JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2014. Vol. **8**(Spl. Edn. 1), p. 35-41

Characterization of Phage PA26 Infecting *Pseudomonas aeruginosa*

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(Received: 21 January 2014; accepted: 06 April 2014)

In this study we isolated a novel bacteriophage PA26 infecting *Pseudomonas aeruginosa* from a water reservoir. PA26 belongs to the *Myoviridae* family. The genome is a double stranded DNA of 72,321 basepair. Eighty eight predicted open reading frames were found, of which fourteen showed functional homology to known proteins. 99% of the phages were adsorbed to the host within 10 minutes after infection. The eclipse period was 10 minutes, and the latent period was 15 minutes, with aburst size of 252. Infectivity was highest at pH 7 and remained stable at temperatures between 4 to 37 °C. The phage inhibited biofilm formation by *P. aeruginosa* and also removed preformed biofilm.

Key words: PA26, bacteriophage, *Pseudomonas aeruginosa*, biofilm, phage therapy.

Pseudomonas aeruginosa is a Gram-negative bacteria found in a number of diverse environments. It is an opportunistic pathogen responsible for infections in burn wounds, respiratory tracts, and even systemic infection[1, 2, 3]. Increasing cases of antibiotic resistance have been reported for this bacterium[4, 5]. Formation of biofilm in catheters, artificial hips, and contact lenses by this bacterium is often a problem[6, 7].

Bacteriophages are viruses infecting specific host bacteria. Theirapplication as an alternative to antibiotics has been previously studied[8]. Phages are good alternatives, especially for biocontrol of antibiotic-resistant bacteria. The advantages of phage therapy over antibiotics include selective removal of target bacteria, a prolonged effect made possible by continued reproduction, and few side effects[8, 9]. Bacteriophages infecting *P. aeruginosa* have been widely reported on[6, 10-13]. There have also been reports describing phage therapy in animal models[14-19].

In this study we have isolated phage PA26 infecting *P. aeruginosa* from a water reservoir in Korea and characterized.

MATERIALS AND METHODS

Bacterial strain

P. aeruginosa was obtained from the American Type Culture Collection (ATCC 27853). The bacteria were grown in Luria-Bertani broth or agar (Duchefa, Nethelands) at 37°C.

Isolation and purification of phage

PA26 was isolated from water obtained from Songhakje reservoir in Naju, Chonranam Do, Korea. The standard double agar layer method was used[20]. For the concentration of phage, glycerol step gradient centrifugation was employed[21]. 200 ml of a mid-exponential phase culture of *P. aeruginosa* was infected with the phage at the multiplicity of infection (MOI) of 1. The phage

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lysate was obtained after complete lysishad occurred. NaCl was added to the lysate at the final concentration of 1 M. After centrifugation at 11,000Xg for 10 minutes, the supernatant was recovered and 10% (w/v) polyethylene glycol (PEG) 8000 was added and the mixture wasincubated at 4°C for 1 hour. After centrifugation at 11,000Xg for 10 minutes, the supernatant was discarded and the pellet was resuspended with 750 ml of SM buffer (100mM NaCl, 8mM $MgSO_4$'"$7H_2O$, 50mM Tris-Cl pH7.5, and 0.002% gelatin). Chloroform was added to the suspension at the ratio of 1:1 (v/v) and the mixture was subjected to another centrifugation at 3,000Xg for 15 minutes. Upper phase was then recovered and added to a polycarbonate tube containing 3 ml of 40% glycerol and 4 ml of 5% glycerol unstirred. After centrifugation at 151,000Xg for 1 hour, the supernatant was discarded and the pellet containing phage particles was resuspended with 400 ml of SM buffer.

Transmission electron microscopy

10^{10} PFU of phage solution was added to a formvarcarcon film (200 mesh copper grids) and negatively stained with 2% uranyl acetate. Morphology was observed under a LIBRA 120 energy-filtering transmission electron microscope (Carl Zeiss, Germany).

Adsorption test

10 ml of *P. aeruginosa* grown to the mid-exponential phase was infected with phage at the MOI of 0.1. The number of free phage was determined at 0, 5, 10, and 20 minutes post infection.

One step growth curve

10 ml of *P. aeruginosa* grown to the mid-exponential phase was infected with phage at the MOI of 0.1. Phage adsorption was allowed to proceed for 5 minutes prior to centrifugation at 11,000Xg for 5 minutes. The supernatant was then discarded and the pelletresuspended in 20 ml of fresh LB broth and further incubated. Two 100 ml cultures were obtained every 5 minutes and chloroform (1% v/v) was added to one of these samples. Phage titer was measured using the standard double agar overlay plaque assay method.

Temperature-stability of phage for infectivity

Phages in SM buffer were incubated at 4, 37, 45, 55, 65, and 80°C for one hour prior to infection. Infectious phage titer was checked using plaque assays.

pH-stability of phage for infectivity

Buffers (2M sodium acetate pH 3, pH 4, pH 5, pH 6, 2M Tris-Cl pH 7, pH 8, and pH9, and 2M glycine, pH 10, pH 11) were prepared. 1 ml of phage solution containing $4X10^6$ PFU was mixed with the same volume of each buffer and the mixture was incubated at room temperature for one hour prior to infection. Infectious phage titer was checked using plaque assays.

Optimal temperature for phage production

10^9 CFU of *P. aeruginosa* was mixed with 5 ml of top agar and the mixture was poured on to an LB agar plate. After 30 minutes' of solidification, 100 ml of phage solutions containing various amounts of phage particles were spotted on the lawn and incubated at 25, 30, 35, 39, or 42°C overnight.

Bacterial biofilm inhibition test

The biofilm test method utilized has been previously described[22]. 10^2, 10^4, or 10^6 CFU of *P. aeruginosa* were inoculated to 100 ml of LB broth in 96 well plates. The plates were then incubated at 37°C for 18 hours with gentle shaking. Planktonic cells were discarded and the wells were washed twice with phosphate-buffered saline followed by air drying. Bacterial cells attached to the wells were fixed by methanol for 15 minutes. After drying the wells were stained with 0.4 % crystal violet solution for 15 minutes and the wells were washed with tap water and air dried. Then 33% acetic acid was added to each well and the plates were incubated for 20 minutes. Absorbance was measured at 595 nm in a 96 well plate reader (BioRad, USA). For biofilm inhibition test, phages were added to each well with bacteria simultaneously. For removal of preformed biofilm, bacterial cells were incubated in each well for 24 hours. Plaktonic cells were removed and wells were washed with phosphate buffered saline two times. 200 ml of phage solution containing various amounts of phages were added to each well and incubated for 24 hours before staining.

RESULTS AND DISCUSSION

PA26 belongs to the *Myoviridae* family and has an icosahedral head of 100 nm in diameter and a long tail of 160 nm in length (Fig. 1a). When the purified phage particle was subjected to SDS-

PAGE analysis (Fig. 1b), the virion was seen to contain at least 14 proteins with sizes ranging from 15 to 130 KDa. The major protein band was noted at the molecular weight of 65 KDa.

The complete genomic DNA sequence of A26 was previously reported[23] (GenBank accession number JX194238). The genomic DNA was readily cut by restriction endonucleases including BamHI, Hind III, and Eco RV (data not shown). This suggests that it is a double stranded DNA. Its length was 72,321 basepairwith GC contents of 54.82%. Based on BLASTN analyses in NCBI, sequence homology exists with *Pseudomonas* phage LIT1 (FN422399) and *Pseudomonas* phage LUZ7 (FN422398)[24]. The max score with LIT1 was 27,091 (94% coverage, 98% max identity), while with LUZ7 it was 1,997 (18% coverage, 97% max identity). No other phage has been found to possess a significant homology with PA26. LIT1 and LUZ7 belong to the*Podoviridae* family[24] while PA26 belong to the *Myoviridae* family, suggesting differences in tail structures. ORF54 of PA26 was a putative tail protein (Fig. 2). BLASTP analyses revealed that the sequence similarity between ORF54 of PA26 and gp53 (tail protein) of LIT1 was 74% identity in 63% query coverage based. This could explain the difference in tail structures. Eighty eight predicted ORFs were found in the PA26 genome, amongst which fourteen were functionally predicted and seventy four were hypothetical.

The first step of phage infection is adsorption and we examined how fast thisoccurred (Fig. 3a). Less than 10% of added phages were freely available in phage-host mixtures at 5 minutes postinfection and less than 1% of added phages were freely available at 10 minutes postinfection. Phage production occurred 10 minutes postinfection (eclipse period) and phage release occurred 15 minutes postinfection (latent period)

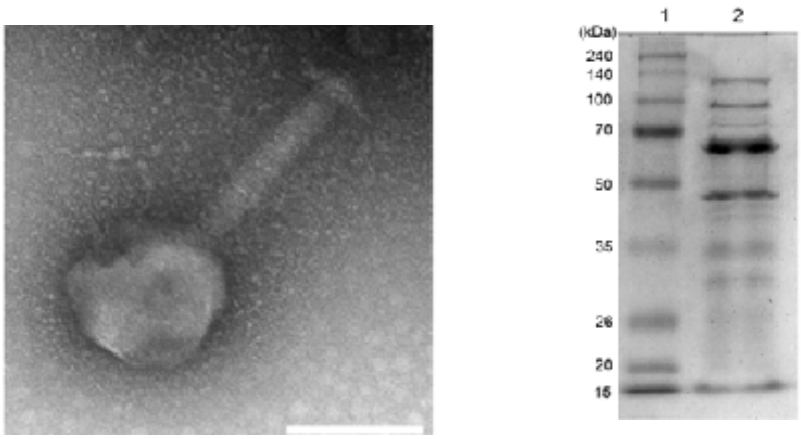

Fig. 1. (a) Transmission electron micrograph of PA26. White bar represents 100 nm. (b) Virion proteins of PA26. Purified phage particles were loaded on an SDS-PAGE and stained with commassie blue.

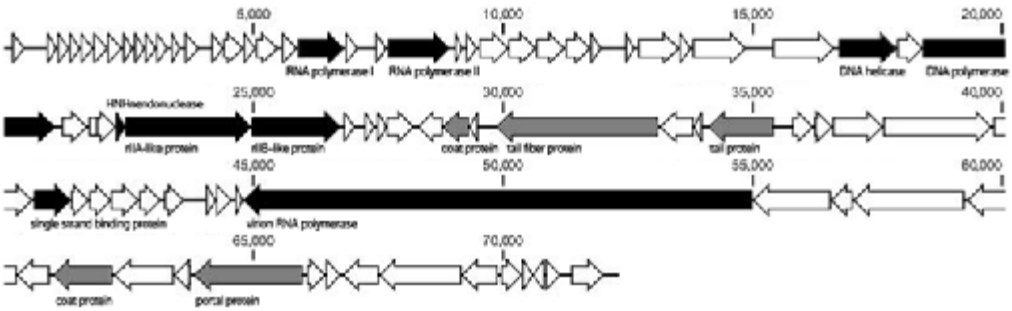

Fig. 2. Genomic map of PA26. Arrows represent open reading frames. White: hypothetical protein; black: replication and regulation protein; grey: structural protein

(Fig. 3b). The burst size was 252. The calculation was based on natural release of phages without adding chloroform.

For potential use as an alternative to antibiotics, phage stabilityafter exposure to differing temperatures and pHs is of concern. The stability of PA26 at a range of temperatures from 4 to37°C was optimal (Fig. 4a). However, a 30% decrease at 45°C and 99% decrease at 65°C were observed. After incubating PA26 in buffers of various pHs for one hour, phage infectivity was checked (Fig. 4b). PA26 remained most stable at pH7, while 90% of its infectivity was maintained at pHs 6 and 8. At higher or lower ranges of pHs, infectivity gradually decreased, and was completely lost at pH 3.

P. aeruginosa grows in a temperature range of 25-42 °C[25]. When 100 ml phage solution containing 10^9 PFU of PA26 was spotted on a lawn of *P. aeruginosa*, a clear zone appeared across all

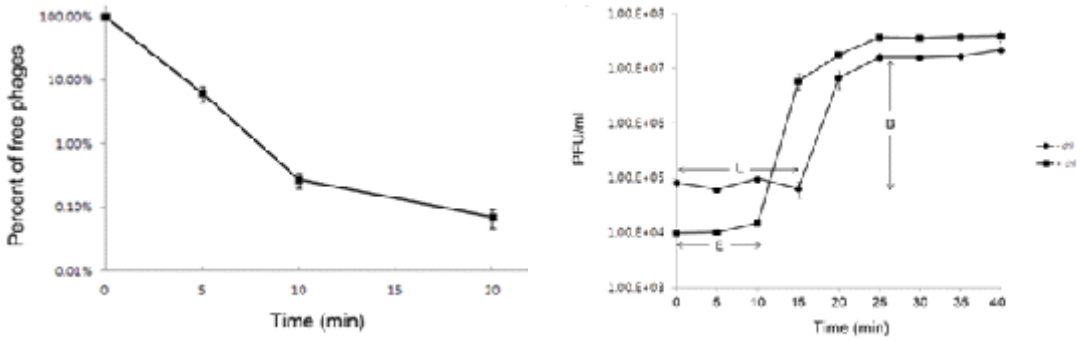

Fig. 3. (a) Adsorption kinetics of PA26. *P. aeruginosa* was infected with PA26 at the MOI of 0.1 and free phages were counted at indicated time points. (b) One step growth curve of PA26.The experiment was performed in triplicate

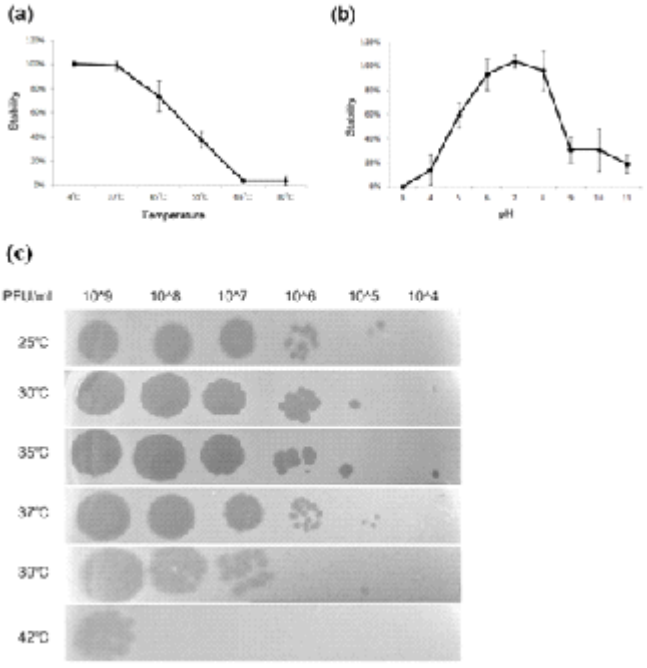

Fig.4. (a) Temperature stability test of PA26. Phages were incubated at indicated temperatures for one hour prior to infection. The experiment was performed in triplicate. (b) pH stability test of PA26. Phages were incubated at indicated pHs for one hour prior to infection. The experiment was performed in triplicate. (c) Optimal temperature test for phage production. Indicated amount of phages were spotted onto a lawn of *P. aeruginosa* and appearance of clear zones was observed

the temperature ranges (Fig. 4c). However, no clear zone appeared with < 10^8 PFU at 42°C, and no clear zone appeared with <10^6 PFU at 39°C. At temperatures of 25, 30, 35, and 37°C, clear zones appeared with as low as 10^5 PFU of phages. Thus it can be said that the optimal temperature range for PA26 production is 25-37°C.

P. aeruginosa is a bacterium which forms biofilm on various surfaces. We tested for the inhibition of biofilm formation on a glass surface where the bacteria and phages were grown together (Fig. 5a). Staining with crystal violet revealed a notable decrease in biofilm formation when *P. aeruginosa* was grown in the presence of PA26 (Fig. 5b). Next, we tested if the phage could remove preformed bacterial biofilm (Fig. 5c). It was noted that PA26 could remove biofilm formed by bacteria grown overnight to various degrees. In both cases, the degree of inhibition and removal depended on the amount of phage added.

97% of previously reported *Pseudomonas* phages have been double stranded DNA phages with tail structures (*Myoviridae*,*Siphoviridae, and Podoviridae*)[26]. The remaining 3% were observed as being PFP phages (polyhedral, filamentous, and pleomorphic). Amongst the tailed phages, 28% were*Myoviridae*, 48% were*Siphoviridae*, and 24% were*Podoviridae*[26, 27]. Morphologically, PA26 is*Myoviridae*, suggesting it comprises one of the major groupings. Although the genomic DNA sequence of PA26 and LIT1 are significantly similar, their tail proteins revealed a certain differences in amino acid sequences, which explains the different tail structures of the two phages. Based on morphology and genomic DNA sequences, we suggest that PA26 is a novel phage infecting *P. aeruginosa*.

For potential use of a phage as an alternative to antibiotics, the infection should be very efficient. PA26 produces progeny phages quickly after infection with a high burst size, suggesting it would be a good candidate for potential phage therapy. As the phage is most stable between temperatures ranging from 4 to 37°C

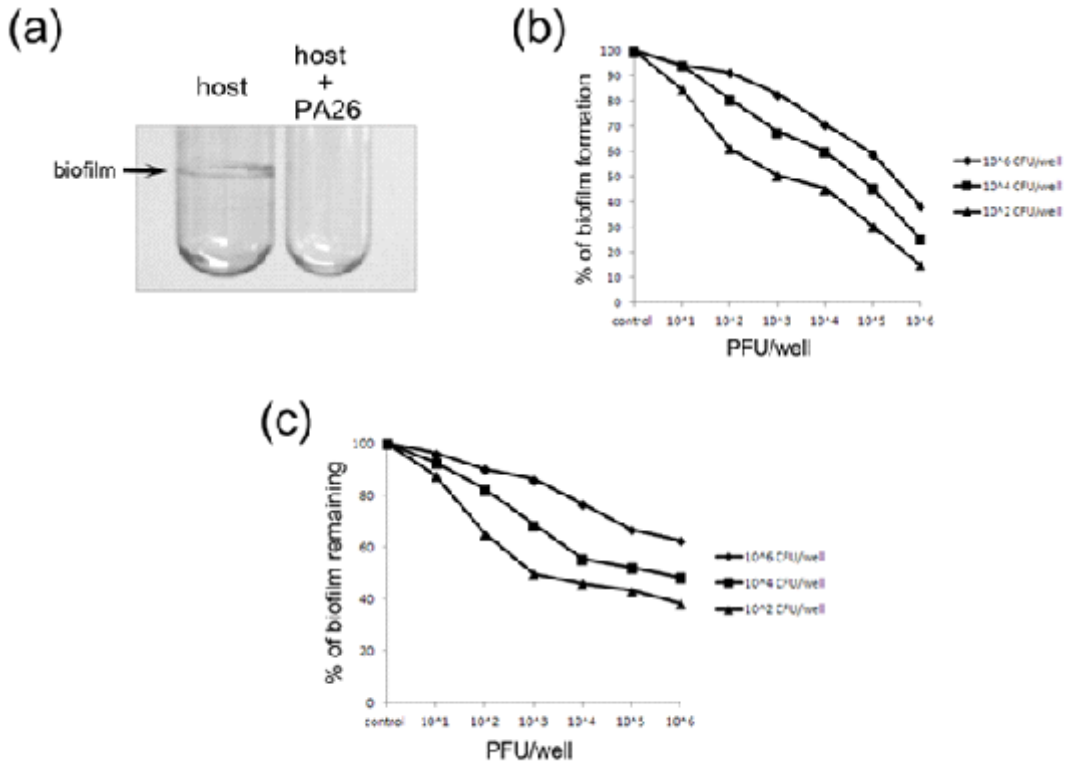

Fig. 5. Biofilm inhibition by PA26. (a) Inhibition of biofilm was seen in a glass tube containing *P. aeruginosa* and PA26 (right). (b) Inhibition of biofilm formation when *P. aeruginosa* was cultured in the presence of PA26. (c) Removal of preformed biofilm when PA26 was added after biofilm formation.

and pHs ranging from 6 to 8, this potential alternative to antibiotics can be handled at a room temperature and neutral pH providing for practical advantages. Although *P. aeruginosa* is known to grow at temperatures ranging from 25 to 42°C, temperatures higher than 37°C are hardly observed in human. Pragmatically speaking, the growth temperature of *P. aeruginosa*as a human pathogen should therefore be 25 to 37°C, the temperature at which PA26 exhibits an optimal production. In addition, PA26 can inhibit biofilm formation by *P. aeruginosa*, and can even remove preformed biofilm. Thus, we suggest that PA26 is an excellentcandidate for a potential phage therapy agent.

ACKNOWLEDGEMENTS

This study was supported by HUFS Research Fund of 2014.

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