



Development of Teicoplanin Tolerance by *Staphylococcus epidermidis* and Increased Susceptibility to Bacteriophage Type 92 by Methicillin-Resistant *Staphylococcus aureus* in Polymicrobial Biofilms

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Abstract

Staphylococcus epidermidis is a commensal commonly found in polymicrobial biofilms with *Staphylococcus aureus*. Given the increasing drug resistance in Staphylococci, we explored combination therapy with teicoplanin and bacteriophage type 92 (Siphoviridae) on the survival and antibiotic tolerance of methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis* to teicoplanin and bacteriophage in 48-h pure and mixed culture biofilms. The combination of teicoplanin and bacteriophage was more effective against MRSA, but not *S. epidermidis* monocultures, than the use of either teicoplanin or phage alone. In polymicrobial biofilms, however, MRSA acquired increased susceptibility to phage infection and *S. epidermidis* acquired increased tolerance to teicoplanin, as well as increased fitness. The results demonstrate bacteriophage alone was more effective against MRSA and *S. epidermidis* in polymicrobial biofilms compared to the combination of bacteriophage and teicoplanin.

Keywords

Biofilms, Bacteriophage, Antibiotic, Tolerance, MRSA, Mixed culture

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as one of the most dangerous and widely publicized pathogens in the United States since its initial identification [1,2]. Originally limited to nosocomial, or healthcare-associated infections (HA-MRSA), MRSA has since been identified as a serious public health threat to persons with no affiliation with hospital settings [3]. In the 2000s, MRSA emerged in community settings [4] including USA college campuses associated with clinical programs resulting in the distinction between HA-MRSA and community-acquired MRSA (CA-MRSA) [5]. The gene responsible for methicillin resistance in *S. aureus*, *mecA*, is not found on methicillin-susceptible strains of *S. aureus* [4]. A number of infections are associated with MRSA including those associated with medical devices, soft tissue abscess infections and occasionally more invasive conditions. MRSA infections are often polymicrobial and frequently include *Staphylococcus epidermidis* [6]. In medical

device infections, MRSA often grows as surface-adherent biofilm communities. Biofilm growth results in increased antimicrobial resistance, particularly in a polymicrobial community [7].

Beta-lactam resistant staphylococcal infections are often treated with glycopeptide antibiotics, such as vancomycin and teicoplanin [8]. While similar to vancomycin in bactericidal spectrum, teicoplanin is not currently FDA-approved for use in the United States [8]. Outside the US, several studies have investigated the efficacy and potential side effects of teicoplanin in comparison to other glycopeptide antibiotics including vancomycin [9]. Since staphylococcal species easily attain antibiotic resistance, and some bacteriophages are known to degrade biofilm matrices, attention has refocused on the therapeutic use of bacteriophages [10].

Bacteriophages have been used since the earliest 20th century to treat a number of infections [11,12]. In Europe and North America phage therapy has been largely supplanted by antibiotic therapy [11] although it has still been pursued in the former Soviet Union and other eastern countries [13]. With the increasing resistance of bacterial infections to antibiotics, phage therapy is being reexamined. In this context, examples of experimental strategies include the use of phage cocktails targeting multiple bacterial strains [14] and combination phage-antibiotic therapy [15]. A number of phage depolymerase enzymes have been described, which enable phage to penetrate biofilm matrix polymers [10,16]. While these work well in monoculture biofilms, polymicrobial biofilms have variable matrix chemistries and our previous study [17] has shown matrix polymers from a non-target host organism to be capable of interfering with some phage. Here, we investigate the effectiveness of teicoplanin and bacteriophage combination therapy in monoculture and polymicrobial biofilms of methicillin-resistant *S. aureus* and methicillin-susceptible *S. epidermidis*. These two organisms are a frequent cause and commonly co-exist in transcutaneous medical device infections [6,18].

Materials and Methods

Bacterial strains, bacteriophage, media, and culture conditions

Methicillin-resistant *Staphylococcus aureus* (ATCC 37741),

Staphylococcus epidermidis (ATCC 12228), and Bacteriophage type 92 (ATCC 33741-B) [19] were obtained from the American Type Culture Collection (Manassas, VA). *S. aureus* and *S. epidermidis* were grown in Tryptic Soy broth (TSB) (Accumedia Inc., Lansing, Michigan) at 37°C in an orbital rotating shaker water bath. Bacteriophage type 92 was used to infect *S. aureus* and *S. epidermidis*. Bacteriophage 92 stocks were prepared by infecting early log phase *S. aureus* using the agar overlay technique as previously described [20]. Briefly, 350 µL of early log phase *S. aureus* was added to 3.5 mL of 0.38% (w/v) agar in TSB. 100 µL of 10⁶ (plaque-forming units (PFU)/mL) phage was added to yield confluent lysis. Following 24 h at 37°C, phage was eluted from the agar overlay in 5 mL TSB at 4°C. The phage-containing liquid was centrifuged at 4°C for 20 min at 4000g (Eppendorf Centrifuge model 5810R, Hamburg, Germany). Supernatant was filtered (0.45 µm) and phage titers (PFU/mL) were determined by a soft-agar overlay assay [20]. Teicoplanin (Sigma-Aldrich Co. St Louis, MO) stocks were prepared in deionized water followed by filter sterilization. Teicoplanin at a concentration of 10 µg/mL was used for all experiments. During dilution plating of mixed cultures, *S. aureus* and *S. epidermidis* could be easily distinguished by growth on mannitol salt agar. A minimum of three biological replicates was performed for each study.

Biofilm formation

Overnight cultures (18 h) of *S. aureus* or *S. epidermidis* were diluted 1:500 and 200 µL was dispensed into clear, sterile, non-tissue treated 96-well plates (Fisher Scientific Co., Horizon Ridge, CT). For polymicrobial biofilms, 50 µL of each overnight culture were diluted in 50 mL TSB and 200 µL of the diluted culture was dispensed into plates. Plates were incubated for 48 h at 37°C with rocking at 10°C, at 15 rpm (VWR Signature™ Incubating Rocking Platform Shaker, VWR, Houston, TX).

Biofilm bacteriophage and antibiotic susceptibility assays

Supernatant was removed from 48 h *S. aureus* biofilms and biofilms washed with 200 µL PBS. Biofilms were treated with either phage (multiplicity of infection (MOI) 10), teicoplanin (10 µg/mL), or a combination of both contained in 200 µL TSB at 37°C. After 12h treatment, biofilms were sonicated for 1 min at 40 kHz, and colony forming units (CFU) determined by dilution plating on TSA plates.

Determination of increase in tolerance to teicoplanin by *S. epidermidis*.

Susceptibility of *S. epidermidis* to teicoplanin was determined

using the Kirby-Bauer disk diffusion technique, as described by the Clinical and Laboratory Standards Institute [21] with TSA substituted for Mueller-Hinton agar. *S. epidermidis* susceptibility to teicoplanin was tested before and after exposure to *S. aureus* in polymicrobial biofilms. *S. epidermidis* recovered from polymicrobial biofilms was sub-cultured nine successive times and each sub-culture was tested for susceptibility to teicoplanin.

Effect of *S. epidermidis* supernatant on *S. aureus* phage sensitivity

S. aureus sensitivity to phage was determined by growing *S. aureus* biofilms with 100 µL of *S. epidermidis* supernatant for 48 h at 37°C. Culture supernatant was obtained after 18 h planktonic culture from either *S. epidermidis* monoculture, or a mixed *S. epidermidis* – MRSA culture. Supernatants were prepared by centrifugation (3000 g for 20 min) followed by filtration through a 0.45 µm pore-size filter. *S. aureus* biofilms were washed with 200 µL PBS and infected with phage at MOI 10. Biofilms were enumerated by sonication and dilution plating as described above.

Transmission electron microscopy (TEM)

For TEM examination, 5 µL of 10¹⁰ PFU/mL phage were placed on 200 mesh count, Formvar-coated copper grids (Electron Microscopy Sciences, Hatfield, PA) overnight at room temperature. Samples were stained with 2% uranyl acetate for one minute and viewed using a JEM-1200 EXII at an accelerating voltage of 60 KV.

Results

Phage type 92 characterization

Phage type 92 was originally isolated in 1979 from MRSA isolates [19]. TEM examination of phage 92 (Figure 1), revealed icosahedral capsids approximately 62 nm in diameter and long, flexible tails approximately 175 nm in length. On the basis of viral morphology and established taxonomy guidelines [22], phage 92 belongs to the family Siphoviridae.

Effect of bacteriophage and teicoplanin, on monoculture and mixed culture biofilms

Growth of *S. aureus* and *S. epidermidis* following treatment with phage 92, teicoplanin and combination treatment is shown in Figure 2. We also calculated changes in fitness (expressed as a ratio of (log CFU treated culture/log CFU monoculture control)) which is shown in Figure 3. When compared to untreated controls, the most effective



Figure 1: TEM micrograph of Phage 92. Based on the flexible tail and icosahedral head [22], this phage should be classified in the family Siphoviridae.

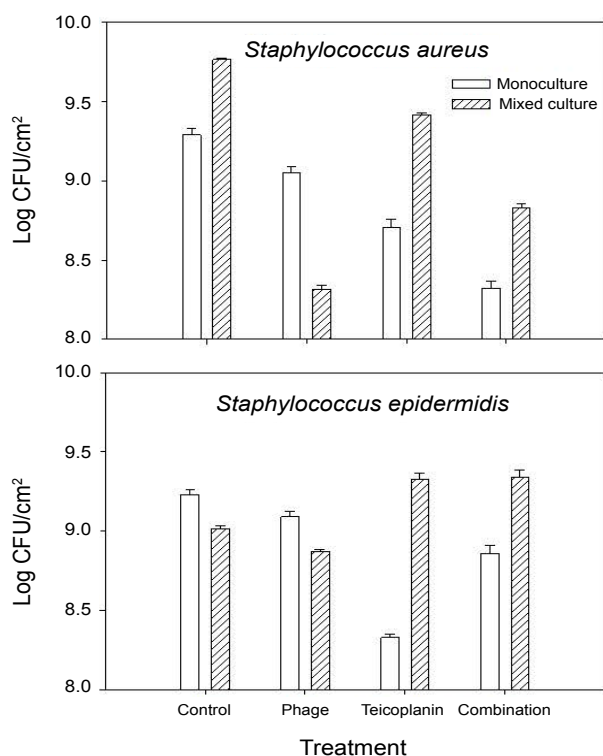


Figure 2: Growth of MRSA (*S. aureus*) and *S. epidermidis* biofilms following exposure to phage 92, teicoplanin, or combination phage 92 and teicoplanin. Details are in the text.

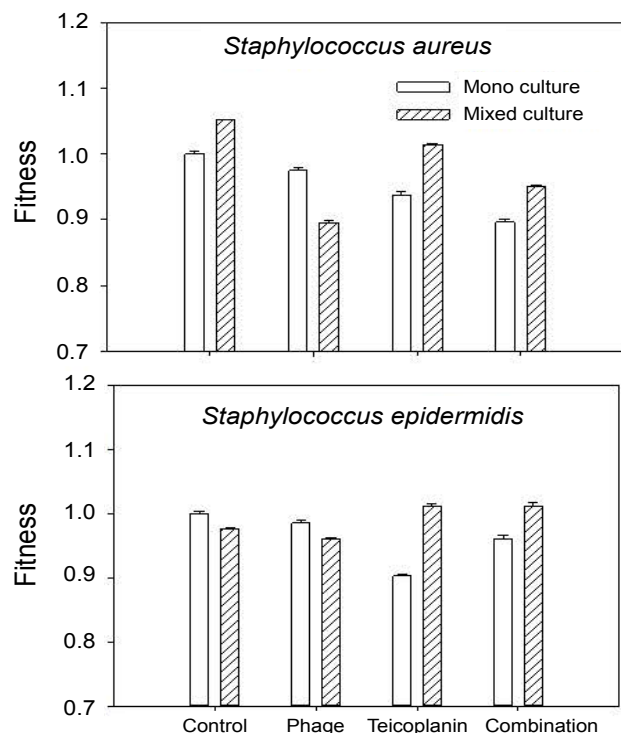


Figure 3: Fitness of *S. aureus* and *S. epidermidis* expressed as the ratio of log CFU treated culture/log CFU untreated (control) monoculture.

treatment of monoculture MRSA biofilm cultures was combination phage and teicoplanin. Since phage require growing host cells in order to replicate, the enhanced success of the combination treatment suggests that teicoplanin did not completely block *S. aureus* growth when added in combination with phage 92. Different results were seen during treatment of mixed culture *S. aureus*. Here, the most effective treatment was phage alone. One likely explanation for this

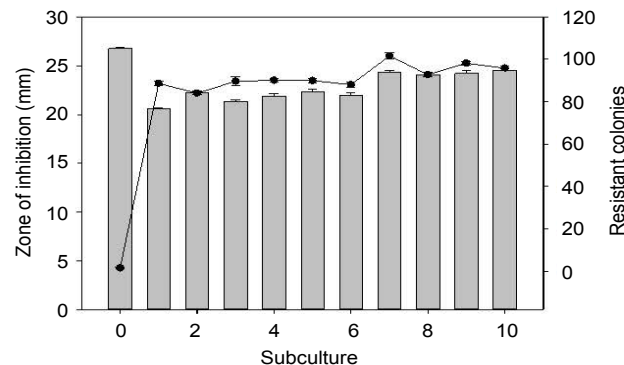


Figure 4: Subculture of *S. epidermidis* with MRSA resulted in a slight increase of tolerance as reflected in a decrease of the zone of inhibition (bar graph). As well the number of resistant colonies (line graph) increased. The notable changes occurred after the organisms were initially grown together (subculture 1), with minimal changes seen during subsequent subcultures.

phenomenon is that phage attack on the host *S. aureus* facilitated competition from *S. epidermidis*, which was minimally affected by the phage during monoculture growth. Of interest, growth in mixed culture resulted in less impact of teicoplanin against MRSA alone or in combination with phage 92.

In monoculture *S. epidermidis* cultures, the most effective treatment was teicoplanin. This is not surprising as teicoplanin has been reported to be effective against this organism [9]. In contrast, mixed culture results were quite different. In untreated mixed-culture biofilms, MRSA outcompeted *S. epidermidis* which is reflected both in culture numbers (Figure 2 control) and fitness calculations (Figure 3 control). Although MRSA is the host organism for phage 92, there was a slight decrease in *S. epidermidis* numbers and fitness ($P < 0.001$) relative to the mixed culture control, suggesting that some phage influence or competition from MRSA was occurring in the presence of phage. In contrast to the reduced fitness seen in *S. epidermidis* monocultures with teicoplanin alone or in combination with phage 92; the fitness of *S. epidermidis* was significantly increased in mixed culture ($P < 0.001$ in comparison to monoculture results) when grown in mixed culture. We also observed that repeated subculture of *S. epidermidis* with MRSA decreased teicoplanin susceptibility and the onset of resistance (Figure 4).

Discussion

The decreased antibiotic susceptibility of monoculture biofilms has been known for over 30 years [23] and a number of mechanisms for biofilm-mediated resistance have been described including altered growth rates within biofilms, biofilm-specific resistance genes and the presence of dormant persister subpopulations (reviewed in [24,25]). Biofilms including MRSA wound infections [6] typically contain polymicrobial communities and there is increasing interest in studying antimicrobial susceptibility in that context. Two studies showed that increased bacterial community diversity resulted in higher biofilm cell density [26,27]. In one of these studies, increased biofilm resistance to disinfection was due to general interactions among all community members [26]. In the second study, biofilm resistance was entirely due to the presence of a single resistant community member [27]. Combination phage-antibiotic treatments have shown promise in the treatment of monoculture biofilms e.g. [28,29], however their effectiveness against mixed cultures is less clear. With planktonic cultures, Harcombe and Bull [30] showed that phage could enhance microbial competition in a two-species system by targeting one component organism. However, biofilm growth does enable many bacteria to persist in spite of competition [31].

Our data would suggest that microbial competition does play a role in the efficacy of some antimicrobial treatments against some biofilms, most notably the phage 92 treatment of MRSA in mixed cultures (Figure 3). Competition was not evident during treatment of mixed culture biofilms with teicoplanin alone or in combination

with phage 92. The increased fitness of both MRSA and *S. epidermidis* during teicoplanin treatment in comparison to the monoculture results ($P < 0.001$) suggests that the two normally competitive organisms, exhibit synergy (facilitation) in the presence of teicoplanin. In previous work with environmental isolates, we found that the presence of one disinfectant (betadine) resistant organism could confer resistance to an entire mixed biofilm population [27] likely through facilitation, so this finding is not unprecedented.

In planktonic monoculture or mixed *Pseudomonas aeruginosa* and *Escherichia coli* cultures, we observed that the administration of sub-lethal concentrations of antibiotics or phage is followed rapidly by the onset of resistance through the planktonic populations likely through mutation and natural selection. In contrast, while biofilm populations of these organisms were protected by this mode of growth, a significant proportion of organisms within biofilms retained susceptibility to phage or antibiotics when released from biofilms by sonication, diluted and cultured on antibiotic- or phage-containing agar [15,17]. We tested the influence of previous co-culture growth on the teicoplanin susceptibility of *S. epidermidis* using a disk diffusion assay. We also measured the number of spontaneous teicoplanin-resistant colonies which grew within the zone of inhibition. During these studies, *S. epidermidis* was sub-cultured overnight up to ten times with MRSA and then purified and tested. As seen in Figure 4, there was a slight but significant ($P < 0.01$) decrease of susceptibility following the first sub-culture. In subsequent subcultures, while there was some variability in the zone of inhibition, it was still significantly less than the original culture (designated as subculture 0) before mixed culture growth. Similarly, there was an increase in the number of resistant colonies seen after the first sub-culture with the numbers increasing from 4 to > 90 in most cases. Although *S. aureus* (ATCC 37741) is resistant to methicillin, it is sensitive to teicoplanin, which would rule out horizontal gene transfer of a pre-existing resistance gene to *S. epidermidis*. Although we do not have a direct explanation for this phenomenon, it is possible that mixed culture growth of *S. epidermidis* with *S. aureus* activated mechanisms that enabled *S. epidermidis* to better tolerate exposure to teicoplanin. Further work will be needed to elucidate the mechanism(s) behind this phenomenon.

Conclusions

Many *S. aureus* infections involve polymicrobial interactions with *S. epidermidis* and biofilm growth [6]. In the current study, interactions between *S. aureus* and *S. epidermidis* strains used affected the efficacy of teicoplanin antibiotic and phage treatment. In some cases, when one organism was targeted (MRSA specific phage 92 in mixed culture), mixed culture interactions enhanced the treatment due to microbial competition, whereas in other cases in which both organisms were targeted by teicoplanin, the efficacy was reduced possibly due to facilitation interactions. Overall these data emphasize the importance of studying mechanisms of antimicrobial treatments in a polymicrobial biofilm growth system.

Acknowledgments

This work was funded by the Homer E. Prince Microbiology Endowment to GM Aron. We thank Alissa Savage for help with TEM.

Conflict of Interest

The authors declare no conflict of interest.

References

- Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, et al. (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* 298: 1763-1771.
- Kuroda M, Ohta T, Uchiyama I, Baba T, Yuzawa H, et al. (2001) Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* 357: 1225-1240.
- Ubukata K, Nonoguchi R, Matsuhashi M, Konno M (1989) Expression and inducibility in *Staphylococcus aureus* of the *mecA* gene, which encodes a methicillin-resistant *S. aureus*-specific penicillin-binding protein. *J Bacteriol* 171: 2882-2885.
- Ma XX, Ito T, Tiensasitorn C, Jamklang M, Chongtrakool P, et al. (2002) Novel

type of staphylococcal cassette chromosome *mec* identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains. *Antimicrob Agents Chemother* 46: 1147-1152.

- Rohde RE, Denham R, Brannon A (2009) Methicillin resistant *Staphylococcus aureus*: carriage rates and characterization of students in a Texas university. *Clinical Laboratory Science* 22: 176-184.
- Peel TN, Cheng AC, Buising KL, Choong PF (2012) Microbiological aetiology, epidemiology, and clinical profile of prosthetic joint infections: are current antibiotic prophylaxis guidelines effective? *Antimicrob Agents Chemother* 56: 2386-2391.
- Pozzi C, Waters EM, Rudkin JK, Schaeffer CR, Lohan AJ, et al. (2012) Methicillin resistance alters the biofilm phenotype and attenuates virulence in *Staphylococcus aureus* device-associated infections. *PLoS Pathog* 8: e1002626.
- Svetitsky S, Leibovici L, Paul M (2009) Comparative efficacy and safety of vancomycin versus teicoplanin: systematic review and meta-analysis. *Antimicrob Agents Chemother* 53: 4069-4079.
- Gallon O, Guillet-Caruba C, Lamy B, Laurent F, Doucet-Populaire F, et al. (2009) In vitro activity of daptomycin against *Staphylococci* isolated from bacteremia and community-onset skin and soft tissue infections in France: data from two nationwide studies. *Eur J Clin Microbiol Infect Dis* 28: 1209-1215.
- Parasion S, Kwiatek M, Gryko R, Mizak L, Malm A (2014) Bacteriophages as an alternative strategy for fighting biofilm development. *Pol J Microbiol* 63: 137-145.
- Hanlon GW (2007) Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *Int J Antimicrob Agents* 30: 118-128.
- d'Herelle F (1931) Bacteriophage as a Treatment in Acute Medical and Surgical Infections. *Bull N Y Acad Med* 7: 329-348.
- Marcuk LM, Nikiforov VN, Scerbak JF, Levitov TA, KotljaroVA RI, et al. (1971) Clinical studies of the use of bacteriophage in the treatment of cholera. *Bull World Health Organ* 45: 77-83.
- Krylov VN (2014) Bacteriophages of *Pseudomonas aeruginosa*: long-term prospects for use in phage therapy. *Adv Virus Res* 88: 227-278.
- Coulter LB, McLean RJ, Rohde RE, Aron GM (2014) Effect of bacteriophage infection in combination with tobramycin on the emergence of resistance in *Escherichia coli* and *Pseudomonas aeruginosa* biofilms. *Viruses* 6: 3778-3786.
- Sutherland IW, Hughes KA, Skillman LC, Tait K (2004) The interaction of phage and biofilms. *FEMS Microbiol Lett* 232: 1-6.
- Kay MK, Erwin TC, McLean RJ, Aron GM (2011) Bacteriophage ecology in *Escherichia coli* and *Pseudomonas aeruginosa* mixed-biofilm communities. *Appl Environ Microbiol* 77: 821-829.
- Krimmer V, Merkert H, von Eiff C, Frosch M, Eulert J, et al. (1999) Detection of *Staphylococcus aureus* and *Staphylococcus epidermidis* in clinical samples by 16S rRNA-directed in situ hybridization. *J Clin Microbiol* 37: 2667-2673.
- Schaeffler S, Perry W, Jones D (1979) Methicillin-resistant strains of *Staphylococcus aureus* phage type 92. *Antimicrob Agents Chemother* 15: 74-80.
- Adams MH (1959) Methods of study of bacterial viruses, Bacteriophages, New York: Interscience Publications.
- Li G, Ma X, Deng L, Zhao X, Wei Y, et al. (2015) Fresh Garlic Extract Enhances the Antimicrobial Activities of Antibiotics on Resistant Strains in Vitro. *Jundishapur J Microbiol* 8: e14814.
- Ackermann HW (2009) Phage classification and characterization. *Methods Mol Biol* 501: 127-140.
- Nickel JC, Ruseska I, Wright JB, Costerton JW (1985) Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. *Antimicrob Agents Chemother* 27: 619-624.
- Lewis K (2007) Persister cells, dormancy and infectious disease. *Nat Rev Microbiol* 5: 48-56.
- Zhang L, Fritsch M, Hammond L, Landreville R, Slatculescu C, et al. (2013) Identification of genes involved in *Pseudomonas aeruginosa* biofilm-specific resistance to antibiotics. *PLoS One* 8: e61625.
- Burmølle M, Webb JS, Rao D, Hansen LH, Sørensen SJ, et al. (2006) Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. *Appl Environ Microbiol* 72: 3916-3923.
- Whiteley M, Ott JR, Weaver EA, McLean RJC (2001) Effects of community composition and growth rate on aquifer biofilm bacteria and their susceptibility to betadine disinfection. *Environ Microbiol* 3: 43-52.
- Bedi MS, Verma V, Chhibber S (2009) Amoxicillin and specific bacteriophage can be used together for eradication of biofilm of *Klebsiella pneumoniae* B5055. *World J Microbiol Biotechnol* 25: 1145-1151.
- Fu W, Forster T, Mayer O, Curtin JJ, Lehman SM, et al. (2010) Bacteriophage cocktail for the prevention of biofilm formation by *Pseudomonas aeruginosa* on catheters in an in vitro model system. *Antimicrob Agents Chemother* 54: 397-404.
- Harcombe WR, Bull JJ (2005) Impact of phages on two-species bacterial communities. *Appl Environ Microbiol* 71: 5254-5259.
- Elias S, Banin E (2012) Multi-species biofilms: living with friendly neighbors. *FEMS Microbiol Rev* 36: 990-1004.