Bacterial multicellular behavior in antiviral defense

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Highlights

- Bacterial multicellular behavior plays an important role in predatory interactions
- Small molecules involved in signalling or chemical defense contribute to phage defense
- Membrane vesicles can act as phage decoys
- Biofilm matrix is an important barrier against phages

Abstract

Multicellular behavior benefits seemingly simple organisms such as bacteria, by improving nutrient uptake, resistance to stresses or by providing advantages in predatory interactions. Several recent studies have shown that this also extends to the defense against bacteriophages, which are omnipresent in almost all habitats. In this review, we summarize strategies conferring protection against phage infection at the multicellular level, covering secretion of small antiphage molecules or membrane vesicles, the role of quorum sensing in phage defense, the development of transient phage resistance and the impact of biofilm components and architecture. Recent studies focusing on these topics push the boundaries of our understanding of the bacterial immune system and set the ground for an appreciation of bacterial multicellular behavior in antiviral defense.

Introduction

"*All for one and one for all, united we stand divided we fall.*" Alexandre Dumas, The Three Musketeers

Viruses infecting bacteria, so-called bacteriophages, represent the most abundant predator on this planet, shaping life in almost all ecosystems. The ongoing 'arms race' between phages and bacteria has led to the evolution of diverse antiphage strategies collectively referred to as the bacterial 'immune system' [1,2]. Classical examples are restriction-modification (RM), CRISPR-Cas and abortive infection encoded by a large fraction of bacterial genomes. Currently, we are experiencing an unprecedented expansion of our understanding of bacterial antiviral immunity driven by the identification of numerous new antiphage systems – several of which are hinting towards a prokaryotic origin of human cell-autonomous innate immune mechanisms [3,4]. These ground-breaking discoveries were made possible by the finding that antiviral systems often co-localize in so-called 'defense islands' in prokaryotic genomes and by intensive screenings based on functional selection [5-8].

Effective antiviral defense of a single bacterial cell also protects the entire population, as it stops the spread of infection and prevents the release of new phages. Most of the systems described recently

function at the intracellular level by targeting phage nucleic acids or by triggering death of the infected cell. However, several studies reported on bacterial antiphage strategies, which are shared by cells in a community and therefore can potentially be considered as antiviral public goods.

In natural environments, microbial communities typically show a high order of organization and emergent properties of bacterial multicellularity. The advantages of multicellular behavior are numerous and include the improved acquisition of nutrients, resistance to physical stresses or antimicrobial molecules, and the protection from predators [9]. In this short review, we will move towards the appreciation of bacterial multicellular behavior in antiviral defense. These mechanisms include the production and secretion of antiphage small molecules, membrane vesicles, quorum sensing-based activation of defense systems, the development of transient phage resistance and the impact of biofilm architecture.

Quorum sensing

The concept of quorum sensing (QS) is at the heart of bacterial multicellularity as it defines the ability of bacterial populations to make group decisions. This communication between cells is mediated via the production and recognition of small molecules, so-called autoinducers, which can affect bacterial behavior associated with virulence, biofilm formation, horizontal gene transfer, and bioluminescence [10,11]. Several studies clearly show that QS also has an effect on the susceptibility of bacteria towards phage infection and on the coordination of phage defense strategies.

In presence of the autoinducers AHL, CAI-1 or AI-2, phage adsorption was shown to be reduced for *Escherichia coli* phages λ and χ, as well as different *Vibrio* phages by downregulation of the respective receptor genes [12-14]. Communication via QS also led to the inactivation of phages via the production of hemagglutinin protease in *Vibrio cholerae* [12]. QS was further shown to affect the adaptive immunity of CRISPR-Cas by activating *cas* gene expression in *Pseudomonas aeruginosa* and *Serratia* [15,16]. Moreover, QS peptides triggered abortive infection in *E. coli* through the *mazEF* toxin-antitoxin module, which was shown to inhibit the spread of phage P1 [17]. Besides this direct impact on phage defense, the influence of QS on phage susceptibility can also be indirect via the downregulation of metabolic activity affecting phage infection [18,19].

Considering the diverse effects of QS on bacterial antiviral defense, it is not surprising that phages also eavesdrop on the communication of their host to optimize their infection strategy. These anti-defense mechanisms include for example receptors binding the host autoinducer and thereby derepressing lysis genes [11,20,21]. Further, *Pseudomonas* phage DMS3 was shown to directly inhibit LasR, the master regulator of quorum sensing, by expressing a small anti-activator protein as counter-defense strategy [22].

Chemical defense

Besides the exchange of information, small molecules produced and secreted by bacteria can also have themselves antiphage properties as recently described for compounds belonging to the classes of anthracyclines and aminoglycosides. Environmental bacteria, especially members of the genus of *Streptomyces*, are prolific producers of bioactive compounds, which are known to provide important fitness advantages in competitive, cooperative as well as predatory interactions [23]. Inhibition of phage infection by bacterial small molecules received first attention in the 1950s and 1960s with a special emphasis on the identification of antiphage molecules applicable in agricultural and medical sectors as summarized in a recent review article [24]. It is, however, striking that their potential role as part of the antiviral immune system of bacteria remained a major blind spot.

Recently, DNA-intercalating molecules belonging to the class of anthracyclines were shown to inhibit infection of several dsDNA phages infecting *Streptomyces coelicolor, E. coli* or *Pseudomonas aeruginosa* [25]. Anthracyclines are naturally produced by *Streptomyces* and are among the most efficient anticancer agents used in clinics [26]. Mechanistically, these compounds were proposed to interfere with phage infection at an early step of the phage life cycle, namely between DNA injection and replication. However, a direct in vivo interaction between the compounds and phage DNA was not shown, yet [25].

A second class of antibiotics, which recently gained interest in the context of phage defense are aminoglycosides [27]. Like anthracyclines, aminoglycosides are mainly produced by *Streptomyces*. These bactericidal, polycationic antibiotics act by targeting the 16S rRNA of the 30S ribosomal subunit, thereby interfering with bacterial protein translation [28]. Inhibition of phages by these compounds was observed for disparate dsDNA phages infecting Gram-positive and Gram-negative bacterial hosts.

It is striking that several – if not most – of the previously described antiphage compounds produced by bacteria have antibacterial properties, too. In nature, producers of antimicrobial molecules typically express a sophisticated set of self-resistance mechanisms [29,30]. This needs to be considered to allow the appreciation of potential antiviral effects of the respective molecule. First insights gained for aminoglycosides suggest that the molecular targets for inhibition of bacteria and phages are distinct. Acetylation of the aminoglycoside antibiotic apramycin abolished the antibacterial activity of the compound, but did not affect its antiphage properties [27].

The ecological relevance of chemically-mediated antiphage defense by small molecules is supported by the inhibitory effect of culture supernatants of natural producer strains secreting the molecules [25,27]. Accordingly, with their excretion into the environment and their broad-spectrum activity, these secondary metabolites – dependent on locally achieved concentrations – could provide a chemical defense against phages at the community level by creating an antiviral milieu. However, resistance to the antibacterial effect of the molecule(s) is prerequisite to be able to benefit from the antiviral properties of the respective molecule. This could select for mutualistic communities thriving by a division of labor between kin cells or even different species and would exclude random competitors without the respective resistance [31].

Apart from a direct interference of bacterial small molecules with phage infection, bacteriostatic protein translation inhibitors can also provide protection against phage infection by increasing the efficiency of CRIPSR-Cas immunity. This can be achieved either by decelerating phage reproduction, which extends the time for the acquisition of adaptive CRISPR immunity [32], or by interfering with the production of phage-encoded anti-defense proteins [33].

Development of transiently resistant phenotypes

Recent studies revealed the development of transient resistance as an important factor involved in the containment of phage infection, especially in structured environments. For *Bacillus subtilis* it was shown that neighboring cells respond to phage infection by D-alanylation of their cell wall-teichoic acids, thereby rendering their peptidoglycan layer resistant to phage attachment enabling active growth and constriction of plaque size [34]. Also in *Streptomyces*, cellular development of transiently phage resistant mycelium was shown to be important for the containment of viral infections. *S. venezuelae* mutants restricted to vegetative growth showed larger plaques and no constriction of plaque size[35]. These studies highlight the importance of cell envelope modifications and cellular development to counteract phage spread. Remarkably, it was now shown for several species that complete cell wall shedding represents a ubiquitous strategy to endure phage attack in osmoprotective environments [36,37].

Considering the role of cellular development for the containment of infections, it is likely to assume that phages fight back by manipulating cellular development for their own purposes. At early stages of phage Alderaan infection, transcriptome analysis of *Streptomyces* cells at the plaque interface revealed a downregulation of genes involved in cell development [35]. Further, the excision of *B. subtilis* prophage SPβ was show to reconstitute the polysaccharide synthesis gene *spsM*, thereby contributing to spore dispersal [38].

Membrane Vesicles

Another protective shield has been shown to be provided by the secretion of membrane vesicles (MVs), which are generated by all living cells. As ubiquitous components of the extracellular space and integral parts of biofilms, membrane vesicles affect intercellular interaction in manifold ways including DNA transfer, metabolite export, virulence and cell-cell communication. Although the most extensively studied membrane vesicles are derived from the outer membrane of Gram-negative bacteria, there are different routes and triggers of membrane vesicle formation in both Gram-negative and -positive cells [39,40].

Several recent studies revealed that MVs may protect from viral predation by acting as phage decoys leading to adsorption of phages and resulting in less productive phage infections of the population [41-45]. Phage-induced lysis of bacterial cells is supposed to contribute extensively to the formation of membrane vesicles in nature [46]. This raises the possibility that membrane vesicles may serve as a defense mechanism by transporting signaling molecules such as quorum sensing molecules in a concentrated manner, as has been suggested for *Paracoccus denitrificans* [47,48]. This could help

bacteria communicate and coordinate their defense against phages more effectively. Further research is needed to understand the exact role of membrane vesicles in bacterial antiviral defense.

Biofilms

Among the huge body of studies that have addressed the interaction of phages with their hosts, most of the data – in particular studies on molecular mechanisms underlying the phage-host tug-of-war – stems from planktonic cultures, where diffusing phages have more or less free access to their prey cells. However, in most environments the majority of bacteria exists in biofilms, where the bacteria are physically associated with each other in a self-produced matrix engulfing the community [49-51]. This results in a number of inherent properties of the biofilm community that drastically increases the tolerance of the bacterial populations against all kinds of environmental stresses. It has been observed that, most times, the inherent recalcitrance of cells in biofilms also extends to phage predation.

Intuitively, a local accumulation of potential prey cells may seem beneficial for phage predation, however, the prey clustering is likely to result in increased co-infections causing a drop in phage propagation per particle [52]. In addition, all biofilm communities are characterized by a pronounced metabolic stratification due to different access to metabolites including oxygen. Thus, many cells within the community exhibit a reduced metabolic activity up to the point of dormancy [53]. Although there are some phages that are able to infect and lyse dormant cells, many if not most phages do not proliferate efficiently at low metabolic activity of the host cells [54-57]. Such dormant, dead or phage-resistant cells may still efficiently bind phage particles and thus serve as potent phage absorbers or a shield that protect their susceptible counterparts [58-64].

Another important factor that governs phage-biofilm interactions is the biofilm matrix, which is encasing the bacterial cells and commonly consists of various polymeric substances such as exopolysaccharides, various proteins, nucleic acids and lipids [50]. Intuitively and as predicted by a biofilm simulation network [65], the interaction of phages and bacteria depends on the ability of the rather large phage particles to diffuse through the biofilm and, thus, to a significant part on the interaction of phages with the matrix. Correspondingly, several studies demonstrate that the biofilm matrix limits phage diffusion and viral predation of the cells [66-70]. An exopolysaccharide named stewartan showed concentration-dependent limiting of phage diffusion, unless the phages were decorated with corresponding depolymerases to counteract this defense [71]. Another study showed that active phages are captured by extracellular proteinaceous assemblages referred to as curli. Notably, the tight binding of phages by these structures implicates that matrix components may have evolved to efficiently absorb specific intruding phages [72]. Phages can thus be efficiently retained in the biofilm, and it has been shown that this not only prevents the embedded cells from phage contact

and infection but may also turn the captured phages into a protective barrier against other susceptible invading or evading bacteria [72-74].

Taken together, cells in biofilms are generally more recalcitrant towards phage assaults. This can mainly be attributed to the biofilm structure and the resulting spatial and metabolic stratification, which in concert limit access to the prey cells and successful phage proliferation. The studies implicate that, as a whole, a biofilm community is a reservoir of a multitude of phage-host interactions, which we are only beginning to understand [75,76].

Conclusions and future perspectives

The recent discovery of numerous new systems impressively demonstrates the gaps in our understanding of the bacterial immune system [1,3]. In this short review, we summarize strategies conferring protection at the multicellular level by the secretion of small molecules, membrane vesicles or via components of the biofilm matrix. In the past, antiviral defense has been mainly studied at the level of isolated systems. It is, however, the interaction between different lines of defense, which ultimately shapes the immune system – a notion which is well accepted for antiviral immunity in eukaryotes, but still very underdeveloped for the prokaryotic world. Technological advances now enable the spatiotemporal analysis of the interaction and complementation of different antiviral mechanisms providing unprecedented insights into their interaction and interdependencies within bacterial species [77,78].

The majority of microbial interactions take place in spatially structured environments. Consequently, several factors, like the physiological status of neighbouring cells and the structure and individual components of the biofilm, have important implications for the range of microbial interactions. Several recent studies reveal the predominance of short-range interactions in densely packed bacterial communities and the impact of system architecture and cell permeability on spatial scales [79,80]. Consequently, these factors likely also shape the dimensions of antiviral defense provided through the secretion of small molecules or membrane vesicles. The spatiotemporal analysis of antiviral defense at the multicellular level will also allow to study the response of bacterial populations to viral infection and represents a powerful approach for the identification of new multicellular strategies involved in the communication between cells and the establishment of complex interaction networks through genetic and/or phenotypic diversification [81,82].

These recent examples provide first insights into the multiple dimensions of bacterial multicellular behavior and its relevance for antiviral defense. Combining molecular mechanistic, evolutionary, and ecological approaches is now essential for a comprehensive understanding of the ecological relevance of these systems in the context of microbial interaction.

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Annotations

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This study combines simulations and in vivo work to show how phage-resistant cells can protect clusters of susceptible cells, which promotes the (co)existance of susceptible cells in the presence of phages.

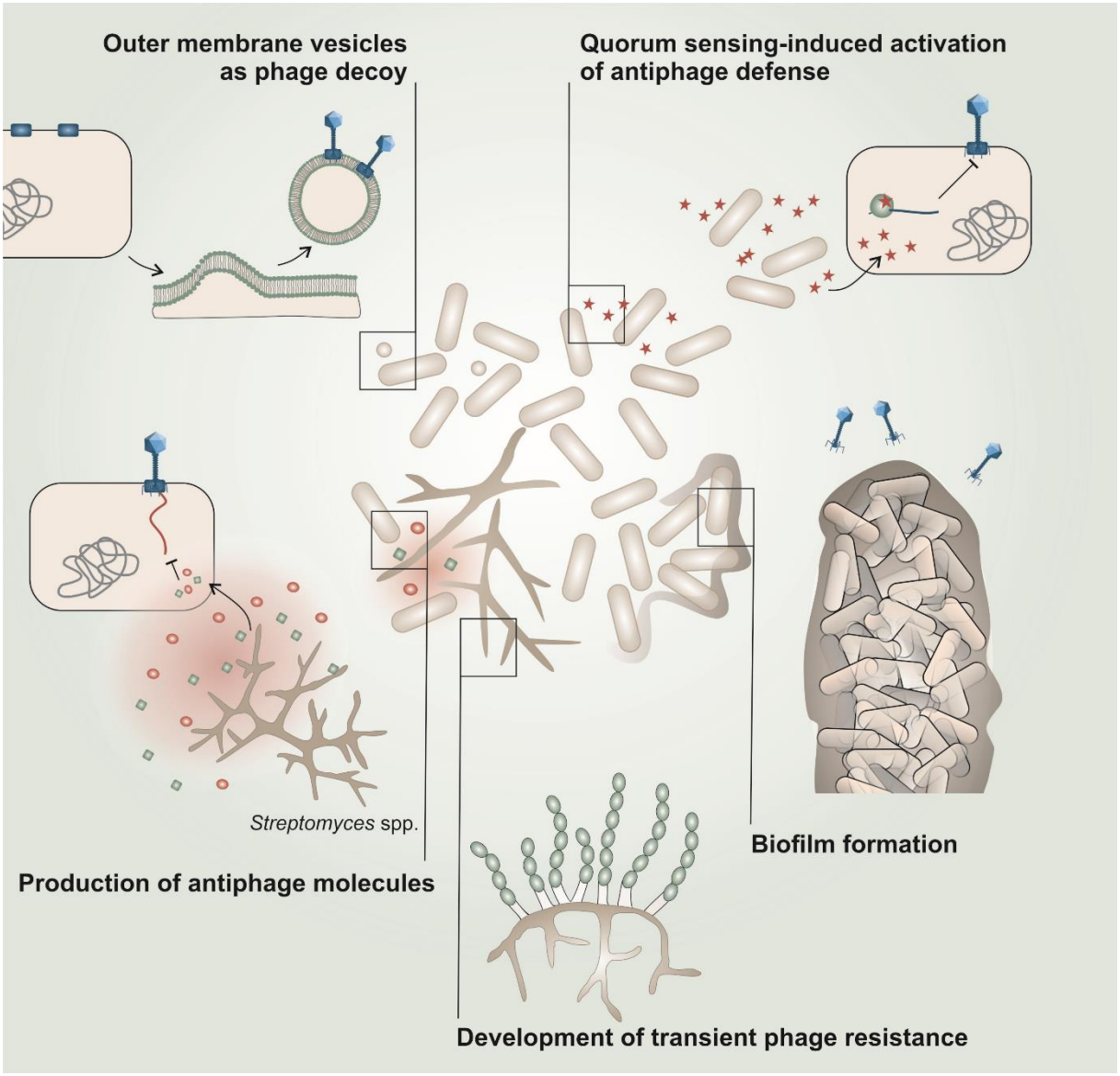

Figure 1: Bacterial multicellular strategies in antiviral defense. Protection against phages on a multicellular level can be mediated by i) extrusion of outer membrane vesicles sequestering phages, which prevents attachment to susceptible cells. ii) Quorum sensing-mediated antiphage defense systems rely on autoinducers (red stars) for transcriptional activation of RM-, CRISPR-, Abi- and other systems. iii) Biofilm formation and trapping of phages via interaction with components of the extracellular matrix reduces diffusivity of phage particles and propagation upon infection. iv) Secretion of antiphage molecules (e.g. anthracyclines or aminoglycosides) confers a chemical defense by inhibiting an early step in the infection cycle. v) Cellular development allows for the emergence of transient phage resistance due to reduced susceptibility of distinct developmental stages.