Journal of Current Science and Technology, September - December 2023 Vol. 13 No. 3, pp. 551-563

ISSN 2630-0656 (Online)

Cite this article: Sawaengwong, T., Sunthornthummas, S., Surachat, K., Atithep, T., Rangsiruji, A., Sarawaneeyaruk, S., & Pringsulaka, O. (2023). Isolation and Characterization of Lytic Bacteriophages against *Aeromonas dhakensis* Isolated from Water in Thailand. *Journal of Current Science and Technology*, *13*(3), 551-563. DOI: https://doi.org/10.59796/jcst.V13N3.2023.932

Journal of Current Science and Technology

Journal homepage: https://jcst.rsu.ac.th

Isolation and Characterization of Lytic Bacteriophages against *Aeromonas dhakensis* Isolated from Water in Thailand

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Submitted 4 March, 2023; Revised 10 May, 2023; Accepted 20 May, 2023;
Published online 30 August, 2023

Abstract

Phage therapy has emerged as a potential solution to the issue of multidrug-resistant bacteria. In this study, a novel bacteriophage vB_AdhS_M4 , which infects *Aeromonas dhakensis*, was isolated. *A. dhakensis* strains were isolated from water samples in Thailand and identified by biochemical and 16S rDNA sequence analysis. Four isolates, namely AM, NGP8, AK3, and SBKN4 were identified as *A. dhakensis* and used as hosts for phage isolation. Only 1 phage, vB_AdhS_M4, was obtained using *A. dhakensis* AM as host. Phage vB_AdhS_M4 morphology, host range, one-step growth curve, pH and temperature stability and genome were investigated. Transmission electron microscopy revealed that vB_AdhS_M4 has an icosahedral head of 64.8 ± 0.51 nm and a long tail of 185.4 ± 0.48 nm, suggesting that it belongs to the *Siphoviridae* family. Phage vB_AdhS_M4 had a latent period of 50 mins and a mean burst size of approximately 48 phage particles per infected cell. Our study on host-range determination demonstrated that this phage was specific for infecting *A. dhakensis*. Phage vB_AdhS_M4 was stable within a pH range of 4-7 and at temperatures ranging from 4 to 45 °C. The complete genome of *Aeromonas* phage vB_AdhS_M4 is 61,429 bp in length, with a G+C content of 61.7% and 77 open reading frames. *In vitro A. dhakensis* growth inhibition were observed with vB_AdhS_M4 at various MOIs. The greatest reduction in cell count occurred during the first 6-12 h of incubation with the phage, compared to the uninfected bacterial control, at all MOIs tested. Although the bacteria regrew after 24 h of incubation with the phage, the bacterial count remained lower than that of the control throughout the entire 48-h period. Based on these findings, it appears that vB_AdhS_M4 could be a valuable tool for biocontrol of *A. dhakensis* in aquaculture.

***Keywords*:** *Aeromonas dhakensis; bacteriophage; biocontrol; lytic bacteriophages*

1. Introduction

Aeromonas dhakensis (previously named *A. aquariorum*) is a Gram-negative bacterium that belongs to the *Aeromonas* genus. It was first isolated from patients with diarrhea in Dhaka, Bangladesh (India) during 1993–1994 (Huys et al., 2002; Kühn et al., 1997). Since then, *A. dhakensis* has been identified in various environments, including soil, water, and food, as well as in clinical samples from both humans and animals (Esteve et al., 2012). *A. dhakensis* is often clinically misidentified as *A. hydrophila* by phenotypic methods (Figueras et al., 2009). Both extensive biochemical tests and molecular methods can be employed to accurately identify and differentiate *A. dhakensis* from other clinical aeromonads, such as *A. hydrophila* subsp. *hydrophila, A. caviae, A. veronii* biovars *veronii* and *sobria, A. trota, A. schubertii,* and *A. jandaei* (Sinha et al., 2004; Chen et al., 2016). Previous research indicates that *A. dhakensis* is the most virulent species of *Aeromonas* in tropical and subtropical regions (Pu et al., 2019). It can cause a range of infections in humans, including gastroenteritis, wound infections, and septicemia. *A. dhakensis* is also known to be resistant to various antibiotics (Chen et al., 2016), which can make treatment of infections more challenging .

Bacteriophages (phages) are viruses that specifically infect and kill bacterial cells. Phages have been studied as a potential alternative to antibiotics for controlling bacterial infections, including those caused by *Aeromonas* species. Bacteriophages that infect *Aeromonas* species have been isolated and characterized from various sources, such as sewage, freshwater, and fish farms. Previous studies have demonstrated that *Aeromonas* phages are effective in reducing the number of *Aeromonas* in both laboratory and field settings, and can be used for prophylaxis or treatment of *Aeromonas* infections in aquatic animals. However, to date no studies have reported on the isolation of phage against *A. dhakensis* and its efficacy in controlling this species. Therefore, this study represents the first report on the isolation and characterization of lytic bacteriophages specific to *A. dhakensis*, and their effectiveness in controlling *A. dhakensis* at the laboratory level.

2. Objectives

The objectives of this study were to isolate and characterize lytic bacteriophages specific to *A. dhakensis*, as well as to assess their ability to inhibit *A. dhakensis* at the laboratory level.

3. Materials and methods

3.1 Isolation and identification of *Aeromonas* spp.

Aeromonas spp. strains were isolated from water samples collected from the river and canal in Bangkok, Thailand, Approximately 1 liter of water was collected from a depth of 0.1-1.0 meters below the water surface, using a sterile glass bottle. Serial dilution was then performed, and the diluted samples were spread onto *Aeromonas* isolation base medium supplemented with ampicillin (Himedia, India) to facilitate the isolation of *Aeromonas* strains. Colonies that appeared dark green, opaque, and with a dark center, resembling *Aeromonas* sp., were selected and restreaked on fresh *Aeromonas* isolation medium until a pure culture was obtained. Gram staining, nitrate reduction test, glucose OF (oxidation-fermentation) test, oxidase and catalase test were performed on the pure isolates according to Bergey's Manual of Systematic Bacteriology (Martin-Carnahan & Joseph, 2005). Other biochemical tests were employed to differentiate between the different *Aeromonas* genera. L-arabinose fermentation was used to distinguish between *A. hydrophila* and *A. dhakensis* , while salicin fermentation allowed differentiation between *A. hydrophila* and *A. dhakensis* from *A. hydrophila* subsp. *ranae* (Beaz-Hidalgo et al., 2013). Additionally, a hemolysis test was conducted on Columbia agar supplemented with sheep blood. To confirm at the species level, further identification was carried out using 16S rDNA sequence analysis. DNA was extracted using the method described by Sambrook et al. (1989) and the extracted DNA was used as a template for polymerase chain reaction (PCR) to amplify the 16S rDNA region of the bacteria using universal primers with the following base sequences: Forward primer (27F): 5' AGAGTTTGA TC (A/C)TGGCTCAG 3', Reverse primer (1492R): 5'TACGG(C/T)TACCTTGTTACGAC TT 3' (Lane, 1991). PCR amplifications were carried out in a Thermal Cycler Gradient TC1000-S (Scilogex, USA). The PCR product was subjected to agarose gel electrophoresis for analysis, followed by purification using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany). The purified product was then sent to Apical Scientific in Malaysia for sequencing. The obtained sequence data were compared with 16S rDNA sequences in the National Center for Biotechnology Information

(NCBI) GenBank database using the Blastn program (http://www.ncbi.nlm.nih.gov/BLAST) for further analysis.

3.2 Bacteriophage isolation

The isolated *A. dhakensis* was cultured in 25 mL of double-strength NB in a 250-mL Erlenmeyer flask for 4-6 h. Then, 25 mL of canal and fishpond samples were added and further incubated at 37°C for 18-24 h. The sample was then centrifuged at 12,000 ×*g* for 15 mins at 4 °C, and the supernatant or phage lysate was filtered through a 0.45 µm membrane filter. The presence of phage was then determined by performing a double-layer agar assay (Adams, 1959). The presence of a clear zone or plaque was purified by transferring it to 300 µL of NB medium containing early exponential phase and incubating at 37°C overnight. This step was repeated at least three times for purification. To prepare phage stocks, the purified phages were propagated in NB medium supplemented with 30 mM $CaCl_2$ (NB-Ca), and stored in a dark bottle at 4°C.

3.3 Electron microscopy

Transmission electron microscopy (TEM) was used to examine the phage morphology. Grid preparation and staining followed the protocol of Pringsulaka et al. (2011). Phage morphology was examined using a TECNAI 20 TWIN transmission electron microscope at 120 kV.

3.4 Host-range determination

One hundred microliters of *A. dhakensis* suspension and reference strains of *Aeromonas* spp. including *A. hydrophila* DMST 2798, *A. hydrophila* DMST 21250, *A. hydrophila* DMST 25194, *A. hydrophila* TISTR 1321, *A. caviae* DMST 25498, *A. sobria* DMST 25185, *A. sobria* DMST 12440, *A. trota* ATCC 49657, and *A. veronii* ATCC 35624 were inoculated into liquefied NB soft agar (NB broth with 0.5% agar). Subsequently, 10 microliters of the isolated phage were spotted onto the surface of the agar previously inoculated with the bacteria, and the mixture was incubated overnight at 37 °C. The clear zone indicating phage lysis was observed at the site where the phage was added.

3.5 One-step growth curve experiments

One-step growth curve experiment was conducted following the method of Sunthornthummas et al. (2017). One milliliter of the sample was collected at 5-min intervals until 70 mins and filtered through a 0.45 µm membrane filter. The phage titer was determined using the double-layer agar method, and the latent period, and burst size were calculated from the resulting one-step growth curve, following the method described by Adams (1959).

3.6 Determination of optimal multiplicity of infection (MOI) of phage

A. dkakensis was cultured in NB medium at 37°C for 18-24 h. Cell suspension with an OD_{600} of 0.4 (equivalent to 1 x 10^8 CFU/mL) was added to a 25 mL centrifuge tube containing 8 mL of NB medium. Then, 1 mL of phage was added to each tube to achieve a MOI of 0.01, 0.1, 1, and 10. A control tube without host cells was also prepared. The tubes were incubated at 37°C for 2 h and then centrifuged at 12,000 ×*g* for 15 mins. The supernatant was filtered through a 0.45 µm membrane filter and the phage titer was determined using the double-layer agar method (Wongyoo et al, 2023).

3.7 Effects of pH and temperature on phage stability

The stability of the selected phage was evaluated under different pH and temperature conditions (Wongyoo et al., 2023). To determine the pH stability, the pH was adjusted to 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 in 5 mL of NB medium using either 1 M hydrochloric acid (HCl) or 1 M sodium hydroxide (NaOH). A concentration of 10^7 PFU/mL of the phage was added and incubated for 1 h at 37 °C, with NB medium at pH 7 used as a control. For the temperature stability test, the phage was incubated at different temperatures of 4, 25, 37, 45, 65, and 75°C, at a concentration of 10^7 PFU/mL, for 1 h. After incubation, the phage titer was determined using the double-layer agar method.

3.8 Whole genome analyses

The genomic DNA of the phage was extracted using the phenol-chloroform method as described by Sambrook et al.(1989). The purified DNA was then sent to the Beijing Genomics Institute (BGI) in China for short-read sequencing. Once the whole genome sequence data of the phage was constructed using SPAdes 3.12 (Bankevich et al., 2012), the quality of the base sequence was assessed using the FastQC program and trimmed using Trimmomatic 0.39 (Bolger et al., 2014). Open reading frames (ORFs) were identified using

Prokka v1.14 (Seemann, 2014). The amino acid sequence was then compared to the National Center for Biotechnology Information: GenBank database using the Blastp program (E-value cutoff = 10^{-3}) to search for genes related to the lysogenic life cycle (integrase gene, excisionase gene, and CI repressor gene), toxin-related genes, antibiotic resistance genes, and genes of virulent factor. The transfer RNA genes were identified using tRNAscan-SE (Chan & Lowe, 2019). Finally, the genome of the phage was visualized using the CG-view server (Grant & Stothard, 2008).

3.9 Phylogenetic analyses

BLASTP was used to search for amino acid sequences of the terminase large subunit of vB_AdhS_M4 in the NCBI nr database, and then used the ClustalW program in MEGA 11.0 software to align the sequences and construct phylogenetic trees based on the aligned sequences using the neighbor-joining method and 1,000 bootstrap replications.

3.10 Effect of single phage in pre- and post-treatment to control *A. dhakensis* AM growth

The inhibition of *A. dkakensis* by phage in laboratory level was carried out at various MOIs (10, 1, 0.1, 0.01, 0.001, and 0.0001). The experiment was divided into two sets: a pre-treatment experiment and a post-treatment experiment. In the pre-treatment, *A. dhakensis* suspension with an OD_{600} of 0.4 was incubated with phage at the above MOIs at 37 °C and 150 rpm. In the post-treatment experiment, *A. dhakensis* suspension with an OD_{600} of 0.4 was incubated at 37 °C and 150 rpm for 3 h, and then phage was added to achieve MOIs of 10, 1, 0.1, 0.01, 0.001, and 0.0001. The samples in each sets were collected at 0, 6, 12, 24, and 48 h and subsequently centrifuged at 12,000 ×*g* for 10 mins, with the supernatant being filtered through a 0.45 µm membrane filter. Two control samples, the bacterial control and the phage control, were included for each MOI. The bacterial control sample did not contain any phages, while the phage control sample did not contain any bacteria. These control samples were incubated alongside the experimental samples. Bacterial titer was counted in NA medium and expressed as CFU/mL, and phage titer was determined by the double-layer agar method.

3.11 Statistical analysis

Each experiment was conducted in triplicate, and the data were presented as mean ± SD. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 20. One-way ANOVA with Tukey's post-hoc test was used to determine statistically significant differences among groups, with statistical significance set at $P \leq 0.05$.

4. Results and discussion

4.1 Isolation and identification of *Aeromonas* spp.

A total of 78 *Aeromonas* isolates were obtained from water samples using *Aeromonas* isolation medium. Biochemical tests were conducted to further differentiate the isolates. Only six isolates (AM, NGP8, TP3, KSS5, AK3, and SBKN4) were identified as *A. dhakensis* based on the test results shown in Table 1. Based on 16S rDNA sequence analysis, 4 isolates, namely AM, NGP8, AK3, and SBKN4 were identified *A. dhakensis* with similarity values of 99% (Table 2). All of the 4 strains were used as a host for the isolation of the phage.

4.2 Phage isolation and morphology

Only 1 phage, namely vB_AdhS_M4 was obtained when A. dhakensis AM was used as the host. This phage produced clear plaque with a diameter of 0.1 ± 0.02 cm (Figure 1). Transmission electron micrograph revealed that vB_AdhS_M4 is a member of the Casjensviridae family, possessing a head with a diameter of 64.8 ± 0.51 nm and a long tail with a length of 185.4 ± 0.48 nm (Figure 1). Phages that infect Aeromonas spp. are classified within the Order Caudovirales and exhibit a diverse range of morphologies. Most Aeromonas phages have been classified within the Myoviridae family, with several others falling under the Siphoviridae family. Examples of Aeromonas phages belonging to the Siphoviridae family include A. hydrophila phage Akh-2 (Akmal et al., 2020) A. hydrophila phage Lah7 (Kabwe et al., 2020), A. hydrophila phages AhSzq-1 and AhSzw-1 (Yuan et al., 2018), A. hydrophila 4L372X, A. salmonicida AsXd-1, and Aeromonas SD04, pIS4-A (Bai et al., 2019).

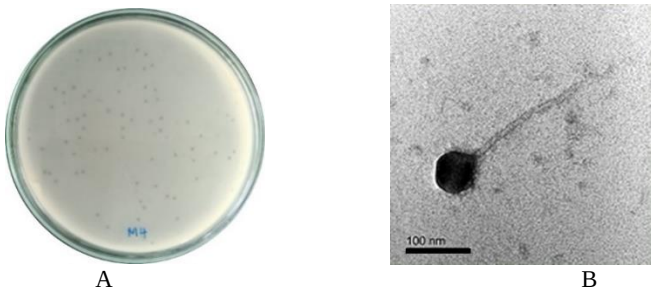
Table 1 Biochemical tests of the isolated *A. dhakensis*

Biochemical tests	***A. dhakensis* isolates**					
	AM	**AK3**	**NGP8**	**TP3**	**KSS5**	**SBKN4**
Indole	+	+	+	+	+	+
Methyl red	+	+	+	+	+	+
Voges-proskauer	+	+	+	+	+	+
Citrate	+	+	+	+	+	+
Hemolysis	β	β	β	β	β	β
Deoxyribonuclease	+	+	+	+	+	+
Gelatinase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Triple sugar iron (TSI) (acid/alkali)	A/A	A/A	A/A	A/A	A/A	A/A
Oxidative/fermentation glucose	F	F	F	F	F	F
Lactose	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+
L-arabinose	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+
Salicin	+	+	+	+	+	+

+ represents positive, - represents negative, and F represents fermentation. β represents β-hemolysis (complete hemolysis), A/A represents Acid/Acid.

Table 2 16S rDNA-based identification of *Aeromonas* isolates

***Aeromonas* spp.**	**Identification results**	**Blast results**	**GenBank accession number**	**% similarity**
AM	*A. dhakensis*	*A. dhakensis* strain P21	NR_042155.1	99.93
NGP8	*A. dhakensis*	*A. dhakensis* strain P21	NR_042155.1	99.37
KSS5	*Aeromonas* sp.	*A. dhakensis* strain P21	NR_042155.1	96.55
AK3	*A. dhakensis*	*A. dhakensis* strain P21	NR_042155.1	99.63
SBKN4	*A. dhakensis*	*A. dhakensis* strain P21	NR_042155.1	99.85



A B

Figure 1 Morphological characterization of phage vB_AdhS_M4 . (A) Plaque morphology analysis was performed using the double overlay method. (B) Phage morphology was observed by transmission electron microscopy. Scale bar = 100 nm.

4.3 Host-range determination and phage stability

Host-range determination revealed that vB_AdhS_M4 was able to infect only *A. dhakensis* AM. Most of *Aeromonas* phages have a narrow host range, for example, phage AhyVDH1 (Cheng et al., 2021), Ahp1 (Wang et al., 2016). The phage was shown to be stable within a pH range of 4-7 and at temperatures ranging from 4 to 45°C. However, exposure to temperatures above 65°C resulted in a decrease of approximately 4 log PFU/mL in its titer, and complete inactivation occurred at temperatures above 75°C (Figure 2).

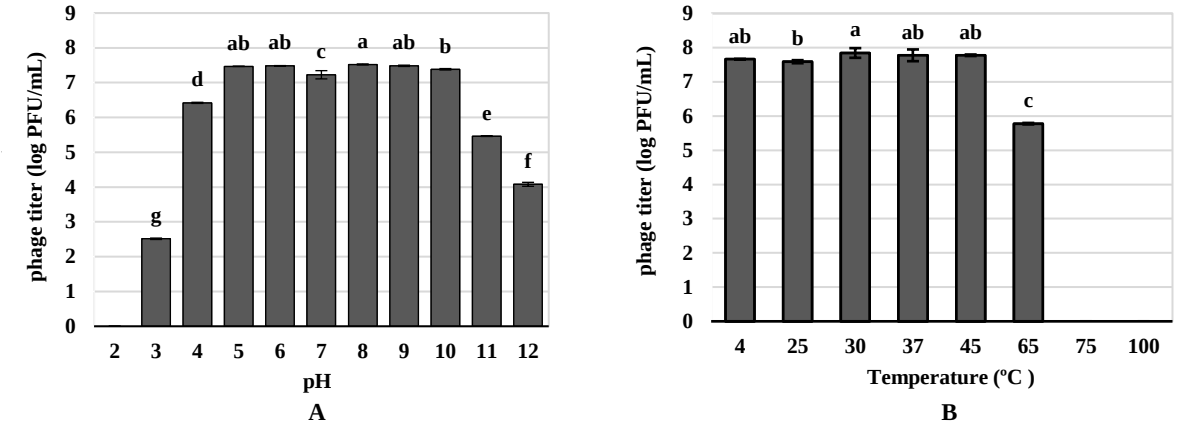


Figure 2 pH stability) A (and thermal stability (B) of phage vB_AdhS_M4 .Values are means of 3 readings with ± SD . Nutrient broth without adjusted were use as a control. pH and thermal stability were analyzed by one-way ANOVA. The means of the triplicate for each incubation were compared by Tukey's test ($p \leq 0.05$).

4.4 Optimal multiplicity of infection (MOI) and one-step growth curve

The optimal MOIs of phage vB_AdhS_M4, which produced the highest titers, was 10, resulting in a maximum titer of 8.85 ± 0.25 log PFU/mL (Table 2). A one-step growth curve of vB_AdhS_M4 showed a latent period of 50 mins and an average burst size of approximately 144 phage particles per infected cell (Figure 3). The latent period of this phage is within the range of 50 mins for phage AhSzq-1 and 60 mins for phage AhSzw-1 but shorter than phage AhSzq-1, which has a latent period of 10 mins. In terms of burst size, phage vB_AdhS_M4 has a similar burst size to phage AhSzq-1, with a value of 45 PFU/infected cell (Yuan et al., 2018).

Table 2 The optimal MOI of phage vB_AdhS_M4

Bacteria (log_{10} CFU/mL)	Phages (log_{10} PFU/mL)	MOI	Phage titer after 2 h (log_{10} PFU/mL)
8	9	10	8.85±0.25
8	8	1	7.38±0.06
8	7	0.1	6.17±0.03
8	6	0.01	5.73±0.06

Data are expressed as average values ± standard deviation (SD) with three replicates.

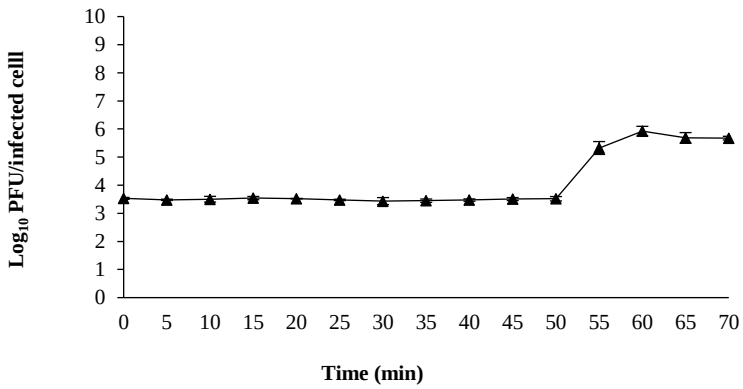


Figure 3 One-step growth curve of ΦTIS1. Data are the mean of triplicate independent experiments with standard deviation.

4.5 Whole-genome sequencing of phages and *in silico* analysis

The complete genome of *Aeromonas* phage vB_AdhS_M4 is 61,429 bp in length, with a G+C content of 61.7% (Figure 4). Upon BLASTn analysis, it was found that this phage shares the highest similarity (96.79%) with *Aeromonas* phage BUCT551 (Accession no. NC_052986.1). The genome was further analyzed for open reading frames (ORFs), and a total of 77 putative ORFs were identified, with 51 on the direct strand and 26 on the complementary strand. Among these, 6 ORFs start with GTG, and 71 ORFs start with ATG. Upon performing BLASTp analysis, it was revealed that 29 proteins in the genome could be annotated with predicted functions, whereas the remaining 46 had unknown functions. Based on their functions, the annotated proteins were classified into three groups, namely phage structure and DNA packaging (ORFs 10, 11, 12, 13, 14, 15, 16, 20, 22, 24, 25, 26, and 27), DNA metabolism and replication (ORFs 1, 2, 6, 7, 8, 9, 39, 41, 43, 44, 46, 50, 53, 58, and 77), and host lysis (ORF 33). No tRNA encoding genes, integrase genes, or virulence genes were found in the genome of vB_AdhS_M4. Phage vB_AdhS_M4 was found to have similar genome characteristics to siphophage LAh7, including a genome size of 61,426 bp with a GC content of 61.90% and 75 ORFs, as reported by Kabwe et al. (2020). However, the genome size of vB_AdhS_M4 is smaller than that of phage AhSzq-1 and AhSzw-1, which have a genome size of 112,558 and 115,739 bp, respectively, and a G+C content of 43.86% and 43.82%, respectively (Yuan et al., 2018).

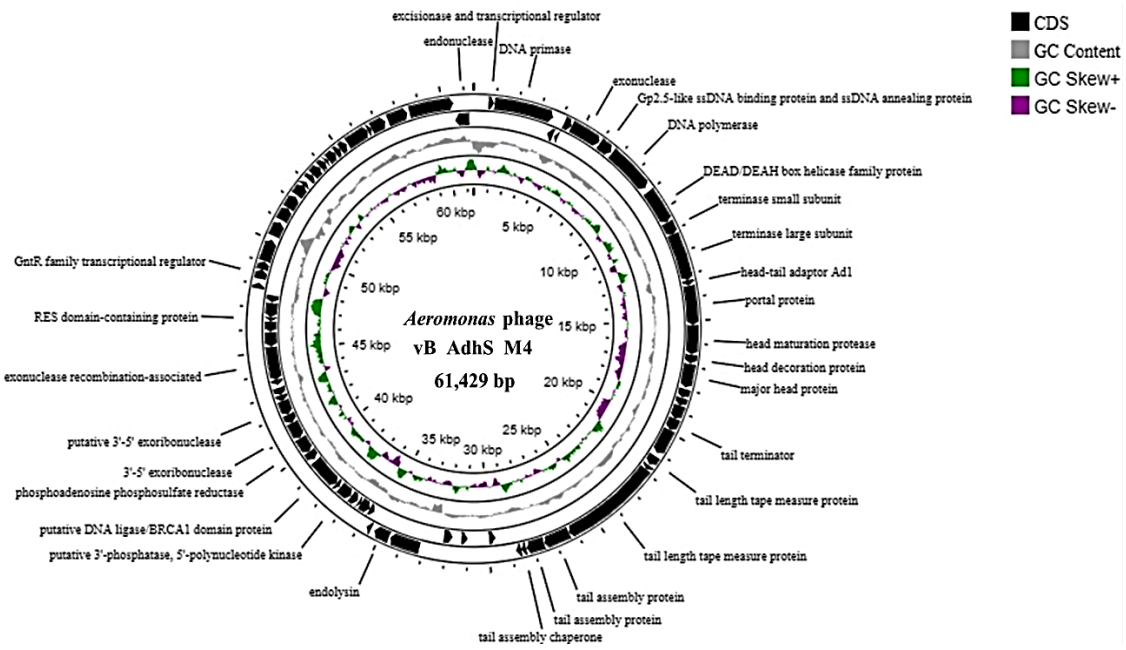


Figure 4 The circular representation of the vB_AdhS_M4 genome shows a diagram with four circles, where each circle represents different information. Starting from the outside to the center, the circles display the following: Circle 1 - coding regions (CDS) on the forward strand, Circle 2 - coding regions (CDS) on the reverse strand, Circle 3 - GC content, and Circle 4 - GC skew. In Circle 4, G + C content is represented in green, while G-C content is represented in purple.

Table 3 The ORFs of putative functional protein in phage vB_AdhS_M4 genome and best similarity with databases

ORF	Position		Strand	Predicted function	E-value	Identity (%)	GenBank ID
	Start	Stop					
1	672	914	+	excisionase and transcriptional regulator	4.00×10^{-52}	100.00	YP_009998390.1
2	933	3614	+	DNA primase	0.00	99.66	YP_009998389.1
6	4529	5938	+	exonuclease	0.00	98.93	YP_009998386.1
7	5989	6618	+	Gp2.5-like ssDNA binding protein and ssDNA annealing protein	3.00×10^{-146}	98.09	YP_009998385.1
8	6679	8787	+	DNA polymerase	0.00	99.15	YP_009998384.1
9	9003	10574	+	DEAD/DEAH box helicase family protein	0.00	98.85	UIS24818.1
10	10583	11182	+	terminase small subunit	1.00×10^{-147}	100.00	YP_009998381.1
11	11,148	13,250	+	terminase large subunit	0.00	99.71	YP_009998380.1
12	13,247	13,486	+	head-tail adaptor Ad1	7.00×10^{-51}	100.00	YP_009998379.1
13	13,486	15,201	+	portal protein	0.00	100.00	YP_009998378.1
14	15,198	16,481	+	head maturation protease	0.00	100.00	YP_009998377.1
15	16,484	16,873	+	head decoration protein	2.00×10^{-88}	99.22	YP_009998376.1
16	16,889	17,929	+	major head protein	0.00	99.42	YP_009998375.1
20	19,348	19,884	+	tail terminator	2.00×10^{-126}	99.44	YP_009998371.1
22	21,190	21,639	+	tail length tape measure protein	1.00×10^{-100}	99.33	YP_009998369.1
24	21,889	26,325	+	tail length tape measure protein	0.00	95.94	YP_009998367.1
25	26,325	27,518	+	tail assembly protein	0.00	88.66	YP_009998366.1
26	27,515	28,336	+	tail assembly protein	0.00	99.27	YP_009998365.1
27	28,346	28,570	+	tail assembly chaperone	1.00×10^{-45}	98.65	YP_009998364.1
33	34,594	35,301	+	endolysin	3.00×10^{-161}	96.17	YP_009998361.1
39	37,053	37,700	-	putative 3'-phosphatase, 5'-polynucleotide kinase	7.00×10^{-158}	99.53	YP_009998355.1
41	37,968	39,413	-	putative DNA ligase/BRCA1 domain protein	0.00	84.95	YP_009998353.1
43	39,951	40,712	-	phosphoadenosine phosphosulfate reductase	0.00	100.00	YP_009998351.1
44	40,726	41,523	-	3'-5' exoribonuclease	0.00	95.09	YP_009998350.1
46	41,959	42,639	-	putative 3'-5' exoribonuclease	1.00×10^{-162}	97.35	YP_009998348.1
50	43,660	45,222	-	exonuclease recombination-associated	0.00	89.66	YP_009998344.1
53	46,401	46,616	-	RES domain-containing protein	3.00E-45	100.00	YP_009998341.1
58	48,656	49,030	+	GntR family transcriptional regulator	8.00E-86	99.19	YP_009998336.1
77	60,559	61,215	+	endonuclease	4.00E-149	96.79	YP_009998317.1

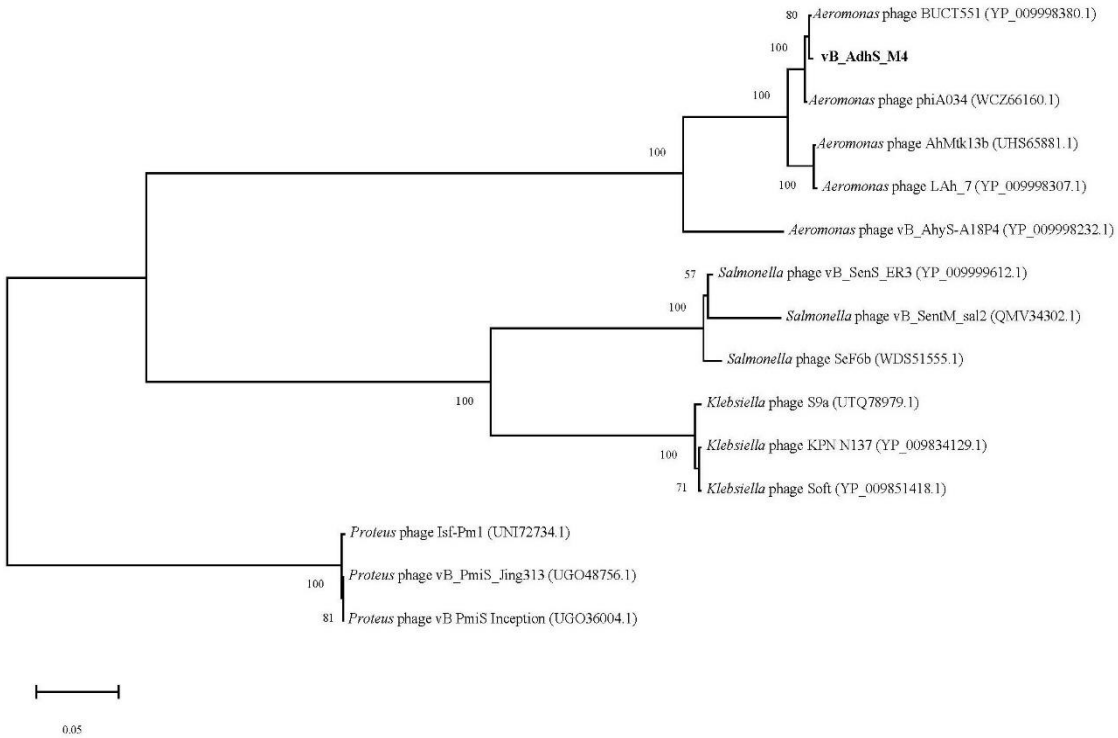


Figure 5 Phylogenetic tree based on the amino acid sequence of the terminase large subunit of phage vB_AdhS_M4. The tree was constructed using MEGA11 and the neighbour-joining method with 1,000 bootstrap replicates. The bootstrap values are shown at the nodes of the tree.

4.6 Phylogenetic analysis

Structure proteins, including terminase, are frequently used for phages taxonomy. These proteins are conserved among phages and provide insights into their evolutionary relationships. The larger subunit of the terminase holoenzyme facilitates the translocation of the cleaved DNA into the empty prohead during phage packaging (Ding et al., 2020). Through the alignment of the large terminase subunit of vB_AdhS_M4 with sequences of closely related phages from the NCBI database and constructing phylogenetic trees based on this alignment, it was discovered that vB_AdhS_M4 is closely related to *Aeromonas* phages of BUCT551 (Figure 5).

4.7 Effect of phage in pre- and post-treatment to control *A. dhakensis* AM growth

The lytic effect of phage on the growth of *A. dhakensis* AM was evaluated at different MOIs (Figure 6, 7). Both pre-and post-treatment, the maximum cell decrease for all phages was observed during 6-12 h of incubation at all MOIs compared with the uninfected bacterial control. The pre-treatment with phages vB_AdhS_M4 reduced the maximum bacterial count by 6.85, 7.32, 7.60, 7.55 and 7.43 log CFU/mL at MOIs 10, 1, 0.1, 0.01, and 0.001, respectively, after 6 h of incubation. In post-treatment, the maximum inactivation was achieved at 6-12 h with the log reduction number of 7.07, 5.94, 7.49, 6.55 and 3.73 log CFU/mL, respectively. Moreover, the higher MOI resulted in the effective to reduce the number than low MOI. The regrowth of bacteria cultured with phages was gradually increased after 24 h in all treatments. However, compared to the bacterial control, the incubation of *A. dhakensis* with phages resulted in a lower bacterial count throughout the entire 48-h period. The phage number in all treatments increased, reaching a maximum at 6 h and gradually decreasing thereafter. The phage count in the phage control group remained unchanged during the 48-hour period across all treatments. Furthermore, post-treatment was more effective in controlling *A. dhakensis* AM than pre-treatment. Our results showed that phage vB_AdhS_M4 had the potential to control *A. dhakensis*. Although, the phage resistant mutants were observed when longer incubation, but the number was still less than the control without phage. Resistance mutations against

a single phage typically occur at a high frequency. Due to the phage's ability to kill susceptible genotypes, these resistance mutations will rapidly spread throughout the bacterial population (Wright et al., 2019; Gurney et al., 2017). As a result, phage therapy often involves the use of combinations of multiple phages. This approach is believed to limit the emergence of resistance while also targeting a wider range of bacterial genotype (Chan et al., 2013; Wright et al., 2019).

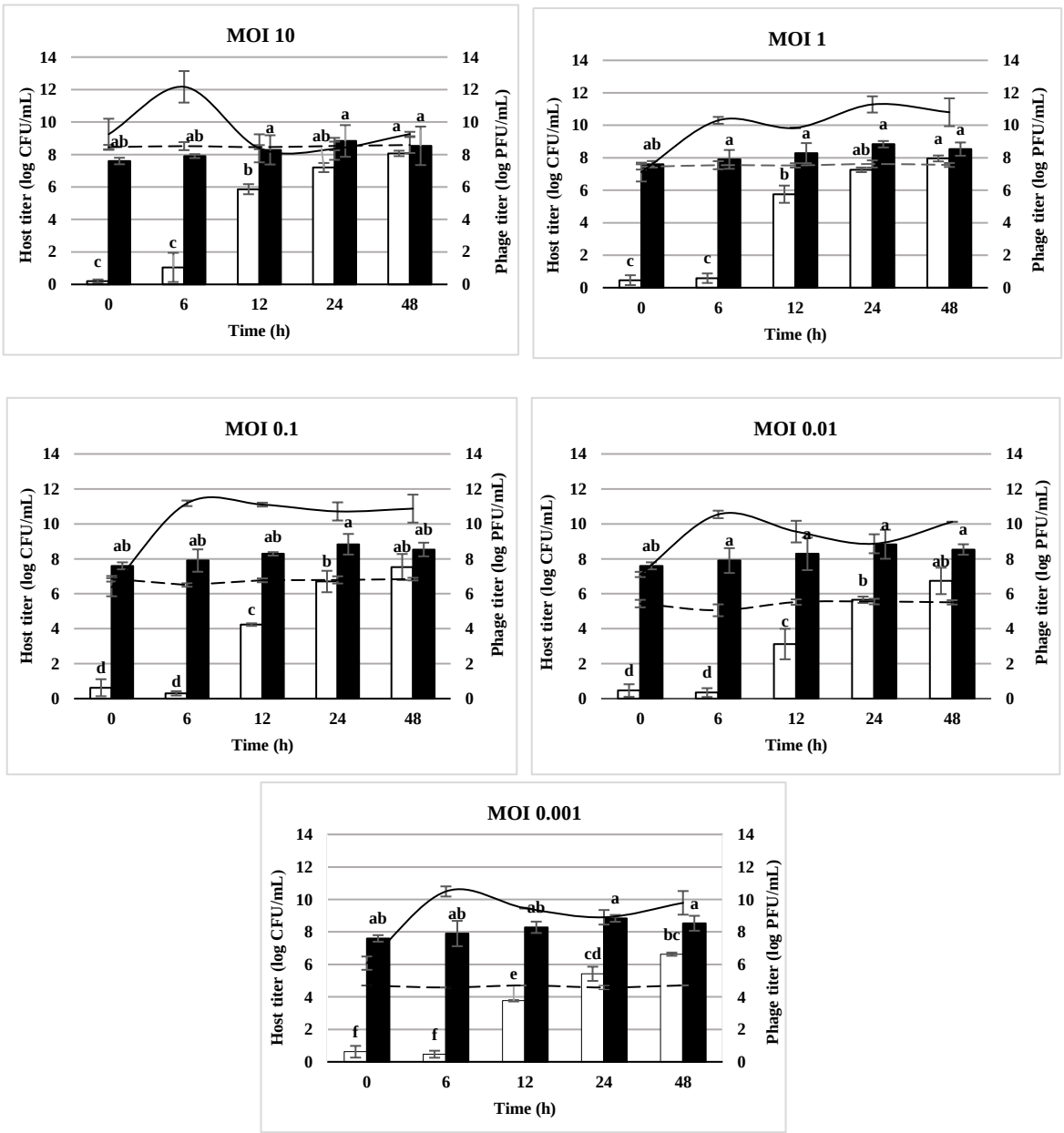


Figure 6 Inactivation of *A. dhakensis* AM by phage vB_AdhS_M4 during 48 h at different MOIs in pre-treatment. Black bars represent host titers in the host control; light bars represent host titers with phage; dashed lines represent the number of plaques counted in the control; solid lines represent the number of plaques counted in the phage treatment. Values represent the mean of three experiments; error bars represent the standard deviation. The data were analyzed using one-way ANOVA. The means of the triplicate for each incubation were compared by Tukey's test ($p \leq 0.05$).

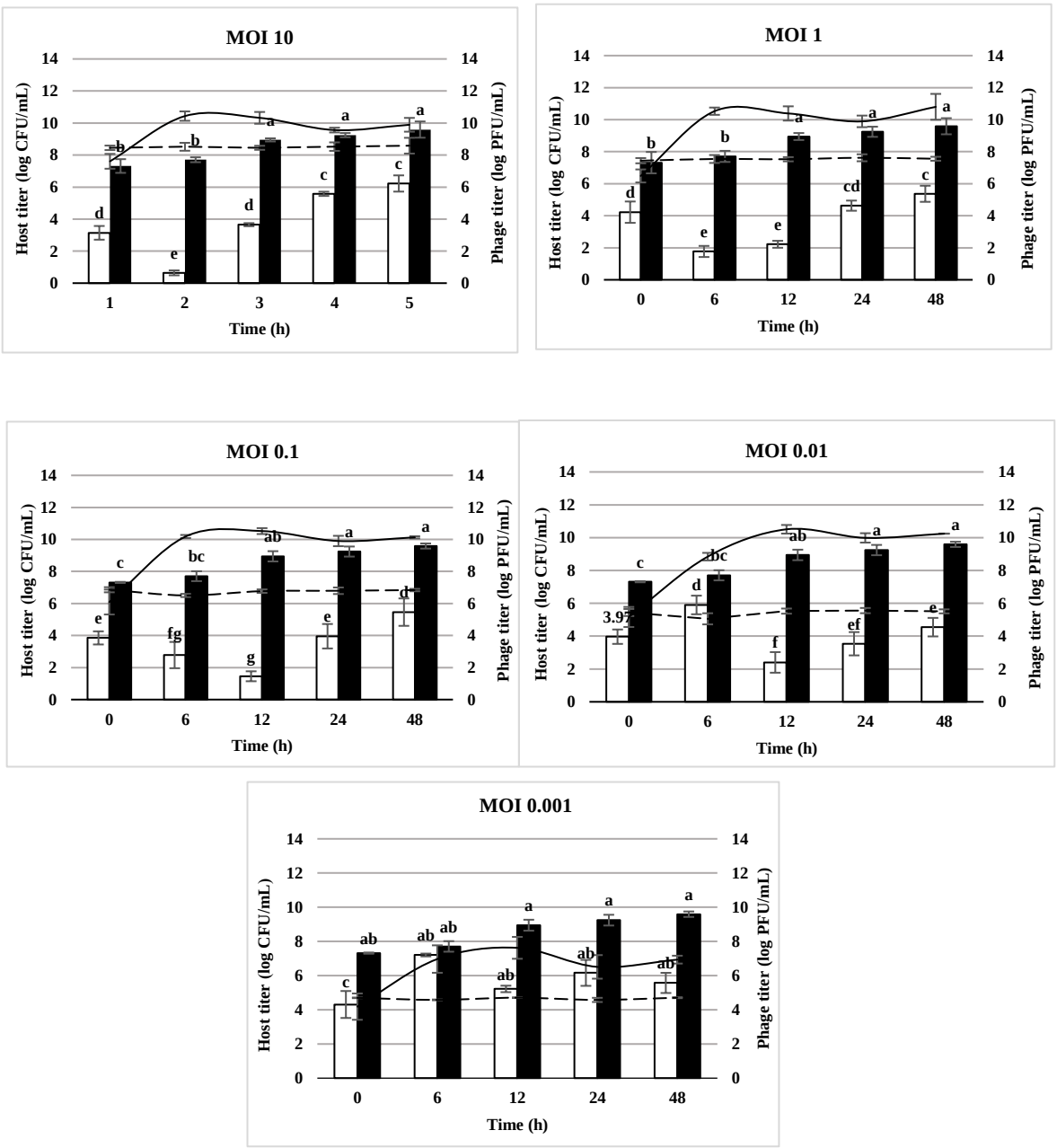


Figure 7 Inactivation of *A. dhakensis* AM by phage vB_AdhS_M4 during 48 h at different MOIs in post-treatement. Black bars represent host titers in the host control; light bars represent host titers with phage; dashed lines represent the number of plaques counted in the control; solid lines represent the number of plaques counted in the phage treatment. Values represent the mean of three experiments; error bars represent the standard deviation. The data were analyzed using one-way ANOVA. The means of the triplicate for each incubation were compared by Tukey's test ($p \leq 0.05$).

5. Conclusion

In this study, a siphophage named vB_AdhS_M4 that can infect *A. dhakensis* AM was isolated and characterized. The phage was found to be stable across a broad range of pH and temperatures, and its complete genome was 61,429 bp long with a G+C content of 61.7%. Notably, the genome lacked any genes coding for antibiotic

resistance, virulent factors, or toxins, and did not possess any necessary genes for a lysogenic cycle. These results suggest that vB_AdhS_M4 may be a promising tool for biocontrol of *A. dhakensis* in aquaculture, and could potentially be used as an alternative to antibiotics for the treatment of *A. dhakensis* infections.

6. Acknowledgement

This work was supported by an annual government statement of expenditure, Srinakharinwirot University (grant number 031/2564), and graduate school fund, Faculty of Science, Srinakarinwirot University.

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