Trends in Microbiology

CellPress
REVIEWS

Review

Big Impact of the Tiny: Bacteriophage–Bacteria Interactions in Biofilms

Mads Frederik Hansen,[1] Sine Lo Svenningsen,[2] Henriette Lyng Røder,[1] Mathias Middelboe,[3,*] and Mette Burmølle[1,*]

Bacteriophages (phages) have been shaping bacterial ecology and evolution for millions of years, for example, by selecting for defence strategies. Evidence supports that bacterial biofilm formation is one such strategy and that biofilm-mediated protection against phage infection depends on maturation and composition of the extracellular matrix. Interestingly, studies have revealed that phages can induce and strengthen biofilms. Here we review interactions between bacteria and phages in biofilms, discuss the underlying mechanisms, the potential of phage therapy for biofilm control, and emphasize the importance of considering biofilms in future phage research. This is especially relevant as biofilms are associated with increased tolerance towards antibiotics and are implicated in the majority of chronic infections.

Highlights

Biofilm maturation and composition is crucial for phage susceptibility of bacteria.

The therapeutic use of phages and their enzymes is a promising supplement to conventional treatment strategies of bacterial infections.

Both prophages and free phages can promote biofilm formation, which may increase protection and diversification of both phages and bacterial hosts.

Future investigations of phage–host dynamics in multispecies consortia are required to elucidate how interspecies interactions and community-intrinsic properties affect phage infections.

The Protective Nature of Biofilms and Significance of Phage Encounters

Bacteriophages (phages) are ubiquitous viruses of bacteria and the most abundant and diverse biological entity on Earth [1,2]. As agents of **horizontal gene transfer (HGT)** (see Glossary) [3,4], and due to the mortality and selection for resistance imposed on the bacterial prey, phages are a driving factor in bacterial diversification and community composition [5–7]. Traditionally phage–bacteria dynamics have been studied in planktonic cultures without consideration of spatial organization and heterogeneity [8]. The realization that bacteria primarily live in structured communities with varying activity has, however, triggered a pronounced interest in addressing interactions between phages and bacteria within such environments. Spatially structured bacterial communities are involved in several aspects of microbial ecology and interactions, ranging from hot spots of elevated metabolic activity [9] and species succession at the microscale on organic particles [10] to assembly of larger and more complex communities termed biofilms. In biofilms cells adhere to a surface or to each other as free-floating aggregates embedded within a matrix, mainly comprised of polysaccharides, lipids, extracellular DNA, and proteins [11,12]. Downstream of adherence, the development of biofilms is hallmarked by characteristic stages, referred to as commitment, early development, maturation, and dispersal (reviewed in [13]). These communities are of special interest as the architecture and matrix provide protection and structural rigidity, and they enable orchestration of collective behaviour [14,15]. In addition, the biofilm-associated cells express traits and activities that are not possible alone or outside of the biofilm, often referred to as emergent properties. These emergent properties include retention of enzymes, long-term cell–cell interactions, and establishment of gradients of nutrients, pH, and oxygen generating microhabitats and a high level of heterogeneity [16].

Biofilms have represented a protective mode of microbial life against harsh environments for millions of years [17], and it appears that biofilms are also a beneficial trait in pathogenesis, as the majority of chronic infections are caused by cells organized into biofilms. Biofilm-encased bacteria exhibit increased tolerance towards antibiotics and have proven difficult to

[1]Section of Microbiology, Department of Biology, University of Copenhagen, Denmark
[2]Section for Biomolecular Sciences, Department of Biology, University of Copenhagen, Denmark
[3]Marine Biological Section, Department of Biology, University of Copenhagen, Denmark

*Correspondence:
mmiddelboe@bio.ku.dk (M. Middelboe) and burmolle@bio.ku.dk (M. Burmølle).

eradicate using conventional treatment strategies [18–20]. While the biofilm environment may also provide some protection against phage infections, encounters between matrix-embedded bacteria and phages have assuredly been frequent throughout evolutionary history. Here, we focus on how biofilm formation contributes to the coexistence between bacterial communities and their viral predators and address the relevance of spatial structure in the context of re-examining phages as **antimicrobial agents**. Finally, research demonstrating that biofilm formation can be induced by phages under certain conditions is highlighted, as this is of great importance for elucidating the dynamics between phages and biofilm-forming bacteria.

Mechanisms Underlying Biofilm-Mediated Protection against Phage Attacks

The underlying mechanisms of coexistence between phages and biofilm communities are challenging to study due to their high complexity, and they therefore remain largely undescribed. In this context, computational simulations are a great tool, due to the ability to study the consequences of changing one parameter at a time. Recently, a biofilm stimulation framework found that the fraction of phage infection simulations resulting in biofilm survival increased when the diffusivity parameter was lowered, implying that phage diffusion within the biofilm is critical for the outcome of phage infection [21]. Another mathematical model predicted that spherical bacterial **microcolonies** could outgrow ongoing phage killing on the microcolony surface, provided that the microcolony had reached a critical size prior to the initial phage encounter [22]. The concept was demonstrated experimentally, and the extent of microcolony growth prior to phage exposure did indeed correlate with the number of microcolonies that survived infection after overnight incubation. Further, half of the surviving microcolonies contained mainly phage-susceptible cells, indicating that mutation to phage resistance did not play a role in their survival. In this model, a biofilm matrix was not explicitly modelled, so phage movement within the microcolony was limited by phage adsorption to the tightly packed bacteria. In keeping with the predicted consequence of a high diffusion rate in the study by Simmons *et al.* [21], a low adsorption rate was predicted to reduce the protective effect of the microcolony structure [22]. Other simulations have also predicted that organization into such spatial refuges leads to spatially separated areas where the bacterial and viral densities, respectively, are increasing. Such separation on the microscale could contribute to sustained coexistence between infective phages and unattainable sensitive bacteria [23].

A recent 2D model has suggested that exopolysaccharides and cell debris can form phage sinks around areas of high bacterial density in spatially structured environments such as biofilms; as phages irreversibly adsorb to these components, they are sequestered from living cells and hence infections are reduced [8] (Figure 1, Key Figure). In the model, high levels of extracellular polymeric substances (EPSs) and large **burst sizes** had the greatest positive influence on the cell density obtained in a spatially structured environment relative to the cell density obtained under otherwise comparable well-mixed conditions (where phage–bacterial dynamics are governed by the law of **mass action**). The nonintuitive observation of increasing burst size leading to higher relative bacterial densities was explained by an increased association with EPS causing a greater loss of the released progeny phages [8].

Biofilm-mediated entrapment of phages was also shown in experimental work *in vitro* [24,25] (Figure 2D), and irreversible phage adsorption was found to be facilitated by the production of outer-membrane vesicles containing phage receptors in both *Escherichia coli* [26] and *Vibrio cholerae* [27]. Additionally, it was revealed that biofilm composition and maturation have a decisive impact on susceptibility towards phage infection. In an *in vitro* model with synthetic sputum medium that promotes *Pseudomonas aeruginosa* aggregates, as found in cystic fibrosis patients, phages were able to prevent aggregate formation when added

Glossary

Amyloid fibres: major extracellular proteinaceous aggregates with high stability and physical robustness.
Antimicrobial agents: agents able to prevent microbial growth or kill microbial organisms.
Autotransduction: a mechanism facilitating horizontal gene transfer. Progeny phage particles released by prophage induction in a fraction of the population can infect other susceptible bacteria and form transducing particles packed with bacterial DNA, not associated with phage genome, and hence introduce novel DNA to the remaining population of lysogens.
Burst size: the number of phage virions released per infected cell.
Community-intrinsic properties: properties emerging in communities which are unpredictable by studying the individual parts in isolation.
Conjugative plasmids: self-transmissible plasmids encoding the components required for conjugation.
Efflux pump: a promiscuous active transporter of various compounds including toxic substrates, for example antibiotics.
Horizontal gene transfer (HGT): transfer of genetic material between organisms, for example, by uptake of free DNA (transformation), mediated by phages (transduction) or mediated by mobilizable plasmids (conjugation).
Lysis–lysogeny decision: the probability of temperate phages to either produce progeny phage particles or establish lysogeny, depending on cues, for example, the level of arbitrium peptides.
Mass action: a well-mixed system in which collisions occur at random.
Microcolonies: cell aggregates confined within a matrix of polymeric substances.
Obligate lytic phage: a phage without the genes required for prophage establishment, which consistently lyses a susceptible host upon infection.
Persister cells: stochastically arising metabolically inactive variants which enable reoccurrence of chronic infections due to high antibiotic tolerance.
Phage therapy: the use of phages for therapeutic applications, for example, to treat bacterial infections in humans.
Prophage: phage genome integrated into a bacterial host chromosome.
Quorum sensing (QS): a system that enables bacteria to monitor population

Key Figure

The Various Possible Outcomes of Phage–Biofilm Encounters

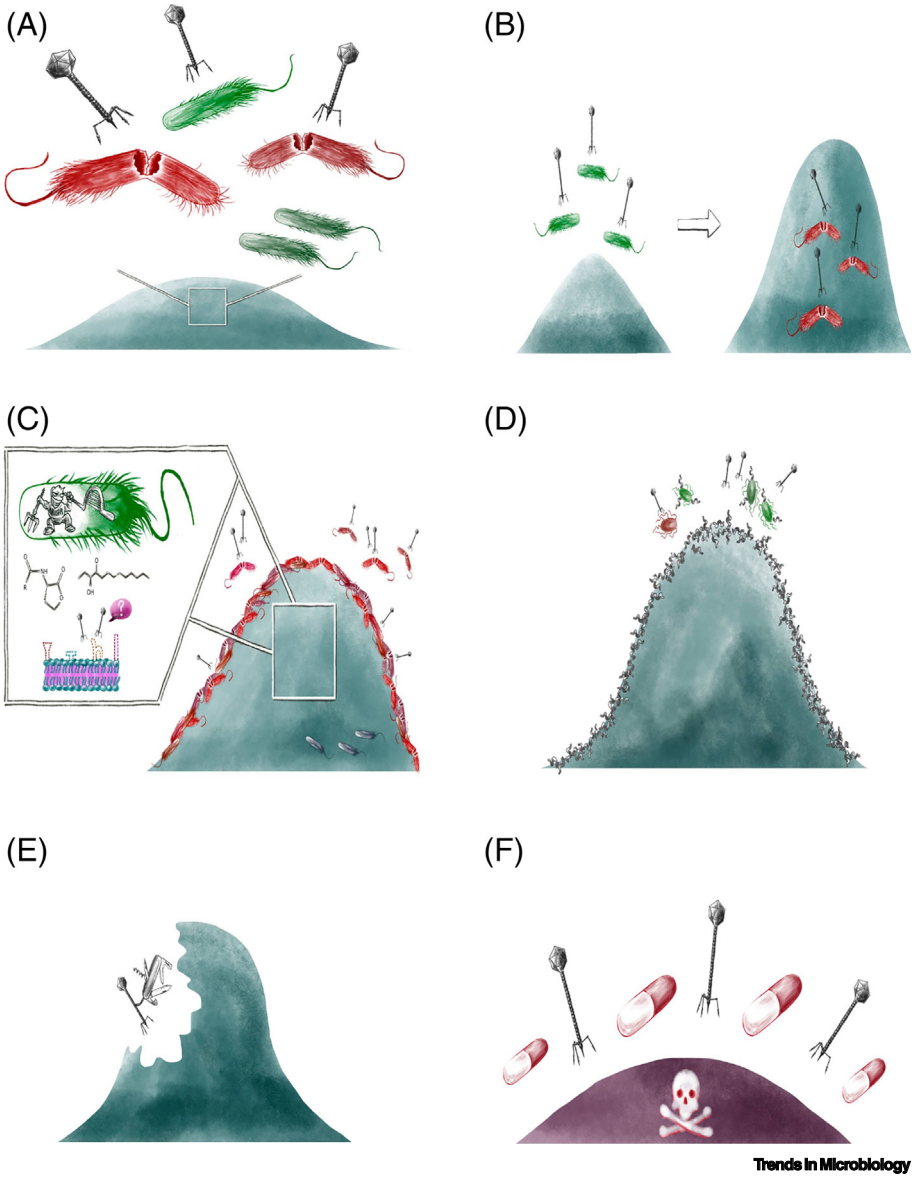

Figure 1. (A) Phages infecting living cells (green) result in an increased number of dead cells (red), which has been exploited therapeutically to reduce biofilm formation [61–66]. (B) Some phages have, however, been shown to induce biofilm formation, for example, by selecting for a mucoid phenotype [79,90] or by releasing matrix components by bacterial cell lysis due to prophage induction [97–99]. (C) The spatial organization of a biofilm community reduces the number of successful infections as phages adsorb to exopolysaccharides or cell debris and multiple phages infect the same host [8]. Also, the emergence of metabolically inactive persister cells (grey) in biofilms delay phage proliferation [35–37]. Cell density affects the bacterial antiphage strategy, and high levels of autoinducers (illustrated as acylhomoserine lactone and *Vibrio cholerae* CAI-1) upregulate the expression of CRISPR-associated genes [42,43] and downregulate the expression of phage

(Figure legend continued at the bottom of the next page.)

density and regulate gene expression accordingly based on the production and detection of specific signal molecules, termed autoinducers.
Synergistic effects: when the outcome is higher than the additive sum of individual effects.
Temperate phages: phages with the ability to integrate into a host chromosome, establish a prophage, and excise, producing progeny phages, at a later timepoint.

simultaneously with a bacterial inoculum. When added postaggregation, phages were able to inhibit dispersal of migrating bacteria and establishment of new aggregates, but they were unable to eradicate well-established aggregates. This protection against phage killing in already established aggregates was demonstrated to be mediated by exopolysaccharides, as mutants unable to produce these were still capable of forming aggregates, but were significantly more susceptible to phage infection [28].

Bacterial appendages, for example, fimbria and pili, are known to promote adherence and biofilm formation, and their expression has been shown to be affected by **prophages** [29] and **conjugative plasmids** [30,31]. Interestingly, *E. coli* carrying a conjugative plasmid encoding F pili did not establish a biofilm when exposed to a nonlytic filamentous phage, f1, known to bind to the tip of these pili. However, 1-day-old biofilms were uninhibited when exposed to f1 phages, which correlated with decreased expression of F pili. Further, the **amyloid fibre** curli was implicated in the failure of phage infection, as mature biofilms produced by a curli-fibre-deficient mutant were more susceptible to phage infection, compared with the wild type or to a curli over-producing strain [32]. Interestingly, Vidakovic *et al*. [33] recently showed that curli-encased planktonic cells were protected during phage exposure. Using an array of single-gene matrix component knockout mutants, they also discovered that expression of curli enabled community-level protection. Curli fibres were identified in the space between cells and covering the outer edge of the community, which enabled greater cell density, prevented phage diffusion, and generated a collective defence with phages retained at the outer periphery (Figure 1). Further, promoter mutations leading to overexpression of biofilm matrix and amyloid fibres resulted in phage protection after 24 h instead of 48–60 h where curli production was initiated in the wild type, emphasizing the temporal and compositional importance of biofilm-mediated protection.

Other factors important for phage infections, for example, coinfection, resource concentration, and gene expression, also suggest other protective contributions mediated by the spatial organization of biofilm communities. The spatial structure leads to localized growth and limited mobility, creating concentrated clusters of cells, where several phages are infecting the same host, resulting in a decreased number of progeny virions [34]. Further, nutrient gradients and a high local concentration of bacteria within a biofilm limit resource availability and is known to lead to dormant **persister cells** [16]. Since all viruses require the transcriptional and translational machinery of a host in order to propagate, phage growth is thereby inhibited in metabolically inactive cells [35,36]. However, coliphage lambda successfully infected persisters and resumed lytic steps upon exiting dormancy and becoming metabolically active again, which means that the process may only be delayed [37].

Bacteria produce, release, and detect extracellular signal molecules known as autoinducers in order to coordinate gene expression according to population density, a phenomenon known as **quorum sensing** (**QS**) [38]. This density-dependent regulation is of high relevance to bacteria–phage dynamics as it enables orchestration of biofilm development [39,40], the evolution of phage resistance [41], and regulation of antiphage strategies, for example, by regulating the expression of CRISPR-associated (*cas*) genes [42,43], phage receptors [44,45],

receptors [44,45]. (D) The production of curli enables protection against phage infection at both individual and community level. In mature biofilms, curli fibres enhance matrix density and are identified at the outer biofilm periphery, retaining phages [33]. (E) Some phages encode depolymerases, enabling them to access and infect otherwise biofilm-sheltered bacteria by degrading matrix components [69,72,73]. (F) Combinations of antibiotics and phage therapy have shown promising, synergistic effects regarding eradication of biofilm [75,76,78,79]. The size ratios between elements in this illustration are not representative of actual dimensions.

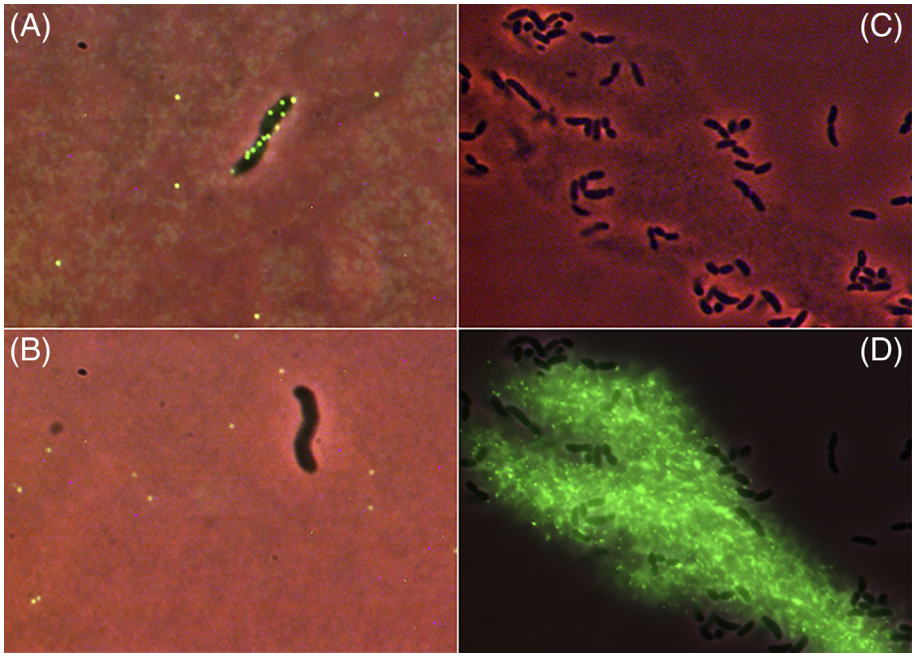

Figure 2. Visualization of Two Different Responses to Phage Exposure in *Vibrio anguillarum* BA35 and PF430-3 Using SYBR Gold-Labelled Phages and Phase-Contrast Epifluorescence Microscopy. (A) Adsorption of phage φH20 is observed in wild-type BA35 cells. (B) Phage adsorption is not observed in BA35 phage lysates, where surviving cells exhibit reduced physiological performance, that is, the ability to utilize fewer substrates, indicating mutational derived resistance. (C) A transit phenotype with reduced phage susceptibility in the PF430-3 lysate, where cells do not exhibit changed substrate pattern, indicates biofilm-mediated tolerance as a response to phage KVP40 exposure (image is without fluorescence). (D) PF430-3 lysate with fluorescence indicates that phages do not adsorb to the cells, but are instead trapped in the matrix, which is supported by the identification of phage-sensitive cells surviving outside the aggregate formation. Modified, with permission, from Tan *et al*. [25].

and phage inactivating proteases [46,47] (Figure 1). Interestingly, a recent study by Silpe and Bassler [48] described how *Vibrio* phage VP882 encodes a QS receptor that enables the phage to inactivate the lytic repressor and enter the lytic cycle in response to high levels of bacterial autoinducers. Thus, this *Vibrio* phage can induce lysis by eavesdropping on bacterial QS. A family of **temperate phages** infecting *Bacillus subtilis* also exploits the bacterial QS system to guide their **lysis–lysogeny decision** [49]. This phage family encodes an autoinducer-like peptide, termed arbitrium, on the phage genome, so that only infected bacteria produce the signal. Accumulation of the arbitrium peptide causes a preference for lysogeny via inhibition of the lysogeny inhibitor AimX. It is therefore expected that the phages will show a preference for lysis at the early stages of infection of a colony or biofilm, while later infection events would increasingly show a lysogenic outcome [49]. These examples of density-dependent phage–bacterium interactions further emphasize the complexity and variable nature of phage–host dynamics during biofilm development.

In addition to autoinducers, bacteria also produce other compounds, for example, secondary metabolites, which were recently shown to inhibit infection by a broad range of phages in Gram-positive *Streptomyces*. Interestingly, these compounds, produced by *Streptomyces*, were found to have antiphage activity towards lambda phage infections in Gram-negative *E. coli* as well, indicating that some bacteria can create a chemical antiphage defence which

can also benefit other members of the community [50]. The protective role of mixed-species communities has also been supported by reduced phage efficiency against *Enterobacter cloacae* when grown in a dual-species biofilm with *Enterobacter agglomerans* [51].

Studies and simulations suggest that organization of spatially structured communities and production of specific matrix components are key factors of coexistence between bacteria and phages and that biofilm formation plays a crucial role in the survival of otherwise phage-sensitive bacteria. However, our understanding relies on a limited number of studies, and we have just recently started to address the importance and potential of coordinated phage behaviour.

Renewed Interest in Phage Therapy for Biofilm Control

Bacteriophages were discovered independently by Frederick Twort and Felix d'Hérelle more than a century ago, and the therapeutic potential of phages as agents for biological control has been studied ever since. The general life cycle and properties of lytic phages suggest several benefits from a therapeutic perspective, that is, considerable specificity, self-propagation at the site of infection, rapid clearance, great diversity, relatively easy isolation for a range of pathogens, and the opportunity to make genetic modifications [52]. Since phages have relatively narrow host ranges and their efficiency depends on the targeted host strain, cocktails of distinct phages are often applied to compensate for laborious identification and simultaneously decrease the risk of the pathogen evading infection [53]. Despite promising results for some applications, **phage therapy** has been overshadowed by the successful development of antibiotics [54–56]. However, modern medicine relies heavily on the ability to treat bacterial infections effectively, and the emergence of antibiotic resistance in pathogenic bacteria is posing a serious threat to human health worldwide [57,58]. Thus, interest has renewed in phage therapy in recent years, and it has been suggested as an alternative therapeutic option [54–56]. The potential of biological control of microbial infections by phage therapy has also shown a broad range of applications with promising results within fields other than human health, for example aquaculture [59] and agriculture [60].

The traditional strategy for phage therapy has been to target pathogenic bacteria with **obligate lytic phages**, and the lytic potential of phage cocktails has been exploited to reduce both monospecies [61–64] and dual-species [65,66] biofilms. As described above, phage infections are, however, often restricted by the biofilm matrix, which means that complete eradication of biofilms by phage therapy has proven challenging. Lu and Collins [67] managed to engineer a T7 phage to encode a matrix-degrading depolymerase and exploited the lytic potential and enzymatic activity to markedly increase the efficiency of phage-mediated eradication of bacterial cells as well as biofilm matrix.

Phages frequently evolve counter-strategies in order to circumvent bacterial defences [68], and some naturally encode similar depolymerases enabling degradation of polymers and thereby weaken the physical barriers of the biofilm matrix and the capsular polysaccharides in order to gain access to cell membranes and receptors (Figure 1). The use of such enzymes in therapeutic contexts is further supported by the fact that the primary function of many of these enzymes is to target the cell wall and compromise bacterial cells [69]. Olsen *et al.* [70] recently found that the polysaccharide depolymerase DA7 and the purified endolysin LysK are both potent antibiofilm agents for a wide range of *Staphylococcus aureus* strains under both static and flow growth conditions, and in combination the two enzymes showed **synergistic effects** on biofilm eradication. Phage cocktails comprised of phages with depolymerase activity may also enhance access, and, under the right conditions, augment the activity of other phages, leading to synergistic removal of pathogens [71]. Other studies with depolymerase-encoding phages and purification of their

enzymes support their use as promising agents for different applications, including antibiofilm control (reviewed in [72,73]).

Phages and Other Agents in a Multitargeted Therapeutic Approach

The establishment, development, and mature properties of biofilms are highly dependent on the bacterial species involved [11]. Therefore, the increased tolerance towards antibiotics and phage predation in biofilm varies widely and depends on the rather complex nature of the community, that is, spatial organization, cell–cell interactions, and the nature of the matrix components. Thus, biofilm elimination has been suggested to require a broader approach, targeting multiple targets simultaneously, utilizing agents with different modes of action [74]. Combinations of phage therapy and conventional antibiotic treatment have shown promising results [75,76] and so have combinations of phage therapy with some disinfectants [51,77]. Recently, studies in *Pseudomonas aeruginosa* have highlighted some of the advantages, as attempted treatment of *P. aeruginosa* biofilms with phages or different antibiotics only showed restricted success. In contrast, combinations of these treatments resulted in markedly synergistic effects, and in some cases the efficiency was increased by phage pretreatment [78,79] (Figure 1). This was suggested to be mediated by phage removal of peripheral cells, leading to increased resource availability for the remaining cells residing in the biofilm and therefore fewer metabolically inactive cells, which are recalcitrant to antibiotic treatment [78]. Another hypothesis suggests that the efficiency of phages is highest before antibiotic treatment, as a larger population size better supports phage multiplication [79]. Further, Chaudhry *et al.* [78] found a reduced development of antibiotic resistance when combining the therapeutic agents. Applying a combinational approach using different targets of inhibitory action may thus introduce an additional advantage as the risk of resistance development is reduced due to the fitness cost associated with resistance against multiple factors [80]. This include phages targeting **efflux pump** receptor sites, where phage resistance modifications have been shown to increase the sensitivity towards several classes of antibiotic [81].

The protective and persistent nature of biofilm formation causes severe therapeutic difficulties. However, re-examination of the lytic potential and diversity of phages and their enzymes is cause for optimism, creating encouraging future prospects. Although phage therapy is not necessarily destined to replace conventional treatment strategies, it may provide a future supplement or alternative when required in the battle against persistent infections.

Phage Exposure as a Trigger of Increased Biofilm Formation

Despite the increasing number of studies describing reduced biofilm formation and enhanced eradication efficiency for treatment strategies involving phages, there are also examples of the opposite.

A study of phage predation in *E. coli* found that it led to increased aggregation, surface adhesion, and production of fimbria, which in turn increased the tolerance toward subsequent phage attacks as penetration of phages through the biofilm was inhibited [82]. Interestingly, repeated treatments with a broad host range *P. aeruginosa* phage cocktail showed lower efficiency in biofilm eradication than single treatments, as the increased phage pressure lead to larger aggregates [79] (Figure 1).

Hosseinidoust *et al.* [83] raised the concern that phage exposure could lead to an unintended induction of biofilm formation in therapeutic settings and tested respective phages towards three well known bacterial pathogens: *P. aeruginosa, Salmonella enterica* Typhimurium, and *S. aureus*. Their results revealed that exposure to some phages stimulated biofilm formation, emphasizing that this response was restricted to some specific phages. The specificity of phage–

host interactions was also evident, when Tan *et al*. [25] found that phages φH20 and KVP40 affected biofilm formation differentially in the fish pathogen *Vibrio anguillarum*. φH20 efficiently inhibited and disrupted biofilm formation in *V. anguillarum* BA35, while KVP40 exposure promoted aggregation and biofilm formation, which trapped the phages in *V. anguillarum* PF430-3 (Figure 2).

The intraspecies variation in phage-defence mechanisms, including differences in the role of biofilms for protection against phage infection, is speculated to be dependent on which receptors are targeted [25,47,83]. The antagonistic coexistence between phages and their hosts consistently results in a rebound of resistant bacterial subpopulations through a wide range of bacterial antiviral strategies, for example, modifications of specific receptors [84,85]. Some receptors have essential roles for cell function, nutrient acquisition, or niche specialization, and modifications can be associated with a permanent cost affecting the ability to utilize various organic compounds [25,86] and lowering of the growth rate [65,87,88]. Other defence mechanisms (e.g., CRISPR-Cas immunity) are inducible and hence only linked to a fitness cost upon phage infection. The prevalence between fixed and inducible strategies depends on resource availability and phage density [89], and it has been suggested that increased biofilm formation could offer a beneficial and flexible strategy for phage protection under conditions where specific phage-targeted receptors are required for maintaining key host functions and other strategies are too costly or inefficient [45,47]. In support of this, Scanlan and Buckling [90] found that *Pseudomonas fluorescens* consistently evolved a mucoid phenotype with costly overproduction of alginate only under nutrient-replete conditions with daily replacement of nutrients by dilution in shaken cultures with constant exposure to phages. A slow-growing, alginate-overproducing mucoid phenotype surviving phage treatment was also identified in *P. aeruginosa* biofilms, where sequencing disclosed that mutations in *mucA*, the negative regulator of alginate production, were present in all mucoid isolates [79]. Another aspect to consider in this context is the strain-specific variation in susceptibility to specific phages, which means that the required density of phages needed in order to lyse a host is relative and varies between strains. Consequently, the selective pressure driving the development of antiphage strategies varies between strains. A recent global transcriptomic analysis of an *S. aureus* biofilm found that low-level phage predation triggered the stringent response, leading to changed bacterial morphology, accumulation of extracellular DNA in the matrix, and production of a thicker biofilm. The same trend was observed in another strain, tolerating a higher phage concentration when exposed to the same phage, but did however require an increase in the phage concentrations, implying that this response requires some degree of successful infection [91]. The induction of a protective biofilm environment during stressful conditions is a common bacterial strategy, and induction of biofilm formation is well known from experiments with subinhibitory concentrations of antibiotics [92,93].

Another perspective is that the phage in some scenarios may benefit from increased biofilm formation, so that the stimulation of biofilm formation by phages can be viewed as an evolutionary adaptation of the phages. The interactions between phages and their hosts are increasingly being classified as mutualistic (Box 1), and entrapment of phages in the biofilm matrix has been suggested to provide protection against hostile environmental factors, for example, radiation, for the phage as well as for the neighbouring bacteria [24]. This view is supported by experiments showing that phages encapsulated in biofilm matrix can cope with higher concentrations of disinfectants than their unprotected counterparts [77] and that phage genomes are shielded from environmental stresses (UV, pH, and temperature) in a pseudolysogenic manner in endospores [94].

Our current knowledge regarding the processes mediating increased biofilm in response to phage exposure is limited, and the ecological, clinical, and evolutionary implications are still speculative. So is the question regarding whether this mechanism and subsequent entrapment of

phages is mutualistic or even beneficial for any of the parts involved (see Outstanding Questions). Nonetheless, it is important to emphasize that experiments conducted under biofilm-related settings are required before applying phages for therapeutic purposes, as even pronounced lytic candidates might trigger an unintended and undesired bacterial response.

Prophage Induction Releases Matrix Components

Phages are increasingly being considered as potential mutualists rather than obligate parasites, which especially has been due to recognition of the benefits obtained by bacteria by harbouring prophages (Box 1). Counterintuitively, prophage induction leading to cell death can also benefit host population fitness, for example, by enabling **autotransduction** [95] or releasing intracellular compounds such as enzymes, toxins, or biofilm components [96]. The latter has been shown to affect matrix composition and enhance biofilm formation, especially through accumulation of extracellular DNA [97–99]. By use of DNase enzyme treatment and microscopy analyses, it was demonstrated that extracellular DNA is a fundamental matrix component with a structural role for establishment of biofilms [100–102]. The release of DNA has also been shown to stabilize biofilm formation in a QS-dependent manner in *Streptococcus mutants* [103]. Prophage knockout mutants revealed that three prophages collectively contributed to greater biofilm formation by the release of DNA in *Shewanella oneidensis* [98]. Similarly, a prophage-cured *Actinomyces odontolyticus* produced less biofilm than the wild type, and treatment with DNase reduced biofilm formation only in the latter [99]. Interestingly, Turnbull *et al*. [104] found that subpopulation cell lysis could be governed by a cryptic prophage endolysin, leading to the release of components, including DNA, which was required for the assembly of membrane vesicles and proper biofilm development.

The Importance of Inoviridae in *P. aeruginosa* Biofilms

Although the interactions between *P. aeruginosa* and Pf-like prophages are distinct from the aforementioned, they have still contributed to the understanding and acknowledgement of phages as important for biofilm formation. Pf-like phages are filamentous, have single-stranded DNA genomes, and belong to the *Inovirus* genus; they normally do not kill their host upon excision, as progeny virions are released by secretion and not bacterial lysis [105]. Interestingly,

Box 1. From a Parasitic to a Mutualistic Relationship

The continuous arms race between phages and bacteria has supported the traditional view of viruses as obligate parasites that exploit their host for own gain. This conservative perspective is, however, currently challenged, as the number of mutualistic interactions identified between viruses and a various range of hosts, that is, insects, plants, fungi, animals, and bacteria, are increasing (reviewed in [121]). Temperate phages are characterized by encoding modules, which enable lysogenization, that is, integration of the genome into the host chromosome. This provides protection of the phage inside the cell with the potential to enter a lytic stage and kill the host. However, it also provides genetic information to the host, which may contribute to bacterial niche partitioning and dissemination of beneficial phenotypic characteristics by HGT [3,4], for example, through transfer and integration of auxiliary metabolic genes [122]. Once integrated, the phage is termed prophage, and if it is not providing a fitness advantage, accumulation of mutations will likely render it defective, due to the supplementary biosynthetic burden. Notably, prophages are frequently associated with superinfection exclusion or pathogenicity, as virulence factors such as toxins, enzymes, or superantigens can be encoded by prophages [123–125]. One of the classic examples is the causative agent of cholera, *V. cholerae*, in which the potent cholera toxin (enterotoxin) is encoded by the filamentous phage CTXφ [126]. A recent sequence database analysis additionally revealed that prophage-encoded virulence and antibiotic resistance is widely distributed within this bacterial genus, ranging from clinical isolates to strains from deep subseafloor sediments [127].

Prophages can be integrated at specific bacterial attachment sites (*attB*) matching identical phage attachment sites (*attP*) mediated by recombinases, or they can exhibit a random positioning as known from the widely distributed Mu-like prophages [128]. In either case, the location sometimes disrupts and inactivates bacterial genes. As these genes can reassemble once the phage is excised, prophages can function as regulatory switches at specific cues and provide a temporal regulation of different bacterial processes [129]. This process has been shown to enable escape from phagosomes of otherwise trapped and doomed *Listeria monocytogenes*, as excision of prophage A118 was fundamental for reconstitution of the competence system necessary for escape [130].

however, a filamentous Pf1 prophage was found entering a superinfective form, leading to cell death in mature *P. aeruginosa* biofilms, which was shown to be essential for the biofilm development and structure (Figure 3). In mutants lacking the common phage receptors, type IV pili and flagella, prophages were induced but this did not result in cell death, indicating that cell death was due to secondary infections. This was verified as exogenous addition of purified superinfective phages restored wild-type-like biofilm formation and cell death in a prophage knockout mutant. The cell death caused by these phages occurred only in mature biofilms and correlated with accumulation of reactive oxygen species (ROS) within the centre of microcolonies and generated hollow voids, enabling dispersal of a resistant subpopulation [106]. DNA damage was later verified as triggering this coordinated induction of Pf-like prophages [107]. This phenomenon is frequently observed for many prophages, as their lytic repressors share the same Ala-Gly restriction site as the SOS repressor, LexA, which is cleaved by the coprotease RecA once DNA damage is sensed [108,109].

This centralized cell death as a result of phage activity and subsequent dispersal of surviving cells was found in five out of five clinical cystic fibrosis isolates, and the generation of phenotypic variants in the dispersed population was reflected in the phage titres [110]. Rice *et al*. [111] also demonstrated that filamentous Pf-like prophages are fundamental for the biofilm development process in *P. aeruginosa* as a Pf4 prophage knockout mutant did not undergo coordinated cell death or generate hollow voids, as the wild type did. Further, mice challenged with the prophage knockout outlived wild-type infected mice, indicating that the prophage significantly contributed to virulence. Biofilm formation by a prophage knockout strain showed decreased tolerance toward sodium dodecyl sulphate, and since few genes related to virulence could be identified on the prophage genome, virulence was suggested to depend on prophage-mediated biofilm development, compositional change of the matrix, and the formation of phenotypic variants. In support of the latter suggestion, the small colony variants associated with the surviving, dispersed sub-

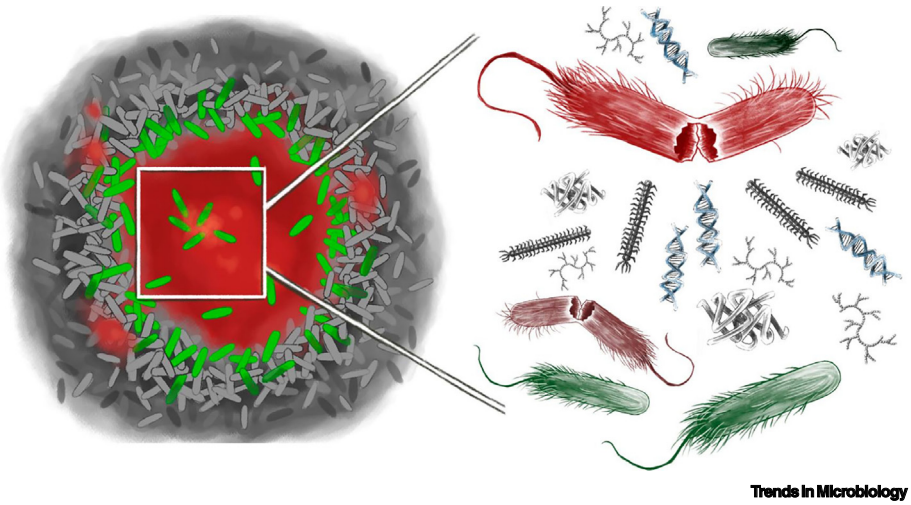

Figure 3. Superinfective Filamentous Phages in Biofilm Development and Function. Prophages of otherwise nonlytic filamentous Pf-like phages are found entering a superinfective form when induced in mature biofilms of *Pseudomonas aeruginosa* [106]. Induction stimulates biofilm formation by releasing matrix components, for example, polysaccharides, proteins, and especially extracellular DNA, from compromised cells (red) [111]. Simultaneously, the phage activity creates hollow voids from where the surviving subpopulation (green) can disperse [106]. The filamentous phages also strengthen the biofilm by promoting liquid crystalline organization and by functioning as matrix components themselves, resulting in increased viscosity and tolerance towards antibiotics and desiccation [113]. The size ratios between elements in this illustration are not representative of actual dimensions.

population released large amounts of the Pf-like phages [112,113] and, additionally, surface adherence and antibiotic tolerance was enhanced [114]. The long negatively charged filamentous virions were found to enhance biofilm functionality by interacting with matrix polymers, promoting liquid crystalline organization, increasing viscosity, and augmenting tolerance towards desiccation and antibiotics [113].

Our understanding of the temporal development of matrix structure, the components and their chemical and physical properties, is still not complete [12]. Biofilm formation serves many purposes and confers several advantages, and as emphasized above, in their interaction with phages, biofilms do not exclusively serve as a defence against viral predators. Phages have the potential to strengthen matrix composition and are increasingly acknowledged as important for biofilm development and properties. The impact they have on cell death and phenotypic variance, and their function as matrix components has, in some cases, proven pivotal for virulence and persistence and might also shelter the phages themselves (see Outstanding Questions).

Concluding Remarks

As pointed out by Clokie *et al.* [1], this is really an exciting time to be a phage biologist, and especially since recent advances within the rising field of 'omics' have revealed otherwise unimagined bacterial responses to phage exposure, that is, altered replication, expression, metabolism, and biosynthesis (reviewed in [115]). These phenotypic adaptions mediated by phages are increasingly classified as mutualistic, benefitting the host as well as the phage (Box 1). Although phage–biofilm encounters have been ubiquitous throughout history in vast and diverse environments, the majority of the research has been conducted in liquid cultures, and hence coevolution between phages and sessile matrix-embedded hosts is understudied. Our understanding of phage nature, predation, and importance for genetic and functional diversity is also limited to a fraction of phage families due to methodological biases [116]. We do, however, hypothesize that biofilm formation may benefit the phage by means other than protection against environmental stressors: phage-mediated accumulation of matrix compounds, that is, DNA accommodated with the high cell density and natural competence in biofilms, grants a hotspot for HGT [117], which could prove a key factor for the diversification and general mosaic structure of phage genomes [2] and hence accelerate evolution of phages (see Outstanding Questions).

A common factor of many of the aspects discussed above is that our experience is mainly based on experiments conducted in relatively simple monospecies communities. While studying phage–host interactions in biofilms is challenging in itself due to their microscale environmental and biological heterogeneity, the complexity is additionally increased when including interspecies interactions. Nevertheless, such systems are of great relevance [118], and even relatively low-abundance species can affect the spatial organization, stability, and composition of biofilms [119] and potentially promote **community-intrinsic properties** [120]. Thus, to elucidate the natural dynamics between phages and bacteria, further experiments and understanding of these bacterial interspecies interactions and their impact on the interplay between phages and bacteria in biofilms are required. Addressing these aspects will also enlighten the actual potential of phage therapy and might improve the prospect of successfully targeting and eradicating infectious biofilms.

Outstanding Questions

Can phage-mediated promotion of biofilms be classified as a mutualistic action, benefitting both phage and host?

What is the impact of coordinated phage behaviour on bacterial evolution and biofilm development?

What is the role of quorum sensing on the regulation of phage–host interactions in biofilms?

Are some phages specialized in provoking increased biofilm formation, and which underlying nonmutational and mutational mechanisms are driving this response?

Does biofilm formation function as a hotspot for phage diversification by increasing competence, having elevated rates of prophage induction and retaining phage DNA in the matrix?

How do interspecies interactions affect phage sensitivity, and are they relevant for the potential of phage therapy?

Acknowledgments

We thank artist Camilla Gunborg Pedersen for her drawings presented in the key figure and Figure 3. This work was supported by the Villum Foundation, Young Investigator Programme (ref. no. 10098) to M.B., the BONUS FLAVOPHAGE project (BONUS Art 185) funded jointly by EU and Innovation Fund Denmark to M.M., The Danish Council for Independent Research (DFF – 7014-00080) to M.M., and support from the Danish National Research Foundation (DNRF120) to S.L.S.

References

1. Clokie, M.R. *et al.* (2011) Phages in nature. *Bacteriophage* 1, 31–45
2. Hatfull, G.F. and Hendrix, R.W. (2011) Bacteriophages and their genomes. *Curr. Opin. Virol.* 1, 298–303
3. Frost, L.S. *et al.* (2005) Mobile genetic elements: the agents of open source evolution. *Nat. Rev. Microbiol.* 3, 722–732
4. Penadés, J.R. *et al.* (2015) Bacteriophage-mediated spread of bacterial virulence genes. *Curr. Opin. Microbiol.* 23, 171–178
5. Martiny, J.B.H. *et al.* (2014) Antagonistic coevolution of marine planktonic viruses and their hosts. *Annu. Rev. Mar. Sci.* 6, 393–414
6. Suttle, C.A. (2007) Marine viruses – major players in the global ecosystem. *Nat. Rev. Microbiol.* 5, 801–812
7. Koskella, B. and Brockhurst, M.A. (2014) Bacteria–phage coevolution as a driver of ecological and evolutionary processes in microbial communities. *FEMS Microbiol. Rev.* 38, 916–931
8. Bull, J. *et al.* (2018) Phage–bacterial dynamics with spatial structure: self organization around phage sinks can promote increased cell densities. *Antibiotics* 7, 8
9. Carreira, C. *et al.* (2013) Heterogeneous distribution of prokaryotes and viruses at the microscale in a tidal sediment. *Aquat. Microb. Ecol.* 69, 183–192
10. Datta, M.S. *et al.* (2016) Microbial interactions lead to rapid micro-scale successions on model marine particles. *Nat. Commun.* 7, 1–7
11. Hobley, L. *et al.* (2015) Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. *FEMS Microbiol. Rev.* 39, 649–669
12. Seviour, T. *et al.* (2018) Extracellular polymeric substances of biofilms: suffering from an identity crisis. *Water Res.* 151, 1–7
13. Stoodley, P. *et al.* (2002) Biofilms as complex differentiated communities. *Annu. Rev. Microbiol.* 56, 187–209
14. Morris, C.E. and Monier, J.-M. (2003) The ecological significance of biofilm formation by plant-associated bacteria. *Annu. Rev. Phytopathol.* 41, 429–453
15. Dragoš, A. and Kovács, Á.T. (2017) The peculiar functions of the bacterial extracellular matrix. *Trends Microbiol.* 25, 257–266
16. Flemming, H.C. *et al.* (2016) Biofilms: an emergent form of bacterial life. *Nat. Rev. Microbiol.* 14, 563–575
17. Hall-Stoodley, L. *et al.* (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* 2, 95–108
18. Costerton, J.W. *et al.* (1999) Bacterial biofilms: a common cause of persistent infections. *Science* 284, 1318–1322
19. Stewart, P.S. and Costerton, J.W. (2001) Antibiotic resistance of bacteria in biofilms. *Lancet* 358, 135–138
20. Burmølle, M. *et al.* (2010) Biofilms in chronic infections – a matter of opportunity – monospecies biofilms in multispecies infections. *FEMS Immunol. Med. Microbiol.* 59, 324–336
21. Simmons, M. *et al.* (2018) Phage mobility is a core determinant of phage–bacteria coexistence in biofilms. *ISME J.* 12, 532–543
22. Eriksen, R.S. *et al.* (2018) A growing microcolony can survive and support persistent propagation of virulent phages. *Proc. Natl. Acad. Sci. U. S. A.* 115, 337–342
23. Heilmann, S. *et al.* (2012) Coexistence of phage and bacteria on the boundary of self-organized refuges. *Proc. Natl. Acad. Sci. U. S. A.* 109, 12828–12833
24. Briandet, R. *et al.* (2008) Fluorescence correlation spectroscopy to study diffusion and reaction of bacteriophages inside biofilms. *Appl. Environ. Microbiol.* 74, 2135–2143
25. Tan, D. *et al.* (2015) Vibriophages differentially influence biofilm formation by *Vibrio anguillarum* strains. *Appl. Environ. Microbiol.* 81, 4489–4497
26. Manning, A.J. and Kuehn, M.J. (2011) Contribution of bacterial outer membrane vesicles to innate bacterial defense. *BMC Microbiol.* 11, 258
27. Reyes-Robles, T. *et al.* (2018) *Vibrio cholerae* outer membrane vesicles inhibit bacteriophage infection. *J. Bacteriol.* 200, JB.00792-17
28. Darch, S.E. *et al.* (2017) Phage inhibit pathogen dissemination by targeting bacterial migrants in a chronic infection model. *mBio* 8, 1–15
29. Chung, I.-Y. *et al.* (2014) A phage protein that inhibits the bacterial ATPase required for type IV pilus assembly. *Proc. Natl. Acad. Sci. U. S. A.* 111, 11503–11508
30. Burmølle, M. *et al.* (2008) Type 3 fimbriae, encoded by the conjugative plasmid pOLA52, enhance biofilm formation and transfer frequencies in Enterobacteriaceae strains. *Microbiology* 154, 187–195
31. Madsen, J.S. *et al.* (2016) Type 3 fimbriae encoded on plasmids are expressed from a unique promoter without affecting host motility, facilitating an exceptional phenotype that enhances conjugal plasmid transfer. *PLoS One* 11, e0162390
32. May, T. *et al.* (2011) Exposure of conjugative plasmid carrying *Escherichia coli* biofilms to male-specific bacteriophages. *ISME J.* 5, 771–775
33. Vidakovic, L. *et al.* (2018) Dynamic biofilm architecture confers individual and collective mechanisms of viral protection. *Nat. Microbiol.* 3, 26–31
34. Taylor, B.P. *et al.* (2017) Emergence of increased frequency and severity of multiple infections by viruses due to spatial clustering of hosts. *Phys. Biol.* 13, 066014
35. Łoś, M. *et al.* (2007) Effective inhibition of lytic development of bacteriophages λ, P1 and T4 by starvation of their host, *Escherichia coli*. *BMC Biotechnol.* 7, 1–6
36. Cerca, N. *et al.* (2007) Susceptibility of *Staphylococcus epidermidis* planktonic cells and biofilms to the lytic action of staphylococcus bacteriophage K. *Lett. Appl. Microbiol.* 45, 313–317
37. Pearl, S. *et al.* (2008) Nongenetic individuality in the host-phage interaction. *PLoS Biol.* 6, e120
38. Ng, W.-L. and Bassler, B.L. (2009) Bacterial quorum-sensing network architectures. *Annu. Rev. Genet.* 43, 197–222
39. Parsek, M.R. and Greenberg, E.P. (2005) Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends Microbiol.* 13, 27–33
40. Srivastava, D. and Waters, C.M. (2012) A tangled web: regulatory connections between quorum sensing and cyclic di-GMP. *J. Bacteriol.* 194, 4485–4493
41. Moreau, P. *et al.* (2017) Bacterial cell-to-cell signaling promotes the evolution of resistance to parasitic bacteriophages. *Ecol. Evol.* 7, 1936–1941
42. Patterson, A.G. *et al.* (2016) Quorum sensing controls adaptive immunity through the regulation of multiple CRISPR-Cas systems. *Mol. Cell* 64, 1102–1108
43. Høyland-Kroghsbo, N.M. *et al.* (2017) Quorum sensing controls the *Pseudomonas aeruginosa* CRISPR-Cas adaptive immune system. *Proc. Natl. Acad. Sci. U. S. A.* 114, 131–135
44. Høyland-Kroghsbo, N.M. *et al.* (2013) A quorum-sensing-induced bacteriophage defense mechanism. *mBio* 4, 1–8
45. Tan, D. *et al.* (2015) Quorum sensing determines the choice of antiphage defense strategy in *Vibrio anguillarum*. *mBio* 6, e00627
46. Hoque, M.M. *et al.* (2016) Quorum regulated resistance of *Vibrio cholerae* against environmental bacteriophages. *Sci. Rep.* 6, 37956
47. Castillo, D. *et al.* (2019) Phage defense mechanisms and their genomic and phenotypic implications in the fish pathogen *Vibrio anguillarum*. *FEMS Microbiol. Ecol.* 95, fiz004
48. Silpe, J.E. and Bassler, B.L. (2019) A host-produced quorum-sensing autoinducer controls a phage lysis-lysogeny decision. *Cell* 176, 268–280.e13
49. Erez, Z. *et al.* (2017) Communication between viruses guides lysis–lysogeny decisions. *Nature* 541, 488–493
50. Kronheim, S. *et al.* (2018) A chemical defence against phage infection. *Nature* 564, 283–286
51. Tait, K. *et al.* (2002) The efficacy of bacteriophage as a method of biofilm eradication. *Biofouling* 18, 305–311
52. Nobrega, F.L. *et al.* (2015) Revisiting phage therapy: new applications for old resources. *Trends Microbiol.* 23, 185–191
53. Chan, B.K. *et al.* (2013) Phage cocktails and the future of phage therapy. *Future Microbiol* 8, 769–783
54. Sulakvelidze, A. *et al.* (2001) Bacteriophage therapy. *Antimicrob. Agents Chemother.* 45, 649–659

55. Abedon, S.T. *et al.* (2011) Phage treatment of human infections. *Bacteriophage* 1, 66–85
56. Moelling, K. *et al.* (2018) A wake-up call: we need phage therapy now. *Viruses* 10, 688
57. Davies, J. and Davies, D. (2010) Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev.* 74, 417–433
58. Forslund, K. *et al.* (2013) Country-specific antibiotic use practices impact the human gut resistome. *Genome* 23, 1163–1169
59. Rørbo, N. *et al.* (2018) Exploring the effect of phage therapy in preventing *Vibrio anguillarum* infections in cod and turbot larvae. *Antibiotics* 7, 42
60. Carstens, A. *et al.* (2018) Unlocking the potential of 46 new bacteriophages for biocontrol of *Dickeya solani*. *Viruses* 10, 621
61. Curtin, J.J. and Donlan, R.M. (2006) Using bacteriophages to reduce formation of catheter-associated biofilms by *Staphylococcus epidermidis*. *Antimicrob. Agents Chemother.* 50, 1268–1275
62. Fu, W. *et al.* (2010) Bacteriophage cocktail for the prevention of biofilm formation by *Pseudomonas aeruginosa* on catheters in an *in vitro* model system. *Antimicrob. Agents Chemother.* 54, 397–404
63. Alves, D.R. *et al.* (2016) A novel bacteriophage cocktail reduces and disperses *Pseudomonas aeruginosa* biofilms under static and flow conditions. *Microb. Biotechnol.* 9, 61–74
64. Melo, L.D.R. *et al.* (2016) Development of a phage cocktail to control *Proteus mirabilis* catheter-associated urinary tract infections. *Front. Microbiol.* 7, 1024
65. Gutiérrez, D. *et al.* (2015) Two phages, phiIPLA-RODI and phiIPLA-C1C, lyse mono- and dual-species staphylococcal biofilms. *Appl. Environ. Microbiol.* 81, 3336–3348
66. Lehman, S.M. and Donlan, R.M. (2015) Bacteriophage-mediated control of a two-species biofilm formed by microorganisms causing catheter-associated urinary tract infections in an *in vitro* urinary catheter model. *Antimicrob. Agents Chemother.* 59, 1127–1137
67. Lu, T.K. and Collins, J.J. (2007) Dispersing biofilms with engineered enzymatic bacteriophage. *Proc. Natl. Acad. Sci. U. S. A.* 104, 11197–11202
68. Samson, J.E. *et al.* (2013) Revenge of the phages: defeating bacterial defences. *Nat. Rev. Microbiol.* 11, 675–687
69. Harper, D. *et al.* (2014) Bacteriophages and biofilms. *Antibiotics* 3, 270–284
70. Olsen, N.M.C. *et al.* (2018) Synergistic removal of static and dynamic *Staphylococcus aureus* biofilms by combined treatment with a bacteriophage endolysin and a polysaccharide depolymerase. *Viruses* 10, 1–17
71. Schmerer, M. *et al.* (2014) Synergy as a rationale for phage therapy using phage cocktails. *PeerJ* 2, e590
72. Chan, B. and Abedon, S. (2015) Bacteriophages and their enzymes in biofilm control. *Curr. Pharm. Des.* 21, 85–99
73. Pires, D.P. *et al.* (2016) Bacteriophage-encoded depolymerases: their diversity and biotechnological applications. *Appl. Microbiol. Biotechnol.* 100, 2141–2151
74. Koo, H. *et al.* (2017) Targeting microbial biofilms: current and prospective therapeutic strategies. *Nat. Rev. Microbiol.* 15, 740–755
75. Bedi, M.S. *et al.* (2009) Amoxicillin and specific bacteriophage can be used together for eradication of biofilm of *Klebsiella pneumoniae* B5055. *World J. Microbiol. Biotechnol.* 25, 1145–1151
76. Rahman, M. *et al.* (2011) Characterization of induced *Staphylococcus aureus* bacteriophage SAP-26 and its anti-biofilm activity with rifampicin. *Biofouling* 27, 1087–1093
77. Agún, S. *et al.* (2018) Study of the interactions between bacteriophage phiIPLA-RODI and four chemical disinfectants for the elimination of *Staphylococcus aureus* contamination. *Viruses* 10, E103
78. Chaudhry, W.N. *et al.* (2017) Synergy and order effects of antibiotics and phages in killing *Pseudomonas aeruginosa* biofilms. *PLoS One* 12, 1–16
79. Henriksen, K. *et al.* (2019) *P. aeruginosa* flow-cell biofilms are enhanced by repeated phage treatments but can be eradicated by phage-ciprofloxacin combination. *Pathog. Dis.* 77, ftz011
80. Torres-Barceló, C. and Hochberg, M.E. (2016) Evolutionary rationale for phages as complements of antibiotics. *Trends Microbiol.* 24, 249–256
81. Chan, B.K. *et al.* (2016) Phage selection restores antibiotic sensitivity in MDR *Pseudomonas aeruginosa*. *Sci. Rep.* 6, 26717
82. Lacqua, A. *et al.* (2006) Emergence of biofilm-forming subpopulations upon exposure of *Escherichia coli* to environmental bacteriophages. *Appl. Environ. Microbiol.* 72, 956–959
83. Hosseinidoust, Z. *et al.* (2013) Formation of biofilms under phage predation: considerations concerning a biofilm increase. *Biofouling* 29, 457–468
84. Dy, R.L. *et al.* (2014) Remarkable mechanisms in microbes to resist phage infections. *Annu. Rev. Virol.* 1, 307–331
85. Seed, K.D. (2015) Battling phages: how bacteria defend against viral attack. *PLoS Pathog.* 11, 1–5
86. Middelboe, M. *et al.* (2009) Bacteriophages drive strain diversification in a marine *Flavobacterium*: implications for phage resistance and physiological properties. *Environ. Microbiol.* 11, 1971–1982
87. Koskella, B. *et al.* (2012) The costs of evolving resistance in heterogeneous parasite environments. *Proc. Biol. Sci.* 279, 1896–1903
88. Hall, A.R. *et al.* (2012) Effects of sequential and simultaneous applications of bacteriophages on populations of *Pseudomonas aeruginosa in vitro* and in wax moth larvae. *Appl. Environ. Microbiol.* 78, 5646–5652
89. Westra, E.R. *et al.* (2015) Parasite exposure drives selective evolution of constitutive versus inducible defense. *Curr. Biol.* 25, 1043–1049
90. Scanlan, P.D. and Buckling, A. (2012) Co-evolution with lytic phage selects for the mucoid phenotype of *Pseudomonas fluorescens* SBW25. *ISME J.* 6, 1148–1158
91. Fernández, L. *et al.* (2017) Low-level predation by lytic phage phiIPLA-RODI promotes biofilm formation and triggers the stringent response in *Staphylococcus aureus*. *Sci. Rep.* 7, 1–14
92. Andersson, D.I. and Hughes, D. (2014) Microbiological effects of sublethal levels of antibiotics. *Nat. Rev. Microbiol.* 12, 465–478
93. Leiker, K. and Weitao, T. (2016) The SOS response of biofilms. *Int. J. Clin. Med. Microbiol.* 1, 1–7
94. Gabiatti, N. *et al.* (2018) Bacterial endospores as phage genome carriers and protective shells. *Appl. Environ. Microbiol.* 84, 1–12
95. Haaber, J. *et al.* (2016) Bacterial viruses enable their host to acquire antibiotic resistance genes from neighbouring cells. *Nat. Commun.* 7, 13333
96. Nanda, A.M. *et al.* (2015) Impact of spontaneous prophage induction on the fitness of bacterial populations and host–microbe interactions. *J. Bacteriol.* 197, 410–419
97. Carrolo, M. *et al.* (2010) Prophage spontaneous activation promotes DNA release enhancing biofilm formation in *Streptococcus pneumoniae*. *PLoS One* 5, e15678
98. Gödeke, J. *et al.* (2011) Phage-induced lysis enhances biofilm formation in *Shewanella oneidensis* MR-1. *ISME J.* 5, 613–626
99. Shen, M. *et al.* (2018) A linear plasmid-like prophage of *Actinomyces odontolyticus* promotes biofilm assembly. *Appl. Environ. Microbiol.* 84, e01263-18
100. Whitchurch, C.B. *et al.* (2002) Extracellular DNA required for bacterial biofilm formation. *Science* 295, 1487
101. Harmsen, M. *et al.* (2010) Role of extracellular DNA during biofilm formation by *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 76, 2271–2279
102. Lawrence, J.R. *et al.* (2003) Scanning transmission X-ray, laser scanning and transmission electron microscopy mapping of the exopolymeric matrix of microbiol biofilms. *Appl. Environ. Microbiol.* 69, 5543–5554
103. Petersen, F.C. *et al.* (2005) DNA binding-uptake system: a link between cell-to-cell communication and biofilm formation. *J. Bacteriol.* 187, 4392–4400
104. Turnbull, L. *et al.* (2016) Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat. Commun.* 7, 11220

105. Rakonjac, J. *et al.* (2011) Filamentous bacteriophage: biology, phage display and nanotechnology applications. *Curr. Issues Mol. Biol.* 13, 51–76
106. Webb, J.S. *et al.* (2003) Cell death in *Pseudomonas aeruginosa* biofilm development. *J. Bacteriol.* 185, 4585–4592
107. Hui, J.G.K. *et al.* (2014) Environmental cues and genes involved in establishment of the superinfective Pf4 phage of *Pseudomonas aeruginosa*. *Front. Microbiol.* 5, 654
108. Little, J.W. (1991) Mechanism of specific LexA cleavage: autodigestion and the role of RecA coprotease. *Biochimie* 73, 411–421
109. Quinones, M. *et al.* (2005) LexA cleavage is required for CTX prophage induction. *Mol. Cell* 17, 291–300
110. Kirov, S.M. *et al.* (2007) Biofilm differentiation and dispersal in mucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *Microbiology* 153, 3264–3274
111. Rice, S.A. *et al.* (2009) The biofilm life cycle and virulence of *Pseudomonas aeruginosa* are dependent on a filamentous prophage. *ISME J.* 3, 271–282
112. Webb, J.S. *et al.* (2004) Bacteriophage and phenotypic variation in *Pseudomonas aeruginosa* biofilm development. *J. Bacteriol.* 186, 8066–8073
113. Secor, P.R. *et al.* (2015) Filamentous bacteriophage promote biofilm assembly and function. *Cell Host Microbe* 18, 549–559
114. Häussler, S. (2004) Biofilm formation by the small colony variant phenotype of *Pseudomonas aeruginosa*. *Environ. Microbiol.* 6, 546–551
115. Fernández, L. *et al.* (2018) Phage or foe: an insight into the impact of viral predation on microbial communities. *ISME J.* 12, 1171–1179
116. Kauffman, K.M. *et al.* (2018) A major lineage of non-tailed dsDNA viruses as unrecognized killers of marine bacteria. *Nature* 554, 118–122
117. Madsen, J.S. *et al.* (2012) The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunol. Med. Microbiol.* 65, 183–195
118. Burmølle, M. *et al.* (2014) Interactions in multispecies biofilms: do they actually matter? *Trends Microbiol.* 22, 84–91
119. Liu, W. *et al.* (2017) Low-abundant species facilitates specific spatial organization that promotes multispecies biofilm formation. *Environ. Microbiol.* 19, 2893–2905
120. Madsen, J.S. *et al.* (2018) Bacterial social interactions and the emergence of community-intrinsic properties. *Curr. Opin. Microbiol.* 42, 104–109
121. Roossinck, M.J. (2011) The good viruses: viral mutualistic symbioses. *Nat. Rev. Microbiol.* 9, 99–108
122. Breitbart, M. (2012) Marine viruses: truth or dare. *Annu. Rev. Mar. Sci.* 4, 425–448
123. Boyd, E.F. and Brüssow, H. (2002) Common themes among bacteriophage-encoded virulence factors and diversity among the bacteriophages involved. *Trends Microbiol.* 10, 521–529
124. Bondy-Denomy, J. and Davidson, A.R. (2014) When a virus is not a parasite: the beneficial effects of prophages on bacterial fitness. *J. Microbiol.* 52, 235–242
125. Bondy-Denomy, J. *et al.* (2016) Prophages mediate defense against phage infection through diverse mechanisms. *ISME J.* 10, 2854–2866
126. Waldor, M.K. and Mekalanos, J.J. (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* 272, 1910–1914
127. Castillo, D. *et al.* (2018) Widespread distribution of prophage-encoded virulence factors in marine *Vibrio* communities. *Sci. Rep.* 8, 9973
128. Morgan, G.J. *et al.* (2002) Bacteriophage Mu genome sequence: analysis and comparison with Mu-like prophages in *Haemophilus*, *Neisseria* and *Deinococcus*. *J. Mol. Biol.* 317, 337–359
129. Feiner, R. *et al.* (2015) A new perspective on lysogeny: prophages as active regulatory switches of bacteria. *Nat. Rev. Microbiol.* 13, 641–650
130. Rabinovich, L. *et al.* (2012) Prophage excision activates listeria competence genes that promote phagosomal escape and virulence. *Cell* 150, 792–802