The Use of Phages for the Removal of Infectious Biofilms

J. Azeredo[*1] and I. W.Sutherland[2]

[1]*IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Braga, Portugal;* [2]Institute of Cell & Molecular Biology, Kings Buildings, Edinburgh University, Edinburgh EH9 3JR Scotland

Abstract: Biofilm formation occurs spontaneously on both inert and living systems and is an important bacterial survival strategy. In humans bioflms are responsible for many pathologies, most of them associated with the use of medical devices. A major problem of biofilms is their inherent tolerance to host defences and antibiotic therapies; there is therefore an urgent need to develop alternative ways to prevent and control biofilm-associated clinical infections. Several *in vitro* experiments have shown that phages are able to infect biofilm cells and that those phages inducing the production of depolymerases have an advantage since they can penetrate the inner layers of the biofilm by degrading components of the biofilm exopolymeric matrix. In practice clinically relevant biofilms and especially those associated with the use of medical devices can possibly be controlled for example by the topic application or the impregnation of the surface of the device with a phage solution. Another interesting approach has been the use of a phage encoding a phage polysaccharide lyase to treat *Pseudomonas aeruginosa* biofilms in cystic fibrosis patients by aerosol administration. All these strategies require prior identification of the phage and/or polysaccharide depolymerase capable of infecting the bacterial cells and degrading the polysaccharide within the biofilm, respectively. The biofilm organisms must therefore be isolated and screened against a bank of phages. This procedure is essential and raises important biotechnological challenges: the existence of a bank of phages well characterised (physiologically and genetically) whose efficacy *in vivo* has been tested and pharmacokinetics studied; the existence of economical and safe production protocols and purification methods (e.g. the presence of endotoxins in a phage preparation may compromise phage therapy). It is however important to consider the fact that the chances of getting a specific phage with a high lytic capability and preferential expressing a relevant exopolymer degrading enzyme is likely to be low. Genetically engineered phages can play an important role in this process. Phages can be genetically manipulated to alter their host range and to induce the production of depolymerases. It is therefore important to reinforce the application of synthetic biology to engineer phages able to efficiently degrade medical biofilms. It is also important to develop efficient methods of phage delivery and to study "*in vivo*" the phage performance against biofilms. It is still not clear how effective the biofilm can be in protecting the phages against the immune system. Efficient and economic phage production and purification protocols need also to be addressed before one can hope to use phage treatment to prevent or control infectious biofilms.

INTRODUCTION

Biofilm formation occurs spontaneously on both inert and living systems and is an important bacterial survival strategy. Biofilms are microbial structures consisting of microbial cells surrounded by an exopolymeric matrix and can occur within an animal host where they can potentially lead to severe infection. In humans a number of pathologies such as endocarditis, urinary tract infections, burn infections, chronic otitis media, chronic bacterial prostatitis, respiratory infections in cystic fibrosis patients and periodontitis have been clearly associated with biofilm associated infections. These microbial structures can also be formed on both indwelling and subcutaneous biomedical implants, such as cardiac pacemakers, prosthetic heart valves, urinary tract prosthesis, peritoneal membrane and peritoneal dialysis catheters, tracheal and ventilator tubing, vascular grafts and stents, joint prostheses and cerebrospinal fluid shunts [1,2]. Those biofilms which develop on indwelling medical devices are a potential source for infections [3]. One of the consequences of biofilm formation on these devices is the constitution of a microbial reservoir that often leads to symptomatic but nonculturable inflammation, recurrent or persistent infection and spread of infectious emboli. One of the major problems of biofilms is their inherent tolerance to host defences and antibiotic therapies. As we earlier suggested, biofilms provide an immobilized but dynamic environment in which the bacterial cells are able to attain homeostasis [4]. While many infectious biofilms are composed of a single bacterial species, the possibility of multispecies infectious biofilms must also be considered. In these, synergistic reactions between the bacteria may lead to enhanced biofilm formation and to increased resistance to antimicrobial agents [5].

Antibiotic efficacy has been predicted *in vivo* by evaluating the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) *in vitro*. According to the standard procedures (NCCLS) these determinations are made using planktonic bacteria in the exponential phase of growth, therefore it can be said that MIC and MBC only predict antibiotic efficacy against rapidly dividing bacteria in acute infections, such as septicemia. Biofilm bacteria are able to tolerate significantly higher levels of antibiotics than planktonic bacteria, antibiotic resistance is reportedly

*Address correspondence to this author at the IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Braga, Portugal; E-mail: jazeredo@deb.uminho.pt

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up to 1000-fold greater in biofilm-bacterial cells. However, reliable methods to compare the antibiotic sensitivities of planktonic bacteria to cells in biofilms are lacking [6]. A recent study in which the same cell density was used to compare the susceptibility of planktonic vs biofilm bacteria to antibiotics reported a 10 fold higher resistance in biofilm bacteria [7]. Nevertheless, very often, the antibiotic concentration needed to eradicate the biofilm is above the peak serum concentration of the antibiotic [8], rendering it ineffective in treating biofilm infections. The clinical relevance of this phenomenon is underscored by the occurrence of biofilm-related infections that are tolerant to antibiotic therapy [8,9]. It has even been observed that in the case of *Pseudomonas aeruginosa* biofilms, aminoglycosides induced the Lon protease which is involved in biofilm formation [10].

The mechanisms of biofilm tolerance to antibiotherapy are still not totally understood. Several explanations lie on the diffusion limitation of the antibiotics through the biofilm matrix [11-13]. However, it seems that this mechanism can only partially explain the increased resistance phenotype generally present in clinically-relevant biofilms [14]. Other mechanisms have been suggested, including slow growth of the cells within the biofilm [15], activation of the general stress response [16], emergence of a biofilm-specific phenotype [17] and persister cells [18]. The failure of antibiotherapy to treat biofilm associated infections has encouraged the search for alternative methods to eradicate biofilms and the therapeutic use of bacteriophages (phages) has been considered a potentially valuable approach.

PHAGE BIOLOGY

Phages are viruses that infect bacteria and they are among the simplest and most abundant organisms on earth; it is estimated that there are 10 phages for each bacterial cell. Some phages are extremely specific while others have a very broad host range. Like all virus, phages are only able to replicate inside their host. There are two major types of phages, virulent (or lytic) and temperate. For therapeutic purposes, only the lytic phages are a good choice because they cause bacterial cell lysis and do not integrate into the host DNA. Phages are classified by the International Committee on Taxonomy of Viruses (ICTV) according to morphology and nucleic acid type (dsDNA, ssDNA, dsRNA, ssRNA). The ICTV presently recognizes one order, 13 families and 31 genera of phages. The dsDNA tailed phages, or *Caudovirales*, account for 96% of all the phages reported in the scientific literature, and possibly make up the majority of phages on the planet (at least 5360 tailed phages are known). These phages can be grouped in three different families according to their tail type. For example *Myoviridae* have a contractile tail whereas *Syphoviridae* has a non-contractile tail and *Podoviridae* family has no tail. There are several other approaches for phage classification based on phage genome, most of which, however, categorize phages within the same group in which they are currently classified by ICTV [19].

During an infection, the lytic phage attaches to a bacterium and injects its genetic material into the host cell, where it directs the expression of genes and proteins responsible for the assembly of new phage particles. The progeny phages burst from the host cell (lysing it) and infect more bacteria. The production and subsequent release of phage particles allows successive phage infection of additional host strain bacteria in a rapid and exponential pattern. The initial contact between phages and bacteria is usually possible by diffusion, collision or attachment. To enter a host cell, phages attach to specific receptors on the surface of bacteria, including lipopolysaccharides, teichoic acids, proteins or even flagella. This specificity means that a phage can only infect certain bacteria having receptors to which they can bind, thus determining the host range of the phage. The infection process can be rather complex. T-like bacteriophages, for example use a syringe-like motion to inject their genetic material into the cell. After making contact with the appropriate receptor, the tail fibers bring the base plate closer to the surface of the cell. Once attached completely, the tail contracts, possibly with the help of ATP present in the tail injecting the genetic material inside the cell cytoplasma. During this process the cell wall is locally digested by a lysozyme domain located at that needlelike structure. The release of the new viral particles from the infected host is made possible by cell lysis through the production of phage induced enzymes. In case of ds DNA phages, a system of holins-endolysins is used to disrupt the cells. Holins cause holes in the cell membrane allowing endolysins to lyse the cell wall peptidoglycan. In case of ssDNA phages an enzyme called "amurein" that blocks the synthesis of murein is produced [20].

PHAGES AS THERAPEUTIC AGENTS

Phages, by their nature, are good candidates for antibacterial therapy on account of their high specificity, affecting only the target bacterium and their non-toxicity to animals and plants. They do not affect eukaryotic cells and possess self-reproducing capability as long as the corresponding host bacteria are present. Moreover, phages can potentially be targeted to receptors on the bacterial surface which are involved in pathogenesis so that any phage-resistant bacterial mutants are attenuated in virulence. Additionally, bacteriological studies have indicated that phages are able to multiply *in vivo* in animal tissues and are able to penetrate into the human organism [21]. Finally, phage production is simple, rapid and relatively inexpensive.

There is a considerable number of publications suggesting the use of phages with veterinary applicability and reporting the high performance of such preparations in the control of animal bacterial infections [22]. However, phage therapy in humans has been postponed partially due to the lack of consistence of the scientific studies that could support it, mainly published by Polish and Russian investigators [23]. Nevertheless, encouraging publications reporting the success of experiments conducted with phage lysins to combat streptococcal and pneumococal infections [24] and a phage cocktail against *Staphylococci, Streptococci, P. aeruginosa, Proteus spp and E.coli* for the treatment and prophylaxis of purulent wound infections [25,26] have renewed the interest in phage human therapy.

Phage Lysins as Therapeutic Agents

Phage lysins also called endolysins are enzymes that impair cell wall integrity. These enzymes are produced by a

sensitive bacterial host at the end of the life cycle of a dsDNA phage and are composed of at least two distinctly separate functional domains: a C-terminal cell wall binding domain and a N-terminal catalytic domain. The catalytic domain can comprise endopeptidases, muramidases (lysozyme), N-acetylmuramoyl-L-alanine amidases and glucoamidases [20]. When a lysin is added to a sensitive host, in the absence of the phage, it lyses the cell wall producing a phenomenon known as "lysis from without" [27]. The *in vivo* efficacy of lysins to treat *Streptococcus pyogens* [28], *Bacillus anthracis* [29], group B streptococci [30] and drug resistant strains of *S. aureus* [31] has already been established.

PHAGE/BIOFILM INTERACTIONS AND PHAGE INFECTION OF BIOFILMS

The interaction between phage and biofilms is a rather complex process [32]. Theoretically, a biofilm should be rapidly infected because cells are more close to each other and this fact can enhance phage replication, when compared to the less accessible bacteria of planktonic cultures [33]. On the other hand, the structure and composition of the biofilm as well as the physiology of the biofilm cells may impose some limitations to biofilm infection. In fact, recent evidence that bacterial strains tolerant to phage infection increase their biofilm formation ability, has suggested that the biofilm phenotype might be an additional strategy of bacteria to escape phage infection [34].

Under suitable conditions the presence of bacterial cells in a biofilm does not prevent access to and infection by phage particles. The results of such interactions do however depend on a number of different factors which include whether or not the phage also possesses associated polysaccharide-degrading enzymes [32]. Flemming *et al.* [35] have drawn attention to the fact that the polymeric matrix in which the microbial cells are embedded consists of various polymers in addition to exopolysaccharides. Thus the phage must either be able to penetrate the matrix by diffusion or enzymes associated with the viral particles and must destroy enough of the matrix if they are to release the embedded bacteria and make them vulnerable to the phage themselves. Their effectiveness will depend on the extent to which the matrix is composed of polysaccharide but also the way in which that interacts with all the other polymers and ions in the matrix. The ability of polysaccharides to interact synergistically to enhance biofilm structure has been suggested. In the example of the plant pathogen *Pseudomonas syringae*, Laue *et al.* [36] suggested that both alginate and the polyfructan levan contributed to biofilm formation by this bacterium although a third unidentified polysaccharide might also play a significant role. A role has also been proposed for DNA released through the action of autolysins in the establishment of biofilms by *Staphylococcus epidermidis* [37]. Similar examples may well be found in other bacteria pathogenic for humans and animals. Both physiological and genetic changes within bacteria can make significant alterations to the biofilm structure [38] and thus to the effectiveness or otherwise of phage attack.

In many clinical situations, single-species biofilms will present problems but when multi-species biofilms occur the interactions between phage and bacterium may be much more complicated and there may also be antagonistic interactions resulting from bacteriocin production [39]. Certainly Tait *et al.* [40] found that attempts to eliminate biofilms formed from dual enterobacterial species with phage and associated polysaccharide depolymerase were unsuccessful. The phage and bacteria could apparently coexist stably within these particular biofilms.

OBSTACLES TO PHAGE BIOFILM INFECTION

The bioflm matrix can pose a serious obstacle for the penetration of the phage. In fact, Doolittle *et al.* [41] reported a partial infection of a *Pseudomonas aeruginosa* biofilm by the phage E27 in which the phage particles were not able to access the deeper layers of the biofilm. Additionally, the biofilm matrix is also a reservoir of proteolytic enzymes as well as endoglucanases which can lead to bacteriophage inactivation. The diffusion limitation imposed by the biofilm matrix is obviously very dependent on the biofilm itself, which in turn is affected by the biofilm formation conditions [42]. Many biofilms possess an open architecture with water-filled channels, which would allow the phage access to the inner biofilm layers [32]. Conversely, a dense biofilm structure would render phage penetration difficult. The presence of dead cells can also be considered a difficulty for the progression of the infection, as they are potential viral receptor sites. Usually, the number of dead cells increases with biofilm age, which makes older biofilms usually more difficult to infect. Another important property of the biofilm matrix that may influence phage action is its charge and hydrophobicity [32].

Another obstacle for the success of biofilm infection is the reduced metabolic activity of biofilm cells, especially those in the inner layers which are possibly oxygen and nutrient deprived. As phage infection and life cycle generally strongly depend on the growth stage of the host bacterium [43,44] the treatment of slowly growing cells in biofilms is a challenge. Sillankorva *et al.* [45] have shown that planktonic cells are more rapidly lysed than biofilm cells of *Pseudomonas fluorescens*. Cerca *et al.* [46] also demonstrated that the infection of a suspended culture of *S. epidermidis* by phage K is faster than that of a biofilm. These authors also assessed the rate of infection of dispersed biofilm cells and concluded that the rate of biomass reduction was dependent on the physiological state of the cells rather than on the diffusion barrier imposed by the biofilm matrix. Nevertheless, in both studies the authors reported equivalent biomass reductions in biofilm and planktonic cultures.

Many infectious biofilms are of dual or multispecies and the presence of more than one species may render biofilm infection difficult. This is in part due to the co-aggregation phenomena, common in biofilm communities from a variety of habitats, in which the cell-to-cell binding may occlude phage receptors.

STRATEGIES FOR THE APPLICATION OF PHAGES TO CONTROL INFECTIOUS BIOFILMS

Many phages, but not all, may produce polysaccharases or polysaccharide lyases that degrade the biofilm matrix helping the phages to gain access to the entire biofilm. Suth-

erland and co-workers were the first to suggest the application of phages and phage induced depolymerases to eradicate bacterial biofilms. In one of their reports these authors described a complete eradication of a biofilm of *Enterobacter cloace* by a cocktail of three phages and their associated polysaccharide depolymerases [40]. These phage enzymes are able to degrade the exopolymeric matrix facilitating the migration of the phage through the biofilm. Hanlon *et al.* [47] found that a *P. aeruginosa* bacteriophage (F116) was able to penetrate the inner layers of the biofilm due to the reduction of the viscosity of the alginate matrix by enzymatic degradation.

In practice how can we control clinically relevant biofilms? Phages are now being successfully used to treat wounds or chronic ulcers by topic application with a phage suspension. The impregnation of the surface of a medical device with a phage solution is another strategy suggested by Curtin and Donlan [48] to control infectious biofilms associated with the use of indwelling devices. These authors have shown that incorporating a coagulase-negative staphylococcus phage into the hydrogel coating of a catheter can significantly reduce the attachment and biofilm formation by *Staphylococcus epidermidis* (the phage host). Another interesting approach is the use of a phage encoding a phage polymerase and a phage polymerase itself to treat *Pseudomonas aeruginosa* biofilms in cystic fibrosis patients by aerosol administration [49]. All these approaches are based on the direct application of phage particles onto the infectious biofilm. If phages were able to pass the gut barrier reaching the peripheral lymph and blood, invasive administration methods would be avoided. This would be of particular importance for the treatment of infections caused by biofilms formed on internal implants such as pacemakers and internal prosthesis. Górski *et al.* [21] suggested that phages can translocate by a process similar to the well-known bacterial translocation. This fact is supported by several studies in which phage particles were detected in human body such as saliva, urine, faeces and human sera [50]. Bruttin and Brussow [51] were however unable to detect T4 coliphages in the blood of volunteers following oral administration. According to Górski *et al.* [52] the phage translocation may be determined by the phage type.

BIOTECHNOLOGICAL CHALLENGES OF PHAGE THERAPY

All the above mentioned strategies need to have a previous identification of the bacteriophage and/or polysaccharide depolymerise capable of infecting the bacterial cells and degrade the polysaccharide within the biofilm, respectively. So, the biofilm organisms must be isolated and screened against a bank of phages. This procedure is essential and raises important biotechnological challenges: the existence of a bank of phages well characterised (physiologically and genetically) whose efficacy *in vivo* has been tested and pharmacokinetics studied; the existence of economically and safe production protocols and purification methods (the presence of endotoxins in a phage preparation may compromise phage therapy).

The application of phages as therapeutic or prophylactic agents against biofilms may however be limited by two aspects: The phage induced immune response and secondly, the phage specificity. There are few studies reporting the interaction of phages with the immune system, however it is clear that phages are antigenic and elicit a serum antibody response that will neutralise the phage particles. However, as phages can quickly be associated with the biofilm structure when introduced into contact with the biofilm it is likely that biofilm matrix would provide an environment protecting the phages. It may also be the case that even if much of the phage and phage-induced enzyme is neutralised by the action of antibodies, sufficient activity remains to disperse much of the biofilm. Most phages have a narrow lytic spectra (there are however phages capable of infecting different strains of different species [53]) and the ability to infect is greatly influenced by the bacterial growth environment and the physiological state of the host bacterial cells [43,44]. Therefore as already indicated, a therapeutic phage must be selected after screening the bacterial infectious agent, grown in conditions close to that found in their actual environment, against a pool of phages. This procedure may delay the beginning of the treatment; nevertheless, a biofilm infection does not proliferate as fast as an acute infection. We should also consider the possibility that certain medical biofilms are difficult to access making impossible the isolation of the strain or strains responsible for the biofilm formation.

THE IMPORTANCE OF ENGINEERING PHAGES

As stated above, a phage product designed "a la carte" based on the identification of the pathogens present in the biofilm is one of the strategies of phage therapy. However, it is important to consider the fact that the chances of getting a specific phage with a high lytic capability and preferential expressing a relevant exopolymeric degrading enzyme are likely to be low [54]. Engineered phages can play an important role in this process. Phages can be genetically manipulated to alter their host range and to induce the production of depolymerases. Cao *et al.* [55] modified the filamentous phage M13 specific for *E. coli* to be able to bind to *Helicobacter pylori*. Another example is the modification of a T7 phage to express K1-5 endosialidase in order to allow it to infect an *E.coli* strain, which is able to produce the K1 polysaccharide capsule and thus is normally resistant to T7 infection [56]. Very recently, Lu and Collins [57] described a methodology to engineer phages to express biofilm-degrading enzymes and used it to modify a T7 phage to express DspB intracellularly during phage infection of *E. coli* cells. This enzyme is released into the extracellular environment during cell lysis and is able to hydrolyse β-1,6-N-acetyl-D-glucosamine [58] which is a polysaccharide adhesin involved in cell-to-cell adhesion in different species of bacteria [59]. A further type of phage/bacterium interaction was found by Kirov *et al.* [60] in *P. aeruginosa* isolates from CF patients. Maturing biofilms demonstrated release of phage within the biofilm on bacterial cell lysis with consequent degradation of localized areas of the matrix.

CONCLUSION REMARKS AND FUTURE PERSPECTIVES

The development of new methods to attack the bacterial biofilms associated with clinical infections is urgently needed. *In vitro* experiments have shown that phages are

able to infect biofilm cells and those viral particles carrying depolymerases have the advantage that they can penetrate the inner layers of biofilms through the degrative action of their associated enzymes on the exopolymeric matrix. Several approaches offer potential. The genetic engineering of phages to enable them to infect a wider range of host bacterial strains and species is one. Another is to introduce new genes into the phages to enable them to degrade the polymers found in the biofilm matrix. The new genes could be specific polysaccharide depolymerases, DNase or proteases. Alternatively bacteriocins effecting elimination of the bacteria may be worth further investigation. It is however important to develop efficient mechanisms for phage delivery. This requires a study of "*in vivo*" phage performance against biofilms and of the effectiveness or otherwise of biofilms in protecting the phages against the human immune system. Efficient and economic phage production and purification protocols needs also to be addressed before one can hope to use phage treatment to prevent or control infectious biofilms.

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