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ORIGINAL PAPER

Isolation and characterization of a new bacteriophage MMP17 from *Meiothermus*

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Abstract Thermophiles and their viruses are extraordinarily important because of their roles in processes of evolution, biogeochemistry and ecology. Species of the genus *Meiothermus* share close relationship with genus *Thermus*, but no *Meiothermus* bacteriophage has been reported yet. In this work, a new thermophilic bacteriophage named MMP17 (*Meiothermus Myoviridae* phage 17) was isolated from a *Meiothermus* strain and was further characterized. MMP17 was a typical myovirus with an icosahedral head (42 nm in diameter) and a tail (120 nm in length and 17 nm in width). Its DNA was about 33.5–39.5 kb in size. MMP17 was very stable at 55–60°C and pH 6–7. According to the one-step growth curve, the latent period and the burst period were 60 and 30 min, respectively. An average of 15 phage particles was released from each infected cell. Four major bands were detected in purified virion preparation by SDS-PAGE. As MMP17 was a thermophilic bacteriophage with lower production temperature, its characterization and the relationship between MMP17 and *Thermus* bacteriophages deserved for further study.

Keywords Thermophile · Bacteriophages · *Meiothermus* · Myovirus · Eryuan hot spring

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Introduction

Bacteriophages and viruses infect prokaryotes including Bacteria and Archaea. With the rapid development of bioinformatics and genomics, an increasing lines of evidences show that viruses play an essential part in horizontal gene transfer among different species (Jessup and Forde 2008; Abedon 2009). Thermophiles and their phages or viruses are extraordinarily important for their roles in processes of evolution, biogeochemistry and ecology (Prangishvili et al. 2001, 2006; Abedon 2009). In approximately 5,600 known viruses, only a few have been isolated from thermophiles, and most of their hosts belong to four genera: *Bacillus*, *Thermus*, *Sulfolobus* and *Thermoproteus* (Prangishvili et al. 2001; Prangishvili and Garrett 2004; Liu et al. 2006; Yu et al. 2006). Among them, *Thermus* bacteriophages belong to the *Myoviridae*, *Siphoviridae*, *Tectiviridae* and *Inoviridae* families (Yu et al. 2006), and *Sulfolobus* viruses belong to the *Rudiviridae*, *Lipothrixviridae*, *Fuselloviridae* and *Guttaviridae* families (Prangishvili et al. 1999, 2001; Arnold et al. 2000). Species of the genus *Meiothermus* are closely related to those of the genus *Thermus*. They are widely distributed in hot natural or artificial aqueous environments. Their optimum growth temperatures are 50–65°C. Formerly belonging to genus *Thermus*, these species have been reclassified from genus *Thermus* later according to their lower growth temperature range, the presence of moderate levels of 2-OH fatty acids, 16S rDNA sequence divergence and formation of red or yellow pigmented colonies (Nobre et al. 1996). Six species of the genus *Meiothermus*: *Meiothermus ruber*, *Meiothermus chliarophilus*, *Meiothermus silvanus*, *Meiothermus cerbereus*, *Meiothermus taiwanensis* and *Meiothermus timidus* have been described (Nobre et al. 1996; Pires et al. 2005). To date, no *Meiothermus* bacteriophage has been reported.

Eryuan is the famous geothermic region in Yunnan Province of China and possesses abundant geothermal resources. In this investigation, a new bacteriophage was isolated from a *Meiothermus* strain from Eryuan hot springs and its characteristics were studied.

Methods

Medium

The medium was prepared as the following (per liter) (Wiegel 1986): Nitrilotriacetic acid 100 mg, NaCl 8 mg, Na_2HPO_3 111 mg, $MgSO_4 \cdot 7H_2O$ 100 mg, KNO_3 103 mg, $NaNO_3$ 689 mg, tryptone 1 g, yeast extract 1 g, $Na_2MoO_4 \cdot 2H_2O$ 0.025 mg, $FeCl_3$ 0.28 mg, $CuSO_4$ 0.016 mg, $MnSO_4 \cdot H_2O$ 2.2 mg, H_3BO_3 0.5 mg, $ZnSO_4 \cdot 7H_2O$ 0.5 mg, $CoCl_2 \cdot 6H_2O$ 0.046 mg, $CaSO_4 \cdot H_2O$ 60 mg, with pH adjusted to 7.5 using 3 M of NaOH solution. To prepare a solid or semi-solid medium, 30 or 8 g of agar, respectively, was added to 1 l of the medium.

Strains

Water samples were collected from hot spring (26°15′04″N, 99°57′28″E, pH 7.3 and 63°C) in Eryuan county, Yunnan Province, China at the height of 2,084 m in February 2009, and 100 µl of sample was spread on solid plates. After incubation for 3 days at 55°C, red pigment producing colonies were picked up and purified by streaking on fresh plates for three times. 16S rRNA genes of the strains were amplified by PCR using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Liu et al. 2006). The PCR products were cloned into pMD-18T vector (Takara, Dalian) and sequenced by Shanghai Sangon Biological Engineering Technology and Service in China.

Isolation of phage

A 10-ml water sample collected from the hot spring mentioned above was added to 100 ml of liquid medium. After shaking at 55°C for 24 h, an aliquot of culture (10 ml) was centrifuged for 5 min at 13,000×*g* (Beckman Avanti J-25, CA, USA) to remove the cells, and the supernatant was filtered using a 0.22 µm pore size filter (Millipore Corp., Bedford, Mass). The filtrate was kept as a phage stock and kept at 4°C.

Phages were isolated by double-layer agar technique (Epstein and Campbell 1975). 100 µl of the phage stock was mixed with 500 µl of the strain TG17 culture, which had been grown in liquid medium to the logarithmic phase (OD_{600} = 0.4–0.5). After incubation at 25°C for 10 min for the adsorption of phage particles, the mixture was added to 3 ml of the liquid semi-solid medium, and the mixture was then poured onto the pre-prepared solid monolayer plate. The double-layer plates were incubated right side up at 55°C overnight in a sealed plastic bag and were examined for the presence of plaques. Single plaques were picked up with toothpicks. The process was repeated three times to isolate the phage.

Purification of phage particles

Phage particles were purified according to the method of Xiang et. al. (2005). The culture containing 5 × 10^9 PFU/ml phages was centrifuged (12,000*g*, 30 min, 4°C) to remove the cells. DNase I and RNase A (Sigma-Aldrich, St. Louis, MO) were added to the supernatant to give a final concentration of 1 unit/ml and 1 µg/ml, respectively. The mixture was incubated at 37°C for 30 min. To precipitate phage particles, PEG 8000 and NaCl were added to the supernatant to final concentrations of 10% (w/v) and 1 M, respectively. After incubation on ice for 3 h, phage particles were collected by centrifugation at 12,000*g* for 15 min at 4°C (Beckman Avanti J-25, CA, USA). The pellet was resuspended in double-distilled water. Cell debris were extracted from the phage suspension by adding an equal volume of chloroform and centrifuged at 12,000*g* for 15 min at 4°C (Beckman Avanti J-25, CA, USA). The supernatant was transferred to a new tube and kept as phage stock. To further purify the phage particles, solid CsCl was added to the phage stock to a concentration of 0.45 g/ml and dissolved by gentle mixing. After centrifugation at 38,000 rpm (Ti70 rotor, Beckman XL-90, CA, USA) for 24 h at 4°C, phage particles were collected, and the phage suspension was dialyzed twice at 4°C overnight against double-distilled water. The phage particles were detected by the double-layer agar technique (Epstein and Campbell 1975).

Electron microscopy

The purified phage suspension was deposited on copper grids with carbon-coated Formvar film, and was stained with 2% (wt/vol) uranyl acetate. Phage particles were examined in a JEM-1400 (JEOL, Tokyo, Japan) electron microscope.

Effect of temperature on phage production and thermostability

To investigate the effect of temperature on phage production, 80 µl of strains TG17 (1 × 10^6 cell/ml) were infected with MMP17 at MOI of 0.1. After incubation at room temperature for 20 min, they were cultured at different

temperatures (40, 42.5, 45, 50, 55, 60, 62.5, or 65°C) in 8 ml of liquid medium for 6 h. Phage titer in the culture was measured by the double-layer agar technique (Epstein and Campbell 1975).

To examine the thermostability of the phage, phage stock (1×10^4 PFU/ml) was incubated at 45, 50, 55, 60, 70, 80 or 90°C for 30 min, respectively. The rate of survival of each treated phage sample was determined by the double-layer agar technique (Epstein and Campbell 1975). Plates were incubated at 55°C for 24 h.

pH sensitivity of phage particles

In order to keep the pH steady, 10 µl of phage stock (1×10^7 PFU/ml) was added into 10 ml modified liquid medium (pH values were adjusted from 2 to 12 by adding 1 M HCl or 1 M NaOH) and incubated for 30 min. The rates of survival of the phage samples were determined by the double-layer agar technique (Epstein and Campbell 1975). Plates were incubated at 55°C for 24 h.

One-step growth curve

A phage sample was mixed with 0.333 ml of a fresh TG17 culture (3×10^8 cell/ml) to obtain an MOI of 10 (Ellis and Delbruck 1939) and the mixture was incubated at 55°C for 10 min. Cells were collected by centrifugation at 13,000*g* for 2 min and were resuspended in 1 ml of fresh medium. This process was repeated 3 times to remove unadsorbed phage particles. A sample (10 µl) of the cell suspension was added to the liquid medium (100 ml) and incubated with shaking at 55°C for 100 min. Phage titer in the culture was measured by the double-layer agar technique at a 10-min interval.

Extraction and restriction endonuclease digestion (RED) of phage DNA

SDS and proteinase K were added to a phage suspension to give final concentrations of 1% and 1.25 mg/ml, respectively. After incubation at 56°C for 3 h, the mixture was extracted twice with phenol–chloroform (1:1), and the DNA was precipitated from the aqueous phase by 2 volume of ethanol. Following centrifugation at 13,000*g* for 15 min, the DNA pellet was washed with 70% ethanol. The air-dried DNA pellet was dissolved in 10 mM Tris–HCl, 5 mM EDTA, pH 8.0. The viral DNA was digested with *Apa*I, *Eco*RI or *Pst*I (TaKaRa, Dalian, China), respectively.

Protein analysis

Purified phage particles were mixed with the loading buffer for SDS-PAGE. After boiling for 5 min, the samples were subjected to SDS-PAGE (12%). The gel was stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA).

Results and discussion

Isolation and morphology

Red-pigment producing strain TG17 was isolated and was determined as *Meiothermus* sp. (named *Meiothermus* TG17, GenBank accession number GU329951) by phylogenetic analysis. *Meiothermus* TG17 was found to be a heterotrophic, aerobic, gram-negative, rod-shaped, red-pigment producing, non-spore forming and thermophilic bacterium that grows at 42–68°C, with optimal growth occurring at 55–60°C.

A phage infecting *Meiothermus* TG17 was isolated and named MMP17 (*Meiothermus Myoviridae* phage 17). When assayed on a double-layer agar plate, MMP17 formed clear plaques of 0.5–1 mm in diameter on the lawn of *Meiothermus* TG17 after incubation at 55°C for 12 h. EM analysis showed that MMP17 had an icosahedral head of 42 nm in diameter and a tail of 120 nm in length and 17 nm in width (Fig. 1). A distinctive narrow neck of 12 nm in width was found at the junction between the head and the tail. These morphological characteristics suggest that MMP17 belonged to the *Myoviridae* family.

Phage production

Following infection with MMP17, *Meiothermus* TG17 was able to produce progeny phage particles when incubated at temperatures between 42.5 and 62.5°C. Phage production occurred optimally at 55–60°C (Fig. 2). According to the one-step growth curve (Fig. 3), the latent period and the burst period were 60 and 30 min, respectively, and 15 phage particles were released from each of infected cell.

Thermostability and pH sensitivity

MMP17 was very stable at 55–60°C (Fig. 4). However, the ability of MMP17 to infect the host had fallen fast when the temperature exceeded 70°C. On the other hand, MMP17 was most stable at pH 6.0–7.0 and was sensitive to treatment at acidic pHs. The survival pH range was from pH 5 to 11 (Fig. 5).

Analysis of phage DNA and protein

MMP17 DNA was isolated and digested with *Apa*I, *Eco*RI or *Pst*I (Fig. 6). The patterns of digestion with *Apa*I show that MMP17 contained double-stranded DNA with an

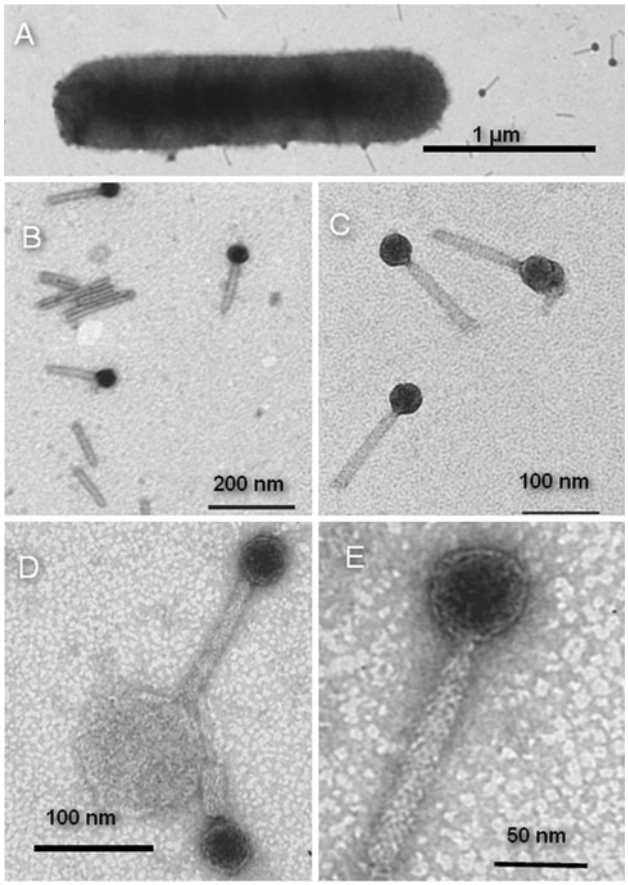

Fig. 1 Transmission electron micrographs of MMP17 and its host cell A: **a** *Meiothermus* TG17 Cell and MMP17 particles. **b** MMP17 particles and separate tails. **c**, **e** Close view of MMP17. **d** Adsorption of MMP17 on cell debris

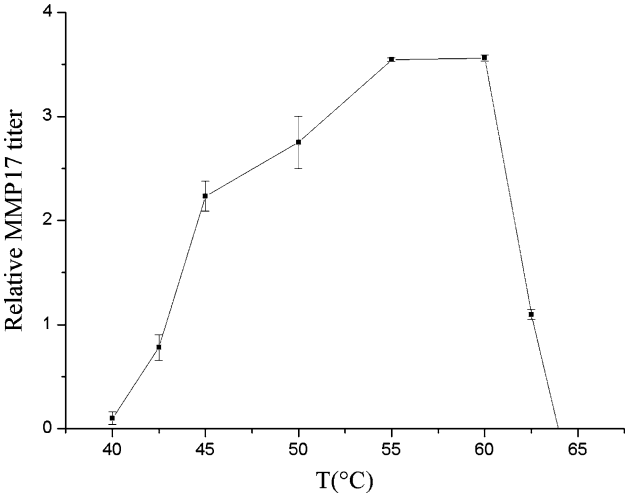

Fig. 2 Effect of temperature on phage production. Relative MMP17 titer: Log P/P_0 is plotted, P_0 being the MMP17 initial titer and P being the mean titer from triplicate assays at different temperature

estimated size about 33.5–39.5 kb. Purified phage particles were subjected to analysis by SDS-PAGE (Fig. 7). Four major bands with estimated molecular masses of 50, 40, 31

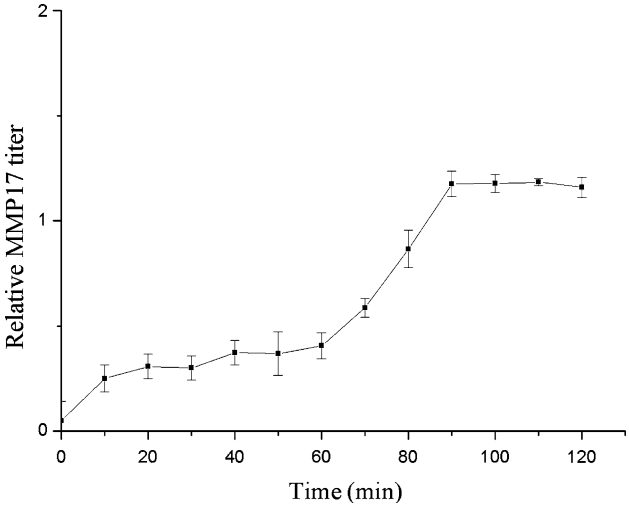

Fig. 3 One-step growth curve of MMP17 relative MMP17 titer: Log P/P_0 is plotted, P_0 being the initial titer of infected TG17 cells and P being the mean titer from triplicate assays at time t

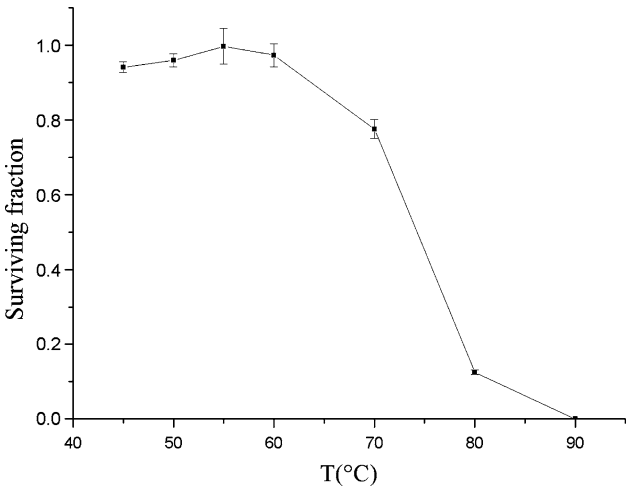

Fig. 4 Thermostability of MM17 surviving fraction: P/P_0 is plotted, P_0 being the MMP17 initial titer and P being the mean titer from triplicate assays after incubation for 30 min at different temperature

and 26 kDa, respectively, were found. Among them, the 31-kDa protein was most abundant.

Thermus phages have been isolated from hot springs in Iceland, Russia, Japan, USA and China (Sakaki and Oshima 1975; Yu et al. 2006; Lin et al. 2010). These phages belonged to *Myoviridae*, *Siphoviridae*, *Tectiviridae*, and *Inoviridae* families. The genomes of *Thermus* bacteriophages *Myophage* ΦYS40 (Naryshkina et al. 2006), *Siphoviruses* P23-45, P74-26 (Minakhin et al. 2008) and phage IN93 (Matsushita and Yanase 2009) have been sequenced. As myovirus, MMP17 also had an icosahedral head and a tail, but it was significantly different from *Thermus* myoviruses (Table 1) by its capsid and genomic size, tail length, major protein bands and growth temperature range.

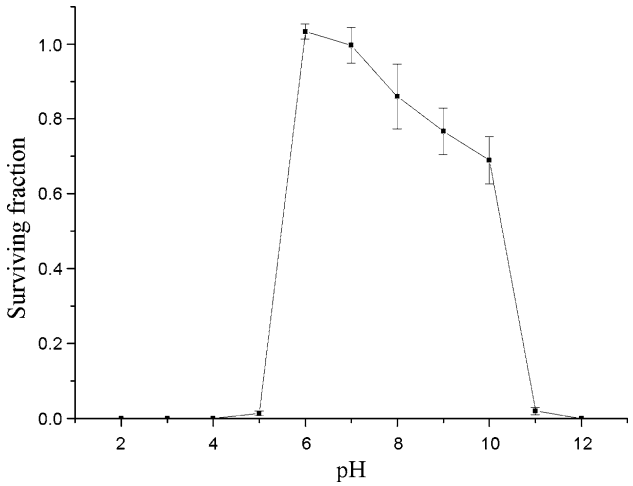

Fig. 5 pH sensitivity of MM17 surviving fraction: P/P_0 is plotted, P_0 being the MMP17 initial titer and P being the mean titer from triplicate assays after incubation for 30 min at different pH

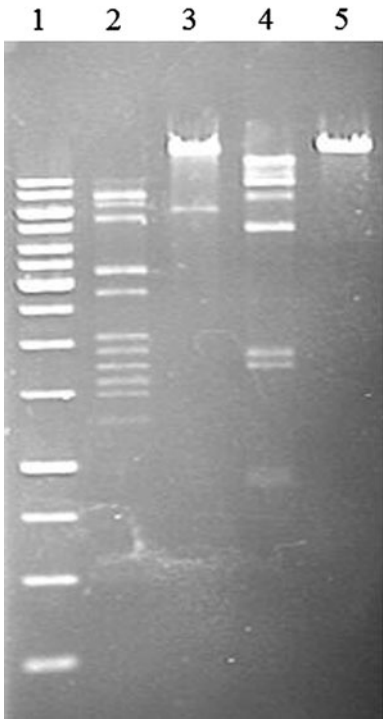

Fig. 6 Patterns of restriction endonuclease digestion of MMP17 DNA. *Lane 1* Size markers (from *top* to *bottom*: 10, 8, 6, 5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.75, 0.5 and 0.25 kb). *Lane 2* MMP17 DNA digested with *Apa*I. *Lane 3*: MMP17 DNA digested with *Eco*RI. *Lane 4* MMP17 DNA digested with *Pst*I. *Lane 5* MMP17 DNA

The production temperature range of MMP17 was from 45 to 65°C, and growth temperature range of MMP17 host cell *Meiothermus* TG17 was from 42 to 68°C. The lower phage production temperature than that of the *Thermus* phages might be related to the low growth temperature of it host cell.

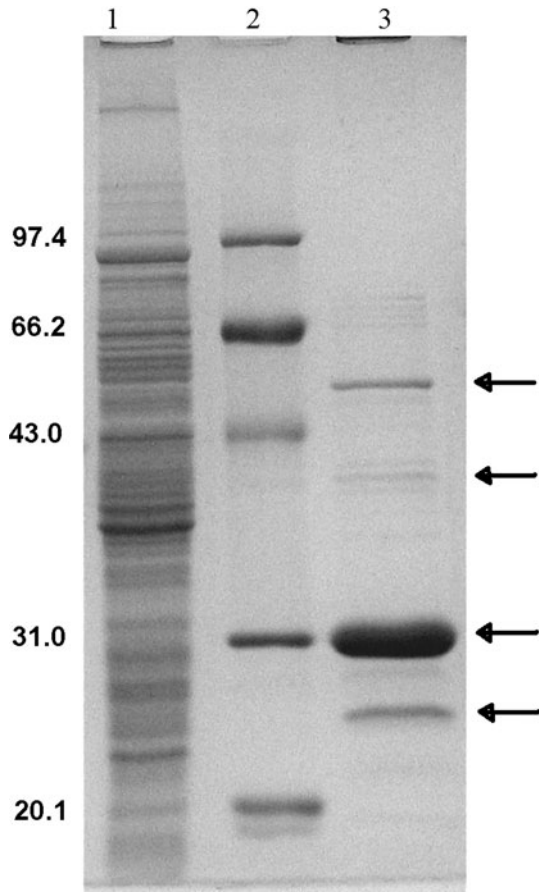

Fig. 7 SDS-PAGE of purified virions. *Lane 1* Host cell. *Lane 2* Protein marker (from *top* to *bottom*: 97.4, 66.2, 43.0, 31.0, 20.1 and 14.4 kDa). *Lane 3* Purified virions. The *arrows* showed the major viral proteins

Table 1 Characterizations of myoviruses MM17, ΦYS40 and P23-22

Phage	MMP17	ΦYS40	P23-22
Host	*Meiothermus* TG17	*T. thermophilus* HB8	*T. flavus* ATCC 33923
			Thermus sp. ATCC 27978
Capsid (nm)	42	125	62
Tail	120 nm × 17 nm	178 nm × 27 nm	135 nm × 18 nm
Size of genome	33.5–39.5 kb	152,372 bp	28–34 kb
The major protein bands	About 31 kDa	About 50 kDa	
		37–50 kDa	
Growth temperature (°C)	42.5–62.5	55–78	70
Optimum growth temperature (°C)	55–60	65	

As Thermophiles, species of genus *Meiothermus* which showed closer relationship with the type strains of genus *Thermus* grew at lower temperature, as compared with species of other thermophilic genera. Species of the genus

Thermus and their phages have been used to study the mechanisms of the evolution and thermophilicity of Thermophiles (Cava et al. 2009). The lower growth temperature of *Meiothermus* species and their phages would contribute to explore the relationship and difference between mesophilic and thermophilic phages, providing a transitional model for the evolution of thermophilic adaptation.

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