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Targeting biofilms using phages and their enzymes

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The complex biofilm architecture composed of extracellular polymeric structures (EPS) provides a protective shield to physiologically diverse bacterial cells immersed in its structure. The evolutionary interplay between bacteria and their viruses (phages) forced the latter ones to develop specific strategies to overcome the biofilm defensive barriers and kill sessile cells. Phages are equipped with a wide panel of enzyme-degrading EPS macromolecules which together are powerful weapons to combat biofilms. Antibiofilm performance can be achieved by combining phages or phage-borne enzymes with other antimicrobials such as antibiotics. Nevertheless, a variety of enzymes encoded in phage genomes still need to be explored. To advance in biofilm control strategies we must deepen the understanding of the biofilm biology itself, as well as discover and better exploit the unlimited antibacterial potential of phages.

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Introduction

Biofilms are microbial communities adhering to surfaces or formed on air–water interfaces. These communities are encased in a dense self-produced polymeric and highly hydrated matrix composed mainly of polysaccharides, proteins, lipids, and extracellular DNA (eDNA) [1]. A peculiar feature of the biofilm population is the fact that cells are organized in specialized functions, ensuring the adaptation of the community to diverse environments. On the other hand, the biofilm matrix confers the biofilm extra protection against dehydration, starvation, and predation. For these reasons the biofilm phenotype has greater adaptive advantages than the planktonic lifestyle and therefore biofilms dominate in all habitats on Earth, accounting for ~80% of bacterial and archaeal cells in the biosphere [2]. The competitive advantages of biofilms are even more striking in adverse conditions, such as those found in the human body. Human natural defences are programmed to eliminate and prevent microbial colonization in internal organs and therefore invasive microorganisms need to display an arsenal of virulence factors to be able to survive and proliferate. In these cases, biofilm formation is an important strategy of microbial survival. The protective effect of the biofilm matrix, together with the resilience of the biofilm-associated cells, contributes to high tolerance to antibiotics and immune clearance. For this reason, the majority of bacterial chronic infections are caused by biofilms, with an estimate of around 65% of all infections, according to the Center for Disease Control (CDC), and 80% according to the National Institutes of Health (NIH) [3]. Biofilms can be formed on human tissues causing endocarditis, cystic fibrosis, periodontitis, rhinosinusitis, osteomyelitis, non-healing chronic wounds, meningitis, and kidney infections. Biofilms can also be formed on surfaces of biomedical materials such as prostheses and implantable devices, generating indwelling device-associated infections [4]. The negative implications of biofilms on human health are tremendous and therefore effective methods to control biofilm-associated diseases are urgently needed.

Bacteriophages (phages) are the natural enemies of bacteria and as such, they can infect bacteria-forming biofilms [5]. There is a growing scientific interest in phages because of the need to discover and develop alternative, or complementary, antimicrobial strategies to counteract the increasing resistance to conventional antibiotic therapy [6]. In particular, the interaction of phages with biofilms has been subject to research in many scientific publications, a number which has grown exponentially in the last 10 years (source PubMed). The increased awareness of the implications of biofilms on human health is one of the reasons for this rising interest. Another reason is the increasing amount of evidence that phages are more efficient antibiofilm agents than traditional antibiotics. Indeed, the majority of the scientific publications concerning phages and biofilms report the successful application of phages against mono and multispecies biofilms, particularly formed by *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (also implicated in the majority of biofilm-related infections). These studies

highlight the ability of phages to control biofilms but also emphasize important concerns about their limitations and suggest possible ways to improve phage effectiveness [5,7•,8–10,11••,12].

In addition to phages, phage-encoded proteins have also been widely exploited as powerful antibacterial weapons. Phages use enzymes such as virion-associated peptidoglycan hydrolases (VAPGHs), endolysins, and depolymerases to interact and kill their hosts. Some of these enzymes are already utilized as recombinant proteins offering a great antibacterial tool to effectively combat biofilms. In this review, we discuss the existing research regarding the biofilm as a complex structure, and the probability of defeat by its natural bacterial enemy (phages) and its enzymatic weapons. We also illustrate here future perspectives on how phages and their evolutionary strategies may help us design new antibiofilm therapies.

Challenges associated with biofilm control

Biofilm eradication and prevention is a huge challenge for current and future medicine, agriculture, the food industry, animal husbandry, and a wide variety of human activity. Biofilm has a complex architecture with integrated biomolecules and mineral components called extracellular polymeric structures (EPS), and biodiverse microbial cells, all perfectly protected from external harsh conditions, antibiotics, and the immune system response (Figure 1). The thick EPS, partially hydrophobic or hydrophilic, significantly limits the diffusion rate of chemicals thereby reducing their local toxic concentration. Moreover, biofilm conglomerate is organized in well separated and protected microhabitats providing a variability of oxygen and nutrient conditions [13,14].

Diversity of biofilm-residing population

The structural organization of biofilm creates an oxygen and nutrient gradient forcing embedded organisms to adaptat to diverse conditions and thus to physiological heterogeneity [1]. Bacteria may adopt different physiologies within the biofilm structure; we may find metabolically active, dormant, and persister physiological states. The cross-talk of signal sensing regulatory networks such as (i) two-component systems (TCS), (ii) diguanylate cyclase systems (DGC), and (iii) quorum sensing (QS), enable bacteria to have a dynamic response to environmental changes [15,16]. The low availability of oxygen and nutrients results in the arrest of bacterial metabolism-transferring cells into dormant forms making them tolerant to many antibiotics and chemicals [17]. On the other hand, hypoxia and nutrient depletion induce the switching to anaerobic respiration or a lag phase, changing the bacterial susceptibility to particular drugs. Sessile cells exhibit adaptive temporary tolerance to antibiotic exposition called the persister phenotype. Moreover, the physiological modification to biofilm conditions might induce active resistance mechanisms such as antibiotic inactivation, antibiotic target modification, or efflux pump overproduction [18]. All the above complex aspects make biofilm-residing bacteria a problematic diverse target for antimicrobials.

Extracellular polymeric structures

eDNA

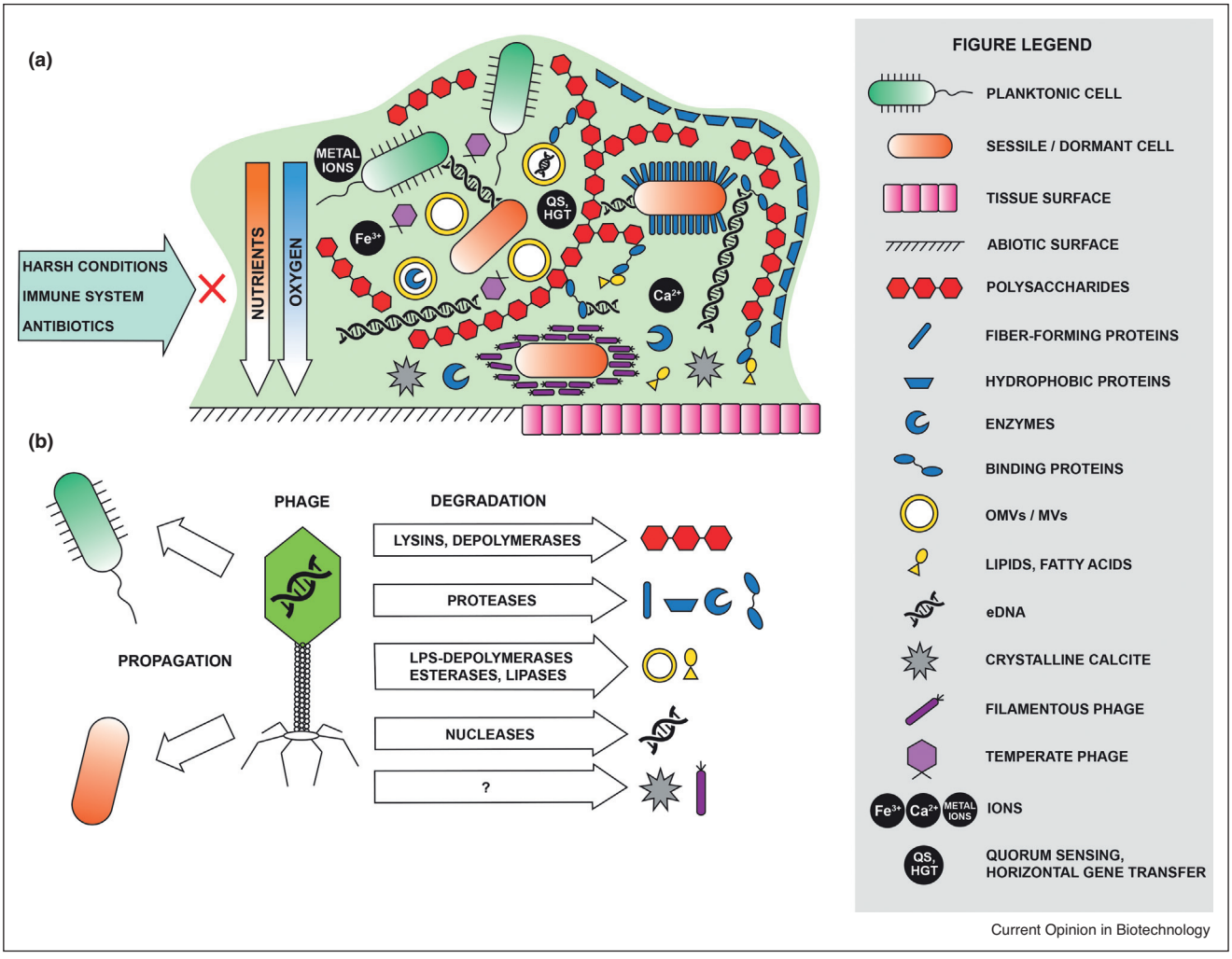
DNA forms long chains and is a perfect macromolecule to build the spatial network for biofilm-embedded cells and other matrix components.

Extracellular DNA (eDNA), as a negatively charged structure, may interact with proteins localized on the cell surface, such as outer membrane vesicles (OMVs), or those immersed in the matrix. It may involve Type IV pili (T4P), enzymes, innate immune elements, as well as toxins. Moreover, the exopolysaccharides produced intensively by sessile cells can colocalize with eDNA chains giving the biofilm structure more strength. It is worth mentioning that macromolecules providing a high amount of negative charge protect the biofilm-living bacteria from the toxic effect of metals or, vice versa, accumulate desired ferric and calcium ions in the matrix [19]. The eDNA can be delivered from enzymatically lysed cells, OMVs transporting a genetic cargo, cell debris left from phage propagation, or actively released DNA via a T4SS-like system, as has been found in *Haemophilus influenzae* [20]. The role of eDNA is not only limited to structural purposes. It also serves in horizontal gene transfer (HGT), driving the biodiversity and evolution of biofilm-residing microbial populations. Some bacteria like *Vibrio cholerae* can genetically benefit from close cell-to-cell localization in the matrix milieu, and actively collect foreign DNA from neighbouring bacteria via type VI secretion system (T6SS) [21].

Polysaccharides

Polysaccharides are produced by all kinds of bacteria as an element of cell surface structures — such as capsules, lipopolysaccharide (LPS), peptidoglycan, or extracellular products and also slime released into the biofilm matrix. Those glycans serve bacteria in many ways — adhesion; cell-to-cell interactions; evasion from the immune system response; mimicking the macromolecules of the infected host; protection against desiccation, and neutralizing toxic compounds. Thanks to the formation of long chains, sticky features, and being an easily accessible source of energy, exopolysaccharides are perfect molecules for biofilm structure [14]. Microorganisms mainly utilize glucans, galactans, fructans, mannans or poly-*N*-acetylglucosamine (PIA) to create biofilm EPS. *P. aeruginosa* even produces three different polysaccharides (Psl, Pel, alginate) promoting a sessile style of life. It was found that Pel is composed of partially acetylated $1 \rightarrow 4$ glycosidic linkages of N-acetylgalactosamine and N-acetylglucosamine and, as a cationic macromolecule, in the acidic

Figure 1



The complex biofilm structure formed on abiotic and biotic surfaces is encompassed by diverse microbial cells (planktonic, dormant, sessile) immersed in extracellular polymeric structures (EPS). EPS causing oxygen and nutrient gradient, is composed of polysaccharides, eDNA, fiber-forming proteins, glycan-binding proteins, outer membrane vesicles/membrane vesicles (OMVs/MVs), lipids, liquid-phage crystalline, and mineral macromolecules, stabilizing biofilm conglomerate, and protecting embedded bacteria from external unfavourable conditions. The close cell-to-cell interactions enable horizontal gene transfer (HGT) and quorum sensing (QS), as well as prophage induction driving the biodiversity and evolution of biofilm-residing microbial populations **(a)**. Phages are a self-producing weapon killing biofilm-living bacteria, as well as a source of already known efficient enzymes degrading EPS macromolecules. The question mark refers to not yet discovered phage-based agents targeting biofilm crystallines. Synthetic biology and deepened knowledge about biofilm biology would give new insight into future anti-biofilm perspectives **(b)**.

environment, interacts with negatively charged eDNA increasing the physical durability of biofilm structure [22]. Several studies showed that exopolysaccharides increase the biofilm tolerance to different antibiotics, but there are no clues to the exact mechanism, as the ionic interactions could be excluded in the case of uncharged *P. aeruginosa* Psl. Contradictory results were also obtained for the drug penetration ability within the biofilm milieu [23].

Proteins

An important element providing the structural stability of formed biofilm is based on proteins which are able to polymerize into higher-order structures, called amyloid-like fibers or fiber-forming proteins. There are several examples studied so far, including curli composed of CsgA units produced by *E. coli;* TasA/TapA fibers formed by *Bacillus subtilis;* PSM polymers found in *S. aureus;* FapB/FapC/FapE nucleated and aggregated structures in *Pseudomonas*, and more simple conglomerates built of self-assembled units, Bap in *S. aureus* or P1 adhesin (AgI/II) in *Streptococcus mutans* [24,25]. The main function of amyloid-like fibers is to form a barrier protecting a bristling cell from physical interaction with the exterior and preventing phage adsorption, or serum complement deposition, on the cell wall surface. Protein fibers together with the abundance of exopolysaccharides also serve structural purposes. As the biofilm is a dynamic

form, specific mechanisms control the self-assembly of amyloid-like fibers, both at the cell-dependent level and a physical rate-limiting level, enabling the step-by-step growth of biofilm structure [26•]. An interesting example of structural functioning protein is BslA produced by *Bacillus subtilis*. This protein forms a hydrophobic protective layer of dimers and tetramers when exposed to oxygen, whereas it exists as a hydrophilic monomer in anoxic conditions deep in the biofilm. This highlights yet another mechanism of bacterial response to variation in redox conditions and a fast adaptation to the biofilm style of life [27].

There is a panel of other surface structure proteins including Type IV pili (T4P), flagella, lectins, glycan-binding proteins, lectins, as well as polysaccharide-modification enzymes which all are engaged in the dynamic steps of the biofilm attachment and maturation process. Recent reports present the complex interactions of *P. aeruginosa* outer-membrane protein OprF with other biofilm elements, providing detailed insight into cellular-macromolecule interplay. The OprF localized in the cell and OMVs, together with lectin LecB, is involved in the attachment process. Both proteins condition the tissue-bacteria/OMV adhesion, the formation of the bacteria-bacteria/OMV complex, as well as bacterial/OMV-polysaccharide interactions in the matrix. It is also proposed that OMVs bearing OprF are a decoy to sequestrate neutrophil elastase thus protecting sessile cells from the innate immune-mediated lysis [28].

Lipids

Lipids as a component of glycolipids, sphingolipids, teichoic acids (wall teichoic acids, WTA, and lipoteichoic acids, LTA) and mycolic acids build the bacterial cell wall structure, and therefore are also found in the biofilm matrix when released from lysed cells. As most of them are long-chain hydrophobic molecules they enhance the autoaggregation of bacteria hidden in the biofilm and trigger the attachment/adhesion process, especially to abiotic surfaces [29].

OMVs and MVs

Outer membrane vesicles (OMVs) produced by Gram-negative bacteria are a sophisticated weapon used for different physiological and pathogenesic purposes. These lipoprotein vesicles, produced under the control of quorum-sensing systems, are released in response to environmental stress. Bacteria use OMVs to transfer specific cargos to distant places. These vesicles, mimicking the original bacterial cell, serve as a bait for antibodies, antimicrobial peptides, or lytic phages, and induce apoptosis and inflammation, and attract immune response away from its producer. OMVs carrying QS molecules, antibiotic degrading enzymes, DNA, or specific toxins to defeat competitive microorganisms, are important for HGT, interspecies interactions, or communication. The strategy to utilize OMVs enables both the planktonic or sessile style of life [30]. OMVs containing lytic enzymes enhance the release of eDNA into the matrix providing the material for the biofilm spatial structure. Recent studies provide interesting data on the OMVs role in biofilm formation and maintenance as well, and show that the composition of biofilm- and planktonic-derived vesicles differ in terms of glycoproteins and lipids [28,31]. The *P. aeruginosa* vesiculation process consumes the hydrophilic B-band LPS for OMV formation leaving bacteria enriched in the hydrophobic A-band LPS. Simultaneously, the isomerization of cis- into trans- unsaturated fatty acids in the inner membrane is observed. Both mechanisms make the cell surface more hydrophobic and the membrane more rigid which make bacteria prone to self-aggregation. It was found that hypoxia forces bacteria towards intensive OMV production, thus inducing biofilm and microcolony formation. This suggests that the vesiculation process, at least in an intensive biofilm producer *P. aeruginosa*, is in some way a self-featured process driven by the low oxygen conditions prevailing within the biofilm milieu [31]. In other words, this mechanism allows for fast transformation of bacteria from planktonic to biofilm residents by a rapid increase in cell surface hydrophobicity due to the release of OMVs.

Bacterial cell lysis, caused by phage propagation, lytic enzymes, or other external agents, enriches the EPS with membrane vesicles (MV) as cell leftovers, which further serve as a source of nutrients, DNA pool in the matrix, and analogously to OMVs which may also be targeted as a decoy by the immune system or phages.

Crystallines

The biofilm structure may be stabilized and solidified by inorganic components forming crystallines. Common urinary tract pathogens including *Proteus* species, *Morganella morganii*, *Klebsiella pneumoniae, P. aeruginosa*, and staphylococci, produce urease-triggering external pH. That leads to the biomineralization of $CaCO_3$ and the formation of insoluble crystals. The accumulation of mineral crystals can be usually seen on abiotic surfaces, such as plastic urethral catheters in biofilm-borne infections. The presence of crystalline calcite was also observed in Gram-positive bacteria, such as *Bacillus subtilis* and *Mycobacterium smegmatis*, which suggests it being a common biofilm feature. The mineral crystallines interact with the remaining organic structural components of EPS, especially those of negative charge, further stabilizing, and fixing, the whole biofilm structure to a particular surface [32].

Recent studies revealed another type of crystal-like particle formed in the biofilm milieu. It was found that filamentous prophage Pf4 is induced and released by sessile living *P. aeruginosa* cells, and by interaction with the extracellular matrix assembly forms liquid crystalline droplets [33]. These droplets are accumulated around the

cell forming a specific thick proteinous shield protecting bacteria from the external harsh environment, antibiotics, and the immune system attack. As the filamentous inoviruses are commonly found prophages it is proposed that the formation of organic crystalline liquid is a wide-spread strategy utilized by many biofilm-forming bacteria [34].

Phage features used in biofilm control

The ubiquitous nature of biofilms indicates that interactions between matrix-embedded bacteria and phages have certainly been frequent throughout evolutionary history. Consequently, phages and biofilm communities have found many mechanisms of coexistence that remain poorly understood. The protective effect of the biofilm matrix and the physiology of biofilm-living cells are, among other factors, responsible for repressing phage predation. The biofilm density plays an important role in phage/biofilm interaction; when biofilms are sparse, phage/bacteria encounters are less likely to occur, resulting in poor infection efficiency. On the other hand, the success of phage dispersion in dense EPS relies on its mobility [35]. Therefore, phages have developed specific strategies to penetrate the three-dimensional structure of the biofilm, and to cope with different cell physiologies (Figure 1).

Diffusion through biofilm water-channels

Biofilms are highly hydrated structures formed by voids, also called water channels, that help the diffusion of nutrients throughout the biofilm. Phages can diffuse through these void spaces and penetrate the inner biofilm layers by diffusion through gravity. In contrast to antibiotics, where diffusional limitations lead to a depletion of the antibiotic concentration at the inner biofilm layers, phages increase in number due to active replication. This fact leads to the lysis of a fraction of the sessile population inhabiting the inner layers, contributing to the disturbance of the biofilm 3D structure [36] (Figure 2).

Enzymatic degradation

Phages are naturally adapted to penetrate biofilm EPS and are equipped with specific enzymes such as VAPGHs, endolysins, and depolymerases which potentiate their anti-biofilm killing efficacy. VAPGHs and endolysins destroy the bacteria cell wall whereas depolymerases are capable of degrading the bacterial surface polysaccharides as well as the EPS components [38]. There is evidence that some phages may even induce depolymerase enzyme expression, however, it is still unclear if this process is controlled at the phage level or as the bacterial response to phage predation [39]. It is important to mention that the cell lysis itself, caused by phage propagation, leads to the release of bacterial cell content directly into the biofilm milieu, thus bacterial enzymes responsible for extracellular polymers resorption and biofilm dispersion are also brought into the EPS degradation action.

Hitchhiking on carrier bacteria

Phages can absorb reversibly to the appendices of motile bacteria. This feature has been shown to occur with phages infecting *Caulobacter crescentus*, where the first interaction with the bacterial flagellum takes place through a filament on the phage head [40]. Therefore phages may develop an active way of penetrating inside biofilm, hitchhiking on motile carrier bacteria.

Tackling persister cells

Unlike antibiotics, phages can infect and kill dormant and persister cells (Figure 1b). Persister cells can be protected from temperate phages (by superinfection immunity), however, they are not protected from strictly lytic infection. Data show that phages can replicate in late stationary cultures known to be mainly composed of growth arrested-cells. The process of replication can initiate immediately, in some cases, after phages enter the target cell [41], or as soon as cells restore their normal growth [42]. Furthermore, the release of intracellular material, and the dispersion of the biofilm, triggers the metabolism of the persister population further activating phage replication.

Strategies to improve phage efficacy

Despite the strategies used by phages to counteract the defensive mechanisms of biofilms, native phages, *per se*, are not able to eradicate the entire biofilm population. To circumvent phage limitations and improve their performance for efficient biofilm control, different approaches have been perused, such as combined therapy (discussed later), or genetic manipulation.

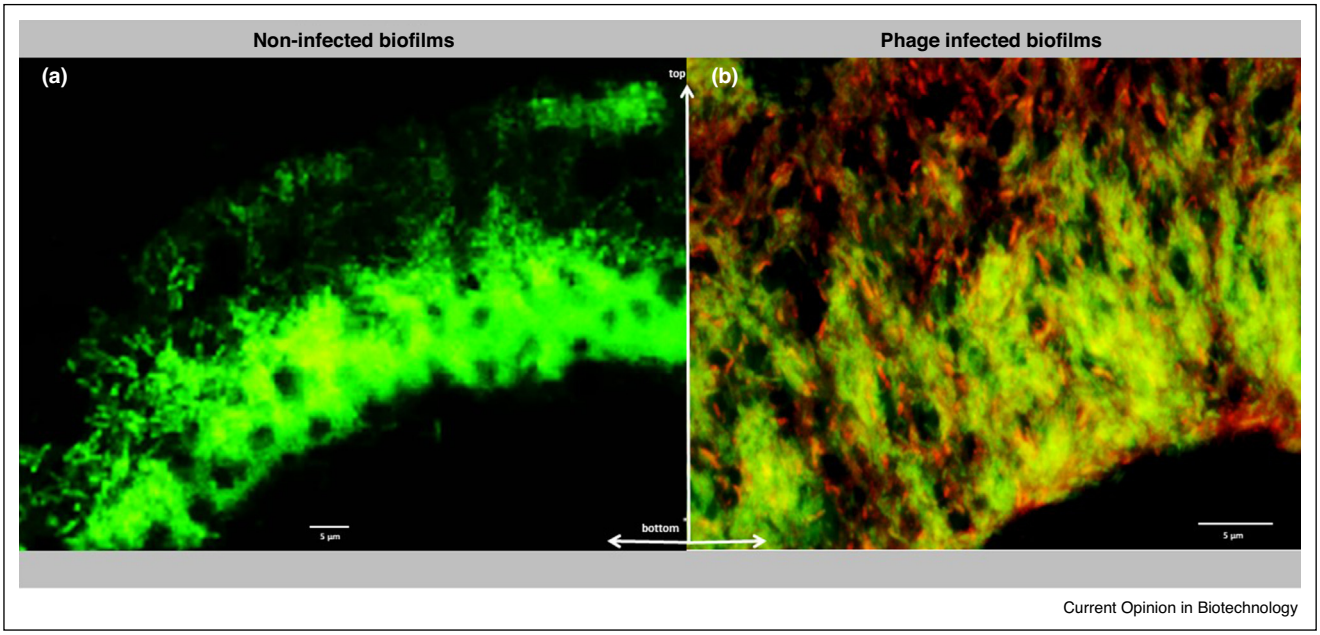
It is important to highlight the potential of synthetic biology tools to rebuild phage genomes displaying enhanced biological properties [43]. Several genome engineering tools have been efficiently applied to phages [44]. It is worth mentioning the recently developed platform for rebooting phages of gram-positive bacteria using Listeria-L-form cells as rebooting compartments [45]. These tools can be particularly useful in the design of engineered phages equipped with matrix-degrading enzymes. Nevertheless, there are still many obstacles in this area such as the incorporation of large gene fragments, for example those encoding depolymerases, into a phage genome. Thus the overall genome size limitation requires the elimination of other genes. However, the decision of the best gene-editing strategy is still a blind operation caused by deficient knowledge of many phage genes function, and their influence on the bacterial host.

Phage derived enzymes

Endolysins and virion-associated peptidoglycan hydrolases

Phage lytic proteins, such as endolysins and virion-associated peptidoglycan hydrolases (VAPGHs) have high

Figure 2



Microscope images of *P. aeruginosa* PAO1 phage-infected biofilm. Forty-eight-hour phage-free biofilm **(a)**, biofilm exposed to a *P. aeruginosa* infecting phage for 5 min **(b)**. The green fluorescence corresponds to *P. aeruginosa* cells and the red fluorescence is *P. aeruginosa* phage infected cells. Images were obtained using the LNA-FISH technique, in which two probes were used; the green fluorescence probe targets the rRNA 23S and the red fluorescence probe targets the mRNA encoding for the major capsid protein of the phages. The images are adapted from Ref. [37].

antimicrobial activity against Gram-positive bacteria when added externally, due to their ability to hydrolyse the peptidoglycan (PG) from the cell wall (Figure 1b) [46,47]. Phages can produce a wide range of PG-degrading enzymes including muramidase, transglycosylase, glucosaminidase, amidase, and endopeptidase. The two latter enzymes are also classified as proteases. Endolysins from Gram-positive infecting phages have a modular design with catalytic activity and substrate recognition separated into two distinct types of functional domains (cell wall binding domains (CBDs) and enzymatically active domains (EADs)). This modularity facilitates domain engineering and production of chimeric enzymes by fusion of catalytic or binding domains from different lytic enzymes, thereby altering enzymatic properties [48]. The application of endolysins against Gram-negative pathogens is impaired by the presence of a protecting outer membrane (OM) layer, however, the combination with membrane permeabilizers turned out to significantly improve lysin efficiency. More recently, genetic engineering allowed us to design the lysin/cationic peptide combination called Artilysins [49]; the lysin/bacteriocin version to obtain Lysocins [50••], and the lysin/phage receptor binding proteins to generate Innolysins, as an anticipated promising strategy [51••].

In terms of anti-biofilm activity, phage lytic proteins offer interesting properties, for example, they easily penetrate the biofilms [52] and are active against both low metabolically active cells and persister cells [53]. Besides these, other valuable antimicrobial characteristics include (i) the lack of resistance development to phage lytic proteins, as peptidoglycan is the crucial and conservative structure; (ii) the lysin activity spectrum is usually broader than the phage host range but narrower than antibiotics/disinfectants); (iii) a simple lysin structure is suitable for modification using synthetic biology tools [54].

The ability of phage lytic proteins to remove biofilms formed by Gram-positive bacteria such as *S. aureus* [55,56], *Streptococcus pneumoniae* [57,58•] and *Listeria monocytogenes* [59] has been confirmed *in vitro*. Moreover, looking at a future application in human therapy, several endolysins have been probed to also be effective *in vivo* using animal models [60].

Notable progress has been made in removing biofilms formed by Gram-negative bacteria, thanks to the study of endolysins with interesting properties. For example, some endolysins are endowed with highly positively charged C-terminal peptides, which can kill bacteria by disrupting the bacterial cytoplasmic membrane [61]; additionally, thermostable endolysins with broad antimicrobial activity, in combination with weak acids, could be useful as an antimicrobial product to control important pathogens such as *Salmonella* or *P. aeruginosa* [62].

Interestingly, some phage lytic proteins seem to exert an effect beyond their lytic activity, as sub-inhibitory concentration downregulated genes, encoding different proteins with autolytic activity, which result in diminished biofilm formation [63]. Indeed, complementary to biofilm removal, phage-derived lysins can be useful for the inhibition of biofilm development, especially on surgical implants and other medical equipment to avoid bacterial colonization. Manufacturing antimicrobial surfaces coated with endolysins and matrix-degrading enzymes is possible to obtain new materials, for example, bioengineered spider silk intended for drug-free sutures for reducing post-implantation infections [64]. All this evidence has led to propose phage lysins as novel antimicrobials to be used in the clinic, and as disinfectants for application in various branches of the economy such as the food industry [65].

Tail-associated proteins with exopolysaccharide degrading activity

Some phages also encode tail-associated proteins with exopolysaccharide degrading activity (depolymerases), and are able to digest polysaccharide forming capsules, lipopolysaccharide, or extracellular polymeric material from the biofilm matrix, conferring the phage with the ability of biofilm invasion and dispersion and, therefore, with potential to be used for biofilm removal (Figure 1b) [39,47]. The data concerning the biofilm dispersion mediated by these proteins is still scarce. Several enzymes with endosialidase, hydrolase, or lyase activity have been identified, mostly in phages infecting Gram-negative bacteria, and only a few in those infecting Gram-positive bacteria [66]. Recombinant depolymerase-targeting capsules have been shown to significantly inhibit biofilm formation and degrade mature biofilm from important pathogens such as *K. pneumoniae* [7•,67] or *S. aureus* [68]. The O-specific polysaccharide lyase cleaving B-band LPS of *P. aeruginosa* was proved to effectively reduce the biofilm mass by targeting both bacterial cells and OMVs embedded in the matrix milieu [69]. Another interesting enzyme (hyaluronidase) is encoded by streptococcal prophages to degrade capsular hyaluronic acid and to reduce biofilm matrix viscosity. Moreover, lysogenic streptococci utilize these prophage-encoded enzymes as a virulence factor, digesting the main component of the tissue extracellular matrix [70].

Similar to endolysins, the antibiofilm activity of these proteins could be enhanced when used in combination with antibiotics, which may represent a promising strategy to combat infections caused by drug-resistant and biofilm-forming pathogens. By degrading the bacterial capsule, depolymerases exhibit an anti-virulent mode of action enhancing the innate immune response [71]. Moreover, a reduction of *in vivo* virulence, along with a significant decrease in the levels of proinflammatory cytokines mediated by treatment with depolymerase enzymes, has been demonstrated in animal models of bacterial infection [72]. Remarkably, in some reports, the exposure of bacterial cultures to phage depolymerase does not promote the development of bacteria refractory to these activities [73••].

Other phage-derived enzymes

Phage derived DNases are usually associated with prophages or prophage-like elements and represent a close evolutionary relationship between bacterial hosts and temperate phages. Streptococcal prophage-encoded DNAses, considered as important virulence factors, are utilized by bacteria to degrade neutrophil extracellular traps and escape the innate immune response [74]. Moreover, these proteins seem to be involved in streptococcal biofilm EPS dispersion, as well as removing biofilms of competitive commensal bacteria inhabiting the same ecological niche (Figure 1b).

Lipases are among other useful enzymes to disperse biofilms by disrupting the lipidic bounds involved in cell-cell or cell-surface interaction. There is little information about the existence of lipid hydrolysis activity in phages, and in fact, lipases represent rare domains found within phage structural components. Nevertheless, a Lipase_GDSL_3 domain has been found in the depolymerases of Cellulophaga phages [39]. Moreover, SGNH hydrolases were found in conserved domains of *Phietalike* viruses within the Siphoviridae family. Although enzymes containing this domain have little sequence homology to true lipases, they are involved in the hydrolysis of fatty acids and aromatic esters [75].

Mycobacteria have a specific cell wall structure with the mycomembrane composed of a mycolyl-arabinogalactan-peptidoglycan complex (mAGP). Therefore mycobacteriophages are provided with two types of cell wall hydrolytic enzymes, LysA (PG hydrolase, discussed above as endolysin) and LysB (mycolylarabinogalactan esterase). The latter is essential for the disruption of mycomembrane by cleaving the ester bond between the arabinogalactan and mycolic acids in the mAGP. LysB is structurally recognised as esterase, cutinase, or lipase and is able to degrade a wide panel of different fatty acids (C4–C16) [76]. The LysB, as lipolytic enzymes, showed antibacterial activity when combined with outer membrane permeabilizers [77].

Overall, the identification of antibiofilm properties of phage-derived proteins requires the use of rapid, reproducible, and accurate technology for screening and comparing their effectiveness. A method based on impedance measurement, using xCelligence real-time cell analyzer (RTCA) equipment [78], and a quartz tuning fork (QTF) system as impedance sensors [79••], were validated to monitor biofilm formation and disruption in different bacterial species.

Combined treatment

Generally, the susceptibility of biofilms to phages and phage-borne enzymes is dependent on biofilm complexity, strain sensitivity, and the biofilm maturation stage. As a consequence, the complete removal of total biomass (adhered cells plus extracellular material) is difficult. Therefore, biofilm eradication might be achieved using a combined treatment as it has been shown that phages and enzymes can be effectively used in cocktails, with antibiotics, and other antimicrobial chemicals [7•,12,80]

The application of phages/lysins with antibiotics, simultaneously or sequentially, has been revealed as particularly effective against biofilms. Indeed, antibiotics can kill the rapidly growing cells (young biofilms) whereas lysins also target stationary phase cells (old biofilms). Synergism occurs because phage-associated bacterial lysis releases the nutrients reactivating the metabolic activity of the growth-arrested cells, that further become sensitive to antibiotics. Lysis also causes a dispersion of the EPS, enhancing the diffusion of the antibiotic to the inner matrix layers, whereas the oxygen availability increases the drug activity [8]. In some cases, phage-resistant cells might be more susceptible to antibiotics [9]. In turn, phages can infect a drug-resistant population, overcoming one of the major limitations of common therapy.

The combined use of phages and matrix dispersing agents is also shown to be very effective. Polysaccharide-degrading enzymes or DNases, whatever the origin, can efficiently break down the biofilm matrix, enhancing the penetration of phages and chemicals, particularly in dense biofilms. Thus the possible application of phage-borne enzymes which are active against EPS components might significantly improve the antibiofilm efficiency of both antibiotics and phages. The use of mechanical debridement improves phage infection as a consequence of better phage and drug accessibility to the biofilm cells (Figure 1b). Moreover, cells released due to debridement also become more susceptible to the treatment. This approach, followed by phage application, has been successfully used in the treatment of chronic wounds [10].

Final considerations and future perspectives

The complex structure of biofilm, encompassing a broad biodiversity of inhabitants, specific niches, cell-to-cell interactions, oxygen, and nutrients at different concentrations, as well as a panel of extracellular macromolecule-stabilizing biofilm conglomerate, present us with a huge challenge in developing effective tools for its eradication. To design improved phage-based methods to remove biofilms, it is important to deepen knowledge in the specific characteristics of biofilms that may play a role in the penetration, diffusion, and propagation of phages through the biofilm. For example, penetration of mixed biofilms is largely dependent on the specific bacterium or combination of bacteria [81] and on phage morphology [82]. Additionally, bacteria-phage dynamics in biofilms promotes the coexistence of phage-susceptible and phage-resistant bacteria due to the susceptible cell cluster protection by the phage-resistant bacteria layer [11••].

Therefore, it seems reasonable to aim to use phages (natural bacterial partners) to learn what kind of weapon or strategies have been evolutionarily developed to successfully infect and invade bacterial populations, especially those hidden and protected by the biofilm structure (Figure 1a). Phages are natural toolboxes offering an arsenal of phage-borne enzymes that can be better exploited as biofilm dispersing agents (Figure 1b). On the other hand, phages harbour genetic determinants that interfere with the host physiology, which could also be explored to target sessile and dormant or persistent cells. Furthermore, we may gear bacterial viruses, both natural or engineered, for efficient infection, bacterial lysis, or physiology modification.

With the modern tools and technologies provided by synthetic biology, and widely available high-throughput sequence techniques, we can look for potential genes and natural systems to design modified, and more effective, antibiofilm agents and complex therapies.

However, we have to keep in mind the possible limitations of future antibiofilm approaches. It is inevitable that bacteria put under selective pressure will find a way to escape novel antibacterials and will develop new types of resistance mechanisms. Nevertheless, evolution has taught us that a constant arms race is an indispensable element of development and progress, in all kinds of aspects of life. The biofilm biology itself has been not fully elucidated yet, hence, the mechanism of intercellular interactions and complex phage-bacteria interplay needs to be deeply explored and understood to ensure the successful progress of the future antibiofilm therapies.

Authors statement

All authors equally contributed to the preparation, creation and presentation of the published work.

Conflict of interest statement

Nothing declared.

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