Antiphage small molecules produced by bacteria – beyond protein-mediated defenses

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Abstract

Bacterial populations face the constant threat of viral predation exerted by bacteriophages (or phages). In response, bacteria have evolved a wide range of defense mechanisms against phage challenges. Yet the vast majority of antiphage defense systems described until now are mediated by proteins or RNA complexes acting at the single-cell level. Here, we review small molecule-based defense strategies against phage infection, with a focus on the antiphage molecules described recently. Importantly, inhibition of phage infection by excreted small molecules has the potential to protect entire bacterial communities, highlighting the ecological significance of these antiphage strategies. Considering the immense repertoire of bacterial metabolites, we envision that the list of antiphage small molecules will be further expanded in the future.

Bacteriophages (see Glossary) (or phages for short) are viruses preying on bacteria and are considered to be the most abundant biological entities in the biosphere [1]. They represent a ubiquitous feature of bacterial existence, as there is virtually no ecosystem where bacteria do not coexist with phages infecting them [1]. The strong evolutionary pressure imposed by phage predation has led to a sophisticated arsenal of antiphage strategies, which have been extensively reviewed elsewhere [2–5]. The repertoire of known defense systems has been significantly expanded through large-scale bioinformatics screenings followed by experimental validation [6,7]. In addition to the already known defense systems such as restriction-modification systems, CRISPR-Cas or abortive infection, antiviral strategies now include the use of cyclic nucleotides as signalling molecules (CBASS [8], Pycsar [9]) and NAD+ depletion as a widespread response to viral infection [10–13]. Scrutiny of these novel

antiphage defense systems revealed striking similarities to eukaryotic immune systems, suggesting that a previously underappreciated fraction of eukaryotic immunity evolved from prokaryotic antiphage defenses [8,10,14–16]. With the accelerating pace of discovery of new antiphage systems, keeping an overview of the currently known antiviral prokaryotic arsenal has become increasingly difficult, but has been facilitated by the development of tools aimed at systematic and comprehensive identification of defense systems in prokaryotic genomes [17,18]. The notion of a bacterial pan-immune system has been recently proposed to recognize phage defense as a community resource distributed between closely related bacteria via horizontal gene transfer (HGT) [19].

In nature, bacteria live in complex, spatially structured and multispecies communities [20], which highlights the need to consider antiphage strategies at the community level. These mechanisms include the release of extracellular vesicles [21,22], formation of protective biofilm structures [23,24] or quorum sensing [25–27]. Chemical inhibition of phages using small molecules secreted in the extracellular space represents another effective multicellular strategy against phage infection, which unlike most defense systems described until now does not rely on proteins or RNA.

The direct inhibition of phage infection by bacterial small molecules was an intense research field in the 1950s and 1960s and has recently regained significant attention. Here, we aim at summarizing the extensive but largely overlooked body of research in the field of antiphage molecules and present the latest developments in this emerging research area. Furthermore, we outline future perspectives for the discovery of novel antiphage metabolites and discuss the ecological significance of this defense strategy.

The present review aims at presenting small molecules other than RNAs and proteins that are produced by bacteria and confer protection against phage infection. As a result, antibiotics preventing phage infection by a primary action on the bacterium are not included.

Chemical defense against phage infection

Overall, the study of antiphage molecules has known two distinct periods of interest – the first one spanning the third quarter of the twentieth century while the second started only a few years ago. The interest to find new compounds active against phages was very strong in the 1950s [28–30], with in some cases heroic screening efforts such as those performed by Schatz and Jones or Asheshov and colleagues—who assessed the antiphage activity of more than 170 and 1000 strains of actinomycetes, respectively [28,29]. In these screenings, the supernatants of 29% (49/176) and 17% (144/1000) of the tested actinomycete isolates caused an inhibition of plaque formation, suggesting that the release of antiphage metabolites is not uncommon in actinobacteria. These two screenings led to the description in follow-up studies of four antiphage compounds (chrysomycin, phagolessin A58, nybomycin and aklavin), the latter being shown to be a close congener of the **anthracycline** aclacinomycin A [31]. The primary goal of these screenings was however not to understand how bacteria defend themselves against phages, but rather to find new antiviral drugs usable in a clinical or agricultural setting [30]. An additional focus was put on substances able to specifically prevent phages from infecting ***Streptomyces*** *griseus* because of the risk phages posed to industrial production of streptomycin by this important production host [32].

Over the decades, a significant number of molecules were described to have antiphage properties. We listed these antiphage compounds in **Table 1**, which includes the phages inhibited and their bacterial hosts. In the following, we focus on the three main classes of antiphage small molecules described to date: anthracyclines, **aminoglycosides** and modified nucleotides produced by prokaryotic **viperins**.

88 **Table 1 | Small molecules with known antiphage properties** (*the classification of
89 aminoglycosides as protein synthesis inhibitors is based on their antibacterial action)

Class	Compound	Phages affected	Bacterial host	Phage family	Genome	Reference
DNA-intercalating agents						
Alkaloid	Ellipticine	λ	*E. coli*	*Siphoviridae*	linear dsDNA	[33]
Fluorochrome	Propidium iodide	λ	*E. coli*	*Siphoviridae*	linear dsDNA	[33]
		ϕScoe2	*S. coelicolor*	*Siphoviridae*	linear dsDNA	
		ϕScoe25	*S. coelicolor*	*Siphoviridae*	linear dsDNA	
Acridine family compounds	Acriflavine	λ	*E. coli*	*Siphoviridae*	linear dsDNA	[33]
		ϕScoe2	*S. coelicolor*	*Siphoviridae*	linear dsDNA	
		ϕScoe25	*S. coelicolor*	*Siphoviridae*	linear dsDNA	
	Ethacridine lactate	λ	*E. coli*	*Siphoviridae*	linear dsDNA	
Polypeptide antibiotic	Actinomycin D	T2r	*E. coli*	*Myoviridae*	linear dsDNA	[34]
		T4	*E. coli*	*Myoviridae*	linear dsDNA	[35]
Anthracyclines	Rutilantin	Various phages infecting both Gram + and Gram -				[29]
	Aclacinomycin (Aklavin) A and analogues	ϕX174	*E. coli*	*Microviridae*	circular ssDNA	[36]
		λ	*E. coli*	*Siphoviridae*	linear dsDNA	[37]
		Various phages infecting both Gram + and Gram -				[38]
	Daunorubicin (Daunomycin)	ϕX174	*E. coli*	*Microviridae*	circular ssDNA	[36]
		λ	*E. coli*	*Siphoviridae*	linear dsDNA	[33]
		T1	*E. coli*	*Siphoviridae*	linear dsDNA	[39]
		T3	*E. coli*	*Autographiviridae*	linear dsDNA	[39]
		T4	*E. coli*	*Myoviridae*	linear dsDNA	[39]
		T5	*E. coli*	*Siphoviridae*	linear dsDNA	[33]
		T6	*E. coli*	*Myoviridae*	linear dsDNA	[33,39]
		T7	*E. coli*	*Autographiviridae*	linear dsDNA	[33]
		JBD26	*P. aeruginosa*	*Siphoviridae*	linear dsDNA	[33]
		JBD30	*P. aeruginosa*	*Siphoviridae*	linear dsDNA	[33]
		ϕScoe2	*S. coelicolor*	*Siphoviridae*	linear dsDNA	[33]
		ϕScoe25	*S. coelicolor*	*Siphoviridae*	linear dsDNA	[33]
	Doxorubicin (Adriamycin)	ϕX174	*E. coli*	*Microviridae*	circular ssDNA	[36]
		λ	*E. coli*	*Siphoviridae*	linear dsDNA	[33]
		ϕScoe2	*S. coelicolor*	*Siphoviridae*	linear dsDNA	[33]
		ϕScoe25	*S. coelicolor*	*Siphoviridae*	linear dsDNA	[33]
		PBS1	*B. subtilis*	*Myoviridae*	linear dsDNA	[40]
		SP10	*B. subtilis*	*Myoviridae*	linear dsDNA	[40]
		ϕScoe2	*S. coelicolor*	*Siphoviridae*	linear dsDNA	[33]
		ϕScoe25	*S. coelicolor*	*Siphoviridae*	linear dsDNA	[33]
	Cosmomycin D	ϕScoe2	*S. coelicolor*	*Siphoviridae*	linear dsDNA	[33]
		ϕScoe25	*S. coelicolor*	*Siphoviridae*	linear dsDNA	
	Epirubicin	λ	*E. coli*	*Siphoviridae*	linear dsDNA	[33]
	Idarubicin	λ	*E. coli*	*Siphoviridae*	linear dsDNA	[33]
	Mitoxantrone	λ	*E. coli*	*Siphoviridae*	linear dsDNA	[33]

Protein biosynthesis inhibitors*						
Aminoglycosides	Streptomycin	MS-2	*E. coli*	*Leviviridae*	linear ssRNA	[41]
		P9	*Streptococcus faecium*	*Siphoviridae*	linear dsDNA	[42,43]
		f2	*E. coli*	*Leviviridae*	linear ssRNA	[44]
		µ2	*E. coli*	*Leviviridae*	linear ssRNA	[44]
		fd	*E. coli*	*Inoviridae*	circular ssDNA	[44]
		F-WJ-I	-	-	-	[45]
		Legendre	*M. smegmatis*	*Siphoviridae*	linear dsDNA	[45]
		Clark	*M. smegmatis*	*Siphoviridae*	linear dsDNA	[45]
		D29	*M. smegmatis*	*Siphoviridae*	linear dsDNA	[45,46]
		phAE159	*M. smegmatis*	phasmid (derived from TM4 phage)	circular dsDNA	[46]
	Kanamycin	D29	*M. smegmatis*	*Siphoviridae*	linear dsDNA	[46]
		phAE159	*M. smegmatis*	phasmid (derived from TM4 phage)	circular dsDNA	[46]
		Spe2	*C. glutamicum*	*Siphoviridae*	linear dsDNA	[47]
		λ	*E. coli*	*Siphoviridae*	linear dsDNA	[46]
		T3	*E. coli*	*Autographiviridae*	linear dsDNA	[48]
		WSP	*E. coli*	-	-	[48]
		BSP	*B. cereus*	-	-	[48]
	Hygromycin	D29	*M. smegmatis*	*Siphoviridae*	linear dsDNA	[46]
		phAE159	*M. smegmatis*	phasmid (derived from TM4 phage)	circular dsDNA	[46]
		Alderaan	*S. venezuelae*	*Siphoviridae*	linear dsDNA	[47]
	Apramycin	Alderaan	*S. venezuelae*	*Siphoviridae*	linear dsDNA	[47]
		λ	*E. coli*	*Siphoviridae*	linear dsDNA	
	Neomycin	80	*S. aureus*	*Siphoviridae*	linear dsDNA	[49]
		T3	*E. coli*	*Autographiviridae*	linear dsDNA	[48]
		WSP	*E. coli*	-	-	[48]
		BSP	*B. cereus*	-	-	[48]
Others						
Di-benzimidazole	Ro 90-7501	λ	*E. coli*	*Siphoviridae*	linear dsDNA	[33]
Quaternary ammonium	Dequalinium chloride	λ	*E. coli*	*Siphoviridae*	linear dsDNA	[33]
?	"Phagostatin"	T3	*E. coli*	*Autographiviridae*	linear dsDNA	[50]
Cyclopentenone	Sarkomycin	f2	*E. coli*	*Leviviridae*	linear ssRNA	[51]
Naphthocoumarin	Chrysomycin	Diverse phages				[52]
?	"Phagocidin"	T3	*E. coli*	*Autographiviridae*	linear dsDNA	[53,54]
Pyrrolobenzodiazepine	Tomaymycin	T1	*E. coli*	*Siphoviridae*	linear dsDNA	[55]
		T3	*E. coli*	*Autographiviridae*	linear dsDNA	
		M2	*B. subtilis*	*Podoviridae*	linear dsDNA	
		SP10	*B. subtilis*	*Myoviridae*	linear dsDNA	

Heterocyclic anthracene	Nybomycin	15/60 phages tested				[56]
?	"Phagolessin A58"	T1	*E. coli*	*Siphoviridae*	linear dsDNA	[57]
		T3	*E. coli*	*Autographiviridae*	linear dsDNA	
		T7	*E. coli*	*Autographiviridae*	linear dsDNA	
Modified ribonucleotides produced by prokaryotic viperins	ddhCTP, ddhGTP, ddhUTP	T7	*E. coli*	*Autographiviridae*	linear dsDNA	[14]

Anthracyclines

Anthracyclines are secondary metabolites naturally produced by *Streptomyces*—a common genus of soil-dwelling bacteria. Chemically speaking, anthracyclines belong to the family of type II aromatic polyketides and feature an aglycone scaffold decorated by a sugar residue [58]. Soon after their discovery, anthracyclines were shown to possess potent antitumour activity and have since then been used to treat a wide range of cancers [59]. They are still among the most effective anticancer treatments ever developed [60–62]. The precise mechanism behind their cytotoxic effect in eukaryotic cells is still subject to debate. However, their antitumour activity can be broadly attributed to their ability to intercalate into the DNA helix and/or bind covalently to proteins involved in DNA replication and transcription [63]. The DNA-damaging properties of anthracyclines also affect their producer, which as a result evolved several self-resistance mechanisms. In the case of *Streptomyces peucetius*, the toxic effects of daunorubicin and doxorubicin are mitigated by a combination of active efflux by DrrA and DrrB, extracellular sequestration to prevent reimport and dislodgement of intercalated anthracyclines by DrrC [64–66].

Multiple reports described the inhibition of phage infection by anthracyclines such as daunorubicin, doxorubicin or cosmomycin (Table 1). Parisi and Soller assessed the impact of daunomycin on the steps of the lytic cycle and showed a strong impairment of phage DNA

synthesis during phage infection, suggesting a blockage occurring during replication or between injection and replication [39].

A major step forward in the understanding of both the mechanism and biological significance of the antiphage properties of anthracyclines was made more than 40 years later by Kronheim and colleagues [33]. In this study, the authors show that daunorubicin inhibits phage λ in *E. coli* as well as several double-stranded DNA (dsDNA) phages infecting *E. coli*, *Streptomyces coelicolor* or *Pseudomonas aeruginosa* and encompassing the three main families of tailed phages *(Siphoviridae, Podoviridae* and *Myoviridae)*. The exact mechanism of action remains unclear, but inhibition by daunorubicin takes place at an early stage of the infection cycle, namely after injection of the phage genome but before phage replication (**Figure 1**). All dsDNA phages tested - whose incoming genome is linear - are inhibited by daunorubicin. In contrast, the filamentous M13 phage, whose ssDNA genome enters as a circular molecule, is not, suggesting that the circularization of incoming linear dsDNA could be the step blocked by daunorubicin. The anthracyclines doxorubicin and cosmomycin D were also shown to have antiphage properties. Importantly, the inhibition of phage infection could be reproduced with supernatants from natural producers of these anthracyclines (*Streptomyces peucetius* for daunorubicin and doxorubicin; strains of the WAC collection [67] for cosmomycin D, respectively). This observation suggests that phage inhibition by anthracyclines is physiologically relevant in the natural environment.

Kronheim and colleagues also reported the antiphage properties of synthetic DNA-intercalating agents such as propidium iodide or acridine derivatives [33]. Further, the inhibition of *E. coli* phage T2 by actinomycin D - another DNA-intercalating agent produced by

Streptomyces - was already described in 1961 [34]. Altogether, this suggests that intercalation into phage DNA is probably a widespread antiphage strategy (Table 1).

Aminoglycosides

Aminoglycosides are bactericidal antibiotics that are active against Gram-negative and Gram-positive organisms [68,69]. As their name suggests, aminoglycosides are pseudosaccharides that possess several amino and hydroxy functionalities and most of them share a core 2-deoxystreptamine ring [70]. Since the amine groups are typically protonated under physiologically relevant conditions, these antibiotics can be considered as polycationic species featuring a binding affinity for nucleic acids. In bacteria, they disrupt protein biosynthesis by targeting the 30S subunit of the ribosomes, which in turns leads to complete blockage of translation or promotes mistranslation [71]. Aminoglycosides were originally isolated from actinomycetes belonging to the *Streptomyces* and *Micromonospora* genera [72]. In nature, aminoglycoside producers are resistant to these molecules, which is a feature important to keep in mind when screening aminoglycosides—and small molecules in general—for antiviral properties.

Using bacterial hosts expressing plasmid-borne aminoglycoside resistance cassettes, aminoglycosides were recently shown to inhibit phages infecting the Gram-negative bacterium *E. coli* as well as Gram-positive bacteria such as *Corynebacterium glutamicum* and *Streptomyces venezuelae* [47]. Experiments aiming at shedding light on the molecular mechanism of phage infection inhibition revealed that phage DNA was present inside cells in the presence of aminoglycosides. Together with the observation that amplification of phage DNA was strongly impaired, these results suggest that the blockage exerted by

aminoglycosides mostly occurs after DNA injection but before genome replication (Figure 1). These results are in line with those obtained by Jiang and colleagues, who reported the inhibition of the two mycobacteriophages phAE159 and D29 by kanamycin, hygromycin and streptomycin [46]. Following the impact of streptomycin on phage adsorption and amplification of phage DNA, the authors propose that the blockage caused by aminoglycosides occurs between genome circularization and replication.

One important question is whether this inhibition of phage infection by aminoglycosides is relevant in a physiological context. In the case of apramycin, inhibition of the *Streptomyces* phage Alderaan could be reproduced with supernatants of the natural producer of apramycin [47], *Streptoalloteichus tenebrarius* (formerly known as *Streptomyces tenebrarius* [73]). Apparition of the antiphage effect of supernatants coincided with the detection of apramycin in the culture supernatants. In combination with the antiphage effect of purified apramycin, these data strongly suggest that the main molecule behind the antiphage properties of the supernatants of *S. tenebrarius* is apramycin [47]. Additionally, it indicates that aminoglycosides are secreted by producers at levels which prevent infection in neighbouring bacteria, opening the door to community-wide protection.

In a natural context, most bacteria do not possess aminoglycoside-resistance genes, and residual concentrations of antibiotics are pervasive across man-shaped and natural environments alike. Zuo and colleagues studied the impact of sublethal concentrations of aminoglycosides on phage infection in aminoglycoside-sensitive hosts [48]. Phage amplification was strongly impeded by concentrations as low as 3 mg/L. Interestingly, tetracycline, another antibiotic blocking protein synthesis by binding to the 30S ribosomal subunit, had a significantly reduced impact on phage proliferation. These results suggest that

177 blockade of translation alone is not sufficient to efficiently prevent phage replication.
178 Alternatively, the mechanism of translation inhibition may be of importance, and the
179 mistranslation caused by tetracycline could participate in the difference of impact observed
180 with aminoglycosides [48].

181 Although the action of aminoglycosides on the phage life cycle *in vivo* is not fully understood
182 yet, independent *in vitro* studies provide further hints about the basis of aminoglycosides'
183 antiphage properties. Exposure of purified phage λ DNA to aminoglycosides leads to
184 condensation of DNA, presumably coated by aminoglycoside fibers [74]. The same authors
185 later proposed that aminoglycosides form a clamp around the DNA double helix, causing a
186 bend responsible for the formation of structural deformations such as toroids [75].

187 *In vivo* mechanistic studies about the inhibition of phage infection by aminoglycosides are
188 scarce, but Brock and his collaborators contributed work worthy of attention. Using
189 *Streptococcus faecium* and its phage P9, Brock and Wooley investigated the inhibition of
190 phage infection by streptomycin [42]. The authors used resistance to shearing forces as an
191 indicator for DNA injection, under the assumption that the formation of a plaque from an
192 initially infected cell subjected to shearing implies a successful delivery of the phage genome.
193 Using this technique, they proposed that streptomycin inhibits phage infection at an early
194 stage of the phage infection cycle, namely the DNA injection step. They further hypothesized
195 that streptomycin exerts its inhibition by binding phage DNA in the capsid, thus preventing its
196 unfolding necessary for infection. It is however important to note that although phage
197 infection could already be inhibited by concentration of 100 μg/ml, high concentrations of
198 streptomycin were used (1 mg/ml) in most experiments. Such high concentration could cause
199 non-specific effects such as phage precipitation potentially not present at lower

concentrations. Moreover, the streptomycin-resistant bacterial host was reported to bind very low amounts of streptomycin, which suggests modifications of the cell surface that could in turn influence the antiphage properties of streptomycin. In another study, Brock demonstrated the inhibitory effect of streptomycin on the *E. coli* RNA phage MS-2 [41]. Streptomycin inhibited the formation of phage progeny very early in the replication cycle (5 to 10 minutes after infection), and no impact of streptomycin was noticed when added shortly after injection has occurred.

The fact that aminoglycosides possess both antibacterial and antiviral properties raises the question of the interplay between these two facets. In the case of apramycin, acetylation of one of its amino groups by the well-studied apramycin acetyltransferase AAC(3)IV abolished its impact of bacterial growth, while fully retaining its protective effect against phages [47]. This observation suggests that the antibacterial and antiviral actions of apramycin and potentially further aminoglycosides could be decoupled from one another and that the respective molecular targets are distinct.

Taken together, these studies suggest that aminoglycosides are not only used by their producers as toxic molecules against bacterial competitors but could serve as protection against the threat of phage predation at the community level.

Modified ribonucleotides produced by prokaryotic viperins

Viperins are important players of the innate antiviral response in eukaryotes [76]. They produce ddhCTP, a modified ribonucleoside lacking the 3'-hydroxyl group necessary for elongation of the nascent viral mRNA, hence acting as chain terminators [77].

Viperin-like genes were known to be present in prokaryotes too, but the function of these prokaryotic viperin homologs (pVips) remained unknown. Recently, they were shown to protect archaea and bacteria from viral infection and displayed a remarkable conservation between the eukaryotic and prokaryotic kingdoms [14]. pVips use indeed a similar mode of action to their eukaryotic homologs to inhibit viral transcription (Figure 1)—except that pVips produce a wider range of modified ribonucleotides (ddhCTP but also ddhGTP and ddhUTP) [14]. Strikingly, the human viperin, when expressed in *E. coli*, conferred resistance to phage infection, which underlines inhibition of viral transcription as a broad antiviral strategy. Interestingly, inhibition of phage infection was also observed with phages like P1 and λ which do not encode their own RNA polymerases and rely instead on the host polymerase to complete transcription. This raises the possibility that pVips also exert their antiviral activity independently of premature termination of viral transcripts, via mechanisms which remain to be elucidated.

Mirroring the absence of toxic effects caused by human viperin in human cells, expression of pVips in *E. coli* had no effect on host transcription and did not cause toxicity. This observation hints that the bacterial RNA polymerase may be less sensitive than the phage RNA polymerase to ddh-ribonucleotides, as self-resistance to the ddh-ribonucleotides would be favored during co-evolution of bacterial RNA polymerase and pVips. In contrast to anthracyclines and aminoglycosides, the modified nucleotides synthesized by viperins do not show antibacterial activity. Additionally, they are not secreted and protection is thus conferred only to producer cells.

Perspectives

Discovery of novel antiphage small molecules

Until now, the antiphage effect of most molecules were either discovered empirically or based on earlier reports describing antiphage properties of the same or closely related molecules. However, recent progress in the fields of genomics, metabolomics and automation have the potential to greatly accelerate the discovery of new antiviral molecules.

Automated screening allows high-throughput testing of the antiphage properties of molecule libraries (**Figure 2**). To this end, bacteria are cultivated in microtiter plates, either alone, in the presence of phages, or together with both phages and the compounds to be tested. If the addition of a given compound suppresses the phage-mediated lysis of the culture, this hit indicates a probable inhibition of phage infection by this molecule, warranting further investigation. This strategy was successfully used with *E. coli* and phage λ to reveal the antiphage activity of anthracyclines and other DNA-intercalating agents [33]. One major limitation of this approach is that the compounds tested need to not interfere with the growth of the bacterium, since strong growth defects would prevent the detection of antiphage effects.

Alternatively, spotting the molecules of interest on a phage-infected bacterial lawn represents another screening strategy with potential for automation and upscaling (Figure 2). This technique has been used for decades to assess antibacterial activity of antibiotics and has been harnessed by phage researchers too [56,78,79]. It enables the appreciation of antiphage effects (or on the contrary phage antibiotic synergy) despite inhibition of bacterial

growth, as shown by rings devoid of plaque formation—or displaying larger plaques, respectively—around the zone of growth inhibition caused by the candidate molecule.

These two strategies are not restricted to pure compounds and can also be used with complex supernatants from bacterial hosts, enabling the exploration of a vaster metabolic landscape as well as of potential synergistic interactions between candidate molecules. In the case where a supernatant inhibits phage infection, **bioactivity-guided fractionation** followed by liquid chromatography–mass spectrometry (LC-MS) can narrow the antiphage properties of the supernatant down to one or a few compounds [33].

These screening approaches are likely to have a low discovery rate due to their untargeted nature. Screening can be narrowed down by testing in priority metabolites released in reaction to phage infection. For example, phage infection in *Streptomyces coelicolor* leads to the formation of coloured halos around phage plaques. The presence of pigmented compounds at the infection interface suggests that *Streptomyces* reacts to phage infection by releasing these molecules, making them interesting candidates for further analysis [80].

In silico prediction of genomic signatures of gene clusters involved in chemical antiphage defense would allow to rationally identify and test candidate molecules. However, antiphage biosynthetic gene clusters (BGCs) such as the ones encoding aminoglycosides and anthracyclines are not detected using the now well-established “guilty-by-association” approach. This discovery strategy is based on the observation that defense systems are clustered in genomic “defense islands”. Genes markedly enriched in the vicinity of known defense genes are therefore assumed to be also involved in antiphage defense [6]. The use of this concept has led in recent years to a considerable expansion of the known repertoire of antiphage defense systems [6,7,14]. It is however biased towards small and very well

conserved genes, explaining why this approach did not detect large and genus- or sometimes even species-specific BGCs as putative novel antiphage defense systems. Now that tools systematically screening for known defense systems are available [17,18], combining detection of phage defense systems and prediction of BGCs could reveal interesting patterns of co-occurrence and help to define genomic features of antiphage BGCs. In the case of antiphage metabolites fulfilling several roles (e.g. antibacterial and antiviral) such as aminoglycosides, these supplementary functions likely impose further genomic and evolutionary constraints, hindering the establishment of genomic signatures for gene clusters encoding multifunctional molecules.

Importantly, empirical approaches and *in silico* screening are not mutually exclusive; uncovering more antiphage secondary metabolites will help to define genomic signatures for antiphage molecules. *Streptomyces* are considered to encode the largest biosynthetic diversity across bacterial genera, and actinobacteria at large show remarkable diversity in their secondary metabolism [81]. Yet these findings are presumably biased by the extensive knowledge we already have about actinobacteria. Less-well-studied bacterial phyla, such as myxobacteria [82,83] or planctomycetes [84,85] to name only a few, also possess elaborate BGC arsenals which represent promising sources for the discovery of novel antiviral molecules.

Ecological relevance

The ecological significance of antiphage molecules was mostly ignored in the first wave of research focusing on antiphage molecules and has only been recently appreciated. When considering the ecological relevance of antiphage molecules, one key question is: is the

antiphage molecule secreted? If yes, are the concentrations reached high enough to block phage amplification? With the evidence currently available, we can answer in the affirmative to these two questions regarding both anthracyclines and aminoglycosides. Indeed, anthracyclines and aminoglycosides are typically exported from producer cells by ABC-type transporters [66,86] and culture supernatants of producers were shown to inhibit phage replication [33,47].

Contrary to most protein-based defense systems, antiphage molecules described so far display rather broad inhibitory abilities. Anthracyclines and aminoglycosides inhibit seemingly very disparate phages infecting diverse bacteria, Gram-positive and negative alike. So far, the rules behind the sensitivity of a given phage to these two classes of compounds remain unclear, the only common feature of the inhibited phages being their double-stranded DNA genome and tailed morphology. This broad range of inhibition has important ecological implications: depending on their local concentrations and diffusion, these antiphage compounds could serve as 'public goods' and protect not only producer cells but also neighboring, unrelated cells—provided they are resistant to these compounds (**Figure 3**). The fact that non-relatives could benefit from antiphage molecules is debatable under the light of sociomicrobiology. We can imagine that spatial structure and biofilms play a key role in restricting the access to these molecules primarily to genetic kin. Alternatively, the substantial metabolic costs associated to the production of complex compounds like aminoglycosides and anthracyclines combined to the genomic instability in *Streptomyces* [87] may lead to a partial or complete loss of the corresponding BGCs in certain subpopulations, following a division of labor strategy. This loss of BGCs following genetic instability could be offset by the gene flow from related bacteria. For instance, actinobacteria like *Salinospora* maintain a pool

of BGCs at the population level which are shuffled between strains through HGT, following a 'plug-and-play' strategy [88].

The dual function of certain antiphage molecules adds another layer of complexity. For instance, aminoglycosides represent a remarkable example of molecular multitasking, with the same molecules exerting two seemingly unrelated effects—inhibition of bacterial translation and of phage replication.

Furthermore, acquisition of resistance to aminoglycosides by initially sensitive cells is highly beneficial for two reasons: not suffering from their antibacterial effect anymore while benefiting from the inhibition of phage infection. Naturally, the mode of resistance to these antibiotics is of particular importance. Considering that aminoglycosides are thought to act intracellularly to block phage infection, resistance mechanisms based on decreased aminoglycoside intracellular concentration—such as decreasing uptake or expressing efflux pumps—would confer resistance to the antibiotic at the expense of the loss of its protective antiphage effect. Conversely, resistance to aminoglycosides mediated by aminoglycoside-modifying enzymes has the potential to inactivate aminoglycosides' antibacterial activity without reducing intracellular concentrations. With the aminoglycoside apramycin, it was shown that acetylation of one of its amino groups suppresses its antibacterial effect while retaining its antiphage properties [47]. Whether this example is a unique case or is a general feature of aminoglycoside modifications remains to be determined. However, this observation could potentially be one factor contributing to the wide distribution of aminoglycoside-modifying enzymes catalyzing, for example, the acetylation, phosphorylation or adenylation of amino or hydroxyl groups at various positions of the aminoglycoside scaffold [89].

While secreted antiphage metabolites raise important ecological questions, keeping antiphage compounds strictly intracellularly also provides the producer with special advantages. From a metabolic point of view, this obviously suppresses the costs associated with exporting the molecules and the problematics of re-entry in neighboring cells. Privatizing the antiviral molecules also prevents non-related bacteria occupying the same niche from benefitting from this resource. Moreover, the modified ribonucleotides produced by pVips necessitate a single enzyme, which greatly facilitates the spread of this antiviral strategy by HGT as reflected by the scattered phylogenetic distribution of pVips across the main bacterial clades [14]. Lastly, the substrates of viperins (ribonucleotides) are so pervasive across life forms that this antiviral mechanism is applicable against a wide range of viruses, prokaryotic and eukaryotic alike [14]. We anticipate that bacteria have evolved further defense mechanisms acting as molecular 'grains of sand' jamming key steps of the viral machinery such as replication or translation.

To fully appreciate the ecological significance of chemical defense against phages, moving away from the traditional "one phage – one bacterium" approach represents a key step. Building simplified, synthetic communities by increasing phage and/or bacterial diversity can provide decisive insights into the physiology of antiphage defense strategies, as shown for example with the importance of CRISPR-mediated phage resistance over modifications of the phage receptor in complex microbial communities [90]. Yet, additional mechanistic insights are required to understand the impact of antiphage molecules on community interactions.

Finally, one further direction worthy of investigation is the study of the interplay between the different defense systems—small molecule- and protein-based. Producers of antiphage molecules also encode other defense systems, and certain secondary metabolites could serve

as a trigger for other defense strategies. For instance, it was recently shown that the transcription-inhibiting antibiotic rifampicin activates nucleotide-depletion defense, even in the absence of phages [91]. Our current knowledge about how prokaryotes coordinate these diverse antiphage strategies to mount efficient antiviral responses is still in its infancy and needs to be advanced to provide an integrated view of the prokaryotic immune system.

Concluding Remarks

Phage defense systems are often considered at the level of the individual cell, where it is mechanistically described how they protect a bacterium from being infected by an incoming phage. By acting at the single-cell level, antiphage strategies prevent the spread of the infection and thereby protect the broader bacterial community. However, some mechanisms specifically protect several cells or the entire population simultaneously. One of these consists in the release of small molecules into the extracellular environment. The antiphage metabolites described until now predominantly correspond to anthracyclines and aminoglycosides, both inhibiting the early steps of the phage infection cycle. Interestingly, aminoglycosides are well-known antibacterial agents, but were also shown to be potent inhibitors of phage infection, suggesting that evolutionary constraints allowed the development of two seemingly very distinct functions.

From a therapeutic standpoint, antiviral metabolites in bacteria have the potential to fuel the discovery pipeline for novel antiviral drugs in humans. For example, synthetic nucleoside chain terminators are widely used in conditions such as HIV [92,93] or infection with herpes viruses [94] and chain terminators produced by pVips could represent new avenues for

treatments of viral infections in humans [14]. Knowledge gained about small molecule-mediated inhibition of phage infection is also relevant for phage therapy, e.g. to avoid antagonistic effects when administering phage-antibiotic combination treatments.

The repertoire of bacterial secondary metabolites is extremely large, and the physiological function of many of these compounds remains unclear. We can thus hypothesize that the number of described antiphage molecules will keep growing in the future (see Outstanding Questions). For example, molecules triggering death of parts of the bacterial population represent promising candidates, as their release in reaction to phage predation would mimic the effect of protein-mediated abortive infection (Abi) systems.

Phages have developed ways to circumvent most bacterial defense strategies, as part of the arms race in which they are engaged with their bacterial hosts. It is therefore plausible that phages have evolved means to overcome this metabolite-based defense system. Elucidating these adaptations could illuminate phage biology by attributing a function to certain already known phage features and further our understanding of the intricate relationships between phages and their bacterial hosts in the context of chemical defense.

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Glossary

Aminoglycosides

Antibacterials naturally produced by *Streptomyces* and *Micromonosporas* species. They target bacterial translation by binding to the 30S ribosomal subunit. Besides their antibacterial action, additional antiphage properties were recently discovered.

Anthracyclines

DNA-intercalating antibiotics produced by *Streptomyces* having antitumor as well as antiphage properties.

Bacteriophages

Viruses that prey on bacteria.

Bioactivity-guided fractionation

Chromatographic separation of extracts aiming at the isolation of a pure biologically active compound.

Chemical defense

Protection against phage infection via bacterial small molecules.

Streptomyces

Genus of Gram-positive bacteria which belongs to the phylum of Actinobacteria. *Streptomyces* species are mainly found in the soil and are characterized by mycelial development as well as by their complex secondary metabolism. *Streptomyces* are one of the most important producers of bioactive molecules.

Viperins

Virus-inhibitory proteins in eukaryotes which convert ribonucleotides into chain terminators, thereby preventing transcription of viral genes. Viperin homologs are found in prokaryotes and are known as prokaryotic viperins (pVips). pVips inhibit phage infection in a similar mode of action than their eukaryotic counterparts.

Figures & Tables

Table 1 | Small molecules with known antiphage properties (*the classification of aminoglycosides as protein synthesis inhibitors is based on their antibacterial action)

Figure 1 |Mechanism of action of antiphage molecules anthracyclines, aminoglycosides and modified nucleotides produced by prokaryotic viperin homologs (pVips). The phage replication cycle comprises several steps, some of which being targeted by antiphage molecules. Unlike the modified ribonucleotides produced by pVips, anthracyclines and aminoglycosides are secreted by producer cells and can be taken up by neighbouring cells.

Figure 2 | Discovery strategies for the identification of new antiphage molecules. Bioinformatic prediction of candidate biosynthetic gene clusters (BGCs) whose products may act against phages (1) inform large-scale testing of small molecule libraries as well as complex supernatants (2). The elucidation of the antiphage compounds can be achieved by bioactivity-guided fractionation (3 and 4) followed by analytic techniques such as liquid chromatography–mass spectrometry (LC-MS) (5). Results of the screening efforts can be then fed back to the bioinformatic screening to help define genomic features of antiphage BGCs (6).

Figure 3 | Ecological significance of the dual properties of aminoglycosides in a bacterial community. Aminoglycoside producers release aminoglycosides (purple) in their environment. Aminoglycosides kill sensitive bacteria (antibacterial effect, A) while they may protect neighbouring bacteria from phage infection (antiviral effect, B), provided they are resistant to these molecules e.g. via prior horizontal gene transfer (HGT) of resistance genes

479 from producer cells (C). Bystander microorganisms not affected by aminoglycosides are
480 shown in grey.

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