI with accession number KC430113. Based on morphology, physiology and 16S rRNA gene phylogenetic tree analysis (Figure 1), MYB41-22 was identified as *B. cereus* MYB41-22. It was rod-shaped, spore-forming and belonged to the Gram-positive class of bacteria (Figure 2). This psychrophilic bacterium could grow between 4–42 °C, with optimum growth between 15 and 20 °C.

A phage infecting *B. cereus* MYB41-22 was isolated and named VMY22. Clear plaques of 2 mm were observed after incubation of phage with bacteria at 4 °C for 36 h on a double-layer plate. Cold-active bacteriophages can infect and reproduce at temperatures ≤ 4 °C, so it was therefore recognized as a typical cold-active phage. TEM examination revealed that VMY22 has an icosahedral head which is 31.9 nm in diameter and 59.2 nm in length, and a tail that is 43.2 nm in length (Figure 3), a typical morphology characteristic of the family *Podoviridae*.

Phage production

B. cereus MYB41-22 strains could produce clear plaques after infection with VMY22 when incubated at 4–37 °C, and showed maximal phage production at 15–20 °C. Table 1 shows that the optimal MOI of VMY22 was 0.1. Figure 4A shows that VMY22 could lyze almost 100% of MYB41-22 cells in 15 min. A one-step growth curve of VMY22 on MYB41-22 showed that the latent period was 70 min and the burst period was 70 min, with

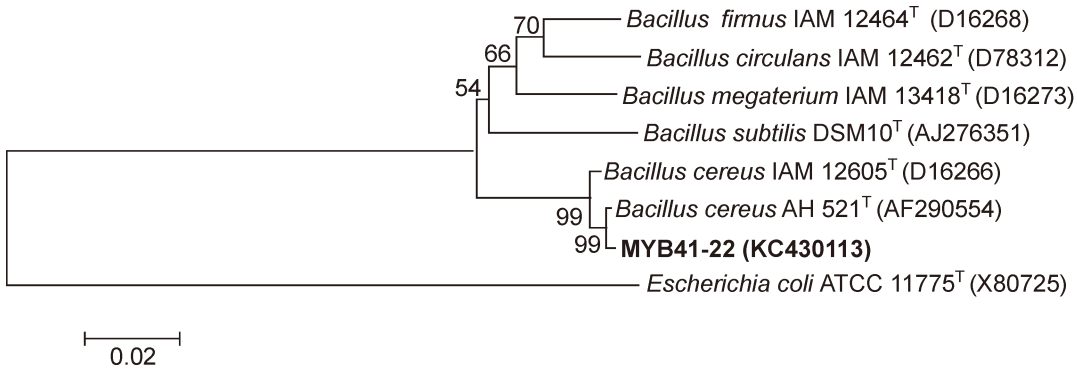

Figure 1. Phylogenetic analysis of strain MYB41-22 based on 16S rRNA gene sequences available from the NCBI GenBank database.

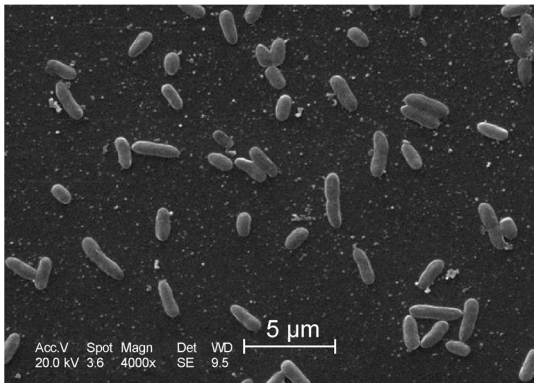

Figure 2. Scanning electron micrograph of strain MYB41-22. The scale bar marked automatically represents 5 μm.

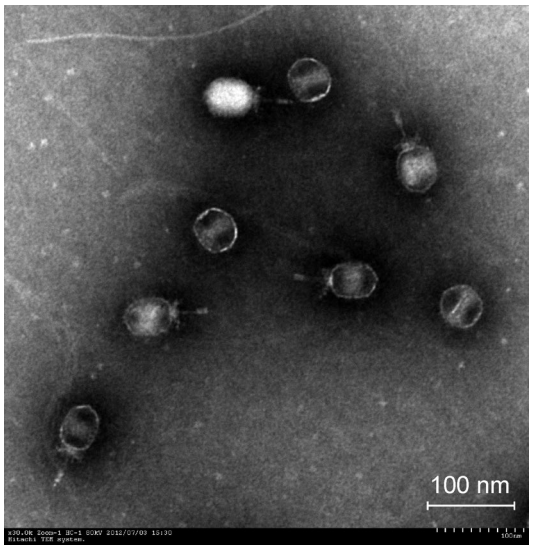

Figure 3. Transmission electron micrograph of *B. cereus* phage VMY22. The scale bar marked automatically represents 100 nm.

Table 1. The optimal multiplicity of infection of *B. cereus* MYB41-22 by VMY22.

Tube	Number of Bacteria (CFU/mL)	Number of Bacteriophages (PFU/mL)	MOI	Titer at 4 h (PFU/mL)
1	2×10^8	2×10^5	0.001	4.3×10^9
2	2×10^8	2×10^6	0.01	5.6×10^{10}
3	2×10^8	2×10^7	0.1	1.4×10^{11}
4	2×10^8	2×10^8	1	3.9×10^9
5	2×10^7	2×10^8	10	2.5×10^9
6	2×10^6	2×10^8	100	1.8×10^9

CFU: colony-forming unit (CFU) is a rough estimate of the number of viable bacteria or fungal cells in a sample.
PFU: plaque-forming unit (PFU) is a measure of the number of particles capable of forming plaques per unit volume.

a burst size of about 78 bacteriophage particles per infected cell (Figure 4B).

Thermolability and pH sensitivity

Thermolability was the most salient physical feature of cold-active phage VMY22 (Figure 4C) (Wells and Deming, 2006). The VMY22 could tolerant more than 60 min at 20 °C with minimal losses, and decreased rapidly when the temperature exceeded 60 °C. VMY22 was shown to be stable between pH 5.0 and 9.0, with maximum survival at pH 8.0 (Figure 4D).

Sensitivity to chloroform and detergents

Sensitivity to organic solvent and detergents were tested, and VMY22 was found insensitive to chloroform; it retained more than 80% infection activity after exposure to 15% chloroform. However, VMY22 infectivity was completely destroyed by treatment with protease K or by

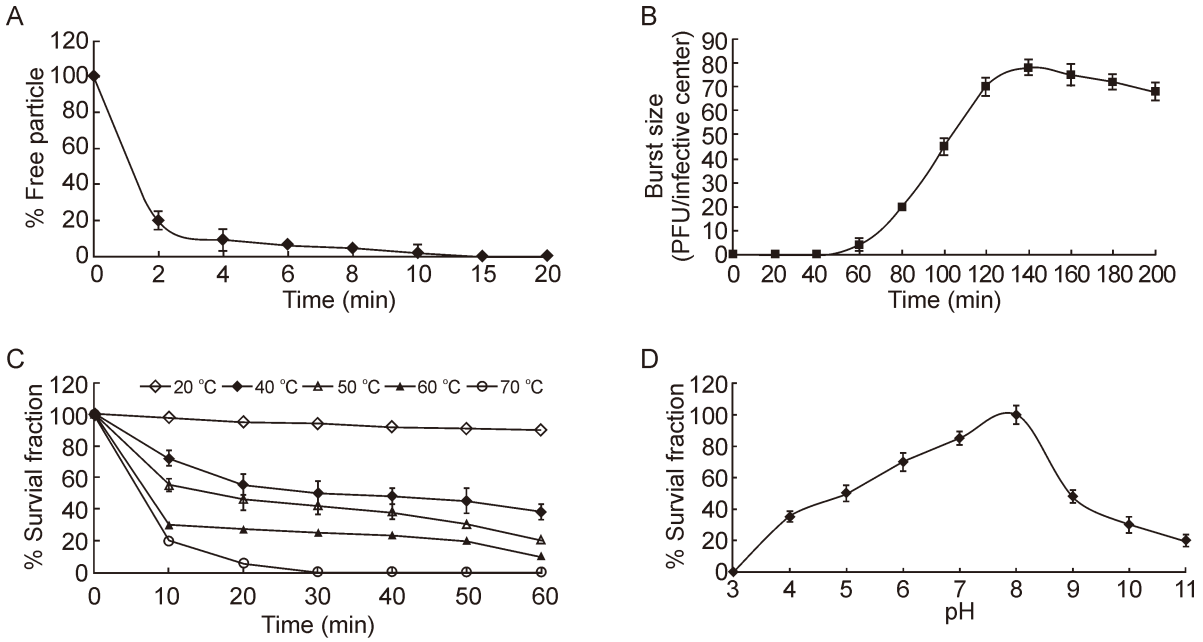

Figure 4. Characterization of VMY22. (A) The adsorption of phage VMY22 to host cells. (B) One-step growth curve of phage VMY22 on *B. cereus* MYB41-22. Values are the mean of three determinations. (C) Thermostability of VMY22 at 20 ℃ (open diamond), 40 ℃ (closed diamond), 50 ℃ (open triangle), 60 ℃ (closed triangle) and 70 ℃ (open circle). Values are the mean of three determinations. (D) pH sensitivity of VMY22. Values are the mean of three determinations.

incubation with SDS or Triton X-100. The insensitivity to chloroform suggested that the capsid of VMY22 did not contain lipids (Wells and Deming, 2006).

Analysis of phage DNA and protein

VMY22 DNA was extracted and digested with restriction endonucleases *Eco*R I, *Bam*H I, *Hin*d III and *Pst* I (Figure 5A). The results showed that VMY22 was dsDNA with an estimated size of 18–20 kb. Purified phage particles were analyzed by SDS-PAGE (Figure 5B). Three main protein bands were observed, with molecular masses 22 kDa, 52 kDa and 57 kDa. Based on the genome sequence of VMY22, these could correspond to lower collar protein, Podovirus_Gp16 superfamily protein and tail protein, respectively (data not shown). The most abundant band was the 52 kDa protein, which may be the major coat protein of cold-active phage VMY22.

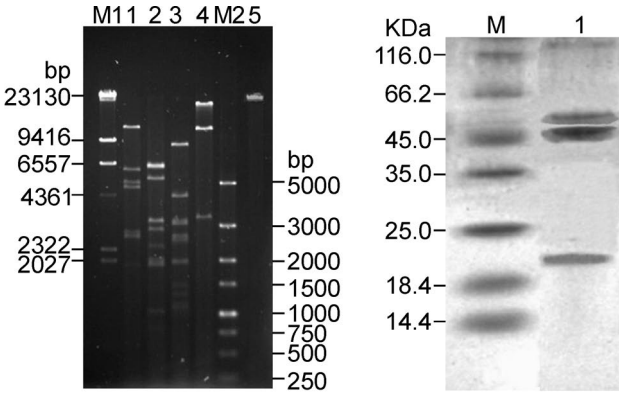

Figure 5. Analysis of VMY22 DNA and protein. (A) Restriction endonuclease digestion patterns of VMY22. M1: DNA marker; 1: VMY22 DNA digested with *Eco*R I; 2: VMY22 DNA digested with *Bam*H I; 3: VMY22 DNA digested with *Hin*d III; 4: VMY22 DNA digested with *Pst* I; M2: DNA marker; 5: Purified VMY22 DNA. (B) SDS-PAGE of purified VMY22. M: protein marker; 1: Purified VMY22.

DISCUSSION

Cold-active phages have been isolated from low temperature environments, such as seawater, food, sewage and sea ice (Anesio and Bellas, 2011; Lopez-Bueno et al., 2009; Rohwer and Thurber, 2009; Sawstrom et al., 2008; Sime-Ngando and Colomber, 2009). To the best of our knowledge, this is the first report on the isolation and characterization of a *B. cereus* lytic cold-active phage from water samples from the Mingyong Glacier.

Of approximately 5,600 known viruses, 136 *Myoviridae*, 203 *Siphoviridae*, 31 *Podoviridae*, 370 tailed bacteriophage and eight polyhedral, filamentous, and pleomorphic (PFP) infecting *Bacillus* have been observed (Ackermann,

2007). While numerous *Bacillus* phages have been isolated, very few cold-active *B. cereus* phages have been characterized in detail. With the exception of phages AP50, AP50-04, AP50-11, AP50-23, AP50-26, AP50-27 and Bam35 belonging to the family *Tectiviridae* (Ackermann et al., 1994), all other *Bacillus* phages belong to the three tailed phage families, *Myoviridae*, *Siphoviridae* and *Podoviridae*. Most *B. cereus* phages belong to *Myoviridae*, for example FWLBc1 (Lee et al., 2011), FWLBc2 (Lee et al., 2011), Bc431v3 (El-Arabi et al., 2013), BCP1-1 (Bandara et al., 2012), and BCP8-2 (Bandara et al., 2012), while *B. cereus* phage TP21-L belongs to the *Siphoviridae* (Klumpp et al., 2010). The *B. cereus* phage VMY22 isolated from Mingyong Glacier in this study belongs to the *Podoviridae*.

The burst size of cold-active phage VMY22 was about 78 PFU/cell at 15 °C, lower than phage FWLBc1 (Lee et al., 2011) (322 PFU/cell at 37 °C), FWLBc2 (300 PFU/cell, at 37 °C) (Lee et al., 2011), and Bc431v3 (318 PFU/cell, at 30 °C) (El-Arabi et al., 2013). Due to being a cold-active phage, the burst size of VMY22 was lower than that of other *B. cereus* phages. In other cold-active phages, reduced burst sizes and protracted latent periods at low temperatures than high temperatures have likewise been reported (Sillankorva et al., 2004; Wells and Deming, 2006), possibly due to bacterial phenotypic responses to the transition to low temperature (Wells and Deming, 2006).

All of the *Bacillus* phages possess dsDNA. Genome analysis indicated that the genome size of phage VMY22 was approximately 18–20 kb, much smaller than TP21-L (37.5 kb) (Klumpp et al., 2010), FWLBc1 (90 kb) (Lee et al., 2011), FWLBc2 (90 kb) (Lee et al., 2011), Bc431v3 (158 kb) (El-Arabi et al., 2013), BCP1-1 (150 kb) (Bandara et al., 2012) and BCP8-2 (150 kb) (Bandara et al., 2012).

B. cereus is ubiquitous and is responsible for a minority of food-borne illnesses. Taking advantage of its ability to compete with *Salmonella* and *Campylobacter*, some harmless *B. cereus* in which animals and/or industries this technique is used to reduce *Salmonella* in the intestines of livestock, thereby improving the growth of the animals, as well as contributing to food safety.

Bacterial contamination can be found in low-temperature environments such as fridges. Bacteriophages could decrease the number of bacterial infections and may be candidate antimicrobial agents against bacterial contamination. Cold-active bacteriophages possess the ability to control pathogens in low-temperature aquaculture and phage therapy has received recent renewed interest (Castillo et al., 2012). As VMY22 is a cold-active bacteriophage with a relatively low reproduction temperature, its characterization and the relationship between MYB41-22 and *B. cereus* phage deserve further study.

ACKNOWLEDGMENTS

This research was supported by the National Natural Science Foundation of China (31160121) and the Yunnan Provincial Education Fund project (2013Z138) and funded by the Open Research Fund Program of the State Key Laboratory of Virology of China (2013002).

COMPLIANCE WITH ETHICS GUIDELINES

All the authors declare that they have no conflicts of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

AUTHOR CONTRIBUTIONS

Ji XL, Tang B, and Wei YL designed the experiments. Ji XL, Zhang CJ, and Fang Y carried out the experiments. Ji XL, Zhang Q, Lin LB, and Wei YL analyzed the data. Ji XL and Wei YL wrote the paper. All authors read and approved the final manuscript.

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