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Biofilm Attenuation by Bacteriophages

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Biofilms are always a major concern in the healthcare field and food industry. The resistant properties of biofilm that allow bacteria to persist are difficult to study. Biofilms are often more resistant to antibiotics than individual planktonic cells. Thus, alternative strategies for managing biofilm formation are needed. Currently, using phages as anti-biofilm agents has been suggested. In this review, some of the diverse strategies, reported in previous studies, for preventing biofilm formation are discussed. Use of phages as anti-biofilm agents can involve phage application prior to biofilm formation, application to biofilms that are already formed, or using phages in association with other mechanisms to physically disrupt the biofilm. The development of novel methods as anti-biofilm agents will add an important dimension to the search for new potent compounds for preventing biofilm-associated infections.

Keywords: Phages, Biofilm, Bacteria, Antibiotics, Resistance.

Biofilm

In general, biofilms are complex composition of bacteria that can be formed either from one or numerous different species living together inside a matrix made of extracellular polymeric substances (EPS) with the capability to attach to numerous surfaces[1]. EPS mainly include polysaccharides, but other biomolecules present include nucleic acids, lipids, and proteins, which form a scaffold that helps bacteria remain attached within the biofilm[2,3]. This matrix displays a modified phenotype, and the regulation of specific drug resistance genes and virulence factors has been observed in bacterial biofilms. Horizontal genetic transfer can occur easily, facilitating cross-breeding of resistance genes[4,5]. Biofilm formation involves five stages 6, as shown in Fig. (1)

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The complex composition of the matrix increases survival ability under extreme conditions, as well as enhances the inflow of nutrients, water, and signaling molecules important for cell communication[8, 9]. Furthermore, the EPS matrix forms a barrier between the external environment and bacteria, which prevent antimicrobials from penetrating the biofilm[10]. Biofilms of Salmonella are more resistant to the triclosan antibiotic than individual planktonic Salmonella cells[11]. Furthermore, negative charges on the EPS can prevent antibiotics from reaching the biofilm[12, 13].

Biofilms play a fundamental role in infectious diseases. Studies have shown that 60–70% of most nosocomial infections are directly linked to the clear presence of biofilms [14]. Bacteria commonly associated with medical devices include *Staphylococcus epidermidis* and *Staphylococcus aureus*, followed by *P. aeruginosa*, as well as other bacteria that opportunistically infect weakened patients[15- 17]. Moreover, bacteria can be present on medical implants including catheters[18, 19].

Bacteria within biofilms exhibit both antibiotic and host defense resistance[19], as well as a decreased growth rate, limited diffusion, and

increased efflux and enzymes responsible for antimicrobial degradation[20, 21]. Generally, the use of antibiotics to treat biofilm-related infections is often not successful[12]. Many studies confirmed that for biofilms, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration were generally higher compared to those for planktonic bacterial cells (approximately 10–1000-fold)[22,24]. Numerous antimicrobials function against actively growing cells, and thus biofilms reduce antimicrobial function. Once bacteria are embedded within a biofilm, various factors such as altered gene expression and quorum sensing increase the resistance to antibiotics[25]. Treating biofilm is difficult and challenging, and thus has been given much attention[26]. Thus, it is extremely important to develop new antimicrobial agents or efficient methods for targeting and destroying the biofilm responsible for infection[27, 28].

Studies of biofilm-phage interactions

Bacteriophages or (phages) in general are viruses that infect bacteria (Figure 2). Some viruses were created to target biofilms[29]. They can either reside in the bacterial host genome in a lysogenic state, or enter a lytic state to destroy bacteria, and thus can be used for therapeutic purposes. Phages show potential as alternatives to antibiotics against bacterial infections and have been widely explored to minimize pathogen loads in food products. Phages may also be safer than antibiotics. Phage isolation is rapid, simple, and inexpensive. Phages are competent against one specific host or a range of hosts, and thus are more effective than the natural microflora which are initially attacked by the biofilm. Phages are biofriendly and not associated with negative side effects[30].

Targeting of biofilms by phages has been examined in numerous studies[31, 32]. Phages have long been used for purposes comparable to those of antibiotics, in addition to treating bacterial infections[33-35]. Biofilm enucleation through phages can involve phage application to prevent biofilm formation, application to biofilms that are already formed, or in association with other mechanisms that may physically disrupt the biofilm.

Promising strategies involving phage against biofilm include the following:

Inhibition of attachment by phages

Phages are capable of affecting the initial adsorption stage of biofilms (or adhered cells). When employing lytic phages, as generally is the case for anti-biofilm phages, phage infection results in the killing and lysis of bacteria. This impacts biofilms structurally and releases new phage virions that can potentially reach and then infect adjacent bacteria[36]. The effect is a cyclical acquisition and killing of biofilm bacteria[34]. Sillankorva *et al*. (2008) reported that single cells on glass surfaces for 60 min were efficiently inhibited by phage φS1. Cell removal was fast and efficient and resulted in a biomass reduction of approximately 90%[37].

Inhibition of EPS matrix by depolymerizing enzymes

It has been reported that some phages are effective at penetrating the EPS matrix by diffusion or through the action of phage-associated enzymes. A large range of enzymes can destroy the biofilm EPS matrix. In the case of phages, these enzymes include those mainly produced to help release phages from the host cell and tail spike proteins that allow infection of bacteria within the biofilm, but in general the activity of these enzymes and proteins are strictly localized. However, studies revealed that proteins with activity limited to the virus particle may be released from lysing cells, affecting the biofilm matrix[38].

Phages are capable of producing depolymerizing enzymes that can degrade the EPS from the host genome. The genome of many phages also contains genes that specialize in producing enzymes that break down the matrix[29,39,40]. Under many conditions, these enzymes target the bacterial cell wall for release from the host cell, but these enzymes can also degrade the biofilm EPS. The T4 and HK620 phages of *Escherichia coli* contain enzymes that exist on the viral tail, and may play a role in degrading the matrix[39, 40]. Polysaccharide depolymerase is a very important part of the phage tail and many tail spike proteins have endoglycosidase activity by breaking down their polysaccharide receptors through hydrolyzation[40]. It has been reported that a phage-induced method of making the biofilm matrix more porous can facilitate the infection process by progeny phage, while a rapid bacterial infection reaction moves away from the focus on infection. Although the presence of polysaccharide depolymerase in phages has been reported, EPS-degrading enzymes are difficult to isolate, and thus

have been reconstructed, including those from T7 phage[41].

Importantly, different species of bacteria produce different EPS components. A depolymerase active that acts on polysaccharides from one species of bacteria may not digest that produced by other bacteria. However, depolymerases likely have broader activity than their parent phages among closely related bacteria, as the complexity and variability in the EPS is lower that of the host bacteria. Son *et al*. (2010) observed this by comparing phage of S. aureus with a specific depolymerase. However, only Staphylococci were affected, suggesting that multiple depolymerases are required to target mixed biofilms and that dynamic depolymerases are needed; haloes may be observed over the phage plaques formed on bacterial cultures, revealing the areas where bacterial polysaccharide has been broken down[42]. Gutiérrez *et al*. (2012) used this approach to detect such activity in two phages infecting S. epidermidis, both of which were then confirmed by sequencing to contain genes for pectin lyases[43] , while Glonti *et al*. (2010) identified haloes in cultures of a phage infecting P. aeruginosa and purified a depolymerase protein from the phage[44]. Yan *et al* (2013) classified phage polysaccharide depolymerases as endorhamnosidases, alginate lyases, endosialidases, and hyaluronidases[40].

Pretreatment of catheter using phages

Another important challenge in medical care for reducing biofilm formation by *S. epidermidis* is pre-treating catheter surfaces with phages (Curtin and Donlan, 2006).

The utilization of phages for treating device-related infections has been examined since the 20th century. Pretreatment of hydrogel-coated catheters by phage was found to inhibit *S. epidermidis* and *P. aeruginosa* biofilm formation[45, 46].

Quorum sensing inhibition (QSI) by phages

One strategy that can be used against biofilm may be to inhibit quorum sensing (QS), a cell-to-cell signaling system that controls the expression of genes necessary for adding virulence factors, such as those responsible for interactions with the host bacteria and regulating biofilm development[47-51]. The key intent behind this strategy is not to kill the pathogens, but to disarm them by making them oversensitive to normal antimicrobial treatments. Furthermore, the QS system does not contribute any mechanisms essential for bacteria survival, but inhibiting this method does not provide firm selective pressure sufficient to cause resistance development[52]. Pei and Lamas-Samanamud (2014) showed that the engineered phage strain T7 which produces metalloenzymes AiiA lactonase have various actions against signaling molecules (acyl homoserine lactones) involved in bacterial quorum sensing and that these molecules are important for biofilm development[53].

Phage growth within biofilms

Experimental data indicate that phages do grow well in *P. aeruginosa* biofilms[53], at least

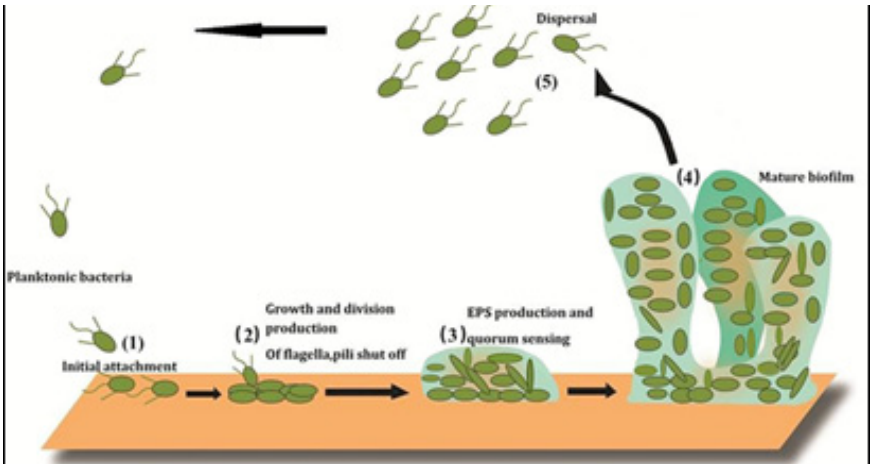

Fig. 1. Biofilm is formed in five stages, these stages are 1) initial, reversible attachment, 2) Irreversible binding and growth, 3) EPS production and inter communication through quorum sensing, 4) Mature biofilm, and 5) dispersal; essential stage for biofilm dispersion and life cycle (adapted and modified from Mizan and others 2015) [7]

in the primary stages of their development. In 2-day-old biofilms [55], of 17 insensitive strains of *P. aeruginosa* phages (therefore, planktonic bacterial hosts were used), 8 strains encouraged the growth of the same phages in the biofilm. However, the effects of antibiotics could be blocked in their initial stages of formation. This agrees with the findings of another study showing that antibiotic resistance begins to appear in the first stages of biofilm formation. Thus, bacteria can be destroyed by phages in cases where antibiotics are ineffective[56].

Previous studies have revealed the processes involved in regulating biofilms, showing that phages effecting *P. aeruginosa* can terminate bacteria in an adult biofilm and (based on their sizes) diffused through thick alginate gel. However, this activity clearly varied from that of highly-restricted tail spike proteins[57]. Sillankorva *et al*. (2004) showed that phages of both *P. fluorescens* and *S. lentus* reduced both single species and mixed biofilms with these agents. The phages of both hosts were completely sequenced, with neither found to code for a polysaccharide depolymerase (although the *P. fluorescens* phage encoded an endopeptidase)[29]. Similarly, Doolittle *et al*. (1996) reported that the *E. coli* phage T4 does not code for polysaccharide depolymerase, except for a restricted tail spike protein released from the phage tail only during host cell penetration. But could spread effectively through biofilm[58].

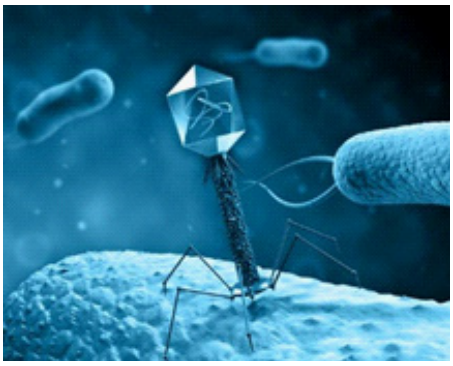

Fig. 2. Bacteriophage targeting bacteria within biofilm

Some studies have shown that phages can penetrate biofilms even if they cannot produce polysaccharide depolymerases, but within biofilm, effective infection has not been observed in most studies. Additionally, some researchers have proposed the existence of EPS-degrading enzymes are extremely important for biofilm-related applications[38]. A study by Tait *et al*. (2002) revealed that using a combination of three phages entirely destroyed a biofilm composed of a single species, while in the presence of other bacterial species which were insensitive, this technique had little effect[59]. A study by Kay *et al*. (2011) also demonstrated that phage efficiency is decreased

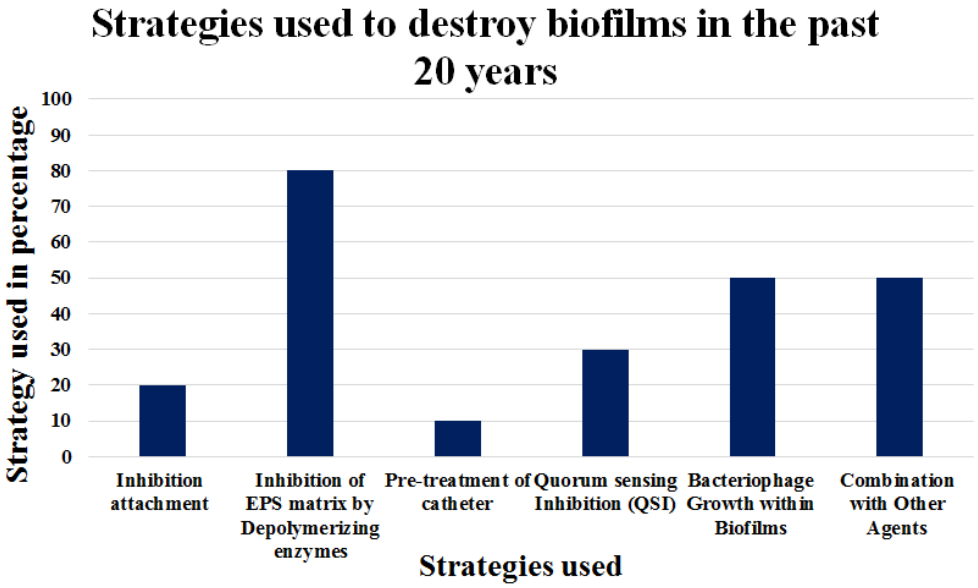

Fig. 3. Phage mediated prevention Biofilm Strategies used within the last few 20 years

in the presence of mixed biofilms[60]. However, Sillankorva *et al*. (2004) reported that efficiency remained high in model biofilms, even when individual bacterial species in the biofilm are targeted by the phage, demonstrating that phages can kill a specific type of bacterial host even when under mixed biofilm conditions. In addition, they reported that phages can effectively target an adult biofilm[29].

Combining phage with other agents

Using phages as mixtures or coupled with antibiotics can completely prevent the development of phage resistance[61]. Verma *et al*. (2010) found that mature biofilms can adapt to antibiotics if lytic phages are used [62], which agrees with the results of some clinical trials of phage activity[63, 64]. Using phages and antibiotics in a combined or sequential manner has shown potential for therapeutic applications. In support of this, Yilmaz et al. (2013) found that using phages coupled with antibiotics to treat biofilms of *S. aureus* was clearly effective[65]. Another study suggested a polysaccharide lyase and DNase enzymes to destroy the matrix can be used in combination with phages[54]. Abedon *et al*. (2011) found similar results, although differential diffusion of phages and co-administered enzymes is difficult. The use of phages can also be combined with physical wound cleaning[33]. Seth *et al*. (2013) used a rabbit ear mold to show that individually removing damaged tissue or foreign objects from a wound and using phage treatment had no effect, while combining these methods was effective. Phages may have similar functions in biocides and sanitizers currently used, but should be applied after the primary cleaning processes to destroy bacteria on the remaining biofilms[66]. Similarly, Ganegama Arachchi *et al*. (2013) found that using a combination of three different phages could clear *Listeria monocytogenes* biofilms effectively from steel surfaces. Thus, when treating biofilms with phages, the biofilm cell surface should be disrupted prior to phage application[67]. Other combinations are also possible for use in biological systems. Liao *et al*. (2012) found that combining phages with commensal bacteria had synergistic effects in preventing biofilm formation on silicone catheter segments[68], while Zhang and Hu (2013) observed that when using phages coupled with biocides such as chlorine, the effects on filters were increased[69]. However, further studies are needed to explore phage activity in a multispecies context, animal models, and in combination with other antimicrobials[70]. Figure 3 shows various strategies that have been used to destroy biofilms within the past 20 years.

CONCLUSIONS

Studies examining interactions between phages and biofilms indicated that phages contain some unique properties and are promising for biofilm control. Different phages have been used to infect numerous bacterial biofilms. The treatment of biofilms using phages is complex, and only strictly lytic phages should be used. Similar to phage infection of planktonic cells, numerous essential steps are required. Phage adsorption to receptors of the targeted bacteria is the initial step of infection. It is also evident that phages express enzymes that can disrupt biofilms. These enzymes are induced from the host genome. While much progress has been made in these methods, more studies are needed. Thus, strategies for destroying biofilm are currently speculative in nature. With additional studies, new and better strategies will be developed.

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