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MINIREVIEW

Bacteriophages as an Alternative Strategy for Fighting Biofilm Development

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Abstract

The ability of microbes to form biofilms is an important element of their pathogenicity, and biofilm formation is a serious challenge for today's medicine. Fighting the clinical complications associated with biofilm formation is very difficult and linked to a high risk of failure, especially in a time of increasing bacterial resistance to antibiotics. Bacterial species most commonly isolated from biofilms include coagulase-negative staphylococci, *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter* spp. The frequent failure of antibiotic therapy led researchers to look for alternative methods and experiment with the use of antibacterial factors with a mechanism of action different from that of antibiotics. Experimental studies with bacteriophages and mixtures thereof, expressing lytic properties against numerous biofilm-forming bacterial species showed that bacteriophages may both prevent biofilm formation and contribute to eradication of biofilm bacteria. A specific role is played here by phage depolymerases, which facilitate the degradation of extracellular polymeric substances (EPS) and thus the permeation of bacteriophages into deeper biofilm layers and lysis of the susceptible bacterial cells. Much hope is placed in genetic modifications of bacteriophages that would allow the equipping bacteriophages with the function of depolymerase synthesis. The use of phage cocktails prevents the development of phage-resistant bacteria.

K e y w o r d s: biofilm, bacteriophage, anti-biofilm activity

Introduction

Biofilms are complex microbial aggregates located at interphases and forming dynamic structures characterized by diverse metabolic processes and interactions between individual components. The ability of biofilm formation is an important factor promoting microbial survival, as well as an important element of microbial pathogenicity. Biofilms are responsible mostly for infections associated with the use of biomaterials, as well as for many chronic diseases not responding to standard antibiotic treatment. For many years, studies were conducted to develop efficient methods of biofilm elimination and prevention. One of the methods involves the use of bacteriophages, *i.e.* bacterial viruses that are their natural enemies. Phage therapy has been used to fight bacterial infections in humans, animals and plants. The therapeutic effects of bacteriophages have been confirmed in many studies and are undoubtedly worthy of more attention as a research subject.

Biofilm as a challenge for today's medicine

The definition of biofilm has changed significantly from the first description of the phenomenon nearly 70 years ago (Zobell, 1943). Currently, biofilm is defined as a multicellular population of prokaryotic and/or eukaryotic cells. These cells are characterized by their spatial order, formation of extracellular polymeric substances (EPS) and increased tolerance to antimicrobial agents (Costerton, 2007; Trafny, 2008). In nature, biofilms may be formed by numerous species of bacteria, fungi, protozoans and algae. However, most attention is paid to bacteria, as it is estimated that 99% of microbes present in ecosystems may grow as metabolically integrated populations, *i.e.* biofilms. The ability to form biofilms also determines the pathogenicity of bacteria and is of great importance in the process of infection (Różalska, 2008; Trafny, 2008). In humans, a number of diseases, such as endocarditis, urinary tract infections, chronic otitis media, chronic bacterial prostatitis,

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respiratory tract infections in cystic fibrosis patients, or periodontal infections have been clearly linked with the associated biofilm development (Costerton *et al.*, 1999; Davey and O'Toole, 2000).

Microbes capable of forming structurally and metabolically ordered cellular aggregates can colonize biomaterials in direct contact with tissues or systemic fluids. Biomaterials are used to manufacture pacemakers and cardiac valves, urinary tract prostheses, articular prostheses, peritoneal membrane implants, some dialysis catheters and respirators. Etiological factors of infections associated with biomaterials may include microorganisms comprising the natural microflora of the skin, oral cavity, urinary and reproductive system, the gastrointestinal tract, as well as exogenous organisms. The most commonly isolated bacterial species include coagulase-negative staphylococci, *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium, Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter* spp. (Donlan and Costerton, 2002; Donlan, 2005; Trafny, 2008). Biofilms formed on various medical devices complicate the healing process and become a serious healthcare issue. Due to the significant increase in the number of antibiotic multiresistant bacterial strains, medical and scientific research is focused on alternative strategies to fight biofilm development. Bacteriophages are the bacteria's natural enemies; therefore, they may be one of the most appealing solutions to this problem (Sutherland *et al.*, 2004; Azeredo and Sutherland, 2008; Donlan, 2009).

Bacteriophages – their prevalence, classification and life cycle

Bacterial viruses were discovered independently about 100 years ago by Ernest Hankin (1896) and Frederick Twort (1915) – they were the first scientists to describe their antibacterial activity. However, it was a French Canadian microbiologist Felix d'Hérelle (1916) who suggested, that these entities were viruses. He gave them the name of bacteriophages and is officially credited with their discovery. D'Hérelle isolated bacteriophages for bacteria responsible for such diseases as cholera, bubonic plague or anthrax and was the first to use phage therapy in the treatment of bacterial dysentery. The first antibiotic – penicillin – was first used twenty years after the official discovery of bacteriophages. This fact, combined with some early clinical failures as well as theoretical and ethical concerns (foreign DNA) led to renouncement of phage therapy in the US and in most Western European Countries.

Bacteriophages are probably the most numerous and most diverse group of viruses. According to one of the hypotheses, at least one phage can be found for each host wherever bacterial development is observed. Soil, water, sewage, human and animal organisms (skin, mouth, saliva, stool, bowels), and even the food we eat are only some examples of environments from which phages have been isolated (Furuse, 1987; Ashelford *et al.*, 2003). The estimated global population of phages is enormously large. It is estimated that the total number of phages in the aquatic environment is above 10^{31}, land ecosystems demonstrate 10^{7} phage particles per 1 gram of soil, and the total number of phages in sewage is 10^{8}–10^{10} per 1 mL. Phages were demonstrated to outnumber bacteria in all tested environments and may constitute the most dominant form of life in the biosphere (Sharp, 2001; Ashelford *et al.*, 2003).

The taxonomic division of bacteriophages was established by the International Committee on the Taxonomy of Viruses (ICTV) on the basis of the morphology of the nucleic acid (dsDNA, ssDNA, dsRNA, ssRNA) (Rohwer and Edwards, 2002; Ackermann, 2006).

Each phage particle consists of nucleic acid (genetic material) and proteins that comprise the main structural elements of phages and exhibit enzymatic activity. There are filamentous phages, isosahedral phages with tails, phages without tails, and several phages with a lipoprotein envelope or contain lipids in the particle shell (Ackermann, 2006). Like all viruses, bacteriophages can proliferate only in living and susceptible bacterial cells. Phages can be divided into two types, depending on the course of infection: virulent, or lytic phages, and temperate, or lysogenic phages. Only lytic phages qualify for therapeutic purposes, as they lead to lysis of the host cell and do not integrate with the host genome (Matsuzaki *et al.*, 2005).

During the lytic cycle, phage particles are adsorbed on the bacterial surface; next, the genetic material of the phage permeates into the cell and takes over the host cell metabolism. This leads to a multiplication of offspring phages, cell lysis and the release of new phages into the environment. The first stage of the lytic cycle, *i.e.* adsorption, is facilitated by viral tail filaments that bind specific receptors on the surface of the cell. Phage receptors may include lipopolysaccharides (LPSs), corresponding proteins, sugar molecules and fimbriae. The specificity of the receptors determines the range of phage host organisms. It is worth mentioning that phages can be divided into monovalent phages, *i.e.* capable of adsorb to specific bacterial species or to specific strains, and polyvalent phages, able to infect across bacterial species or genera. During the lysogenic cycle, the viral genome integrates into the host's replicon and remains as a prophage within the bacterium. This prophage will remain as the lysogen until induction takes place, triggering a shift to the lytic cycle and

the production of new phages in the system (Matsuzaki *et al.*, 2005; Clark and March, 2006).

Phages in therapy

The discovery of phages as bacteria-eliminating factors suggested their possible therapeutic uses. Due to their nature, bacteriophages have many advantages as therapeutic agents. First of all, they are highly specific and efficient against their target bacteria; therefore, in contrast to most antibiotics, they do not cause a reduction or even elimination of natural microflora in host organisms. Phages do not infect human or animal cells, and various administration methods are available; phages replicate in susceptible bacteria as long as they are available (Clark and March, 2006; Skurnik and Strauch, 2006). The mechanism of the action of bacteriophages is different from that of antibiotics, which makes them effective against multiresistant bacteria. In addition, the selection process of phage-resistant bacteria is ten times slower than in case of antibiotic-resistant bacteria (Hanlon, 2007). It should be also mentioned that phage production is easy, fast and relatively unexpensive.

Phages have been and continue to be used to treat infectious diseases in plants (Fox, 2000) and animals (Barrow *et al.*, 1998). Phage therapy has been used in humans in the treatment of such diseases as dysentery, skin infections, pulmonary infections, meningitis, infected wounds or myelitis, caused by numerous diverse organisms, including *Staphylococcus* spp., *Streptococcus* spp., *E. coli*, *P. aeruginosa*, *Shigella* spp. and *Salmonella* spp. A high level of efficacy of phage therapy was observed in murine infections with vancomycin-resistant *Enterococcus faecium* (VRE). A single injection of 3×10^8 pfu (plaque forming units) of an active phage 45 minutes after administration of 10^9 cfu (colony forming units) of VRE was sufficient for the eradication of the pathogen and resolution of clinical symptoms in 100% of tested mice. Even when the phage was administered late, under critical clinical conditions, *ca.* 50% of mice fully recovered (Biswas *et al.*, 2002). Attention is drawn to the possibility of using phages in urinary tract infections. Preliminary clinical trials in patients infected with *E. coli*, *Pseudomonas* spp., *Klebsiella* spp., *Enterobacter* spp. and *S. aureus* suggest a high level of efficacy of phages following oral administration (Dzuliashvili *et al.*, 2007). The spread of multidrug-resistant *P. aeruginosa* infections persuaded researchers to carry out preclinical studies with phage preparations (virulent bacteriophage clone cocktails). The *in vitro* activity of such cocktails was observed for 99.5% out of 206 tested species, and the therapeutic efficacy in studies conducted in white mice was in the range of 80–100% and was higher than that of ciprofloxacin (50–80%). A combination of antibiotic and phage ensured 100% therapeutic efficacy (Dzuliashvili *et al.*, 2007). The use of phages in mice with burn wounds additionally infected by subcutaneous injection of *P. aeruginosa* also produced good therapeutic results, largely dependent on the method of administration of the phage. The most effective route of administration (87%) in generalized infection was intraperitoneal injection; intramuscular and subcutaneous administration were less effective – 28 and 22%, respectively (McVay *et al.*, 2007).

Most cases employing phage therapy in humans were not subject to the strict supervision typical for clinical trials. However, positive treatment results and lack of adverse effects have stimulated an increasing interest in this type of therapy and research in bacteriophage biology (Sulakvelidze *et al.*, 2001; Tenover, 2001). The George Eliava Institute of Bacteriophage, Microbiology and Virology in Tbilisi (Georgia) can boast significant achievements in phage therapy. In their greatest period, the Institute employed about 1,200 workers and produced several tons of phage preparations per day. Also in Poland, the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences in Wrocław has been active in phage therapy applications since 1957. The use of phages in the treatment of various infections may reduce the use of antibiotics, and thus reduce the spread of multidrug-resistant bacteria.

Phage therapy is associated with some problems, mostly regarding the introduction of foreign genetic material into the patient's system, with the possibility of transferring the undesirable traits of gene encoding. Therefore, researchers focus their attention on phage enzymes that take part in degradation of bacterial walls and shells. These are lytic enzymes that hydrolyze peptidoglycans in both the carbohydrate and protein part of the molecule; this enzymes in the case of Gram-plus bacteria are active both outside (environment), and inside the host cells (Brzozowska *et al.*, 2011). The *in vivo* efficacy of lysines was confirmed for *Streptococcus pyogens* (Nelson *et al.*, 2001), *Bacillus anthracis* (Schuch *et al.*, 2002) and antibiotic-resistant *S. aureus* strains (O'Flaherty *et al.*, 2005).

Bacteriophage-biofilm interactions

Numerous phages have been used to fight bacterial biofilms; however, phage-biofilm interactions are relatively complex and very diverse. Bacteriophages may infect biofilm-forming bacteria as well as planktonic bacteria passing into the biofilm upon formation; phages may be trapped non-specifically in the biofilm EPS, as well as produce enzymes that disturb the stability of this extracellular polymer. Biofilm may also show a resistance to phage infection (Sutherland *et al.*, 2004).

In theory, biofilm should become infected faster than planktonic cells, as the vicinity of the cells in the biofilm structure may increase the phage replication rate (Hanlon, 2007). On the other hand, the structure and composition of the biofilm, as well as the physiology of biofilm cells, may impose some limitations in this regard. Various imaging techniques, including confocal microscopy with fluorescent in situ hybridization (FISH) and atomic force microscopy have revealed the heterogeneity of biofilm structures with a diverse distribution of cells, matrix, and water-filled channels and pores. It is worth mentioning that many biofilms have open structures with water-filled channels that facilitate phage access inside the biofilm (Sutherland *et al.*, 2004; Donlan, 2009; Dollittle *et al.*, 1996) demonstrated the radial movement of T4 phage molecule across the biofilm, similar to the process of forming clear patches in the bacterial lawn, suggesting that biofilms may be destroyed by single phage doses. In addition, *Lactococcus* phage c2 has been observed to have the ability to penetrate the biofilm through water channels and cell clusters. Similar phenomenon has also been observed in case of the biofilm of *Stenotrophomonas maltophila*, the cells of which were not sensitive to this phage (Briandet *et al.*, 2008).

Besides the fact that phages are capable of reducing the number of bacterial cells in biofilms, there are several factors that may lead to a reduction in the lytic efficacy of phages (temperature, medium composition, EPS matrix type, *etc.*), resulting in a less effective phage action on the target cells. Also the metabolic status of bacterial cells in biofilms may pose problems for phage treatment, as cells in exponential growth are attacked faster that cells at later phases of growth. However, in some cases, the diversity of the biofilm structure, a deficiency of nutrients and a slow bacterial metabolism are no obstacles for the lytic phage cycle. Hadas *et al.* (1997) demonstrated that the burst size of T4 phages, *i.e.* the number of phages released following their replication was in the range of 12 to 200 pfu, depending on the physiological condition of the host cell (*E. coli*). In addition, the bacteriophage T4 remained capable of infecting bacterial cells even with a limited availability of nutrients. An increase in the amount of nutrients caused an increase in bacterial cell growth, leading to larger burst sizes and a reduction of eclipse and latency periods. Further studies showed that the age of *P. aeruginosa* biofilm only marginally reduced its susceptibility to phage activity F116 (Hanlon *et al.*, 2001). The bacteriophage was active even against biofilms that were 20 days old. For many phages biofilm matrix is a barrier preventing from the phage infection. Doolittle *et al.* (1996), have reported that a *P. aeruginosa* phage was unable to reach the host cells in the deeper layers of a biofilm suggesting that the phage could not penetrate through the biofilm matrix. Additionaly, the biofilm matrix is also a reservoir of proteolytic enzymes which can lead to bacteriophage inactivation.

The first and most fundamental step in bacteriophage infection is the adsorption of phage particles to the specific receptors on the surface of the bacterial cell. In biofilms, where bacterial microcolonies are surrounded by EPS, the matrix is the factor that may pose a problem for phages in reaching their receptors on the target cell surface. However, it has been observed that some phages are able to overcome this obstacle and penetrate the extracellular matrix due to their "accompanying" enzymes. These enzymes hydrolyze the EPS so that bacteriophages can reach the lipopolysaccharide, external membrane proteins or other receptors crucial for the initiation of productive phage infection (Hughes *et al.*, 1998a; Hughes *et al.*, 1998b; Donlan, 2009). Numerous phages that induce enzymes capable of degrading the EPS in many Gram-negative bacteria, including bacteria capable of biofilm formation, have been isolated. On culture plates, such phages are characterized by halos of different sizes, surrounding the plaques obtained after the infection of a single bacterial cell. The halos are formed by bacteria from which the EPS has been removed by excess phage enzyme released during the lysis of infected cells (Sutherland *et al.*, 2004). The activity of polysaccharide depolymerases was observed in the case of phage SF153b acting against *Enterobacter agglomerans* biofilms (Hughes *et al.*, 1998b). Moreover, Hanlon demonstrated the diffusion of the anti-*P. aeruginosa* phage across the alginate gel structure, as well as showing that the mixture of pure, phage-free depolymerases reduces the viscosity of the alginate and EPS in *P. aeruginosa* (Hanlon *et al.*, 2001). Another example of enzyme associated with bacteriophages infecting cystic fibrosis strains of *P. aeruginosa*, able to degrade extracellular alginic acids was described by Glonti and co-workers in 2010 (Glonti *et al.*, 2010). Microscopic analysis of the halo zones formed on *P. putida* lawns by recombinantly purified tail spikes bacteriophage φ15 , which possesses EPS degrading activity, clearly shows that most of bacteria within the halo zones were separated from each other, their EPS material was reduced or completely removed (Cornelissen *et al.*, 2011). A small single dose of endosialidase E, which degrades *N*- acetylneuraminic acid capsule, has therapeutic utility in systemic infections due to *E. coli* K1 strains in neonatal rats (Mushtaq *et al.*, 2005). However, these enzymes are highly specific and rarely effective against more than a few types of polysaccharides.

Depolymerase synthesis in some phages is very useful for biofilm destruction. However, this is not a common trait; therefore, *e.g.* the T7 phage was modified to by genetic engineering methods to include genes that led to the production of EPS-degrading enzymes

(Azeredo and Sutherland, 2008, Donlan, 2009). Unfortunately, this genetic modification led to a reduced anti-biofilm activity compared to the native phage (Lu and Collins, 2007).

The use of bacteriophages to fight biofilms

In recent years, bacteriophages have been more and more frequently used in the treatment of infections in humans and animals; however, studies involving bacteriophages have usually been carried out in standard laboratory cultures, *i.e.* in planktonic cells, not in biofilm-forming cells. The first experiments involving the use of phages in fighting biofilms were published as recently as 1995. Many experiments using various bacteriophages and various bacterial biofilms have been conducted to date (Table I), suggesting that phages are capable of reducing the bacterial population in this particular form of bacterial cultivation.

Two strategies have been identified for the use of bacteriophages in fighting biofilms – prevention, *i.e.* blocking the onset of biofilm development, and eradication, *i.e.* removal of an existing biofilm (Azeredo and Sutherland, 2008; Donlan, 2009; Różalska *et al.*, 2010).

Himba *et al.* (1997) were the first authors to document the use of phages to prevent the development of *Listeria monocytogenes* biofilms on a stainless steel surface. A very important research project on preventing the adhesion and proliferation of bacteria on the surfaces of medical devices was carried out in 2006 by Curtin and Donlan. It was demonstrated that the phage active against *Staphylococcus epidermidis*, introduced into the hydrogel coating of a catheter significantly reduced the formation of the biofilm of this microorganism in *vitro.* Fu *et al.* (2010) made use of a similar *in vitro* model to verify whether phage pre-treatment might reduce the formation of *P. aeruginosa* biofilms. To this end, three experimental approaches were used – phage lysate was passed through the catheters before, immediately after and 24 h after exposure to *P. aeruginosa*. The experiment was conducted in two series. In the first series, a single M4 phage lysate was used, while the second series made use of a phage cocktail consisting of M4 and four other phages selected according to the sensitivity profile of the bacteria comprising the biofilm formed at the first stage of the experiment. It was shown that the treatment of catheters with M4 phage before bacterial exposure significantly reduced bacterial adhesion to the catheter surfaces, and thus biofilm formation (<99%). The biofilm that formed after a period of time was easily eradicated by the phage cocktail, which was more effective than the single phage due to a wider scope of activity and a lower possibility of developing phage resistance by the biofilm bacteria (Fu *et al.*, 2010). These results confirm the legitimacy of using phages to protect medical devices from the formation of biofilms by clinical strains of bacteria.

Of particular note is the potential use of bacteriophages to prevent the formation of biofilms on the surfaces of catheters and other medical biomaterials, although many issues, such as phage infectability and stability, the ability of a particular material to adsorb phage and phage stability in the presence of plasma proteins, require further studies. In addition, careful selection of phage cocktails, matrix optimization and validation of methods *in vitro* and in animal models are very important in the assessment of the usefulness of bacteriophages in fighting biofilms (Donlan, 2009).

Another strategy consists in using lytic bacteriophages to eradicate already formed biofilms. Every year, more and more information is published regarding the biofilm eradication capability of bacteriophages (Table I). Studies showed that some of the most important obstacles that disqualify antibiotics from being used in biofilm treatment, such as the development of tolerance (lack of bactericidal effect), the presence of EPS and the effect of biofilm age, may be overcome by bacteriophages (Sutherland *et al.*, 2004; Donlan, 2009). When a phage makes contact with the biofilm, further interactions depend on the susceptibility of the biofilm-forming bacteria and the availability of receptors on the cell surface. If the bacteriophages are equipped with enzymes that degrade polysaccharides, or if a significant number of cells undergo lysis, the integrity of the biofilm may be broken. Phages producing large amounts of depolimerases, as mentioned in the previous section, are important for eradicating biofilms. Such activity leads to a disturbance of the EPS structural stability and thus to bacteriophage access and adsorption to target cells followed by the infection and lysis of biofilm-forming cells (Hughes *et al.*, 1998a).

Several phages that were used in biofilm eradication have been defined. *E. coli* biofilm was found to be sensitive to bacteriophage T4 (Doolittle *et al.*, 1995). Sharma *et al.* (2005) described the synergistic effect of a commercial alkaline cleanser (Enforce®-Ecolab, Inc., St Paul, MN, USA) and bacteriophage KH1 in the deactivation of biofilm formed by *E. coli* O157:H7 on a stainless steel surface. Sillankorva *et al.* (2004) demonstrated the efficacy of phages in the eradication of *P. fluorescens* biofilm at both early and advanced (5 days) stages of development. A combination of antibiotic therapy with therapeutic phage activity was also found to be more efficient in the removal of *K. pneumoniae* biofilm (Bedi *et al.*, 2009).

As shown in numerous experiments, phages may effectively infect bacteria and cause bacterial lysis in single-species biofilms (Hughes *et al.*, 1998b, Hanlon *et al.*, 2001; Tait *et al.*, 2002; Sillankorva *et al.*, 2004;

Table I
List of experiments illustrating the effects of phages on bacterial biofilms

Biofilm forming bacteria	Phages	Year	Experimental results and reference
E. coli	T4	1995	• eradication of existing biofilm, 28 h biofilms treated with 10^9 or 10^{10} PFU/ml phage for 30 min to 8 h [Doolite *et al.*]
E. coli, *P. aeruginosa*	T4, E79	1996	• eradication of existing biofilm, phage T4 infected both surface-attached and surface-associated *E. coli,* phage E79 adsorbed only to *P. aeruginosa* cells on the surface of the biofilm [Doolite *et al.*]
L. monocytogenes	2307-B1	1997	• eradication of existing biofilm, passive treatment (10^{10} phages/ml) was used, 3 $\log_{10}$ reduction in biofilm viable count within 6 h [Himba *et al.*]
E. agglomerans 53b	SF153b	1998	• eradication of existing biofilm, EPS degradation, 24 h biofilms treated with 10^{10} PFU/ml of phage for 15 min [Hughes *et al.* b]
E. coli K-12	T4	2001	• eradication of existing biofilm, small phage impact, bacterial density after 3 hours was reduced about 1,5 $\log_{10}$ [Corbin *et al.*]
P. aeruginosa	F116		• eradication of existing biofilm, 2-log reduction in the cell, numbers in 20-day-old biofilms, time- and concentration-dependent reduction in alginate viscosity of up to 40% [Hanlon *et al.*]
S. epidermidis	? phage		• eradication of existing biofilm, *in vitro* catherer model [Wood *et al.*]
E. cloace, *E. agglomerans*	11229, φ*Ent*, φ1.15, Blackburn, Philipstown	2002	• eradication of existing dual-species biofilm, treatment of purified EPS depolymerase and various chemical disinfectants [Tait *et al.*]
P. fluorescens	φS1	2004	• eradication of existing biofilm, 5 day biofilms treated with 10^9 PFU/ml phage at different temperatures, approximately one-log reduction (85% after 200 min) [Sillankorva *et al.*]
E. coli O157	KH1	2005	• eradication of existing biofilm, phages as well as various cleanser and disinfectants solutions to kill *E. coli* found in biofilms adhering to stainless steel surface [Sharma *et al.*]
S. epidermidis	456	2006	• prevention of biofilm formation, pre-treatment of catheter surfaces with phages [Curtin and Donlan]
S. aureus	φ11, φ12		• determination of influence of the φ11 and φ12 endolysins on staphylococcal biofilms by a modified biofilm plate assay [Sass and Bierbaum]
S. epidermidis	K	2007	• eradication of existing biofilm, passive treatment with 2×10^8 of phage K, one log biofilm reduction after 24 h [Cerca *et al.*]
E. coli TG1	T7		• engineered phage substantially reduced bacterial biofilm cell counts by 4.5 orders of magnitude (99.997% removal) [Lu and Collins]
S. maltophilia	C2	2008	• studies diffusion and reaction of phages in biofilms; biofilms did not confer resistance to the entrapment of virus-size particles and to their diffusion [Briandet *et al.*]
P. fluorescens	φS1		• eradication of bacterial cells by phages at the early stage of biofilm formation [Sillankorva *et al.*]
P. aeruginosa, *A. johnsonii*, *B. subtilis*	Bacteria-specific phages	2009	• the first report revealing the potential of phage usage as an anti-biofouling factor in membrane processes [Goldmann *et al.*]
K. pneumonia B5055	Phage + antibiotic		• eradication of biofilm, efficacy of bacteriophage alone or in combination with amoxicillin, the combination therapy gives better results than the two therapies alone [Bedi *et al.*]
S. aureus	SAP-2	2010	• biofilm removal activity of bacteriophage and a cell-wall-degrading enzyme (SAL-2), derived from SAP-2; enzyme showed a broader spectrum of activity [Son *et al.*]
L. monocytogenes	P100		• irrespective of the serotype, growth conditions, or biofilm levels, phage significantly reduced *L. monocytogenes* biofilm [Soni and Nannapaneni]
P. fluorescens, *S. lentus*	IBB-PF7A, IBB-SL58B		• studies mono and dual species biofilms formed by *P. fluorescens* and/or *S. lentus*, cocktail of phages effectively killed and removed the hosts from the biofilm [Sillankorva *et al.*]
P. aeruginosa	M4		• pretreating hydrogel-coated catheters with *P. aeruginosa* bacteriophages cocktail on biofilm formation, reduction viable biofilm count about 2.8 $\log_{10}$ CFU cm^2 [Fu *et al.*]

Table I cnontinued

Biofilm forming bacteria	Phages	Year	Experimental results and reference
P. aeruginosa	phiIBB-PAP21, phiIBB-PAA2	2011	• control of both planktonic cultures and biofilms, biofilm cells of *P. aeruginosa* PAO1 acquired resistance to phiIBB-PAP21, phage phiIB-PAA2 continued to destroy biofilm cells, even after 24 h of infection [Pires *et al.*]
P. aeruginosa	BVPaP-3		• control of biofilm, phage at MOI-0.001 could prevent biofilm formation by *P. aeruginosa* hospital strain on the pegs within 24 h [Ahiwale *et al.*]
E. coli, P. aeruginosa	λW60, PB-1		• examination of phage infection on the viability of monoculture and mixed species biofilm and planktonic cultures [Kay *et al.*]
C. jejuni	CP8, CP30		• control of biofilm, phages within the biofilm could not only effectively target and lyse cell but were also able to disperse the EPS forming the biofilm [Siringan *et al.*]

Sharma *et al.*, 2005; Lu and Collins, 2007). However, in many cases the problem is caused by a multispecies biofilm, where phage-biofilm interactions may be more complex (Sutherland *et al.*, 2004; Azeredo and Sutherland, 2008). An attempt to eradicate a biofilm caused by two bacterial species of genus *Enterobacter* by phages and polysaccharide depolymerases failed to achieve the goal (Tait *et al.*, 2002). Recent studies by Sillankorva *et al.* (2010) referred to a case where a biofilm formed by two bacterial species (*S. lentus* and *P. fluorescens*), was successfully subjected to the action of lytic phages – a polyvalent *Staphylococcus* phage and *Pseudomonas* phage known from earlier studies (IBB-PF7A). The authors underlined the importance of the optimization of conditions in which bacteriophages are used to eradicate biofilms. The studies showed that both single- and dual-species biofilms may be effectively controlled by phages; the phages may effectively reach relevant susceptible host cells and cause the lysis of these cells. Further research on simultaneous use of phages and other antimicrobial agents also seems justified (Sillankorva *et al.*, 2010).

Summary

The development of new methods of fighting biofilms is currently one of the major problems in medicine. Numerous *in vitro* experiments have shown that phages can infect bacterial cells within biofilms, and the depolymerases of some phages facilitate the penetration of phages into the inner layers of the biofilm by means of EPS degradation. This leads to the conclusion that the use of bacteriophages might become a new strategy in the prevention and eradication of biofilms. In addition, genetic modifications of phages might bring about a higher efficacy against biofilms, and the use of various phage mix compositions would prevent or minimize phage resistance. However, before bacteriophages can be used to fight biofilms, procedures for the development of phage preparations, as well as procedures for phage administration and dosage regimens must be established to guarantee the efficacy and safety of such treatment.

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