Pseudomonas aeruginosa Bacteriophage PA1Ø Requires Type IV Pili for Infection and Shows Broad Bactericidal and Biofilm Removal Activities

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We isolated a new lytic *Pseudomonas aeruginosa* phage that requires type IV pili for infection. PA1Ø has a broad bactericidal spectrum, covering Gram-positive and Gram-negative bacteria, and can eradicate biofilm cells. PA1Ø may be developed as a therapeutic agent for biofilm-related mixed infections with *P. aeruginosa* and *Staphylococcus aureus.*

Bacteriophages (phages) are bacterial viruses that play an important role in bacterial biology, diversity, and evolution (6). Due to their ability to lyse bacteria, attempts have been made to use lytic phages to treat bacterial infections, and some have shown clinical promise as therapeutic antimicrobial agents (2, 11, 16). However, phages are not commonly used therapeutically due to several problems, such as narrow host range, insufficient purity of phage preparations, poor stability or viability of phage preparations, and a lack of understanding of the heterogeneity and mode of action of phages (17, 18). Despite such problems, the worldwide spread of multidrug-resistant (MDR) pathogenic bacteria has led to the revival of this therapeutic approach (12).

In this study, we isolated a novel lytic phage, PA1Ø, which is capable of lysing MDR pathogenic bacteria, especially *Pseudomonas aeruginosa* and *Staphylococcus aureus*. From sewage samples collected from poultry and pig farms located in Gyeonggi Province, South Korea, several phages specific to *P. aeruginosa* were found. Among the isolated phages, one, PA1Ø, was selected, propagated, and purified successfully according to the method of Merabishvili et al. (13). Concentrated phage solutions were prepared as previously described (5) using cesium chloride (CsCl) gradients (9). Approximately 10^{17} PFU/ml of phage stock solution was obtained and stored at 4°C for future use.

PA1Ø exhibited an icosahedral head and a long noncontractile tail by transmission electron microscopy analysis. The lengths of the icosahedral head and the long tail were approximately 58 and 164 nm, respectively. Morphologically, PA1Ø belongs to the family *Siphoviridae* (1). To assess the host range of PA1Ø, spot tests and plaque formation assays were performed with various species of bacteria (Table 1). In a spot test, PA1Ø formed clear lytic zones on the lawns of *P. aeruginosa*, *Shigella sonnei*, *S. aureus*, *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Streptococcus pneumoniae*, *Streptococcus salivarius*, and *Listeria monocytogenes*. However, no lytic zones were found on the lawns of *Escherichia coli*, *Serratia marcescens*, *Enterobacter aerogenes*, *Acinetobacter baumannii*, or certain *Streptococcus* spp. In the plaque formation assays, PA1Ø formed distinct plaques only when used to infect *P. aeruginosa* or *S. sonnei*. PA1Ø could not produce typical plaques when used to infect *S. aureus*, although it formed distinct clear zones on the lawn of *S. aureus* in spot test with lytic areas two to three times larger than those formed on the lawns of *P. aeruginosa* or *S. sonnei*. This result indicates that PA1Ø can infect and propagate in only *P. aeruginosa* and *S. sonnei*.

The adsorption rate of PA1Ø was determined. About 87% of PA1Ø phage were adsorbed to *P. aeruginosa* PAO1 cells within 2 min. The rate of PA1Ø adsorption to *S. sonnei* was similar to that for *P. aeruginosa*. A one-step growth experiment showed that the latent period of PA1Ø was approximately 10 min. The first burst of PA1Ø was recorded at 15 min, and the burst size was assumed to be approximately 261 PFU/bacterium.

To identify the receptor molecule used by PA1Ø to infect *P. aeruginosa*, we constructed and screened a library of random transposon (Tn) insertion mutants of *P. aeruginosa* strain PAO1 for resistance to PA1Ø treatment. A library of random transposon insertion mutants of *P. aeruginosa* strain PAO1 was constructed by biparental mating using *E. coli* SM10 (λ*pir*) harboring pBTK30 as a donor strain (4). Gentamicin-resistant (Gm^r) transconjugants were pooled, and an aliquot of the pool ($\sim 10^8$ CFU/ml) was inoculated into LB containing 1×10^{11} PFU/ml of PA1Ø. After 16 h growth at 37°C, cultures were diluted 100-fold in fresh LB containing the same titer of PA1Ø to enrich the population of PA1Ø-resistant mutants. This enrichment step was repeated three times, and then culture aliquots were spread on LB agar plates containing 200 μg/ml gentamicin (Gm) to isolate mutants that had potentially acquired resistance to PA1Ø. Under these conditions, three mutants were successfully identified as demonstrating normal growth. As shown in Fig. 1, these mutants exhibited uninterrupted growth in the presence of 1×10^{11} PFU/ml PA1Ø, while the growth of wild-type PAO1 was greatly diminished under the same conditions; the optical cell density of these mutants in the presence of PA1Ø was almost comparable to that of PAO1 in plain LB medium. Further sequence analysis revealed Tn insertion into the *pilY1* (PA4554), *pilB* (PA4526), or *pilA* (PA4525) gene in each of these selected mutants. Since these genes encode core components of the type IV pilus synthetic machinery, this result strongly suggests that PA1Ø binds to the pilus to infect *P. aeruginosa*. The

TABLE 1 Results of spot tests and plaque formation assays

Species	Strain or isolate	Spot test result[a]	Plaque formation	Source
Pseudomonas aeruginosa	PAO1	+	Yes	Yonsei University
	ATCC 29853	+	Yes	ATCC[b]
	PA 1-30	+	Yes	Laboratory collection
	PA 1-67	+	Yes	Laboratory collection
	PA 1-86	+	Yes	Laboratory collection
	PA 2-22	+	Yes	Laboratory collection
	PA 2-35	+	Yes	Laboratory collection
	PA 2-36	+	Yes	Laboratory collection
	PA 2-50	+	Yes	Laboratory collection
Shigella sonnei	NIH1	+	Yes	Laboratory collection
	H9	+	Yes	Laboratory collection
	H114	+	Yes	Laboratory collection
	H131	+	Yes	Laboratory collection
	H151	+	Yes	Laboratory collection
	H1	−	No	Laboratory collection
	H122	−	No	Laboratory collection
Escherichia coli	ATCC 25922	−	No	ATCC
	03K543	−	No	Laboratory collection
	04K743	−	No	Laboratory collection
	03K830	−	No	Laboratory collection
	03K692	−	No	Laboratory collection
Serratia marcescens	ATCC 8100	−	No	ATCC
Acinetobacter baumannii	ATCC 19606	−	No	ATCC
Enterobacter aerogenes	EA7	−	No	Laboratory collection
Staphylococcus aureus	ATCC 29213	++	No	ATCC
	ATCC 25923	++	No	ATCC
	MRSA WS-05	++	No	Laboratory collection
	MRSA D43-a	++	No	Laboratory collection
	KNUH-2	++	No	Laboratory collection
	KNUH-3	++	No	Laboratory collection
	KNUH-5	++	No	Laboratory collection
Staphylococcus epidermidis	KNUH-134	+	No	Laboratory collection
	KNUH-174	+	No	Laboratory collection
	KNUH-175	+	No	Laboratory collection
Staphylococcus hominis	KNUH-328	++	No	Laboratory collection
	KNUH-329	++	No	Laboratory collection
Streptococcus pneumoniae	15	−	No	Laboratory collection
	83	+	No	Laboratory collection
	134	−	No	Laboratory collection
Streptococcus salivarius	65	−	No	Laboratory collection
	68	+	No	Laboratory collection
	84	+	No	Laboratory collection
	133	−	No	Laboratory collection
Streptococcus gordonii	143	++	No	Laboratory collection
Streptococcus agalactiae	188	−	No	Laboratory collection
Listeria monocytogenes	10403S	+	No	Martin Wiedermann

[a] ++, large clear lysis; +, small clear lysis; −, no lysis.
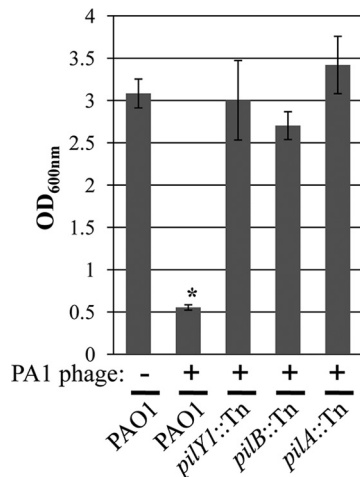[b] ATCC, American Type Culture Collection.

FIG 1 Three pilus mutants grow normally in the presence of PA1Ø. The growth of three pilus-negative strains and wild-type PAO1 was monitored by measuring the optical density (OD) at 600 nm. PAO1 growth in plain LB is included as a control. *, $P < 0.001$ versus all other growth.

adsorption assay using *P. aeruginosa* mutants revealed that PA1Ø was unable to adsorb to the mutants, confirming that the type IV pilus is the receptor molecule used by PA1Ø to infect *P. aeruginosa*.

We assessed the bactericidal activity of PA1Ø against eight bacterial strains (*S. aureus* ATCC 25923, WS-05, and D43-a; *P. aeruginosa* PAO1; *S. hominis* KNUH-328 and KNUH-329; and *S. epidermidis* KNUH-134 and KNUH-174). Overnight bacterial cultures were diluted to an optical density at 600 nm (OD_{600}) of 1, followed by a 10-fold dilution. For treatment of planktonic cultures with phage, 100-μl aliquots (5×10^6 CFU) were inoculated into 96-well microtiter plates, followed by inoculation of 100 μl of PA1Ø samples (1×10^{10} PFU/ml), to a multiplicity of infection (MOI) of 200, into the same wells and a further 6-h incubation at 37°C. Fifty microliters of 0.1% triphenyltetrazolium chloride (TTC) was added, and samples were further incubated at 37°C for 1 h, after which absorbance at 540 nm was determined using an enzyme-

linked immunosorbent assay (ELISA) reader (VersaMax; Molecular Devices, Sunnyvale, CA) (7, 9). For biofilm formation, 100 μl of an overnight bacterial culture (5×10^6 CFU) were inoculated into a 96-well microtiter plate and incubated at 37°C for 1 day in a humidified incubator. After removal of planktonic cells and medium by washing with 100 μl of fresh tryptic soy broth (TSB), 100 μl of PA1Ø (1×10^{10} PFU) was inoculated into the biofilm. After incubation at 37°C for 3 h in a humidified incubator, 50 μl of 0.1% TTC was added and incubated for a further 1 h. Absorbance was recorded at 540 nm. PA1Ø significantly inhibited the bacterial growth in all bacterial strains tested (Fig. 2A). After incubation for 6 h with PA1Ø and *P. aeruginosa* PAO1, the growth of PAO1 was greatly inhibited in comparison with an untreated control. We also measured PA1Ø's biofilm removal activity (Fig. 2B). After treatment of 24-hour-old biofilms of *P. aeruginosa*, *S. aureus*, *S. epidermidis*, and *S. hominis* with PA1Ø, the numbers of viable cells in phage-treated biofilms were greatly decreased in comparison with those in untreated biofilms. Field emission scanning electron microscopy (FESEM) analysis clearly showed PA1Ø's biofilm removal activities (Fig. 3). In phage-treated samples, lysed cells were obvious, and most of the cells adopted a "ghost" morphology (Fig. 3B, D, F, and H). In contrast, no lysed or killed cells were found in the phage-untreated groups (Fig. 3A, C, E, and G).

The genome sequence of PA1Ø was deciphered and announced recently (8). The PA1Ø genome consists of 51 putative protein-coding genes. Since the PA1Ø genome showed a high homology to the D3112 phage genome in the study (8), we performed Dot Matrix View and MAUVE genome alignment analyses to compare the two genomes in detail. The analyses revealed six unmatched regions (Fig. 4). Unmatched region 1, spanning ORF1 to ORF5 of D3112 phage including the *c* repressor protein-coding gene, was not found in the PA1Ø genome. Unmatched region 3 covers the coding regions of host nuclease inhibitor proteins in both phages, and other unmatched regions correspond to genes coding hypothetical proteins of both phages. Three highly conserved regions between PA1Ø and D3112 cover hypothetical proteins (regions A and C), the major head subunit (region B), and the putative tail component (region C).
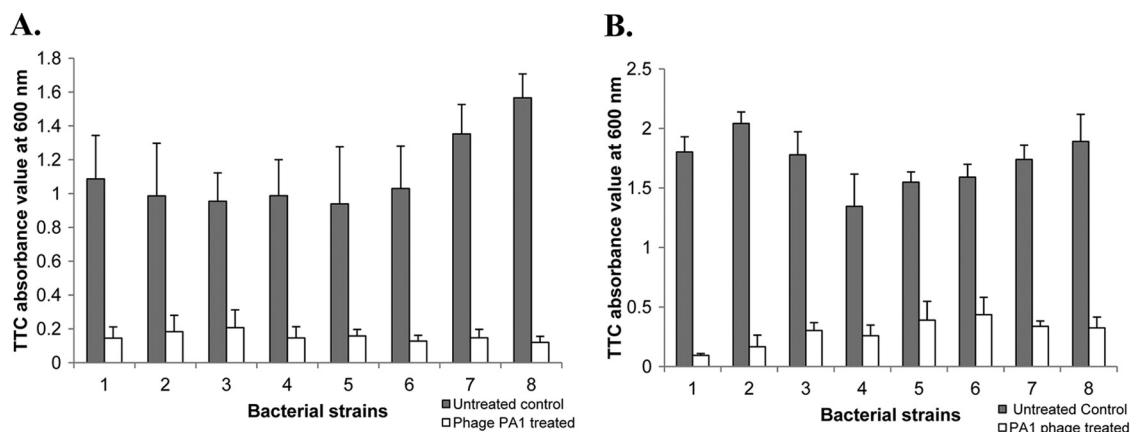

FIG 2 Bactericidal and biofilm removal effects of PA1Ø. Planktonic cultures (A) and 1-day-old biofilms (B) of eight different bacterial species were treated with PA1Ø at an MOI of 200 for 6 h and 1×10^{10} PFU of PA1Ø for 3 h, respectively. Triphenyltetrazolium chloride (TTC) absorbance values at 600 nm were compared with those of non-PA1Ø-treated controls (gray bars). 1, *S. aureus* ATCC 25923; 2, *S. aureus* WS-05; 3, *S. aureus* D43-a; 4, *S. epidermidis* KNUH-134; 5, *S. epidermidis* KNUH-174; 6, *S. hominis* KNUH-328; 7, *S. hominis* KNUH-329; and 8, *P. aeruginosa* PAO1. Experiments were performed three times independently, and values are means and standard deviations.
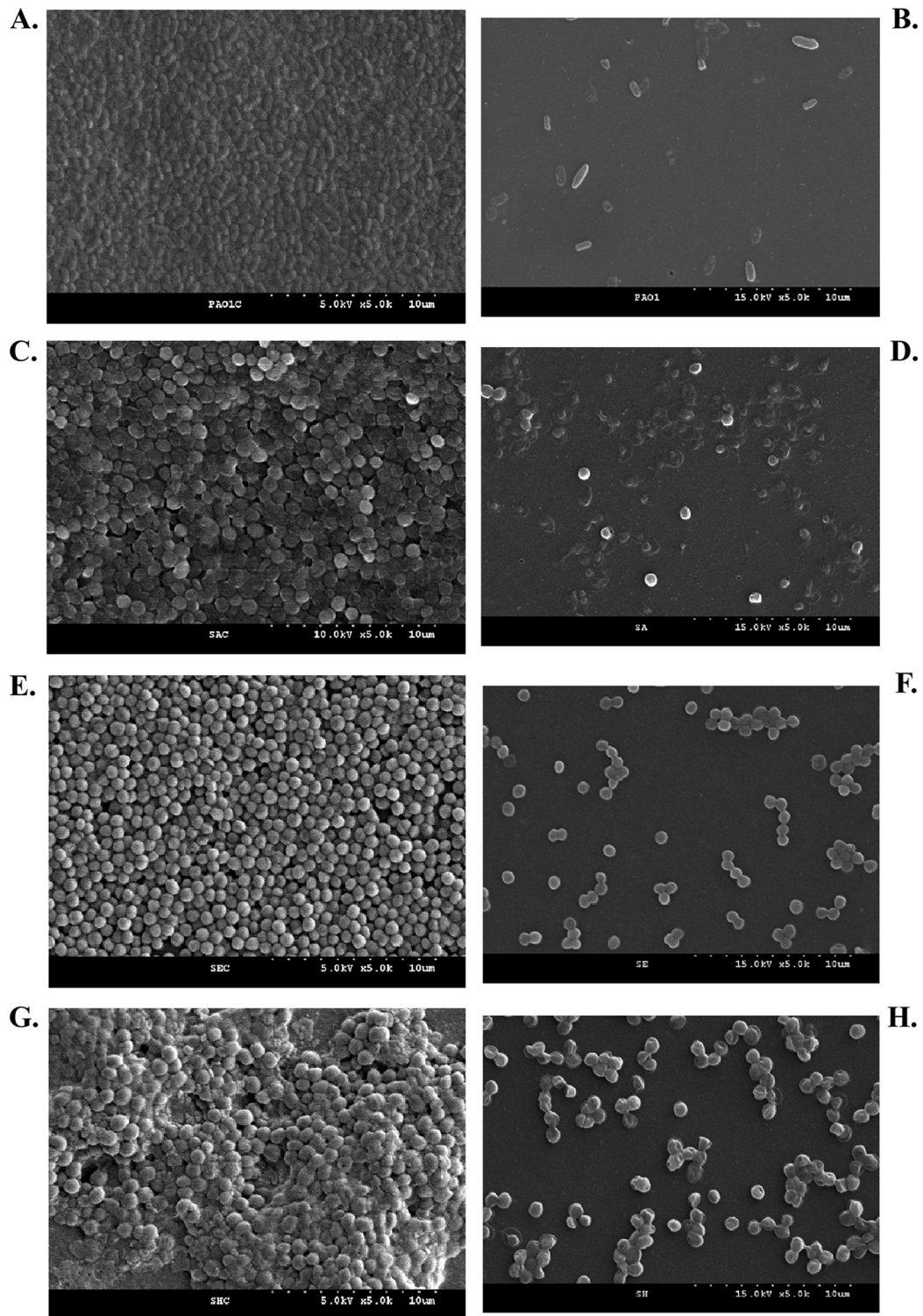
FIG 3 Biofilm removal capacity of PA1Ø. Non-PA1Ø-treated (A, C, E, and G) and treated (B, D, F, and G) biofilms of four bacterial strains were visualized by scanning electron microscopy. (A and B) *P. aeruginosa* PAO1; (C and D) *S. aureus* ATCC 25923; (E and F) *S. epidermidis* KNUH-134; (G and H) *S. hominis* KNUH-329.

In this study, we isolated a novel lytic bacteriophage, PA1Ø, with a broad host range of *P. aeruginosa* and *S. sonnei*. Besides these bacteria, PA1Ø could lyse a number of Gram-positive bacterial pathogens. Since PA1Ø could form plaques only when in-

fecting *P. aeruginosa* and *S. sonnei*, its bactericidal mechanism against Gram-positive bacteria might be different from that against *P. aeruginosa* and *S. sonnei*. One possible explanation is the production of phage-associated lytic enzymes. In fact, Lai et al.
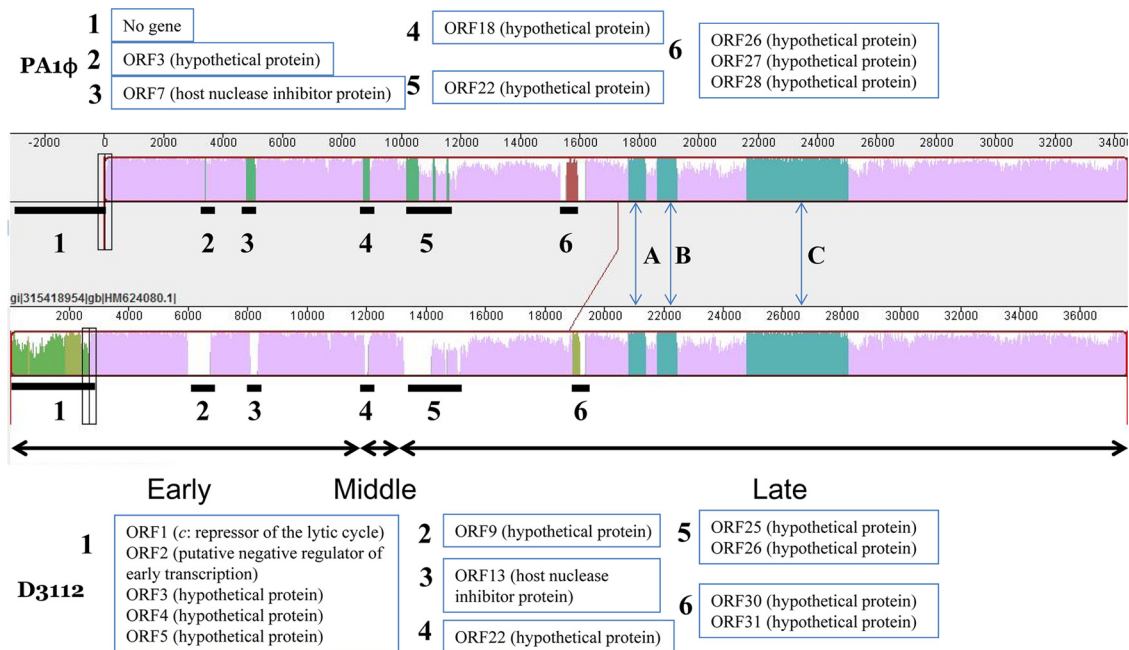
FIG 4 Alignment of the PA1Ø and D3112 genomes. This MAUVE-generated diagram shows the degree of sequence similarity between two phages. Both gene maps are measured against a ruler in base pairs. The degree of sequence similarity between two genomes is indicated by the height of the similarity peaks. The six genomic dissimilarity regions are marked with black bold lines and numbered, and affected genes in both phages are listed in numbered boxes. Regions A to C show highly conserved regions of PA1Ø and phage D3112.

reported that *Acinetobacter baumannii* phage varphiAB2, which shows broad bactericidal activity against Gram-positive and Gram-negative bacteria, produced endolysin (LysAB2), which was responsible for the lysis of *S. aureus* by degrading bacterial cell wall's peptidoglycan (10). Another feature of PA1Ø is its ability to destroy biofilms of *P. aeruginosa*, *S. aureus*, *S. epidermidis*, and *S. hominis*. It could significantly lyse those bacterial cells in the established biofilms within 3 h. To our knowledge, the broad bactericidal spectrum and strong lytic activities against both planktonic and biofilm cells exhibited by PA1Ø have never been demonstrated in other phages.

In several aspects, PA1Ø resembles *P. aeruginosa* phage D3112, which is a well-known temperate phage used for molecular biology studies of *P. aeruginosa* (3, 19). Both phages are *Pseudomonas* phages belonging to the family *Siphoviridae*. Both phages infect *P. aeruginosa* via the type IV pili (14). The genome of PA1Ø is highly homologous to the genome of D3112 phage (GenBank accession number AY394005.1), with overall nucleotide identity of 90%. The difference of PA1Ø from D3112 is the absence of the unmatched region 1 spanning first five ORFs of phage D3112. Because unmatched region 1 includes a *c* repressor-encoding gene, known to play a critical role in the maintenance of the lysogenic state of the D3112 phage in *P. aeruginosa* (15), it seems that PA1Ø lacks the critical lysogenic genes. Thus, it is suggested that PA1Ø is a lytic phage, not a temperate, transposable one.

In conclusion, this study characterized a new lytic *P. aeruginosa* phage that has a broad bactericidal spectrum covering various species of Gram-positive and Gram-negative bacteria. Moreover, PA1Ø can eradicate biofilm cells. Therefore, PA1Ø can be developed as an alternative antimicrobial agent that may be useful in treatment of biofilm-related infections and mixed infections caused by bacteria such as *P. aeruginosa* and *S. aureus*.

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