Characterization of induced *Staphylococcus aureus* bacteriophage SAP-26 and its anti-biofilm activity with rifampicin

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Lytic bacteriophages (phages) have been investigated as treatments for bacterial infectious diseases. An induced phage, SAP-26, was isolated from a clinical isolate of *Staphylococcus aureus*. It belongs to the family Siphoviridae and its genome consists of double-stranded 41,207 bp DNA coding for 63 open reading frames. The phage SAP-26 showed a wide spectrum of lytic activity against both methicillin-resistant *S. aureus* and methicillin-susceptible *S. aureus*. Furthermore, combined treatment with a phage and antimicrobial agents showed a strong biofilm removal effect which induced structural changes in the biofilm matrix and a substantial decrease in the number of bacteria. Such a broad host range in *S. aureus* and biofilm removal activity of the phage SAP-26 suggests the possibility of its use as a therapeutic phage in combination with appropriate antimicrobial agent(s). Among the three antimicrobial agents combined with phage, the combination of rifampicin showed the best biofilm removal effect. To the authors’ knowledge, this study showed for the first time that *S. aureus* biofilm could be efficiently eradicated with the mixture of phage and an antimicrobial agent, especially rifampicin.

**Keywords:** bacteriophage; *Staphylococcus aureus*; biofilm; antimicrobial agent

**Introduction**

*Staphylococcus aureus* is a common and dangerous pathogen causing skin abscesses, wound infection, endocarditis, osteomyelitis, pneumonia and toxic shock syndrome (Lowy 1998; Foster 2005). The number of staphylococcal infections has increased in parallel with the increased use of intravascular devices (Lowy 1998), while the treatment of these infections has been difficult because of the emergence of isolates resistant to multiple antimicrobial agents, including methicillin and vancomycin (Weigel et al. 2003).

Many staphylococcal infections have been associated with biofilms formed on implant surfaces (Costerton 1999). The two consequences of biofilm formation on implant surfaces are increased resistance to antimicrobial agents and frequent failure of conventional therapy. This resistance of bacteria within biofilms is attributed to a possible barrier function of the biofilm caused by binding of the antimicrobial agents within the matrix, and the metabolic change in the bacterial cells. Infection of medical implants is associated with considerable morbidity and cost (Siegenthaler et al. 2003; Darouiche 2004). The increasing multidrug resistant bacteria and biofilm-associated infections are forcing researchers to develop alternative antimicrobial agents.

Bacteriophages (phages) are viruses of bacteria which play a critical role in bacterial biology, diversity, and evolution. Virulent phages have potential as specific microbiological control agents for bacterial pathogens in human, animal and plant diseases (Alisky et al. 1998; Leverentz et al. 2001). Lytic phages have shown clinical promise as therapeutic agents for local or systemic treatment of bacterial infections (Chibani-Chennoufi et al. 2004). Moreover, phages are able to degrade exopolysaccharide matrix, infect the organisms and cause extensive disruption of biofilms (Donlan 2009).

In this study, a phage (SAP-26) was induced from a clinical isolate with mitomycin C and its phenotypic and genotypic characteristics were investigated. The phage SAP-26 has a wide spectrum of lytic activity against *S. aureus* clinical isolates. The efficacy of this phage alone and in combination with clinically relevant antimicrobial agents was evaluated as a tool for eradication of *S. aureus* biofilms.

**Materials and methods**

**Bacterial strains**

A total of 47 clinical isolates of *S. aureus* were used to investigate the induction of *S. aureus* phage. *S. aureus*
WS-05 strain was used for the propagation of isolated phage and one-step growth curve experiments. S. aureus D43-a strain was used for testing the ability of isolated phage to eradicate S. aureus planktonic and biofilm cells. S. aureus WS-05 and S. aureus D43-a strains were susceptible to clindamycin, amikacin, gentamicin, sulfamethoxazole-trimethoprim, tetracycline, azithromycin, rifampicin, and vancomycin, but resistant to amoxicillin and oxacillin. To test the resistance of S. aureus against the induced phage, epidemiologically unrelated clinical isolates of 110 methicillin-resistant S. aureus (MRSA) and 61 methicillin-susceptible S. aureus (MSSA) were used.

**Induction, isolation, purification, and propagation of bacteriophage**

Induction of phage from bacteria was performed as previously described with modifications (Mirold et al. 1999; Frye et al. 2005). In brief, S. aureus was grown in LB broth to reach logarithmic phase and mitomycin C was added at a final concentration of 1 mg l⁻¹. After overnight incubation, bacteria were removed by centrifugation at 4000 × g for 10 min followed by filtration through a 0.45 µm pore size membrane filter. The filtrates were subjected to plaque assay to yield plaque-producing lytic phage. Purification of phages was performed by single plaque isolation using the double layer agar plate method, and this method was repeated three times to yield a pure phage (Ceyssens et al. 2009). The filtrates were then subjected to ultracentrifugation (Beckman SW-55 Ti rotor) at 90,000 × g at 4°C for 2 h. After this, the supernatant was removed and the pellet was dissolved with SM buffer (100 mM NaCl, 8.1 mM MgSO₄·7H₂O, 50 mM Tris·Cl (pH 7.5), 0.01% gelatin). Several drops of chloroform were added to the phage solution and the solution was then stored at 4°C for future use. The induced phage was designated as SAP-26 and deposited in the Korean Collection for Type Cultures under the accession number of KCTC 11665BP. Propagation of phage SAP-26 was performed with the clinical isolate of S. aureus WS-05, which does not harbor the SAP-26 sequence, as shown by PCR in the authors’ laboratory. The primers (5’-TTGCTTAA CAAAACACTCAAG-3’ and 5’-TCAATCACCAG TTTCATTTTC-3’) for the PCR are specific to ORF2, encoding head morphogenesis protein of the SAP-26. Determination of phage titer was performed as follows. After dilution of phage lysate with SM buffer, 0.1 ml of phage lysate and 0.1 ml of log phase host bacteria (10⁸ CFU ml⁻¹) were mixed with 3 ml of LB soft agar and then poured onto a LB agar plate. The plates were incubated overnight at 37°C and the number of plaques was counted. Phage titers were determined as plaque forming unit (PFU).

**Determination of adsorption rate and burst size of the phage SAP-26**

To determine the adsorption rate, S. aureus bacterial cells (WS-05 strain) were grown in LB broth to exponential phase, then infected with the phage SAP-26 at MOI 0.0001, and incubated at room temperature. Samples were taken and centrifuged at 0, 3, 6, 9, 12, 15, and 18 min. The supernatants were used for plaque assays to determine the titers of non-adsorbed phages (Hadas et al. 1997).

The burst size of the phage SAP-26 was determined by performing a single growth curve experiment as follows. S. aureus bacterial cells (WS-05 strain) were harvested by centrifugation and resuspended in 1 ml of fresh LB medium (5 × 10⁶ CFU ml⁻¹). Phage SAP-26 was added at an MOI of 0.0001 and allowed to adsorb for 30 min at 4°C. The mixture was centrifuged at 12,000 × g for 5 min and the pellet was resuspended with 10 ml of fresh LB medium, followed by further incubation at 37°C. Samples were taken at 5 min intervals up to 45 min and immediately diluted and titrated for phage plaque counting. Each of the above experiments was repeated three times, independently.

**Electron microscope observation of phage virion**

After deposition of the phage SAP-26 on carbon coated copper grids, phage particles were negatively stained with 2% uranyl acetate and viewed using a transmission electron microscope (TEM) (Hitachi-7000, Tokyo, Japan) operated at 60 kV.

**Lytic activity of the phage SAP-26 against clinical isolates of S. aureus**

A total of 110 MRSA isolates and 61 MSSA isolates were tested for susceptibility lytic activity of the phage SAP-26 by conducting spot tests. A bacterial lawn was prepared by pouring 4 ml of molten agar (0.75%) containing 100 µl of bacterial culture in stationary phase onto a LB agar plate. After solidification of the overlay, a 15 µl aliquot of phage (∼10¹⁰ PFU ml⁻¹) was spotted directly onto the bacterial lawn. Plates were dried and incubated overnight at 37°C. Plates that showed clear circular lytic zones were assessed as susceptible bacteria.

**Application of the SAP-26 phage with or without azithromycin, vancomycin, and rifampicin for the removal of S. aureus biofilm cells**

The ability of SAP-26 alone or combined with an antimicrobial agent for the removal of S. aureus biofilm cells was assessed as follows. Biofilm formation by bacteria in microtiter plates was performed as previously reported with minor changes (Lee et al.
2008). Briefly, an overnight culture of *S. aureus* D43-a strain was prepared in Mueller-Hinton broth (MHB) to produce a bacterial culture showing OD = 1.0 at 600 nm and was diluted again 1:100 with MHB. The wells of the microtiter plate were aliquoted with 100 µl of *S. aureus* and incubated at 37°C for 24 h. After incubation for 24 h, planktonic cells with medium were removed and the biofilms were exposed to phage alone, antimicrobial agent alone, or a combination of phage and antimicrobial agent. The number of phage used was 10^8 PFU. The antimicrobial agents used were azithromycin (Pfizer Inc., Groton, CT, USA), vancomycin (Sigma Chemical Co., St Louis, MO, USA), and rifampicin (Rifodex®, Chong Kun Dang Pharmaceutical Corp., Seoul, Korea) and concentrations of each drug were 10 fold higher concentrations than the MIC values for each drug, ie rifampicin (0.6 mg l^{-1}), azithromycin (80 mg l^{-1}) and vancomycin (10 mg l^{-1}). After treatment with phage and/or antimicrobial agent, the plates were incubated at 37°C for 2, 4, 8, 12, and 24 h, respectively. Colorimetric measurement with 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma Chemical Co., St Louis, MO, USA) was performed to measure live bacteria after the treatments as described in a previous report (Kim et al. 2010). The reduction of TTC by viable bacteria produced red formazan and bacterial growth was measured quantitatively by colorimetric absorbance at 540 nm. The bacterial load of the treated biofilm was also estimated by viable cell count and expressed as a log_{10} reduction value.

**Field emission scanning electron microscopy (FE-SEM)**

After treatment with phage SAP-26, rifampicin, or combined treatment, the structure of biofilms and bacterial cell populations within the biofilms were visualized by FE-SEM. The biofilms grown on cover slides were washed with phosphate buffer saline (PBS) and then dehydrated through a series of aqueous ethanol washes (20%, 50%, and 70% for 10 min and absolute ethanol for 30 min). Specimens were coated with gold and photographed with a FE-SEM (Hitachi S-4300, Hitachi, Tokyo, Japan).

**Phage DNA purification and genome sequencing**

Extraction and purification of phage genome were carried out with a Lambda Midikit (Qiagen, Valencia, CA, USA) according to instructions provided by the company. The full genomic sequence of the purified DNA was analyzed by shotgun library construction and full genome sequencing (Macrogen, Seoul, Korea). Identification of putative open reading frames (ORFs) and multiple sequence alignments were performed using Sequin application Ver. 9.5 (NCBI). DNA sequence similarity searches were performed using the NCBI BLAST program (http://www.ncbi.nlm.nih.gov) with sequences in the GenBank database. The genomic sequence data of the phage was also deposited in the GenBank under the accession number of GU477322.

**Results**

**Isolation and characterization of phage SAP-26**

Cultures of 47 *S. aureus* strains were treated with mitomycin C and the harvested supernatants were subjected to spot testing and plaque assay. Thirteen of the 47 supernatant samples were positive for a spot test and 6 out of the 13 spot test-positive supernatants were able to produce lytic plaques by a subsequent plaque forming assay. One of the six phages designated as SAP-26 formed lytic spots on almost all *S. aureus* strains tested. The phages also produced clear lytic plaques on their host bacteria, indicating the lytic characteristics of phage SAP-26. After several rounds of plaque assays, a phage isolate was purified and propagated.

Examination of the phage SAP-26 by TEM analysis demonstrated that the phage contained an icosahedral head and a long tail (Figure S1 [Supplementary material is available via a multimedia link on the online article webpage]). The diameter of the phage head was approximately 50 nm and the tail size was approximately 170 nm in length. According to the classification of Ackermann (2009), the phage SAP-26 belongs to the family Siphoviridae.

According to the phage adsorption assay, >95% of SAP-26 could adsorb to a susceptible *S. aureus* strain within 9 min (Figure 1A). The SAP-26 phage, however, could not bind to a non-susceptible *S. aureus* strain (Figure S2 [Supplementary material is available via a multimedia link on the online article webpage]). The results of a one-step growth curve showed that the latent period of SAP-26 was ~15 min and the burst size was ~10^7 phage particles from one bacterium (Figure 1B). This result indicates that SAP-26 is a lytic phage having a relatively large burst size.

**Bactericidal activity of SAP-26 against clinical isolates of S. aureus**

Using the phage SAP-26, *S. aureus* D43-a strain was treated at MOI 0.1, 1, 10 and 100 (Figure S3 [Supplementary material is available via a multimedia link on the online article webpage]). After a 6 h-incubation, the percent OD_{600} values of MOI 10 and 100 treated samples were decreased to ~14% of the untreated control values, and the percent OD_{600} values of MOI 0.1 and 1 treatments were ~60% and 40%, respectively. In a further susceptibility test, the phage
SAP-26 could kill all 110 MRSA isolates tested and 58 out of 61 (95%) of the MSSA isolates.

Biofilm removal activity of SAP-26 alone or in combination with antimicrobial agents

One day-old biofilms formed by *S. aureus* D43-a strain were challenged with phage SAP-26 alone, antimicrobial agent alone, or a combination of phage and antimicrobial agent for 24 h (Figure 2). Phage SAP-26 alone could kill approximately 28% of the biofilm bacteria after 24 h (~72% cells alive after 24 h). Among the three antimicrobial agents used, rifampicin showed the best bactericidal result (~60% cells alive after 24 h). Azithromycin and vancomycin could kill ~25% and 17%, respectively (~75% and 83% cells alive after 24 h, respectively). However, a substantial synergistic effect was observed when the *S. aureus* biofilm was treated with the phage SAP-26 and rifampicin in combination. Approximately 35% of the live cells remained after the treatment which had the best bactericidal effect among other combined treatments. Phage SAP-26/azithromycin and phage SAP-26/vancomycin treatments showed ~40% and 60% cells alive after 24 h, respectively.

In viable bacterial cell count assays, a conventional method to determine the decrease in viable bacteria after the phage and/or rifampicin treatments, phage SAP-26 (10^9 PFU) killed ~log 3 cells of the biofilm bacteria and rifampicin killed ~log 4 cells of the biofilm bacteria (Figure 3), while the co-treatment with phage SAP-26 and rifampicin killed ~log 5 cells of the biofilm bacteria, indicating ~0.001% survival.

The *S. aureus* biofilms which were treated with phage SAP-26 and/or rifampicin were observed and photographed by FE-SEM (Figure 4). In the untreated control group, many cocci were seen as highly dense forms with a biofilm matrix (Figure 4A). In the phage SAP-26 treatment group, grape-like cell clusters and clear free spaces were shown and the cell boundaries were faint and unclear (Figure 4B). The biofilm matrixes were broken down after phage SAP-26 treatment. The cell population of the rifampicin treatment decreased more than that of the phage SAP-26 treatment (Figure 4C), which was consistent with the data from the live cell measurements. In the combination treatment of SAP-26 and rifampicin, a few bacteria and many ghost cells with irregular morphology remained, with many free spaces (Figure 4D), indicating efficient eradication of the biofilm by SAP-26 and rifampicin.

**Genomic characteristics of SAP-26**

The complete genome sequence of SAP-26 was determined. The SAP-26 genome comprises double-stranded DNA with an average GC content of 34.02%. The genome size of SAP-26 was 41207 bp. A total of 63 ORFs were identified by Glimmer (Ver. 3.0) analysis. Among them, phage-related functions were predicted from 22 ORFs, and the others were hypothetical proteins (Supplementary Information Table 1 [Supplementary material is available via a multimedia link on the online article webpage]). Sequence analysis showed that SAP-26 is a close relative of *S. aureus* phage phiNM1 (Bae et al. 2006), showing an overall DNA similarity of 75% throughout the genome. Also, SAP-26 shares 69% similarity with *S. aureus* phage phi11 (Genbank acc. number AF424781) and 67% similarity with phiMR25 (Genbank acc. number AB370205). Most importantly, the genomic sequence has verified that SAP-26 carries neither antibiotic resistant genes nor known toxin genes often associated with certain *S. aureus* phages, such as enterotoxin (Betley and Mekalanos 1985), leukocidin (Kaneko et al. 1997, 1998), and exfoliative toxin (Yamaguchi et al. 2000; Yoshizawa et al. 2000).
Biofilm associated infections have been implicated in 465% of microbial infections (Spoering and Lewis 2001) where microbial cells were found to be 10 to 1000-fold more resistant to antimicrobial agents than their planktonic counterparts (Gilbert et al. 2002). In that situation phages have been suggested as effective antibiofilm agents (Donlan 2009).

There are reports regarding the eradication for *S. aureus* using phages. Lysin originated from a phage has been used for the elimination of MRSA (Rashel et al. 2007) and phages have been tested in vivo to treat *S. aureus* infection (Capparelli et al. 2007). However, limited reports are available regarding *S. aureus* biofilm treatment using phages and combinations of phages and antibiotics.

In the present study, treatment of *S. aureus* biofilm with a new induced lytic phage in combination with an antimicrobial agent resulted in a significant reduction in bacterial numbers. Among the three antimicrobial agents combined with phage, the combination of rifampicin with phage showed the best biofilm removal effect. To the authors’ knowledge, this study showed for the first time that *S. aureus* biofilm could be efficiently eradicated with the mixture of phage and rifampicin. The new phage, SAP-26, was isolated from a clinical *S. aureus* isolate WS-26 by induction. Although it is an induced phage, it could lyse almost all *S. aureus* clinical isolates regardless of whether the isolates were MRSA or MSSA. Such a broad host range in *S. aureus* and the strong biofilm removal activity of the phage SAP-26 suggests its potential as a therapeutic phage when administered in conjunction with an appropriate antimicrobial agent(s). Other scientists have also reported that temperate *S. aureus* phages could have therapeutic potential (Hoshiba et al. 2010). The genome of the SAP-26 is similar to other *S. aureus* phages such as PhiNM1 and Phi MR25. These phage genomes contain some important components such as cell wall hydrolase, phage holin and phage amidase which play an important role in killing bacteria.

In addition to rifampicin, azithromycin and vancomycin in combination with SAP-26 were used. However, those antibiotics did not produce a better bactericidal effect than that of the SAP-26/rifampicin co-treatment. Azithromycin reduced additional biofilm formation by *Staphylococcus epidermidis*, but had little effect on biofilm density, even in combination with other antimicrobial agents (Presterl et al. 2009). Vancomycin was substantially reduced through *S. aureus* biofilms (Singh et al. 2010). Rifampicin penetrated *S. epidermidis* biofilms, but could not efficiently kill the biofilm-embedded bacteria (Zheng and Stewart 2002). In the case of combined treatment of biofilm with antibiotics and phage, it may be assumed that phages are able to penetrate biofilm layers through the pores and channels, thereby causing

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**Discussion**

Biofilm associated infections have been implicated in >65% of microbial infections (Spoering and Lewis 2001) where microbial cells were found to be 10 to 1000-fold more resistant to antimicrobial agents than their planktonic counterparts (Gilbert et al. 2002). In that situation phages have been suggested as effective antibiofilm agents (Donlan 2009).

In the present study, treatment of *S. aureus* biofilm with a new induced lytic phage in combination with an antimicrobial agent resulted in a significant reduction
destruction of the biofilm matrix (Hughes et al. 1998). Both phage and antibiotics can reach and adsorb to cells in different biofilm layers, including the basal layer of the biofilm, causing death of the cells. Due to the destruction of biofilm matrix, the bacterial cells were released as planktonic cells and then attacked by both antibiotics and phages. This could be the reason for the efficient killing of bacterial cells observed in the present study when two agents were applied in combination. Although the efficacy of phages against \textit{S. aureus} biofilm has been documented, few techniques are available that can demonstrate the interaction of biofilms with bacteriophage. In the present study, viable cell counting was performed to measure the number of live bacteria and FE-SEM analysis to visualize biofilm structure. From those analyses, it was observed that phage SAP-26 with rifampicin destroyed the biofilm matrix and killed bacterial cells. Therefore, it was clearly demonstrated that a combined treatment using rifampicin and phage works efficiently against biofilm. In conclusion, phage SAP-26 and rifampicin could simultaneously be used to eradicate as well as to reduce growth of \textit{S. aureus} biofilms.

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