Journal of Microbiology, Immunology and Infection 55 (2022) 634–642

Available online at www.sciencedirect.com

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Original Article

Biological and genomic characterization of two newly isolated *Elizabethkingia anophelis* bacteriophages

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Received 7 March 2022; received in revised form 21 April 2022; accepted 29 May 2022
Available online 6 June 2022

KEYWORDS
Elizabethkingia anophelis;
Bacteriophage;
Siphoviridae;
Antibiotic resistance;
Phage therapy

Abstract *Background: Elizabethkingia anophelis* is an opportunistic pathogen that infects newborns and immunocompromised patients. Because the infection is associated with high mortality as a result of its intrinsic resistance to antibiotics, alternative treatment methods are needed. Our previous study successfully isolated the world's first *E. anophelis* phage, TCUEAP1, which showed beneficial protection to *E. anophelis*-infected mice. More new bacteriophages are needed in order to provide sufficient choices to combat *E. anophelis* infections.
Methods: In the current study, two new phages infecting *E. anophelis* were isolated from wastewater and were designated as TCUEAP2 and TCUEAP3. Further experiments, namely, transmission electron microscopy (TEM), infection assay, host-range analysis, and sequencing were performed to determine their biological and genomic characteristics.
Results: TEM analysis revealed that both TCUEAP2 and TCUEAP3 possess an icosahedral head with a non-contractile tail, and belong to the *Siphoviridae* family. Further experiments revealed that TCUEAP3 has a longer latent period and higher burst size compared to TCUEAP2. Host range analysis showed that both TCUEAP2 and TCUEAP3 have a narrow host range, infecting only their respective hosts. The genomic size of phage TCUEAP2 was 42,403 bps containing 61 predicted open reading frames (ORFs), whereas the genome size of TCUEAP3 was 37,073 bps containing 40 predicted ORFs.

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https://doi.org/10.1016/j.jmii.2022.05.004

Conclusion: Due to the distinct biological characteristics of TCUEAP2 and TCUEAP3, they may be satisfactory for clinical uses such as preparation of phage cocktails or decontamination in clinical settings.

Introduction

Elizabethkingia is a genus of nonfermenting gram-negative bacilli which is widely distributed in natural environments. The genus comprises three pathogenic species including *Elizabethkingia meningoseptica*, *Elizabethkingia anophelis*, and *Elizabethkingia miricola*, which are associated with opportunistic infections, neonatal meningitis, and nosocomial outbreaks.[1—4] *E. meningoseptica* was originally thought to be the predominant species in the genus as it was most commonly isolated from immunocompromised patients and neonates. Until recently, numerous studies found that *E. anophelis* was frequently misidentified as *E. meningoseptica*, suggesting that *E. anophelis* may also be the predominant pathogen.[5—7]

Infections caused by *E. anophelis* are associated with high morbidity and mortality due to its intrinsic resistance towards antibiotics and the lack of information regarding its antibiotic resistance spectrum.[5,7,8] Previous research revealed that *E. anophelis* was highly resistant against multiple antibiotics, including β-lactams, β-lactam/lactamase inhibitors, carbapenems, and aminoglycoside.[9] Several antibiotic-resistant genes have been identified within the *E. anophelis* genomes.[9] However, the reported antibiotic susceptibility of the isolates from different countries varied. For example, *E. anophelis* isolated from Korea and Taiwan were resistant to fluoroquinolones, while isolates from Wisconsin, USA were susceptible to it.[6,9,11,12] Due to its intrinsic resistance, *E. anophelis* infections are difficult to treat.

Bacteriophage (phage) therapy has been proposed as an alternative way to deal with the problem of antibiotic resistance.[13,14] Previously, we have successfully isolated the world's first *E. anophelis* phage, TCUEAP1.[15] Intraperitoneal injection of TCUEAP1 into *E. anophelis*-infected mice effectively reduced the bacterial load in the mice with an enhanced survival rate.[15] However, due to the narrow range of phage hosts, it is necessary to find more new phages for the clinical fight against bacterial infections.[17] In this study, two new phages, TCUEAP2 and TCUEAP3, were isolated and their biological and genomic characteristics evaluated.

Materials and methods

Bacterial strains and growth conditions

The sources of the bacteria strain used in this study are listed in Supplementary Table 1. All bacteria cultures were grown in Luria Bertani (LB) broth or LB agar (BioShop Canada Inc., Burlington) at 37 °C. The growth of bacteria was monitored by measuring the optical density at 600 nm (OD_{600}), where an OD_{600} of 1.0 corresponds to 1×10^9 cells/mL.

Isolation and purification of the phages

Wastewater samples were collected from Tzu Chi University, Hualien, Taiwan and were used for phage screening. Thirty milliliters of each wastewater sample were cleared of debris and bacteria by centrifugation at 10,000×*g* for 10 min and filtered through a 0.22-μm filter. To enrich phages from wastewater samples, a one-milliliter culture of *Elizabethkingia* strains ($OD_{600} = 0.6$) was mixed with 20 mL of wastewater filtrate and incubated at 25 °C for 4 h. After enrichment, the mixtures were centrifuged at 10,000×*g* for 10 min and filtered through 0.22-μm filters. To identify the presence of phage, 10 μL of the filtrate was spotted on soft LB agar (0.6%) containing 100 μL of bacterial culture ($OD_{600} = 0.6$). Plates were incubated overnight at 25 °C and a positive plaque was picked by a sterile Pasteur pipette tip and inoculated into a new culture. The new culture was serially diluted and plated on another bacteria-containing soft agar medium for plaque purification. All phages were purified at least three times to ensure sample homogeneity.

Host range analysis

The host range of the isolated phages was determined by both the double-layer method and the spot test[15] on different *Elizabethkingia* strains listed in Supplementary Table 1. Briefly, the double-layer method was performed by serially 10-fold diluted (10^1—10^9) the phage stock, and 10 μL of each dilution were mixed with 100 μL of the bacterial culture and 5 mL of soft LB agar (0.6%) which then poured over a solid LB agar plate. Plates were incubated at 25 °C overnight. Spot test was performed by spotting 10 μL of phage lysates (10^9 PFU/mL) onto lawns of individual hosts. The plates were incubated at 25 °C overnight, and positive results were based on the detection of any lysis or plaque.

Transmission electron microscopy of the phages

A single drop of the purified phage lysate (containing 10^9 PFU/mL) was dropped on a formvar-coated grid (200-mesh copper grids), followed by negative staining with 2% uranyl acetate. The phage morphology was examined using a Hitachi H7500 transmission electron microscope (Hitachi Company, Japan) operated at an accelerating voltage of 80 kV.

Determination of optimal multiplicity of infection (MOI) for TCUEAP2 and TCUEAP3

The optimal multiplicity of infection (MOI) of the phage was determined by infecting the phage with its host. The host strain was grown in LB broth at 37 °C until it reached the early log phase (OD_{600} = 0.6). Phages at an MOI of 10, 1, 0.1, 0.01, 0.001, and 0.0001 were added and the mixtures were incubated at 37 °C for 3.5 h. After incubation, the mixtures were collected, and the phage titers were determined by the double-layer method.

Infection assay

To investigate the inhibitory effect of phage against *E. anophelis*, the growing culture of host strain (OD_{600} of 0.5) was incubated with phage stock at different MOIs (MOI 10, 1, 0.1, and 0.01) at 25 °C or 37 °C. Control experiment was performed using an equal volume of phage buffer [10 mM Tris—HCl (pH 7.5), 10 mM $MgSO_4$, 68.5 mM NaCl, and 1 mM $CaCl_2$]. Changes in bacterial culture were monitored by measuring OD_{600} at one-hour intervals for eight hours.

One-step growth curve assay

To determine the latent, eclipse, and burst period of phage, one step growth curve analysis was performed as described previously.[15] Briefly, the host strain was incubated at 37 °C until it reached an OD_{600} of 1.0. Then, 3 mL of bacterial culture was mixed with the phage at an MOI of 0.01, and allowed to adsorb for 10 min at room temperature.[18] After adsorption, the mixture was centrifuged and the pellet was re-suspended in 30 mL of fresh LB. Two samples (1 mL for each) were taken every 10 min for 90 min (for TCUEAP2) and every 15 min for 180 min (for TCUEAP3). The first sample was immediately titrated without any treatment (total phage titer), and the second sample was treated with 1% chloroform to release intracellular phages (free phage titer). Each sample was then serially diluted and plated by the double-layer method.

Adsorption assay

Bacterial cultures were prepared to reach an OD_{600} of 1.0, and LB was added to a final volume of 10 mL. Phage stock was then added at an MOI of 0.01 and incubated at room temperature. One-hundred microliter samples were taken every 5 min for 30 min. Supernatants containing the unabsorbed phages were titrated by the double-layer method. The percentage of free phages was calculated by dividing the phage titer in the supernatant to that in the initial phage stock.

Effect of temperature on phage stability

Determination of the thermal stability of the phage was performed as previously described with some modifications.[19] Briefly, 1 mL phage (10^9 PFU/mL) was incubated for one hour at six different temperatures (4 °C, 25 °C, 37 °C, 50 °C, 60 °C, and 70 °C) and phage titers were immediately determined by the double-layer method. A similar experiment was performed by incubating the phage at three different temperatures (4 °C, 25 °C, and 37 °C) for 30 days to determine the optimal condition for long-term storage.

Effect of pH and chloroform on phage stability

To determine how pH and chloroform affect phage stability, 1 mL of phage preparations (10^9 PFU/mL) were treated with various pH buffers (pH 2, 4, 7, and 11) or different concentrations of chloroform (0.5% or 2%) at 25 °C for one hour. Phage titers were then determined by the double-layer method.

Genome sequencing and analysis

Total genomic DNA of phages was extracted as previously described.[20] Purified genomic DNA was subjected to whole-genome sequencing using the Illumina Mi-seq platform. Raw sequence reads were assembled using Bowtie v1.1.1. Prediction of all open reading frames (ORFs) was performed by Prodigal[21] and annotation of predicted ORFs was carried out by the Basic Local Alignment Search Tool (BLAST).[22] The sequence data and annotation information of phage TCUEAP2 and TCUEAP3 were deposited at GenBank under accession numbers OK632025 and OK632026, respectively.

Results

Isolation of new phages infecting *E. anophelis*

Wastewater samples collected from Hualien Tzu Chi University were enriched and screened using 24 strains of *Elizabethkingia*. Two samples formed clear plaques of about 0.5—1 mm in diameter. The two phages isolated from the plaques formed on *E. anophelis* ANO14 and ANO1 were designated as TCUEAP2 and TCUEAP3 respectively. Transmission electron microscopy revealed that both virions belonged to the *Siphoviridae* family, under the order of *Caudovirales*.[23] TCUEAP2 consists of an icosahedral head with a diameter of 57.2 ± 3.3 nm and a non-contractile tail that is 197.8 ± 3.6 nm long and 10.9 ± 2.4 nm in diameter (Fig. 1A); whereas TCUEAP3 has an icosahedral head with a diameter of 43.5 ± 2.7 nm and a non-contractile tail that is 179.0 ± 6.4 nm long and 8.0 ± 1.9 nm in diameter (Fig. 1B).

Optimal multiply of infection (MOI) of TCUEAP2 and TCUEAP3

The optimal MOI was tested from MOI 0.0001 to MOI 10 at 37 °C. The results showed that the optimal MOI for both phages was 1.0, at which the phage titer was about 5×10^{10} PFU/mL for TCUEAP2 (Fig. 2A) and 1×10^{10} PFU/mL for TCUEAP3 (Fig. 2B).

Host range analysis and infection assay

To evaluate phage specificity, 24 *Elizabethkingia* strains were screened by spot test and double-layer agar methods. The results showed that both TCUEAP2 and TCUEAP3 have a

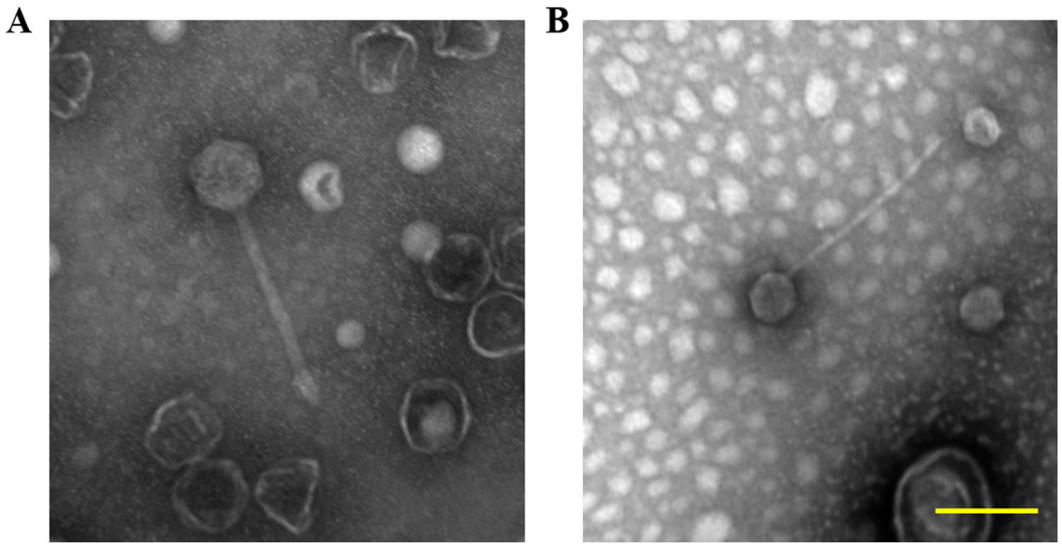

Figure 1. Virion morphologies of TCUEAP2 and TCUEAP3. Transmission electron microscopic (TEM) image of phage (A) TCUEAP2 and (B) TCUEAP3. Both phages showed an icosahedral head with a long tail. Scale bar indicates 100 μm.

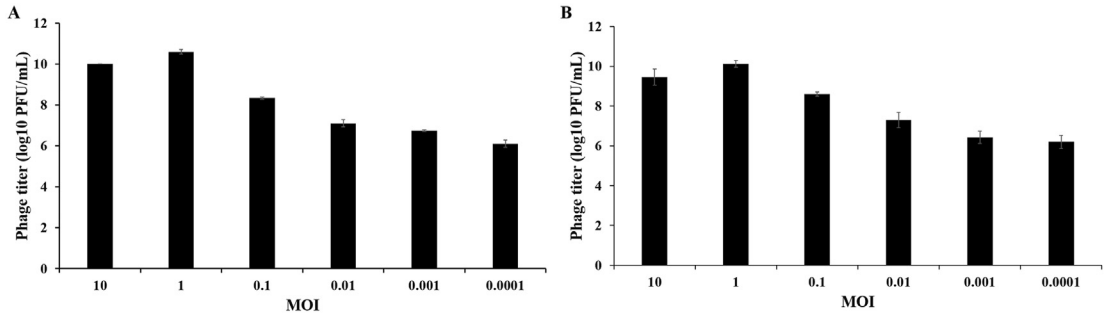

Figure 2. Optimal multiplicity of infection (MOI) of TCUEAP2 and TCUEAP3. The optimal multiplicity of infection (MOI) of (A) TCUEAP2 and (B) TCUEAP3 was determined by infecting the phage with its host. Phage titers were determined after 3.5 h of incubation at different MOI at 37 °C. Data points represent mean ± standard deviations from three replicate experiments.

very narrow host range. Of the 24 bacterial strains tested, TCUEAP2 only formed plaques on its host *E. anophelis* ANO14, and TCUEAP3 on *E. anophelis* ANO1. We then performed infection assays for phage TCUEAP2 and TCUEAP3 against their hosts ANO14 and ANO1. The effect of infection was observed through inoculation of a bacterial culture at exponential phase (OD_{600} of 0.5) at different MOIs (ranged from 0.01 to 10 for TCUEAP2 and from 0.01 to 1.0 for TCUEAP3) at 25 °C and 37 °C. TCUEAP2 reduced the growth of *E. anophelis* ANO14 at both temperatures but only at high MOIs such as MOI 10 and MOI 1.0 (Fig. 3A—B); whereas TCUEAP3 only reduced the growth of *E. anophelis* ANO1 at MOI 1.0 at 25 °C, but not at other lower MOIs or at 37 °C (Fig. 3C—D).

Latency period, burst size, and absorption efficiency

The one-step growth experiment showed that the eclipse and latent periods of TCUEAP2 were about 40 min and 60 min, respectively, and the burst size was approximately 6 phage particles per infected bacterial cell (Fig. 4A). The eclipse and latent periods of TCUEAP3 were about 75 min, and the burst size was about 210 phage particles per infected cell (Fig. 4B). In adsorption analysis, within 10 min, about 80% of the TCUEAP2 phages (Fig. 4C) in contrast to around 70% of the TCUEAP3 phages were adsorbed to their host (Fig. 4D).

Temperature sensitivity of the phages

The thermal stability assay showed that both TCUEAP2 and TCUEAP3 were stable under 37 °C after one hour incubation time, and the phage titer decreased when temperature increased (Fig. 5A—B). To determine the optimal storage condition for the phages, a similar experiment was performed at 4 °C, 25 °C and 37 °C for 30 days. The results showed that TCUEAP2 titer has no reduction at either temperature following a 30-day incubation time (Fig. 5C); TCUEAP3 was only stable at 4 °C as the phage titer decreased when it was stored at 25 °C or 37 °C (Fig. 5D). Phage TCUEAP2 was obviously more stable than TCUEAP3.

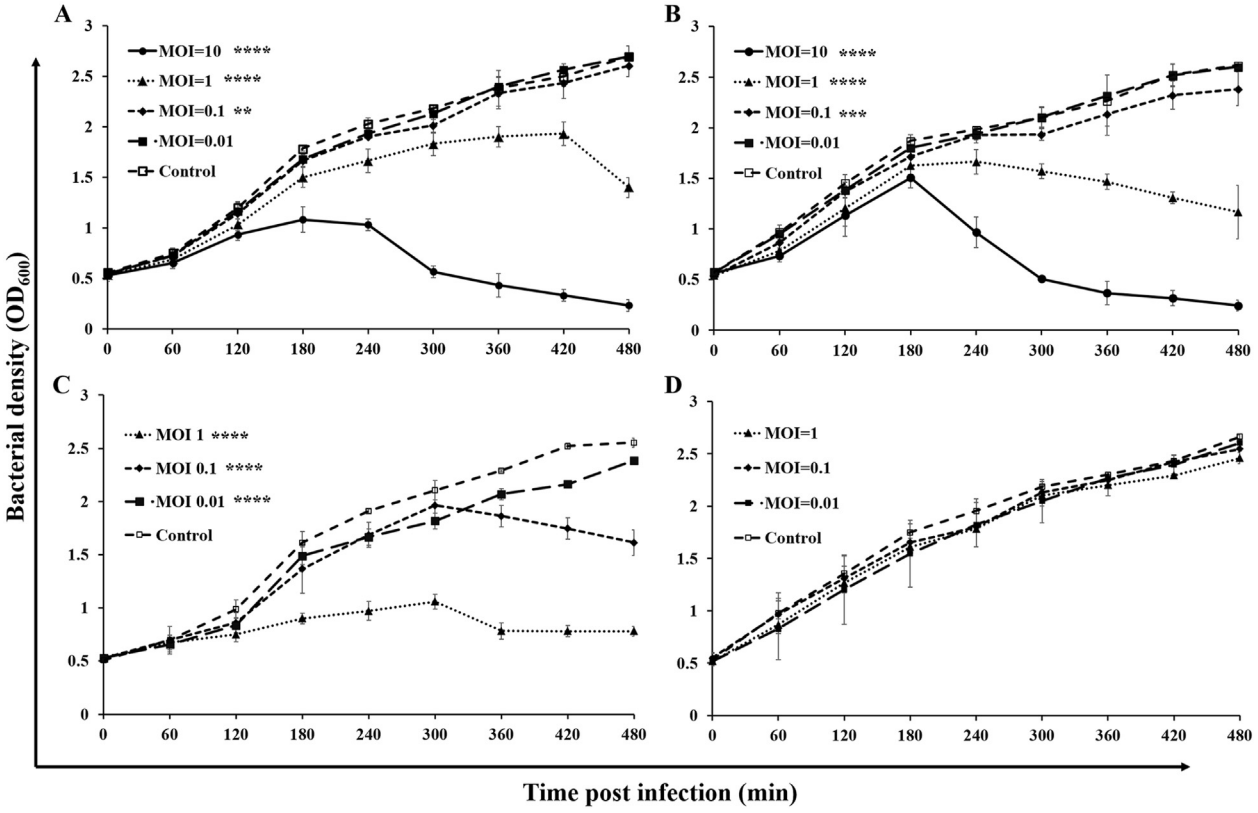

Figure 3. Infection assay of TCUEAP2 and TCUEAP3. Infection assay of TCUEAP2 with their host *E. anophelis* ANO14 at (A) 25 °C and (B) 37 °C. Infection assay of TCUEAP3 with their host *E. anophelis* ANO1 at (C) 25 °C and (D) 37 °C. Data represent mean ± standard deviations from three replicate experiments. ** p-value < 0.01, *** p-value < 0.001, and **** p-value < 0.0001 compared with control group. Significance according to one-way ANOVA.

Effect of pH and chloroform on phage stability

The optimal pH for phage stability was also determined. Results showed that after one hour, phages TCUEAP2 and TCUEAP3 were relatively stable at pH 7, while reduction of phage titer was observed at pH 11. Both phages were completely inactivated at pH 2 and pH 4 (Fig. 5E—F). Chloroform stability testing showed that phage titer was unaffected by chloroform exposure (Fig. 5G—H).

Genomic characterization of TCUEAP2 and TCUEAP3

Restriction analysis of the phage DNA showed that TCUEAP2, TCUEAP3, and our previously isolated TCUEAP1[15] have different chromosome restriction profiles (Supplementary Fig. 1). To gain a deeper understanding of the genomic characteristics of phage TCUEAP2 and TCUEAP3, genome sequencing was performed using the Illumina Mi-seq platform. The genome size of TCUEAP2 was 42,403 bps with a G+C content of 38.8% and 61 predicted open reading frames (ORFs). Of the 61 predicted ORFs, three were predicted to encode capsid related proteins (ORF_7, ORF_9 and ORF_10). Four ORFs predicted to encode tail-related proteins include ORF_6 for tail protein, ORF_14 for tail assembly chaperone, ORF_15 for tail tape measure protein, and ORF_17 for tail fibers. Another three ORFs were predicted to encode DNA packaging-associated products (ORF_5, ORF_30 and ORF_46), and ORF_20 was possibly to encode an N-acetylmuramoyl-L-alanine amidase associated with host cell lysis (Fig. 6A; Supplementary Table 2). For phage TCUEAP3, the genome was 33,037 bps with an overall G+C content of 35.2%, and 40 predicted ORFs. Six ORFs were predicted to be associated with phage structures, including four tail fiber proteins (ORF_21, ORF_36, ORF_37, ORF_38), one tape measure protein (ORF_35), one portal protein (ORF_23), and two capsid proteins (ORF_27 and ORF_28). Genes that are required for phage genome packaging and integration were also identified, including terminase (ORF_25 and ORF_26) and phage Mu-like transposase (ORF_4 and ORF_5). The ORF assigned to the cell host lysis (N-acetylmuramoyl-L-alanine amidase) was predicted at ORF_29 (Fig. 6B; Supplementary Table 3).

Discussion

Elizabethkingia has gained global attention in recent years due to its multidrug resistance.[6,7,24] Numerous studies have recently proved that *E. anophelis*, rather than *E. meningoseptica* is responsible for the majority of human infections and outbreaks.[5,6] In 2020, we reported the world's first *E. anophelis* phage TCUEAP1.[15] For the purpose in developing phage therapy, it is beneficial to prepare a diverse collections of phages. In the present study, two

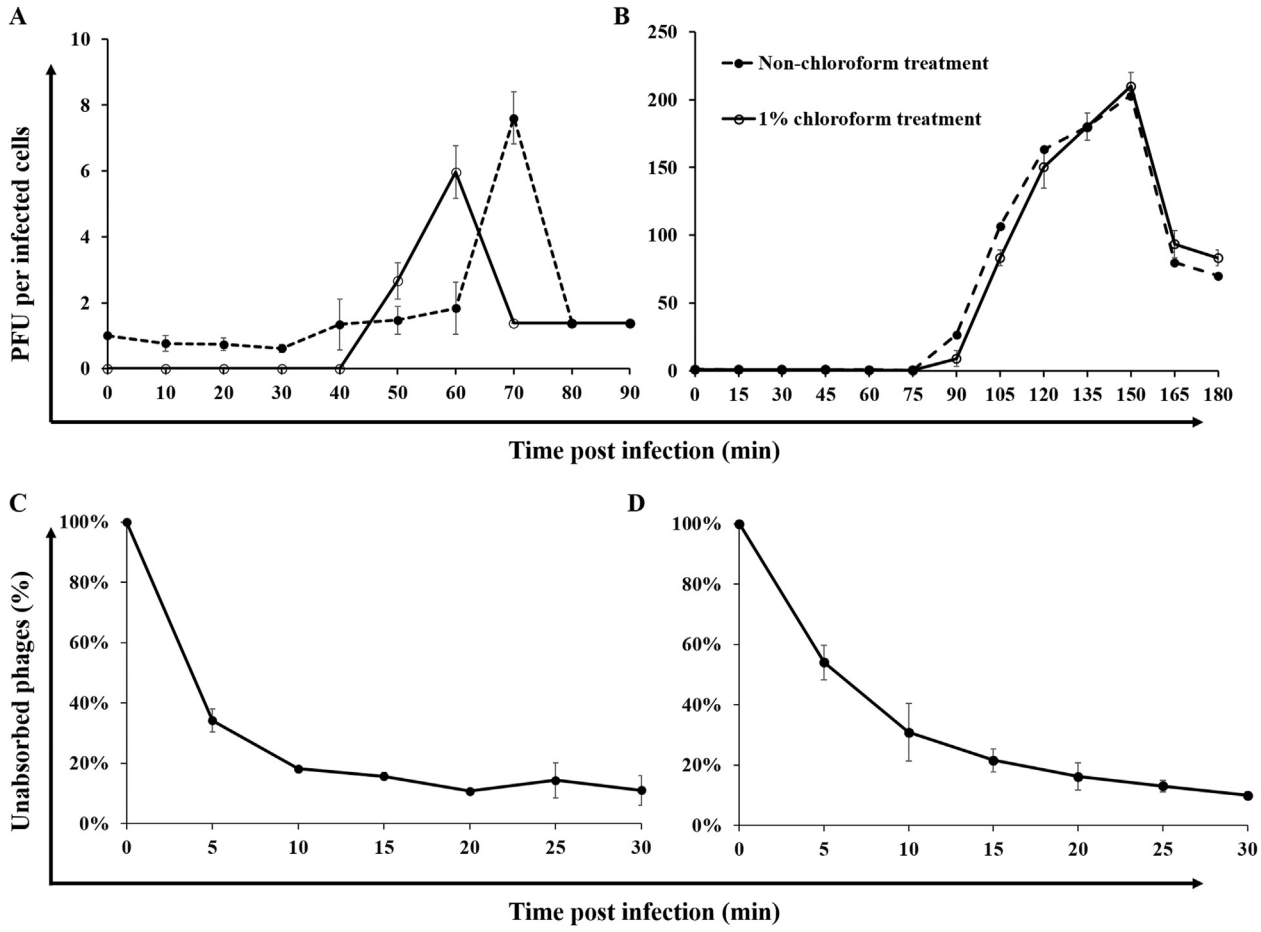

Figure 4. One-step growth curve and adsorption curve of TCUEAP2 and TCUEAP3. One-step growth curve of (A) TCUEAP2 and (B) TCUEAP3. TCUEAP2 and TCUEAP3 were grown in an exponential phase culture of *E. anophelis* ANO14 and ANO1, respectively. Adsorption curve of (C) TCUEAP2 and (D) TCUEAP3 to their bacterial host at MOI 0.01. The percentage of unabsorbed phages was calculated by dividing the phage titer in the supernatant to that in the initial phage stock. Data points represent mean ± standard deviations from three replicate experiments.

additional *E. anophelis* phages were isolated and were designated as TCUEAP2 and TCUEAP3. Though all three phages displayed *Siphoviridae* morphology, the host specificity, genome and biological characteristics are dissimilar. Our results found that TCUEAP2 and TCUEAP3 had a very narrow host range, only infecting their respective host. Although TCUEAP3 had a longer latent period compared to TCUEAP2 and TCUEAP1, it tends to produce a much higher number of virus particles (about 200 PFU/cell) compared to TCUEAP1 and TCUEAP2 (about 10 PFU/cell; Supplementary Table 4). However, regarding growth inhibitory ability, we found that phage TCUEAP3 was able to reduce bacterial growth only at room temperature but not at physiological temperature when tested using the maximal MOI of 1. We were not able to obtain higher phage titer to conduct the host inhibition assays. As TCUEAP3 was isolated from wastewater samples, the lower working temperature may reveal that it is strictly an environmental bacteriophage.[25] Additionally, it seems that a higher burst size doesn't mean the phage will infect the host effectively when the results were compared between TCUEAP2 and TCUEAP3 (Fig. 3A and C). The short latent period might outcompete the long latent period by displaying higher burst sizes.[26]

The genomic size of TCUEAP2 is 42,403 bps containing 61 ORFs. BLAST analysis indicated that some putative products involved in the phage structural components were similar to that from *Bacteroides* phage B124-14 (GenBank accession: HE608841) and B40-8 (GenBank accession: FJ008913.1). Both phages belong to the *Siphoviridae* family and were isolated from urban sewage.[27,28]

The genome size of phage TCUEAP3 is 37,073 bps and contains 40 ORFs. ORF_4 and ORF_5 were predicted to encode a Mu-like transposase. Temperate phages engage three different systems for their lysogenic pathway, and one of them includes Mu-like transposition.[29] Therefore, these transposase genes may be associated with genetic transposition, by integrating a random phage genome into the host genome through a non-replicative ("cut and paste") mechanism, so that the phage can replicate as a prophage.[30] The identification of the genes encoding putative Mu-like transposase suggests that TCUEAP3 may probably be a temperate phage.[29] However, the isolation

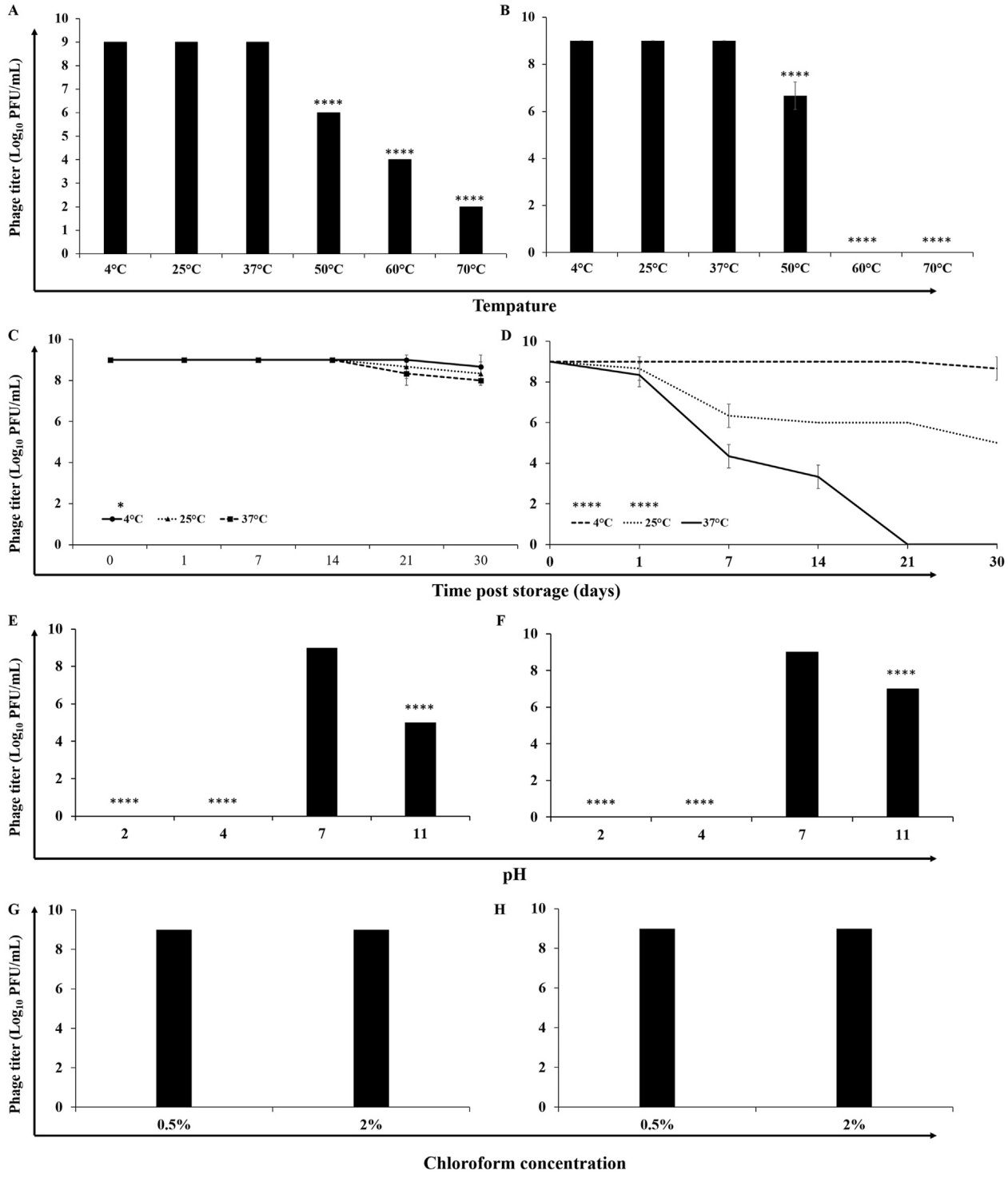

Figure 5. Physical stability of TCUEAP2 and TCUEAP3. For thermal stability, TCUEAP2 and TCUEAP3 were exposed against different temperatures (A—B) for one hour or (C—D) for 30 days. Phages were also exposed to (E—F) different pH or (G—H) different concentrations of chloroform for one hour. Phage titers were then determined by the double-layer method. (A, C, E, G) represents results from TCUEAP2; (B, D, F, H) represents results from TCUEAP3. Data points represent mean ± standard deviations from three replicate experiments. * p-value < 0.05 and **** p-value < 0.0001 compared with (A—D) 37 °C group or (E—F) pH 7 group. Significance according to one-way ANOVA.

of TCUEAP3 in this study was achieved by the liquid enrichment method that is usually used to isolate lytic phages.[31] Generally, temperate phages reduce the growth rate of bacterial hosts, giving rise to the morphology of turbid plaques.[32] Indeed, the plaques of TCUEAP3 presented turbid morphology, a possible sign of being

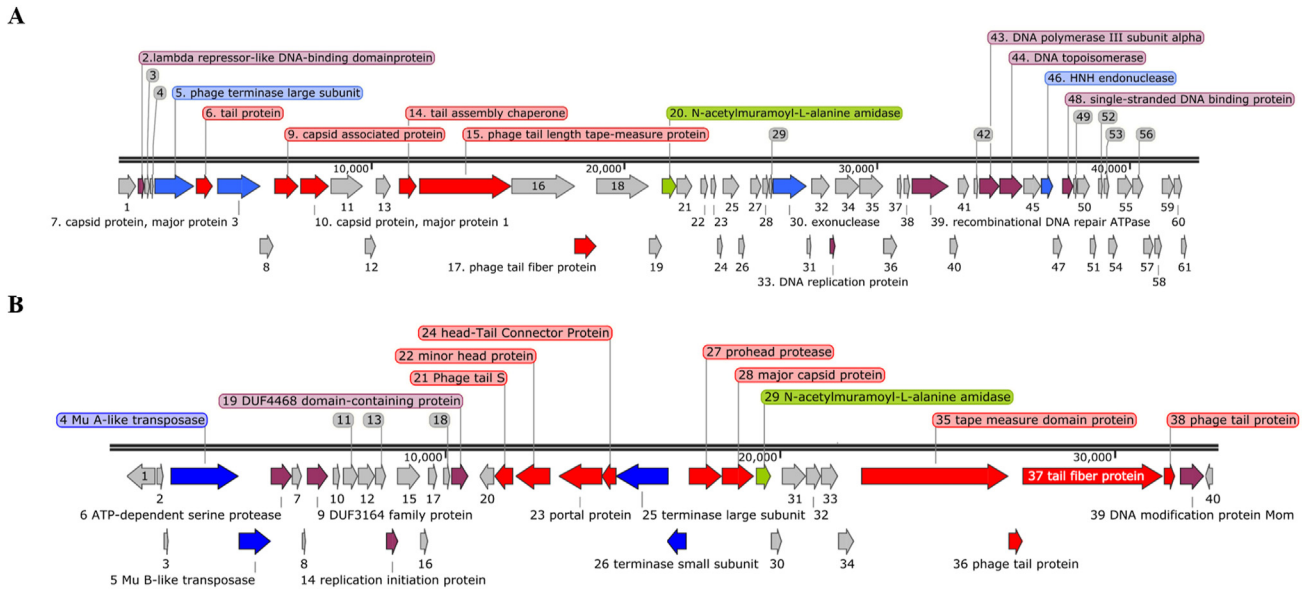

Figure 6. Genomic organization of TCUEAP2 and TCUEAP3. (A) TCUEAP2 contains 42,403 bps and 61 predicted open reading frames (ORFs). (B) TCUEAP3 contains 33,037 bps and 40 predicted ORFs. ORFs coding for structural proteins are marked in red; DNA packaging proteins are marked in blue; lytic proteins are marked in green; proteins involved in DNA replication are marked in magenta; hypothetical proteins are marked in grey. The figure was generated using the SnapGene program, http://www.snapgene.com (accessed on 8 July 2021).

temperate phages. Further investigation is needed to clarify this issue.

Phage-derived endolysin is considered an alternative treatment for infections by antibiotic-resistant bacteria.[33] These enzymes specifically degrade the peptidoglycan in the cell wall of the host bacterium. Based on the BLAST analysis, the protein products of ORF_20 of TCUEAP2 and ORF_29 of TCUEAP_3 were predicted to be N-acetylmuramoyl-L-alanine amidases, which were likely involved in degrading bacterial cell walls leading to the host cell lysis.[34] We therefore cloned the two genes, expressed and purified the proteins, and performed antimicrobial tests. However, our preliminary results indicated that neither of the two recombinant phage products possesses bactericidal abilities (Supplementary Fig. 2). It may be due to the limitations of functional predictions, that is the phages lack well-defined lytic modules, a feature has been observed in some *Siphoviridae* phages.[27] Another possibility is that in the *in vitro* experiment, it lacks assistant proteins that are needed for the lytic modules to exert their antibacterial activity *in vivo*. This is worthy of further exploration.

While developing phage therapy as a potential alternative method in bacterial infections, building a collection of rich and diverse phages is a prerequisite,[35] and in the battle against *E. anophelis* is no exception. So far, we have successfully isolated three distinct *E. anophelis* phages. Although they may not provide immediate satisfactory applicability for clinical needs, they represent a step forward in the preparation of the weapons against *Elizabethkingia* bacteria. We have been conducting phage studies for many years. In our experience, isolation and characterization of the phages against *E. anophelis* has been a strenuous task. We hope our results can encourage more effort on the studies of the phages against this newly emerging bacterial pathogen.

Declaration of competing interest

All authors declare that they have no conflict of interest.

Acknowledgments

We thank the Electron Microscopy Laboratory of the Department of Anatomy of Tzu Chi University for technical assistance. This work was supported by grants 108-2320-B-320-007-MY2 and 110-2320-B-320-005-MY3 from the Ministry of Science and Technology of Taiwan.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2022.05.004.