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ORIGINAL ARTICLE

Characterization and Detection of Endolysin Gene from Three *Acinetobacter baumannii* Bacteriophages Isolated from Sewage Water

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Abstract *Acinetobacter baumannii* is an opportunistic pathogen that exists in hospital environments. The emergence of multidrug resistant *A. baumannii* (MDRAB) has been reported worldwide. It is necessary to find a novel and effective treatment for MDRAB infection. In this study, three bacteriophages, designated as ØABP-01, ØABP-02 and ØABP-04 were selected for analysis. Transmission electron microscopy showed that bacteriophage ØABP-01 belonged to the *Podoviridae* family and bacteriophage ØABP-02 and ØABP-04 are classified into the family *Myoviridae*. ØABP-01 had the widest host range. ØABP-01, ØABP-02 and ØABP-04 exhibited a latent period of 15, 20 and 20 min. The burst sizes of the three bacteriophages were 110, 120 and 150 PFU/cell. DNA restriction analysis using *Eco*RI, *Hin*dIII, *Pst*I, *Sph*I, *Bam*HI and *Sma*I showed different DNA fragment patterns between the three bacteriophages. ØABP-01 and ØABP-04 was positive for the endolysin gene as determined by PCR. In conclusion, bacteriophage ØABP-01 showed broad host-specificity, good lytic activity and a short latency period, making it an appropriate candidate for studying the control and diagnosis associated with MDRAB infections.

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Introduction

Acinetobacter baumannii is a Gram negative coccobacillus which is ubiquitous in the hospital setting. *A. baumannii* causes a diverse range of infections such as ventilator-associated pneumonia, skin and soft-tissue infections, urinary tract infection, wound and blood stream infection. It is an opportunistic pathogen that is a major cause of nosocomial infection which has a high mortality rate [1]. Emergence of multidrug resistant *A. baumannii* (MDRAB) has been reported worldwide [2–4]. The increased incidence of antibiotic resistance has led to the search for an alternative antimicrobial treatment. Phage therapy is one potential candidate for the treatment of multidrug resistant bacteria [5]. Clinical trials of bacteriophages and their derivatives as potential alternative agents for controlling multi drug resistance infection have been described in various bacterial pathogens [6–9]. Bacteriophages are able to replicate in the host cell and produce endolysin to lyse the host cell. Endolysin is a phage peptidoglycan hydrolases produced in the lytic cycle that degrades the bacterial cell wall [10, 11]. Phage endolysins have been studied in a variety of pathogenic bacteria including *A. baumannii* for theirs antibacterial activity [11–13]. In the past three years, *A. baumannii* bacteriophages and endolysin have been isolated and characterized [14–24]. However, there are geographic differences in *A. baumannii* host strains. Depending on their host specificity, bacteriophages and phage endolysin that have been isolated in one place may not be effective in another area. Thus, the aim of this study was to isolate and characterize the *A. baumannii*

bacteriophages from the waste water treatment plant in two hospitals in Thailand.

Materials and Methods

Bacterial Strains and Media

Eleven clinical MDRAB isolates obtained from Buddhachinaraj hospital, Phitsanulok, Thailand were used for screening of *A. baumannii* bacteriophages [25]. All *A. baumannii* were grown in Luria–Bertani broth (LB) or Luria–Bertani Agar (LBA).

Bacteriophage Isolation

Phages were isolated from the inlets and outlets of wastewater treatment plants in two hospitals in Phitsanulok province. Samples were collected three times at monthly intervals from October–December 2010. Samples were centrifuged and filtered. Then, 5 ml each of the filtered supernatants were mixed with 5 ml double strength broth containing overnight culture *A. baumannii*. After 48 h growth at 25 °C, the culture was centrifuged and filtered through a membrane filter (0.22 µm pore size, Sartorius Stedim biotech, Germany). The presence of lytic phage in the filtrate was examined by using the double layer method [26].

Phage Enrichment and Purification

Single plaques was cut out from the soft agar layer, diluted in liquid broth, centrifuged at 12,000 rpm and supernatant was used for phage propagation. Phage was propagated using liquid culture of *A. baumannii* hosts and isolated phages from supernatant. Hundred millilitre of bacterial host cells (OD_{600} of 0.3) was added with isolated phages at a multiplicity of infection (MOI) of 0.5 and incubated at 37 °C until complete lysis. After that, chloroform was added and bacterial debris was pelleted by centrifugation at 4,000 g for 10 min. The supernatants were passed through a membrane filter (0.22 µm pore size, Sartorius Stedim biotech, Germany)). Three repeated rounds of complete lysis were performed and the filtrates were subjected to the double layer method [26].

Host Range Analysis

Host range analysis was determined by spot test using 11 *A. baumannii* clinical isolates [25], *A. baumannii* ATCC19606 and 14 strains of different bacterial species (*Enterococcus faecalis*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella flexneri*, *Vibrio parahaemolyticus*, *Bacillus cereus*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Escherichia coli* and *Pseudomonas aeruginosa*). 100 ul of overnight bacterial host cultures (10^8–10^9 CFU/ml) were added to 2.5 ml of 0.7 % soft agar at 45 °C. This mixture was poured onto 2 % solid agar plate to make double layer agar plates. After solidification, 5 µl aliquots of phage suspension (1.0×10^8 PFU) were spotted on the lawn of host bacteria. Plates were dried and incubated at 37 °C for 24 h. Clearance zone indicating lysis at the spot of phage inoculation implied that the host was sensitive.

One Step Growth

Cells of *A. baumannii* were harvested by centrifugation and then resuspended in fresh LB broth in a concentration of 1×10^9 CFU/ml. Phages were added at an MOI of 0.001 and allowed to absorb for 30 min at 4 °C. The mixtures were then centrifuged at 12,000×g for 10 min and the pellet was resuspended in 20 ml of LB broth. Samples were taken every 5 min over a period of 60 min and immediately assayed for plaque titre by the method described earlier. Latent period, burst time and burst size were calculated from the one-step growth curve. Each of the above experiments was repeated three times with triplicate samples.

Morphology of Phage

A drop of phage suspension (10^{12} PFU/ml) was applied to the surface of a formvar-coated grid and negatively stained with 0.5 % uranyl acetate for 3–5 min. After drying, the preparations were observed in a transmission electron microscope (Philips, Oregon, USA).

Analysis of Bacteriophage Nucleic Acid

Bacteriophage nucleic acid was extracted as described by Kutter and Sulakvelidze [27]. Phage lysate was treated with DNase I and purified using PEG8000. Purified phage particles were treated with SDS (10 %) at 65 °C for 15 min. An equal volume of phenol–chloroform (1:1) was added to remove proteinaceous materials. The extraction was repeated twice, and the nucleic acids were precipitated with 0.1 volume of 3 M sodium acetate and 1 volumes of isopropanol. Phage DNA was resuspended in TE buffer. Precipitation was repeated. The final pellet was washed twice with 70 % ethanol, air dried, and then resuspended in TE buffer. One µg samples of phage DNA was cut with restriction enzymes *Eco*RI, *Hin*dIII, *Pst*I, *Sph*I, *Bam*HI and *Sma*I and analyzed by electrophoresis in 1 % agarose gel containing 0.5 ug/ml ethidium bromide. Pulsed-field gel electrophoresis was performed as described previously [28].

Detection of Endolysin Gene in Bacteriophage Genome

The sequence of endolysin was obtained from the GenBank Database of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Primers specific for the endolysin gene (forward: 5′ATGATTCTGACTAAAG ACGGATTTAGTATT3′ and reverse: 5′CTATAAGCTCC GTAGAGCACGTTC3′) were designed using Biology Workbench (http://workbench.sdsc.edu/). PCR was performed in a DNA thermal cycler using phage DNA ØABP-01, ØABP-02 or ØABP-04 genomic DNA as a template. A PCR was carried out: 2 min at 94 °C, 30 cycles of 20 s at 94 °C, 20 s at 63 °C, and 30 s at 72 °C, followed by 5 min at 72 °C. PCR products were analyzed by electrophoresis in 1 % agarose gel containing 0.5 µg/ml ethidium bromide and sequenced using Applied Biosystems. Phylogenetic analysis was constructed using Molecular Evolutionary Genetics Analysis software (MEGA version 5.05).

Nucleotide Sequence Accession Number

The nucleotide sequences of two endolysin genes have been deposited to the GenBank Database under accession no. KF548002 and KF548003.

Results and Discussion

Phage Isolation, Host Range Analysis and Morphology

In this study, we used previously characterized *A. baumannii* strains to isolate bacteriophage from waste water treatment plants [25]. Fifty-four isolates of *A. baumannii* bacteriophages were collected from the inlets and outlets of the two treatment plants. Most of the bacteriophages isolated were from the inlets of hospital treatment plants (76 %). We determined the host range of 54 phage isolates using spot tests with *A. baumannii* and 14 bacterial species. No clear zone was observed against all 14 bacterial species tested bacteria. ØABP-01, ØABP-02 and ØABP-04 with high lytic activity on a broad range of *A. baumannii* were selected for further characterization. ØABP-01 was able to lyse all *A. baumannii* tested (n = 12). ØABP-02 and ØABP-04 lysed 50 % of *A. baumannii* tested (n = 6). Only ØABP-01 and ØABP-02 could completely lyse *A. baumannii* ATCC 19606. All 3 phages showed different plaque characteristics (Fig. 1). Plaques of ØABP-01 showed large clear zones (3–8 mm). ØABP-02 showed plaques with small clear zones inside (1–2 mm) and large opaque zones surrounding, whereas ØABP-04 showed plaques with clear zones inside and an opaque zone surrounding. The variation in plaque characteristics could be related to different types of bacteriophage. Morphology of the three bacteriophages was observed in a transmission electron microscope as shown in Fig. 2. ØABP-01 was found to have an icosahedral head (78 nm) with a short tail (9 nm), belonging to the *Podoviridae* family. ØABP-02 has an icosahedral head (80–85 nm) with a long tail (67–125 nm) and ØABP-04 has an icosahedral head (72 nm) with a long tail (110 nm). Both were classified into the *Myoviridae* family. All three bacteriophages were tailed bacteriophages and identified as members of order *Caudovirales*. To date, more than 96 % of isolated bacteriophages belong to this order [29]. ØABP-01 was classified in the *Podoviridae* family which is in accordance with previous studies in bacteriophages infecting *A. baumannii* phiAB1, AB2 and AB7-IBB2 [14, 21, 23]. Bacteriophage ØABP-02 and ØABP-04 were classified into the family *Myoviridae*. This family was also reported in bacteriophages ABp53, ZZ1 and AP22 [16, 18, 19].

Phage Physiology

The growth cycle of all three bacteriophages was characterized by one-step growth. Burst size and latent period of three bacteriophages are shown in Fig. 3. The latent periods of ØABP-01, ØABP-02 and ØABP-04 were 15, 20 and 20 min and the burst sizes of ØABP-01, ØABP-02 and ØABP-04 were 110 ± 5.56, 120 ± 17.67 and 150 ± 10.96 PFU/cell. ØABP-01 had a shorter latent period and displayed good lytic activity compared to other bacteriophage isolates. Previous reports of bacteriophages infecting *A. baumannii* showed latent periods ranging from 9 to 25 min and burst size from 22 to 409 PFU/infected cell [14–16, 18–22].

Phage Nucleic Acid Analysis

Analysis of all bacteriophage nucleic acids by digestion with DNaseI revealed that all phage nucleic acids were sensitive to DNase (data not shown). To date, most of *A. baumannii* bacteriophage isolates are DNA phages [14, 15, 16, 18, 19, 22]. PFGE analysis revealed that phage DNA ØABP-01 and ØABP-04 have genome sizes of approximately 30 kb and ØABP-02 showed a larger genome size of approximately 240 kb (Fig. 5a). Restriction analysis of ØABP-01 and ØABP-04 genomes showed they could be cut with restriction enzymes *Bam*HI, *Eco*RI, *Hin*dIII and *Sph*I (Fig. 4b). All six restriction enzymes used in this study appeared unable to cut the ØABP-02 genome (Fig. 4b).

Detection of Endolysin Gene

The endolysin gene was detected in most *A. baumannii* bacteriophages isolated from different regions [13, 23, 25].

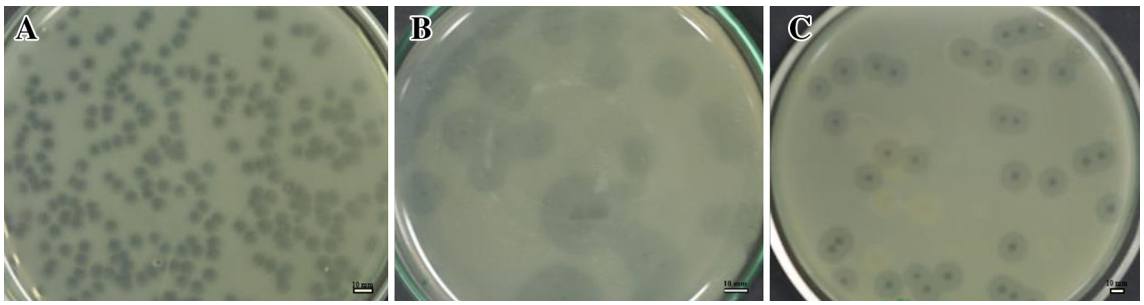

Fig. 1 The appearance of plaques on a bacterial lawn formed by three *A. baumannii* bacteriophages. **a** ØABP-01 plaque characteristic showed large clear zone. **b** ØABP-02 plaque characteristic showed small clear zone inside with large opaque zone surround. **c** ØABP-04 plaque characteristic showed clear zone inside with an opaque zone surround

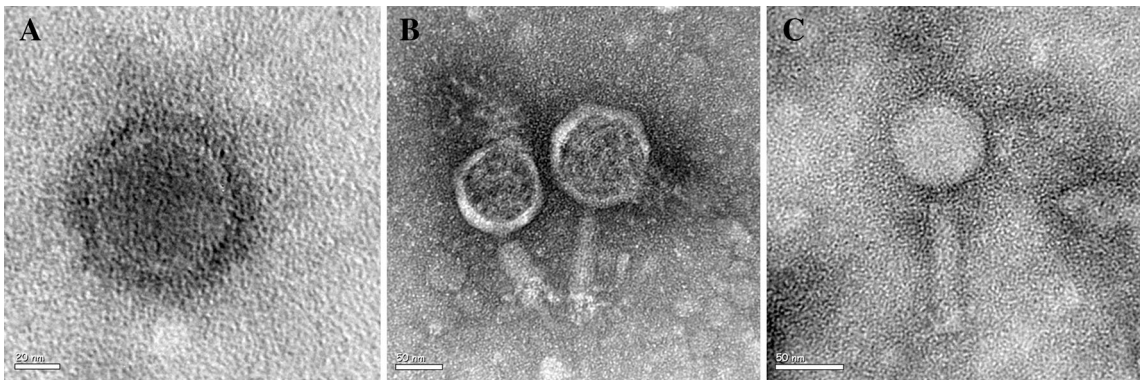

Fig. 2 Electron micrograph of *A. baumannii* bacteriophages. **a** ØABP-01, **b** ØABP-02 and **c** ØABP-04

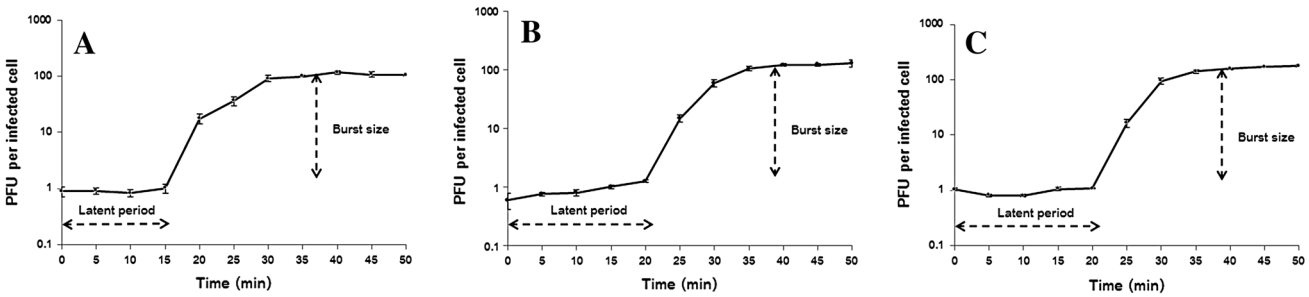

Fig. 3 One step growth of ØABP-01 (**a**), ØABP-02 (**b**) and ØABP-04 (**c**) bacteriophages against *A. baumannii* host strains. All data are shown as mean of three independent experiments. *Error bar* represent the standard error of mean

PCR was utilized to investigate the presence of the endolysin genes as shown in Fig. 5a. The amplified product of the endolysin gene (*lys*) could be detected from ØABP-01 to ØABP-04 genomes. Most double-stranded DNA phages accomplish host cell lysis through the holin-endolysin system. The absence of the endolysin gene in ØABP-02 may be due to different mechanisms to exit the host cell. Some DNA phage can exit the host cells by inhibition of specific host enzymes and impairing peptidoglycan biosynthesis [30]. The mechanism of ØABP-02 host lysis needs further investigation. Analysis of ABP-01 and ABP-04 endolysin sequences showed 558 and 576 bp fragment of the endolysin genes. Open Reading Frame analysis using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) revealed the endolysin protein of ABP-01 and ABP-04 consisting of 185 and 191 amino acid residues. Similarity of bacteriophage endolysin genes is essential for structural analysis which contributes to the potential of using endolysin as an antimicrobial agent. Sequencing the endolysin gene from ØABP-01 yields 94 % sequence identity to the endolysin gene *(lys)* phage from Abp1(Accession: JX658790.1), phiAB1(Accession: HQ186308.1) and phiAB2 (Accession: HM755898.1) and 93 % sequence identity to Acinetobacter phage AB3 (Accession: KC311669.1)

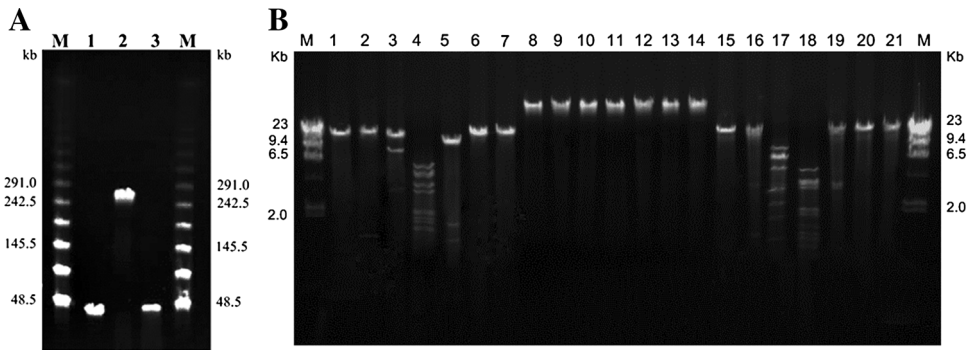

Fig. 4 Nucleic acid analysis of *A. baumannii* bacteriophages. **a** Pulsed-field gel electrophoresis (PFGE) of undigested phages (4.5 V/cm, 30 h). Lambda ladder PFG marker (*lane M*); ØABP-01 (*lane 1*); ØABP-02 (*lane 2*); ØABP-04 (*lane 3*). **b** Restriction pattern of *A. baumannii* bacteriophages. Lambda DNA marker/HindIII (*lane M*); DNA of ØABP-01 uncut (*lane 1*); DNA of ØABP-01 cut with *Bam*HI (*lane 2*), *Eco*RI (*lane 3*), *Hin*dIII (*lane 4*), *Sph*I (*lane 5*), *Pst*I (*lane 6*), *Sma*I (*lane 7*), DNA of ØABP-02 uncut (*lane 8*); DNA of ØABP-02 cut with *Bam*HI (*lane 9*), *Eco*RI (*lane 10*), *Hin*dIII (*lane 11*), *Sph*I (*lane 12*), *Pst*I (*lane 13*), *Sma*I (*lane 14*),DNA of ØABP-04 uncut (*lane 15*); DNA of ØABP-04 cut with *Bam*HI (*lane 16*), *Eco*RI (*lane 17*), *Hin*dIII (*lane 18*), *Sph*I (*lane 19*), *Pst*I (*lane 20*), *Sma*I (*lane 21*)

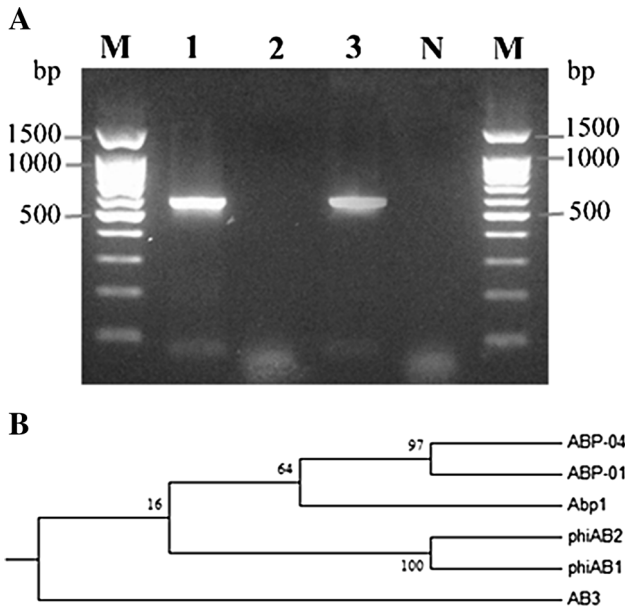

Fig. 5 **a** Amplification of endolysin gene from *A. baumannii* bacteriophages detected by 1 % agarose gel electrophoresis. *Lane M* DNA marker, *Lane 1* ØABP-01, *Lane 2* ØABP-02, *Lane 3* ØABP-04, *Lane 4* Negative control. **b** Phylogenetic tree analysis of endolysin gene of phage ØABP-01, ØABP-04, phiAB, phiAB2, Abp1 and AB3. Phylogenetic analysis was conducted in MEGA version 5.05. Values on branches represent maximum likelihood support values

obtained from GenBank. In addition, the sequence of the fragment of the endolysin gene from ØABP-04 yield 93 % identity to Abp1(Accession: JX658790.1), phiAB1(Acession: HQ186308.1) and phiAB2 (Accession: HM755898.1) and 92 % identity to Acinetobacter phage AB3(Accession: KC311669.1). A phylogenetic tree was constructed based on nucleotide sequences of known phage endolysin genes (Fig. 5b). Sequencing and phylogenetic relationships of the endolysin genes showed that ØABP-01 and ØABP-04 are closely related to phage Abp1, phiAB1 and phiAB2 isolated from China and Taiwan [13, 23, 24]. Despite the different origins of the host strains and the origin of the isolates, this implies that ØABP-01 and ØABP-04 may have common ancestral origins with other phages.

Conclusion

Three bacteriophages classified as *Podoviridae* and *Myoviridae* family members were characterized. Bacteriophages in this study are quite different from those of other reports in genome size, morphology, and one-step growth curve. ØABP-01 has shown good specificity in host range and good lytic activity against *A. baumannii* is a valuable candidate for further study.

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