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REVIEW

Bacteriophages and Lysins in Biofilm Control

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Abstract

To formulate the optimal strategy of combatting bacterial biofilms, in this review we update current knowledge on the growing problem of biofilm formation and its resistance to antibiotics which has spurred the search for new strategies to deal with this complication. Based on recent findings, the role of bacteriophages in the prevention and elimination of biofilm-related infections has been emphasized. *In vitro*, *ex vivo* and *in vivo* biofilm treatment models with single bacteriophages or phage cocktails have been compared. A combined use of bacteriophages with antibiotics *in vitro* or *in vivo* confirms earlier reports of the synergistic effect of these agents in improving biofilm removal. Furthermore, studies on the application of phage-derived lysins *in vitro, ex vivo* or *in vivo* against biofilm-related infections are encouraging. The strategy of combined use of phage and antibiotics seems to be different from using lysins and antibiotics. These findings suggest that phages and lysins alone or in combination with antibiotics may be an efficient weapon against biofilm formation *in vivo* and *ex vivo,* which could be useful in formulating novel strategies to combat bacterial infections. Those findings proved to be relevant in the prevention and destruction of biofilms occurring during urinary tract infections, orthopedic implant-related infections, periodontal and peri-implant infections. In conclusion, it appears that most efficient strategy of eliminating biofilms involves phages or lysins in combination with antibiotics, but the optimal scheme of their administration requires further studies.

Keywords Antibiotics · Bacteriophages · Biofilm · Chronic diseases · Lysins

Introduction

Bacteriophages are viruses that specifically infect and kill their bacterial hosts. The growing threat of antimicrobial resistance (AMR) and the paucity of newly developed antibiotics has revived interest in using bacteriophages (phages) to combat AMR. So far no formal proof of effectiveness of phage therapy has been obtained, therefore it is still carried out as compassionate treatment (experimental therapy). Nevertheless, data from animal models and human clinics strongly suggest that phage therapy is safe and has possible beneficial actions (Górski *et al.* 2019; Hesse and Adhya 2019; McCallin *et al.* 2019).

Biofilms are communities of microorganisms adhered to both biological and abiotic surfaces. It may be involved in the pathogenesis of chronic diseases, especially infections associated with the use of catheters, drains and implant placement (Akanda *et al.* 2018; Maszewska *et al.* 2018; Morris *et al.* 2019; Taha *et al.* 2018). Bacterial cells are embedded in a self-produced matrix of extracellular polymeric substances (polysaccharides, proteins, lipids and nucleic acids). These structures are characterized by low susceptibility to antibiotics (Pires *et al.* 2017b). Increased cell density and physiological changes in a biofilm may be the cause of growing biofilm resistance to antibiotics (Mah and O'Toole 2001). Furthermore, biofilm is characterized by lower metabolic activity, slower growth and greater opportunity for exchange of antibiotic resistance genes,

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which is also responsible for the induction of antibiotic resistance (Akanda *et al.* 2018).

Due to the ineffective antibiotic therapy of biofilms, there has been growing interest in phages as a strategy in preventing biofilm formation and elimination. Some studies on the application of phages as an alternative strategy to prevent and control biofilm are encouraging (Pires *et al.* 2017b). However, the cells located in the deeper layers of biofilm in the absence of oxygen and nutrients show reduced metabolic activity, which affects the lower activity of antibiotics and the lower replication of phages (Pires *et al.* 2017b; Sillankorva and Azeredo 2014). Within the biofilm, acquisition of phage resistance is also observed (Pires *et al.* 2017b). The mechanisms by which bacteria acquire resistance to phage are known: prevention of integration of phage DNA into bacterial DNA; degradation of phage DNA by clustered regularly interspaces short palindromic repeats CRISPR/Cas; blocking phage replication, transcription and translation; prevention of phage adsorption by structural modifications of bacterial receptors or by inaccessibility of receptors due to the presence of matrix biofilm.

The phages can be applied as a single phage preparation or a phage cocktail. Phage cocktails increase the spectrum of phage activity and reduce the development of phage-resistant variants (Pires *et al.* 2017b). Phage cocktails have proven to be efficient in preventing biofilm formation and biofilm eradication (Abedon *et al.* 2017; Abedon 2018). Phages can be used with other antibacterial agents, such as antibiotics, honey and disinfectants, to improve the effectiveness of biofilm elimination (Melo *et al.* 2019). Synergism of phages with mechanical debridement against biofilms has also been observed (Melo *et al.* 2019). Due to their ability to penetrate biofilms, bacteriophages may be applied with an antibiotic or as a substitute antibiotic in biofilm treatment (Abedon *et al.* 2017). Additionally, depolymerases (matrix degrading enzymes encoded by phage) can be used to prevent and disperse biofilm (Abedon *et al.* 2017; Chan and Abedon 2015; Pires *et al.* 2017b). Phage-derived lysins are bacteriophage enzymes that cleave peptidoglycan, the main component of the bacterial cell wall of either Gram-positive or Gram-negative bacteria, inducing lysis of a bacterial cell (Borysowski *et al.* 2011; Fischetti 2018). Recent investigations have indicated that phage-derived lysins can potentially be used as antibacterial agents (Borysowski *et al.* 2011; Gray *et al.* 2018; Lood *et al.* 2015; Sharma *et al.* 2018). This review is intended to explain the application of phages and lysins or in combination with antibiotics against biofilm formation or eradication as a possible strategy for removing bacterial infections.

Phages and Phage Cocktail in Control of Biofilms

Biofilm may form on catheters or implants during chronic infections such as urinary tract infections and orthopedic implant-related infections. During treatment of biofilm with single *Pseudomonas aeruginosa* phage, phage-resistant bacteria may form at 6 h after infection (Pires *et al.* 2017a). Phage-resistant variants have also been observed in human and animal studies (Oechslin 2018). The appearance of bacterial mutations may be the cause of emergence of phage-resistant mutants (Oechslin 2018). Bacteriophage cocktails delay the appearance of phage-resistant bacteria compared to single phages. Application of bacteriophage cocktails that target different host receptors is recommended to prevent phage-resistance (Bai *et al.* 2019). Phages with different ranges of lytic activity can be used as a single phage preparation or a phage cocktail to enhance their lytic activity. Phage cocktails improve the lytic effects by extending the phage host range and increasing the number of target pathogens (Oechslin 2018). According to various authors, phage alone or phage cocktails may be applied to prevent bacterial colonization and biofilm formation on such medical devices (Maszewska *et al.* 2018; Melo *et al.* 2016; Morris *et al.* 2019).

Application of Phages in Biofilm-Related Urinary Tract Infections

Urinary tract infections are the most widespread human infections and about 75% of them are associated with the use of a urinary catheter (Maszewska *et al.* 2018). Phage cocktails or single phages were used *in vitro* to against catheter-associated urinary tract infections causing by *Proteus mirabilis* or *P. aeruginosa* biofilms created on catheters or on a polystyrene plate (Fu *et al.* 2010; Maszewska *et al.* 2018; Melo *et al.* 2016). Comparison of the effects of a single phage and a phage cocktail is important in the treatment of biofilm (Fu *et al.* 2010; Maszewska *et al.* 2018; Melo *et al.* 2016).

A phage cocktail consisting of two phages against *P. mirabilis* was used on a dynamic biofilm model simulating a catheter-associated urinary tract infection (Melo *et al.* 2016). The phage cocktail significantly reduced the number of bacteria after 96 h and 168 h and influenced the lower colonization of bacteria, which were confirmed by fluorescence microscopy and electron microscopy study. Similarly, recent studies *in vitro* but on a polystyrene plate have demonstrated the positive effect of *P. mirabilis* phage cocktails on the prevention of biofilm formation and biofilm destruction (Maszewska *et al.* 2018). After 24 h of action of *P. mirabilis* three-phage cocktail on 24 h-old

biofilm, elimination of *P. mirabilis* causing catheter-associated urinary tract infections at a similar or slightly greater level compared to phage alone was observed. Additionally, phages included in the cocktail did not inhibit each other's activity (Maszewska *et al.* 2018).

Studies *in vitro* compared the application of a single phage and a phage cocktail on biofilm formation of *P. aeruginosa* on hydrogel-coated catheters (Fu *et al.* 2010). Two hours prior to the administration of the bacteria, a single phage of *P. aeruginosa* M4 phage was applied at a dose of 10 log plaque forming units (PFU/mL) on the catheter. Such administration significantly reduced the number of bacteria on 24 h-old biofilm from 6.87 to 4.03 log CFU/cm^2 (colony-forming unit/cm^2). Administration of phage simultaneously with bacterial inoculation significantly reduced the biofilm formation to 4.37 log CFU/cm^2. The reappearance of bacteria occurred between 24 and 48 h, but additional phage administration at 24 h significantly reduced the growth of biofilm. The pretreatment of 48 h-old biofilm with the *P. aeruginosa* phage cocktail resulted in a significant reduction of the number of bacteria from 7.13 to 4.13 log CFU/cm^2, but phage-resistant bacteria were rarely isolated. The potential use of a phage cocktail in reducing the formation of biofilm by clinically important bacteria was suggested (Fu *et al.* 2010). It is worth noting that application *in vitro* of a phage cocktail rather than single phages improves the killing of biofilm by increasing lytic activity of phages and preventing the appearance of phage-resistant bacteria (Fu *et al.* 2010; Maszewska *et al.* 2018). Recently Malik *et al.* (2019) have also highlighted the high efficacy of phage cocktails in combating biofilms and the possibility of enhancing their efficacy by combining with antibiotics and depolymerases.

Application of Phages in Biofilm-Related Orthopedic Infections

The pathogenesis of peri-prosthetic joint infections is associated with adhesion of pathogenic bacteria to orthopedic implants and with formation of biofilm (Taha *et al.* 2018). *Staphylococcus aureus* and coagulase-negative staphylococci contribute to 50%–60% of peri-prosthetic joint infections (Akanda *et al.* 2018). The comparison of the use of phages and antibiotics in the removal of *S. aureus* bacteria is important in the treatment of orthopedic infections (Kaur *et al.* 2016; Morris *et al.* 2019). An *in vitro* study showed that *S. aureus* phage cocktail composed of five phage preparations reduced the number of bacteria from 6.8 log to 6.2 log colony forming units (CFU, $P < 0.01$) in biofilms on titanium surfaces, which is of great importance in orthopedic implant-related infections (Morris *et al.* 2019). No significant reduction of *S. aureus* biofilm was observed after antibiotic action of 100 × MIC (minimum inhibitory concentration) of cefazolin (Morris *et al.* 2019). *In vitro* studies comparing the effect of a phage cocktail with an antibiotic in the treatment of a *S. aureus* biofilm-related orthopedic infection indicated the advantage of using a phage cocktail over an antibiotic (Morris *et al.* 2019). The study *in vivo* involved mice with post arthroplasty model of infection with *S. aureus* successfully treated with phages and linezolid (no mention on the presence of biofilm was supplied) (Kaur *et al.* 2016). Mice were implanted with a wire coated with phage and/or linezolid into the intra-medullary canal of the femur bone followed by inoculation of methicillin resistant *Staphylococcus aureus* (MRSA). Maximum bacterial burden in the surrounding joint tissue in mice implanted with the naked and polymer coated wire was achieved on day 5, reaching $\sim$8 log CFU. Mice with phage coated wires indicated control of the tissue bacterial burden with significant reduction of > 3 log on day 5 and 7 and sterile tissue by day 10. A maximum significant decrease in bacterial load of $\sim$4.5 log in the joint tissue from days 5 and 7 and sterile tissue by day 10 was observed in mice implanted with wires coated with phage and linezolid (mice with dual coated wire). Faster resumption of locomotion in this examined group was observed. Studies showed no formation of resistant mutants. Treating orthopedic device related infections with two agents (phage and antibiotic) is a potential approach to control infections caused by methicillin-resistant *S. aureus* (Kaur *et al.* 2016). However, *in vivo* studies have shown the best effectiveness in removing *S. aureus* bacteria from an orthopedic device, but without forming biofilm using a single phage and an antibiotic simultaneously (Kaur *et al.* 2016).

Application of Phages in Biofilm-Related Oral Infections

Acquired antibiotic resistance genes and biofilm formation are involved in oral diseases such as periodontal and peri-implant disease (Khalifa *et al.* 2016; Pinto *et al.* 2016; Szafrański *et al.* 2017). Phages for the oral bacteria *Actinomyces naeslundii*, *Aggregatibacter actinomycetemcomitans*, *Enterococcus faecalis*, *Fusobacterium nucleatum*, *Lactobacillus spp.*, *Neisseria spp.*, *Streptococcus spp.*, and *Veillonella spp*. have been reported (Szafrański *et al.* 2017). The effect of phages on oral biofilm bacteria was investigated (*A. actinomycetemcomitans, E. faecalis* and *S. mutans*) (Szafrański *et al.* 2017). *A. actinomycetemcomitans* is involved in periodontitis, infective endocarditis and abscesses. *E. faecalis* is involved in tooth root canal infections and implant placement. *S. mutans* is also involved in dental infections such as caries. In all studies, the number of bacteria significantly decreased, by applying

phage alone, from 2.3 log to complete bacterial removal. Phage killed 95% of *A. actinomycetemcomitans* bacteria but did not remove the biofilm matrix. Application of phage against *S. mutans* and *E. faecalis* biofilm *in vitro* reduced the number of bacteria by 5 log. The effectiveness of oral phages was not confirmed *in vivo* in an animal model of biofilm (Szafrański *et al.* 2017).

The biggest problem in recurrent root canal infections is vancomycin-resistant enterococci (VRE). *E. faecalis* phages may be used against biofilm in root canal infections (Khalifa *et al.* 2016). *In vitro* and *ex vivo* studies in the removal of oral *E. faecalis* biofilm have been compared (Khalifa *et al.* 2015). Single *E. faecalis* phage was applied in a 2-week-old biofilm *in vitro* (Khalifa *et al.* 2015). Phages at a dose of 10^7 plaque forming units (PFU/well) were incubated with biofilm for one week. *E. faecalis* phage significantly reduced viable counts by 5 log within 7 days. The effective reduction of *E. faecalis* biofilm *ex vivo* in a human root canal model by treatment with *E. faecalis* phage was achieved. The root canals were contaminated with an *E. faecalis* suspension with OD_{600} 0.1 and phage-treated group teeth were treated with phage 10^8 (PFU/mL). In the phage-treated group the number of *E. faecalis* bacteria was reduced by 7 log after 48 h (Khalifa *et al.* 2015). Compared *in vitro* and *ex vivo* studies of application of single *E. faecalis* phages in the reduction of oral biofilm indicated better phage efficiency in *ex vivo* studies (Khalifa *et al.* 2015).

Phages and Antibiotics in Control of Biofilm

Studies *in vitro* of *P. aeruginosa* Phages and Antibiotics against Biofilm: Simultaneous or Sequential Treatment

Synergy is the interaction between two factors, when the combined effect is greater than the sum of individual effects. One strategy for more effective biofilm removal is the use of phages and antibiotics, observing a synergistic effect between these factors. A synergic effect of *P. aeruginosa* phages and antibiotics against biofilm *in vitro* was observed (Chaudhry *et al.* 2017; Henriksen *et al.* 2019; Issa *et al.* 2019). Two *P. aeruginosa* phages killed 48-h-old biofilm grown on plastic surfaces *in vitro* with better efficacy compared to a single phage (Chaudhry *et al.* 2017). The synergic effect was observed for simultaneous treatment of 48-h-old biofilm on plastic for the next 48 h with phages and some antibiotics, such as ceftazidime ($1 \times$ MIC and $8 \times$ MIC), ciprofloxacin ($1 \times$ MIC), but not for gentamicin and colistin. The simultaneous combination of phages and most examined antibiotics ($1 \times$ MIC) was effective after 12 h of treatment in reducing 8 h-old biofilm grown on layers of epithelial cells. Some antibiotics were more effective at lower doses when combined with phage. Administering phage before some antibiotics may cause better efficacy of killing biofilm than using agents simultaneously. Statistically significant results in killing 48-h-old *P. aeruginosa* biofilm on plastic surfaces were achieved using gentamicin and tobramycin 24 h after phage application (Chaudhry *et al.* 2017).

Other studies also confirm the strong synergistic effect of phages and ciprofloxacin at sub-MIC levels when treating *P. aeruginosa* flow-cell biofilm (Henriksen *et al.* 2019). In this model, a ~ 6 log reduction in the abundance of bacterial cells in biofilms was achieved. The synergy of phage and ciprofloxacin was confirmed by further research of elimination of *P. aeruginosa* biofilm *in vitro* (Issa *et al.* 2019). Combination of phages and ciprofloxacin enhanced the reduction of bacterial load by $\geq 50\%$. The authors suggest an association between biofilm inhibition and smaller plaques of phage formed after high adsorption of phage to bacterial cells. It is worth noting that simultaneous treatment of phages and antibiotics caused a synergistic effect in killing of *P. aeruginosa* biofilm (Chaudhry *et al.* 2017; Henriksen *et al.* 2019; Issa *et al.* 2019), but application of *P. aeruginosa* phage before antibiotics improves elimination of biofilm (Chaudhry *et al.* 2017). Additionally, the combination of phage with an antibiotic in the treatment of biofilm may affect the reduction of an antibiotic dose (Chaudhry *et al.* 2017).

Studies *in vitro* of *S. aureus* Phages and Antibiotics against Biofilm: Simultaneous or Sequential Treatment

Other *in vitro* studies have indicated better reduction of *S. aureus* biofilm after pretreatment with phage and then with an antibiotic (Kumaran *et al.* 2018; Tkhilaishvili *et al.* 2018). Treatment of biofilms with phage, antibiotics or both simultaneously caused minimal reduction of bacterial cells in biofilm. Application of phage treatment before antibiotics caused significant reduction of viable cells by up to 3 log. This effect was evidently stronger for phage with vancomycin and cefazolin at a lower antibiotic dose (Kumaran *et al.* 2018). A synergistic effect of simultaneous treatment with *S. aureus* phage and antibiotics of biofilm *in vitro* was observed (Tkhilaishvili *et al.* 2018). The biofilm was evidently eradicated after simultaneous treatment at a sub-eradicating titer of *S. aureus* phage of 10^5 PFU/mL and at sub-eradicating concentrations of rifampin (64 μg/mL) and daptomycin (32 μg/mL). No synergistic effect was achieved for fosfomycin and vancomycin. Pretreatment with *S. aureus* phage at 10^5 PFU/mL followed by a sub-eradicating dose of antibiotics improved the

synergistic effect in killing biofilm *in vitro*. Additionally, degradation of the matrix exopolysaccharide of biofilm by *S. aureus* phage was observed (Tkhilaishvili *et al.* 2018). It is worth noting that a study on another bacterial species has also demonstrated that an increase of phage from 10^4 to 10^7 PFU/mL may reduce the concentration of cefotaxime from 256 to 32 µg/mL in *Escherichia coli* biofilm eradication *in vitro* (Ryan *et al.* 2012). It is worth emphasizing that simultaneous treatment of some *S. aureus* phage and antibiotics resulted in a synergistic effect (Tkhilaishvili *et al.* 2018), but pretreatment of *S. aureus* phage before antibiotics increases elimination of biofilm (Kumaran *et al.* 2018; Tkhilaishvili *et al.* 2018).

Studies *in vivo* of Phages and Antibiotics against Biofilm: Simultaneous or Sequential Treatment

Studies *in vivo* indicated that a simultaneous combination of *S. aureus* or *P. aeruginosa* phage and antibiotics increases killing of biofilm (Yilmaz *et al.* 2013), but using an antibiotic prophylactically followed by *C. difficile* phage treatment increases reduction of bacteria (Nale *et al.* 2016). A synergistic effect of phage and antibiotics was observed in the treatment of biofilm created by *S. aureus* and *P. aeruginosa* in an osteomyelitis model in rats (Yilmaz *et al.* 2013). An implant-related infection model in rats was treated with phages, antibiotics and a combination of both factors. The MRSA group received Sb-1 phage and teicoplanin while the *P. aeruginosa* group received PAT14 phage and imipenem, cilastatin and amikacin. A reduction of CFU was observed in each treatment group. MRSA biofilm was significantly eliminated by simultaneous treatment with the antibiotic and phage. A significant reduction of bacteria was observed in all *P. aeruginosa* biofilm groups. Greater reduction of *P. aeruginosa* biofilm by simultaneous treatment with the antibiotic and phage was observed (Yilmaz *et al.* 2013). Therapy of *Clostridium difficile* infection with antibiotics often causes failure or recurrent infection (Nale *et al.* 2016). Formation of *C. difficile* biofilms may contribute to this failure. The *C. difficile* phage cocktail alone or in combination with vancomycin reduced *C. difficile* biofilms and prevented colonization in a *Galleria mellonella* larva model (Nale *et al.* 2016). Pretreatment of *Galleria mellonella* larva with vancomycin prophylactically and subsequently with *C. difficile* phage increases reduction of bacteria (Nale *et al.* 2016).

Lysins in the Treatment of Biofilm

Phage-derived endolysins are double-stranded DNA phage-encoded peptidoglycan hydrolases. They are produced in bacterial cells infected by phage at the final stage of the lytic cycle and have the ability to kill replicating and nonreplicating bacteria. Endolysins cleave covalent bonds in the peptidoglycan cell wall of Gram-positive bacteria that induce rapid lysis (Borysowski *et al.* 2006; Borysowski *et al.* 2011; Fischetti 2018). Gram-negative bacteria are strongly resistant against exogenously added lysins due the occurrence of protective outer membranes (Lood *et al.* 2015). However, engineered lysins - artilysins created by adding peptides or other proteins improved antibacterial activity of lysins (Yang *et al.* 2014). Lower activity of endolysin against Gram-negative bacteria can be improved by membrane-destabilizing factors (Lood *et al.* 2015). Lysins against Gram-negative pathogens were discovered recently (Fischetti 2018). Given the above, lysins constitute an alternative to phages and to antibiotics as potential antibacterial agents. They have the potential to kill antibiotic-resistant bacteria. Lysins have an advantage over broad-spectrum antibiotics, because lysins have a narrow antibacterial range, therefore do not disrupt the normal flora. It has been suggested that lysins cause the low chance of inducing bacterial resistance (Borysowski *et al.* 2011; Fischetti 2017; Fischetti 2018; Gray *et al.* 2018; Kropinski 2006). Phage-derived lysins can potentially be used in the elimination of biofilm (Table 1).

Application of Lysins for Gram-Positive Bacteria against Biofilm

Phage lysins that kill *S. aureus* biofilm *in vitro* were described for the first time in 2007 (Sass and Bierbaum 2007). After 2 h incubation of *S. aureus* ϕ11 endolysin with biofilm the reduction of 24 h and 48 h-old *S. aureus* biofilm was observed. The same efficiency was achieved for killing of *S. aureus* biofilm cells by endolysin as lysostaphin, but without activity on *S. epidermidis* biofilm determined by staining of cells with 0.1% safranin. These studies indicated the high specificity of this lysin (Sass and Bierbaum 2007). *In vitro* studies demonstrated that lysins may eliminate biofilms (Poonacha *et al.* 2017; Schuch *et al.* 2017; Sharma *et al.* 2018; Singh *et al.* 2014). Lysins can also eliminate biofilms in animal models in endocarditis and catheter-associated infection (Sharma *et al.* 2018). Lysins could kill persisters that remained after treatment with antibiotics *in vitro* (Sharma *et al.* 2018). A positive antibiofilm effect of lysin *S. aureus* CF-301 was achieved in an *ex vivo* study in human synovial fluid, which forms a strong antibiotic-resistant biofilm, with the conclusion that this lysin may have a potential role in treating joint infections (Schuch *et al.* 2017). *S. aureus* biofilm formed in human synovial fluid was highly susceptible to removal and killing by CF-301 between 4 h and 24 h. Lysin CF-301 removed all biofilm biomass *in vitro* in the catheter tubing after 4 h and decreased the number of bacteria by > 5 log

Table 1 Phages and lysins in biofilm control.

Phages/lysins	Type of infection	Infection model	Place of biofilm formation	Results of treatment	Reference
Phages					
P. mirabilis phage cocktail	Urinary tract infection	In dynamic biofilm model	Foley catheter	Significantly reduced the number of bacteria after 96 h and 168 h	Melo *et al.* (2016)
P. mirabilis phage cocktail	Urinary tract infection	*In vitro*	Polystyrene plate	Eradication of biofilm after 24 h	Maszewska *et al.* (2018)
S. aureus phage cocktail	Orthopedic implant infection	*In vitro*	Titanium surfaces	Significantly reduced the number of bacteria in biofilm	Morris *et al.* (2019)
S. mutants and *E. faecalis* single phages	Oral infection	*In vitro*	Polystyrene plate	Reduction of the number of bacteria by 5 log	Szafrański *et al.* (2017)
E. faecalis single phage	Oral infection	*In vitro*	Polystyrene plate	Reduction of viable counts by 5 log within 7 days	Khalifa *et al.* (2015)
E. faecalis single phage	Oral infection	*Ex vivo*	Root canal	Significantly reduced the number of bacteria by 7 log after 48 h	Khalifa *et al.* (2015)
Lysins					
Lysin *S. aureus* CF-301	Catheter-based infection	*In vitro*	Catheter tubing	Removed biofilm biomass within 4 h; reduction of the number of bacteria > 5 log	Schuch *et al.* (2017)
Lysin *S. aureus* CF-301	Joint infection	*Ex vivo*	Human synovial fluid	Effectively removed biofilm between 4 h and 24 h	Schuch *et al.* (2017)
Lysin *A. baumannii* PlyF307	Catheter-based infection	*In vitro*	Catheter	Reduction by 1.6 log of the number of bacteria after 2 h	Lood *et al.* (2015)
Lysin *A. baumannii* PlyF307	Catheter-based infection	*In vivo* mice model	Catheter sections implanted subcutaneously	2 log reduction of bacterial viability after 3 h	Lood *et al.* (2015)
Lysin *P. aeruginosa* LysPA26	Not assigned type of infection	*In vitro*	Polystyrene plate	Reduction by 1–2 log of the number of bacteria after 2 h	Guo *et al.* (2017)

CFU/mL (Schuch *et al.* 2017). 24-h-old biofilm formed *in vitro* on plates was treated for 6 h with *S. aureus* chimeric lysin P128 (Poonacha *et al.* 2017). P128 has shown bactericidal activity against *S. aureus*. However, strong reduction of biofilm cells by 99% was achieved against *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* biofilms with 15-31 μg/mL P128, whereas a higher concentration of P128, 62.5 μg/mL, was required to reduce *Staphylococcus lugdunensis* biofilm. 48-h-old biofilm formed *in vitro* on catheters was treated for 18 h with P128. 1 × MIC (8 μg/mL) of P128 caused > 2 log CFU reduction of *S. epidermidis* biofilm (Poonacha *et al.* 2017). *S. aureus* chimeric lysin Ply187 was tested *in vitro* for 30 min against *S. aureus* biofilm grown on glass cover slips (Singh *et al.* 2014). Ply187 at 1 × MIC caused 100% killing of *S. aureus* biofilm cells (Singh *et al.* 2014). *S. aureus* lysins proved to be an efficient new agent in the elimination of staphylococcal biofilm infection *in vitro* (Poonacha *et al.* 2017; Schuch *et al.* 2017; Singh *et al.* 2014). Melo *et al.* (2018) described studies of endolysin against *S. aureus* biofilm, which indicated greater activity of lysin against suspended biofilm cells than intact or scraped biofilms. A biofilm matrix may cause reduction of lysin activity. Moreover, in this study endolysin resistance did not appear in biofilm cells.

Combination of Lysins for Gram-Positive Bacteria and Antibiotics and Other Factors against Biofilm

Synergy between lysins and antibiotics in treating MRSA biofilms has been observed (Sharma *et al.* 2018). It is particularly important to explain the importance of using lysins and antibiotics in the treatment of biofilm (Chopra *et al.* 2015; Schuch *et al.* 2017) and the order of administration of these factors (Chopra *et al.* 2015). An *in vitro* study of treating *S. aureus* biofilm with endolysin and an antibiotic was described (Chopra *et al.* 2015). Simultaneous treatment of MRSA biofilm with endolysin MR-10 and minocycline (a broad range tetracycline) at a dose of 4 μg/mL overnight showed no significant decrease of old biofilm from day 4 to

day 7. In a sequential treatment of biofilm, endolysin MR-10 was used for 6 h and next overnight with minocycline at a dose of 4 µg/mL. Compared to simultaneous treatment, a significant decrease of bacterial cells of young biofilm until day 3 was observed. The reduction of old biofilm was achieved to some extent. The use of an antibiotic after endolysin had limited action on mature biofilm cells. Probably the reason for such a result is the lack of metabolically active cells in old biofilm. Endolysins kill rapidly growing cells and non-dividing cells regardless of the metabolic status of cells. They can better penetrate deeper layers of biofilm than antibiotics. A significant decrease of *S. aureus* bacteria in other sequentially treated MRSA biofilm with minocycline and endolysin MR-10 was observed. Minocycline at a dose of 4 µg/mL was applied for 3 h and next endolysin MR-10 overnight was used in MRSA biofilm. Such a sequential combination with tetracycline and endolysin MR-10 efficiently eliminated younger and older biofilm, so using an antibiotic before application of lysin significantly increases the elimination of even a mature biofilm (Chopra *et al.* 2015). Potent antibiofilm *S. aureus* activity of lysin CF-301 *in vitro* was examined (Schuch *et al.* 2017). In addition, we can compare the effect of lysine or antibiotics on biofilm treatment. Biofilm was formed *in vitro* on polystyrene, glass, surgical mesh and catheters. The effect of the action of CF-301 on biofilm was examined by staining with methylene blue and quantitative estimation of biofilm bacteria. Rapid elimination of biofilm that formed on polystyrene, glass and PVC (catheter tubing) by CF-301 was observed. Biofilms of MRSA were grown for 3 days in the catheter lumen. Addition of CF-301 at MIC (32 µg/mL) to 0.01× MIC (0.32 µg/mL) removed all biofilm biomass within 4 h and reduction of bacteria was >5 log CFU/mL. Daptomycin had a weak effect on biomass biofilm from 5000× MIC (5 mg/mL) to MIC (1 µg/mL) and a decrease of bacteria by 1–3 log CFU/mL. CF-301 eradicated all *S. aureus* biofilm on catheters after only 1 h and killed all released bacteria by 6 h (Schuch *et al.* 2017). Evidently, the advantage of lysin CF-301 over the antibiotic to kill biofilm was demonstrated (Schuch *et al.* 2017). The effect of treatment with lysin combined with other factors (lysozyme, proteinase K and lysostaphin) on biofilm reduction has also been investigated (Schuch *et al.* 2017; Simmons *et al.* 2012). The application of the lysin PlyLM against *Listeria monocytogenes* biofilm was examined *in vitro*, and was found to effectively reduce monolayer biofilm similarly to lysozyme and proteinase K, as was examined in crystal violet staining intensity (Simmons *et al.* 2012). A synergistic effect in the elimination of biofilm was achieved for lysin and these two factors (Simmons *et al.* 2012). A combined treatment of biofilm with lysin *S. aureus* CF-301 and the cell wall hydrolase lysostaphin had a synergistic effect (Schuch *et al.* 2017).

Application of Lysins for Gram-Negative Bacteria against Biofilm

The limited progress of research on genetically engineered lysins for Gram-negative bacteria necessitates more studies performed *in vitro* and *in vivo*. *Acinetobacter baumannii* is a Gram-negative bacterium considered to be a common nosocomial pathogen. The studies *in vitro* and *in vivo* of biofilm treatment by *A. baumannii* lysin were compared (Lood *et al.* 2015). Treatment of biofilm growing on catheters with *A. baumannii* lysin PlyF307 significantly reduced biofilm *A. baumannii in vitro* and *in vivo* (Lood *et al.* 2015). *In vitro* lysin PlyF307 reduced by approximately 1.6 log the number of bacteria in a 3-day-old biofilm on a catheter after 2 h of treatment with lysin. *In vivo* catheter sections were implanted with a 2-day-old *A. baumannii* biofilm subcutaneously in the backs of mice. After 24 h, two doses of 1 mg PlyF307 were administered subcutaneously at the site of the implant for 4 h. After 3 h the estimation of bacterial viability was performed. 1 mg of PlyF307 caused a 2 log reduction of bacterial viability in biofilm. A stronger reduction of biofilm cells by lysin (2 log vs 1.6 log) was observed in an *in vivo* study compared to an *in vitro* study. Additionally, mice were infected with 10^8 CFU of *A. baumannii* intraperitoneally (i.p.) and 2 h later received a single dose of 1 mg of lysin PlyF307. The organs were strongly infected by 2 h, suggesting development of systemic infection. PlyF307 rescued 50% of mice from a lethal dose of *A. baumannii* (Lood *et al.* 2015).

Moreover, comparing the activity of *A. baumannii* lysin PlyF307 and two peptide derivatives against biofilm *in vitro* has shown higher activity of peptide derivatives than lysin (Lood *et al.* 2015; Thandar *et al.* 2016). C-terminal amino acids 108 to 138 of PlyF307 lysin named P307. Derivative peptid $P307_{SQ\text{-}8C}$ was engineered from P307 to improved activity (Thandar *et al.* 2016). $P307_{SQ\text{-}8C}$ was obtained by fusing eight amino acids (SQSRESQC) to the C terminus of P307. *A. baumannii* biofilms *in vitro* in PVC catheters were treated with two peptide derivatives P307 and $P307_{SQ\text{-}8C}$ for 2 h and 24 h (Thandar *et al.* 2016). Biofilms treated with P307 and $P307_{SQ\text{-}8C}$ after 2 h were reduced respectively by ~ 3 and 4 log of CFU/mL. An additional decrease by 1.3 log of CFU/mL was observed with P307 and no further decrease with $P307_{SQ\text{-}8C}$ after 24 h (Thandar *et al.* 2016). Lysin PlyF307 caused reduction of biofilm cells *in vitro* on a catheter by 1.6 log after 2 h (Lood *et al.* 2015).

Activity of *P. aeruginosa* lysin LysPA26 against biofilm *in vitro* was similar to the activity of *A. baumannii* lysin PlyF307 *in vitro* (Lood *et al.* 2015; Guo *et al.* 2017). 48-h-old *P. aeruginosa* biofilm formed *in vitro* on a polystyrene plate showed a reduction by 1–2 log of the number of

bacteria after 2 h treatment with lysin LysPA26 (Guo *et al.* 2017). Interestingly, *P. aeruginosa* lysin LysPA26 could destroy other Gram-negative bacteria (*Klebsiella pneumoniae*, *A. baumannii*, *E. coli*), but does not affect Gram-positive bacteria (Guo *et al.* 2017).

Based on the recent reports, potential application of phage-derived lysins for Gram-positive and Gram-negative bacteria as new agents against biofilm-related infections is suggested.

Conclusions

Based on recent discoveries, the potential application of phages as well as phage-derived lysins in the prevention and elimination of biofilm-related infections is a promising therapeutic option. It is important to emphasize the use of a phage cocktail rather than single phages as a positive factor increasing the killing of biofilm and reducing the development of phage-resistant bacteria especially in biofilm-related urinary tract infections. *Ex vivo* studies of treatment of oral biofilm with a single phage demonstrated a greater reduction of biofilm than *in vitro* studies. Combining phages with antibiotics improves antibiofilm properties. Some antibiotics were more effective at lower doses when combined with phage. The sequence of use of antibiotics and phages in killing biofilms may be important. Application of phages before antibiotics increases elimination of biofilm. Interestingly, an advantage of applying a phage cocktail over an antibiotic in killing biofilm-related orthopedic infection was observed. Stronger killing of biofilm cells by lysin against Gram-negative bacteria was observed in an *in vivo* study compared to an *in vitro* study. Combining lysins with antibiotics increases biofilm elimination. Pretreating biofilm with an antibiotic and then with lysin improves elimination of biofilm, but other studies have shown an advantage of using other lysin over an antibiotic against biofilm. Further research of biofilm elimination by different antimicrobial factors is required, especially an *in vivo* biofilm model.

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Compliance with Ethical Standards

Conflict of interest A Górski and B Weber-Dąbrowska are co-inventors of patents owned by the Hirszfeld Institute of Immunology and Experimental Therapy and covering phage preparations. M Łusiak-Szelachowska declares that she has no conflict of interest.

Animal and Human Rights Statement This article does not contain any studies with human participants or animals performed by any of the authors.

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