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Bacteriophages as drivers of bacterial virulence and their potential for biotechnological exploitation

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Abstract

Bacteria-infecting viruses (phages) and their hosts maintain an ancient and complex relationship. Bacterial predation by lytic phages drives an ongoing phage-host arms race, whereas temperate phages initiate mutualistic relationships with their hosts upon lysogenisation as prophages. In human pathogens, these prophages impact bacterial virulence in distinct ways: by secretion of phage-encoded toxins, modulation of the bacterial envelope, mediation of bacterial infectivity and the control of bacterial cell regulation.

This review builds the argument that virulence-influencing prophages hold extensive, unexplored potential for biotechnology. More specifically, it highlights the development potential of novel therapies against infectious diseases, to address the current antibiotic resistance crisis. First, designer bacteriophages may serve to deliver genes encoding cargo proteins which repress bacterial virulence. Secondly, one may develop small molecules mimicking phage-derived proteins targeting central regulators of bacterial virulence. Thirdly, bacteria equipped with phage-derived synthetic circuits which modulate key virulence factors could serve as vaccine candidates to prevent bacterial infections.

The development and exploitation of such antibacterial strategies will depend on the discovery of other prophage-derived, virulence control mechanisms and, more generally, on the dissection of the mutualistic relationship between temperate phages and bacteria, as well as on continuing developments in the synthetic biology field.

Bacteria and bacteriophages, an ancient cooperative relationship

Since their discovery, bacteriophages are often described as strict parasites of their bacterial host [1]. However, this simplified idea of a phage preying on its host is outdated [2,3]. Although phages indeed rely on bacterial cells for their replication and reproduction [4], more research now emphasizes the contribution of phages to the fitness of their hosts [5–7]. The observed impact of bacteriophages differs depending on the replication strategy they pursue; strictly lytic or temperate [8,9]. Although strictly lytic phages immediately produce new virions and subsequently kill the cell, they are drivers of bacterial evolution [1]. This results from a clear-cut predator-prey relationship, in which both populations co-evolve to survive [10]. Bacteria can gain resistance against phage infections, usually via receptor-attachment inhibition, by losing or mutating their phage receptor [11]. In turn, phages circumvent those antiviral strategies and adapt to the modified receptor [12]. By exerting a continuous pressure on the bacterial cells, phages drive the long-term evolution of bacterial populations [13].

Additionally, temperate phages integrate their DNA into the bacterial chromosome or reside in the cell as a plasmid for multiple generations [1]. Phages entering the lysogenic cycle will become prophages and in some cases can even lose their ability to excise, reproduce and/or lyse their host cells, thereby converting into cryptic prophages [14]. During this mutualistic relationship, an interesting interplay takes place between the inhabiting prophage and bacterial host cell [15]. Temperate prophages can either direct the host phenotype towards an optimal virion producing factory, resulting in cell lysis, or they can support the mutualistic relationship [2]. An intriguing example of such a mutualism, is this of Ankyphage-infected bacteria in the marine environment. By expression of prophage-encoded ankyrin proteins,

the immune response of marine sponges is attenuated along with a downregulation of phagocytosis towards bacteria, facilitating the symbiotic relation between bacterial cells and the eukaryotic sponge [16].

Upon integration, a phage will supplement the bacterial genome with genes necessary for virion production, but also additional genes termed “morons” [17]. These morons are independent loci and are usually flanked by regulatory elements [18,19]. Expression of these accessory genes, results in an increased fitness of the bacterial host and thereby, directly, also their own fitness [7]. Needless to say, as phages depend fully on bacteria for their reproduction, the prophage benefits from this enhanced host fitness within a competitive ecosystem [20]. The contribution of phages to bacterial virulence was first shown in 1927 by Frobisher *et al.,* revealing that toxin-encoding genes could be transferred from a toxic *Streptococcus* strain to non-toxic strains [21]. Thirty years later, the contribution of bacteriophages to the conversion of a nontoxic *Corynebacterium diphtheria* into a virulent strain was reported [22–24]. Since then, several research papers have addressed the impact of phage on bacterial virulence [24–28]. In addition, the integration and excision of prophages from bacterial chromosomes are frequently-occurring events and result in the transmission of morons from one bacterial cell to another when prophages enter a lytic cycle upon environmental stress [3,29]. In this manner, bacteriophages drive the evolution of an entire bacterial population. Considering that up to 20 % of the bacterial genome is of phage-origin, the impact of (pro)phages on bacterial ecology and evolution in all environments is significant [30].

The fascinating mutualistic relationship between temperate bacteriophages and bacteria could radically expand our horizons in the field of biotechnology. By investigating and

97 understanding the influence of prophages on bacterial virulence, new insights can be gained
98 on how to exploit this for novel antibacterial strategies. Although the influence of
99 bacteriophage on bacterial fitness is observed in many different niches, this review will only
100 discuss recent findings on how prophages drive bacterial pathogenesis in humans.
101 Furthermore, since tackling infectious diseases caused by antibiotic resistance bacteria has
102 become an emerging societal need, we will emphasize how influencing bacterial virulence by
103 prophage-derived means may serve as a base for novel antibacterial strategies in the future.
104
105 **Prophages reshape the virulence of human pathogens**
106
107 Several pathogens owe their virulence, or at least in part, to bacteriophages [31]. Since the
108 prevalence of phage-derived sequences in human pathogens is high, it is of interest to know
109 their contribution to host fitness. Emerging pathogens causing critical infections include
110 *Pseudomonas aeruginosa, Salmonella enterica, Escherichia coli, Vibrio cholera,*
111 *Staphylococcus spp.* and *Clostridium spp* [32]. For all of these pathogens, bacteriophages have
112 shown to play a role in their observed virulence. Phages are able to alter the phenotype of
113 their host at different levels, including toxin secretion, modification of the bacterial envelope,
114 impacting bacterial infectivity and bacterial cell regulation (Fig. 1 and Table 1).
115
116 *Prophages contribute to the secretion of toxins*
117
118 In the human body, pathogens secrete numerous virulence factors and among these,
119 exotoxins (bacterial toxins) are secreted [32]. Many harmful bacterial toxins are encoded in
120 prophage regions and are thereby easily transferred among the bacterial population, which

121 can even lead to the development of novel diseases [31]. Indeed, even commensal bacteria can
122 rapidly acquire toxic genes, transforming them into human pathogens. Most exotoxins
123 supplied by temperate phages are only expressed when lytic growth is induced, for instance
124 during DNA-damaging antibiotic treatments, although some exceptions exist [34,35]. The best
125 known phage-encoded exotoxin, the cholera toxin, was discovered in 1996 [26,36]. Only in the
126 presence of the CTXϕ prophage, the enteric microbe *V. cholera* displays a pathogenic
127 phenotype, causing the life-treating disease [19]. Furthermore, the botulinum neurotoxins type
128 C1 and D in *Clostridium botulinum* are carried by the temperate bacteriophages CEβ and DEβ,
129 respectively. Exposure to the toxins can lead to neuronal paralysis in humans [37,38]. In another
130 example, lysogenisation of *E. coli* cells by *Stx*-encoding lambdoid phages results in the
131 development of the Shiga toxin producing *E. coli* (STEC) pathogen, with the toxin inhibiting
132 the eukaryotic protein synthesis [35]. Furthermore, the toxic-shock-syndrome toxin-1
133 (*Staphylococcus aureus*) and diphtheria toxin (*Corynebacterium diphtheria*) are also phage-
134 derived [24,39–41]. Similarly, in *Clostridium difficile*, the observed pathogenicity fully depends on
135 the presence of prophage regions. This opportunistic pathogen harbors two principal toxins,
136 TcdA and TcdB, which are most likely of bacteriophage origin. Both are cytotoxic and their
137 expression results in the destruction of the actin cytoskeleton and tight junctions of intestinal
138 epithelial cells. In addition to those two toxins, Riedel *et al*. identified a novel binary toxin
139 locus (CdtLoc), encoded in the genome of prophage PhiSemix9P1. Expression of the region is
140 under control of the *cdtR* gene and results in the production of an ADP-ribosyltransferase
141 CDTa, resembling the cholera toxin, and the transporter protein CDTb. When all three toxins
142 are expressed, a hypervirulent host cell arises [42].

143
144

Table 1 Overview of the effect of prophages on the bacterial virulence. The bacteria, prophage, corresponding phage gene and the observed effect on bacterial virulence are listed.

Bacteria	Prophage	Phage-encoded gene	Effect on bacterial virulence	References
Toxin secretion				
Vibrio cholera	CTXϕ	*ctx*	Cholera toxin production	26, 25, 36
Clostridium botulinum	CEβ, DEβ	*C1, D*	Botulinum toxin production	41, 37
Escherichia coli	933W, H-19B	*stx1, stx2*	Shiga toxin production	172
Staphylococcus aureus	80α	*tst*	Toxic-shock syndrome toxin-1 production	40
Corynebacterium diphtheria	β-phage	*cdtA, cdtB*	Diphtheria toxin production	24, 173
Clostridium difficile	Ancient prophage	*tcdA, tcdB*	TcdA and TcdB toxin production	78
	PhiSemix9P1	*cdt*	CDTa and CDTb toxin production	42
Bacterial envelope				
Pseudomonas aeruginosa	D3	*iap*	Reduction O-chain length by inhibition of the host cell alpha polymerase	47
Salmonella enterica serovar Typhimurium	ε15	*22*	Reduction O-chain length by inhibition of the host cell alpha polymerase	49
Salmonella enterica serovar Typhimurium	P22	*gtr*	Addition of glucose units to the O-chain	51
Escherichia coli	Sp5	Unknown	Downregulation flagellin	58
Escherichia coli	phi4	*EC958_1546*	Increased flagellin expression	57
Pseudomonas aeruginosa	JBD26	Unknown	Inhibition working mechanism Type IV pili	59
Bordetella bronchiseptica	PHB09	Entire phage	Reduced twitching motility due to disruption *pilB*	61
Bacterial infectivity				
Pseudomonas aeruginosa	Pf4	Entire phage	Promotion adhesion of bacterial cells to mucin	64, 65
Neisseria meningitidis	ϕMDA	Entire phage	Promotion adhesion of bacterial cells to epithelial cells	66
Pseudomonas aeruginosa	Pf	Entire phage	Reduced inflammation and phagocytosis	67
Staphylococcus mitis	ϕSM1	*PblA, PblB*	Mediation bacterial interaction with platelets	70
Enterococcus faecalis	pp1, pp4, pp6	*EF0348, EF2001, EF2811, EF2813*	Mediation bacterial adherence to platelets	71
Salmonella enterica serovar Typhimurium	Fels-2	*STM2699*	Crosslinks bacterial cells to the eukaryotic spectrin receptor	72
Vibrio cholera	CTXϕ	*tcp*	Expression of toxin-coregulated pili	36
Staphylococcus aureus	ϕSPβ	*sasX*	Expression of a surface protein promoting bacterial adherence to epithelial cells	73
Salmonella enterica	Gifsy-1	*gogB*	Increased inflammatory response, enhanced tissue damage and increased bacterial colonization	75

Salmonella enterica	Gifsy-1	*sarA*	Lowering production reactive oxygen species by macrophages	76
Clostridium difficile	ϕCD38-2	*cwpV*	Increase in bacterial cell aggregation	77
Staphylococcus aureus	CC398	Unknown	Increased expression of fibronectin-binding proteins	79
Regulation of bacterial virulence				
Staphylococcus aureus	ϕ13	Entire phage	Loss of β-toxin production due to disruption *hlb*	82
Listeria monocytogenes	ϕ10403S	Entire phage	Interruption *comK* gene	83
Pseudomonas aeruginosa	PaP3	*70.1*	Inhibition stress regulator RpoS	86
Staphylococcus aureus	ϕ11, ϕ80α	*cI*	Induction *SigB* regulon, impacting the development of the bacterial infection	87
Clostridium difficile	PhiDHM1	*agrB, agrC, agrD*	Interfering with the quorum sensing system	89
Vibrio cholera	VP882	*vqmA*	Interfering with the quorum sensing system	90
Clostridium difficile	ϕCD119	*repR*	Decrease in β-toxin expression	91
Clostridium difficile	ϕCD27	Unknown	Decrease in β-toxin expression	92
Clostridium difficile	ϕCD38-2	Unknown	Increase in β-toxin expression	93
Clostridium difficile	ϕCD2, ϕCD6, ϕCD8	Unknown	Increase in β-toxin expression	94
Escherichia coli		*cro*	Activation transcription type three secretion system	95
Escherichia coli	SpIE1	*esr41*	Increase in cell motility and resistance against colicin, attenuation of iron uptake	99, 100
Escherichia coli	Sp5	*agxR*	Increase in iron uptake	102
Salmonella enterica serovar Typhimurium	BTP1	*STnc6030*	Provides superinfection exclusion	103

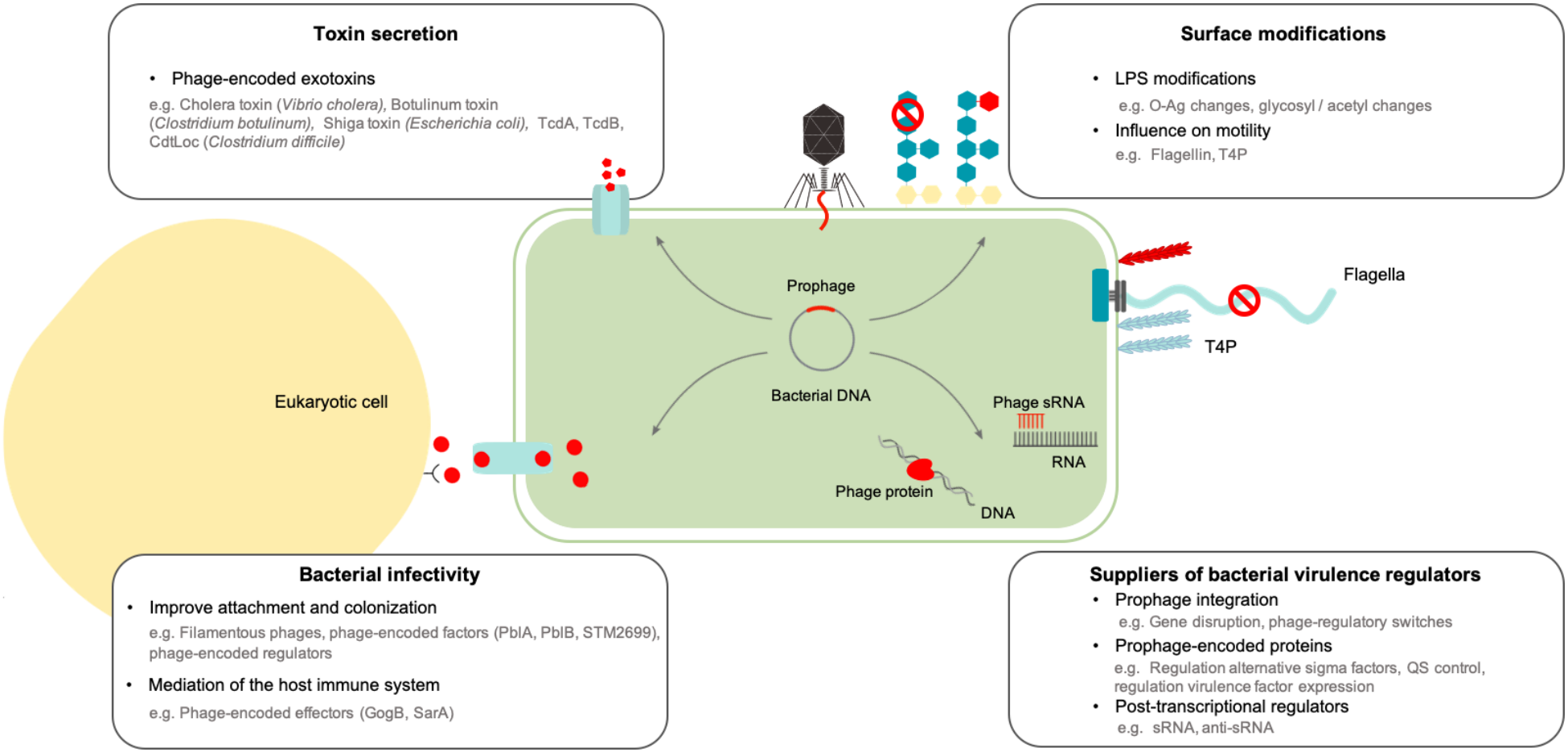

Figure 1: Prophages affect bacterial virulence on different levels. Prophages influence the virulence of their bacterial host at four different key levels: toxin secretion, modification of the bacterial envelope, bacterial infectivity and general bacterial cell regulation. Many temperate phages carry exotoxin-encoding genes and are thereby able to transform avirulent bacterial isolates into pathogenic strains (top left). Second, prophages can modify the envelope of the host cell, by changing the LPS structure or motility moieties, including the flagella and type IV pili (T4P) (top right). Moreover, the bacterial infectivity in the human body can be altered by temperate phages, either by improving tissue colonization or by mediation of the eukaryotic immune response (bottom left). Fourth, prophages have shown to act as general bacterial cell regulators and in this way affect the bacterial virulence, due to insertional effects, phage-encoded protein and RNA regulators (bottom right).

Prophages modify the bacterial envelope

In general, phages can modify the bacterial envelope in two different ways: by changing the lipopolysaccharide (LPS) structure or by influencing bacterial motility moieties. As both components serve as receptors for phage infection, altering their structure serves to ensure superinfection exclusion [43]. Moreover, LPS and motility elements are highly immunogenic [44]. By transforming those antigens, host cells can escape human immune defenses, which benefits both bacteria and phage. LPS is the principal component of the outer membrane as well as a key virulence factor of Gram-negative pathogens [45]. The LPS layer consists of three different domains: a lipid A, a core oligosaccharide and an O-antigen. The O-antigen is a versatile domain and is of importance for recognition by phages and the human immune system as well as for general pathogenicity [46]. As O-antigens often serve as phage receptors, some prophages encode factors altering this chain, blocking phage infection. Two main strategies for LPS modification include the introduction of structural changes in the O-antigen chain and the addition of a sidechain. The former can be accomplished by expression of an α polymerase inhibitor (iap), which mimics the Gram-negative encoded protein Wzz, disrupting the Wzy/Wzz interaction complex [47]. This protein complex controls the number of O subunits of the LPS chain and interference of iap thereby results in a reduction of the O-chain length, provoking serotype conversion [48]. Both *P. aeruginosa* and *S. enterica* serovar Typhimurium prophages contain such a mechanism [47,49,50]. Other phages can alter the LPS structure due to glycosyl or acetyl modification. For instance, in *S. enterica* serovar Typhimurium, the glucosyltransferase operons are located within a prophage region, resulting in the addition of glucose units to the O-region of the LPS chain [51]. Furthermore, prophages can introduce acetyl groups by encoding for a site-specific acetyltransferase [52–54].

Besides LPS modifications, some prophages were demonstrated to influence the motility of their hosts. On the bacterial surface, type IV pili (T4P) and flagella are responsible for cell motility [55,56]. These structures are important for the search of nutrients and play a role in the colonization of host tissue, but they also serve for bacteriophage adhesion [56,57]. Prophage morons of *E. coli* have been identified that affect the flagellar motility of their host cell. For example, the Shiga-toxin encoding temperate phage Sp5 reduces the expression of *fliA*, *fliC* and *flhCD*, resulting in an overall downregulation of flagellin and thereby a reduced swimming motility [58]. In contrast, Kakkanat *et al.* reported that the protein EC958_1546 from prophage phi4 contributes to the motility of uropathogenic *E. coli* (UPEC). Overexpression of this hypothetical protein leads to hyper-motile *E. coli* strains with an increased flagellin expression, most likely via an altered chemotactic response [57]. In other pathogens, prophages have been shown to inhibit the working mechanism of T4P. Bondy-Denomy *et al*. reported the ability of temperate phage JBD26 to inhibit twitching motility in *P. aeruginosa*, without inhibiting the display of the pili structures on the surface [59]. On the other hand, prophages have also shown to prevent the assembly of T4P on the bacterial surface [60]. In *Bordetella bronchiseptica*, the temperate bacteriophage PHB09 disrupts pili formation due to specific integration in the *pilB* gene of the host, leading to a reduced twitching motility and consequently a reduced overall virulence [61].

Prophages impact bacterial infectivity

Bacteria possess several tools for tissue adherence and colonization and to avoid clearance by the immune system, all to facilitate a successful infection of the human body [62]. From this

perspective, inhabiting prophages have the ability to interfere with bacterial infectivity at three different levels, as detailed in the paragraphs below. The first level constitutes entire virions which mediate the bacterial infectivity. The second level involves both structural proteins and secreted effectors, encoded by temperate phages, with key roles in bacterial infection. A third level of interference are phage-encoded regulators, which regulate the induction of bacterial infection factors. Phages might in this manner help bacteria to improve the attachment, colonization and invasion of eukaryotic cells, but as well provoke immune suppression.

First, the prophage-encoded virulence factor can be the bacteriophage itself. Promotion of tissue colonization by virion production has been reported several times for the pathogen *P. aeruginosa*. Here, production of the filamentous prophage Pf4 is induced during biofilm growth. The resulting filamentous particles improve the adhesion of bacterial cells to mucin, a major component of the mucus barrier, and thereby trap the bacteria in the lung environment [63–65]. Similarly, the filamentous prophage ϕMDA in *Neisseria meningitidis* was found to interact with the epithelial cell layer and in this way promotes bacterial cell adherence. Since the produced virions resemble type IV pili structures, they stimulate bacterial cell aggregation and consequently, mucosal colonization [66]. Interfering with the human immune response to sustain the bacterial infection, is yet another reported strategy of prophages. The filamentous Pf phage is often integrated within the *P. aeruginosa* genome and is abundantly produced during the infection stage. A reduced inflammation and phagocytosis are observed at the infection site when Pf phages are present, revealing their importance [67]. It was found that those filamentous phages drive the maturation of macrophages towards a M2-phenotype. M2 macrophages promote, in contrast to M1-type

227 macrophages, tissue repair and possess anti-inflammatory functions [68]. Therefore, *P.*
228 *aeruginosa* cells harboring Pf phages are more resistant to macrophage uptake due to
229 prophage interference [65]. In another more recent study, the same conclusions were drawn.
230 Here, the filamentous phage Pf was shown to impair wound healing and reduce bacterial
231 clearance at the site of infection. After endocytosis of the phage by leukocytes, production of
232 the phage RNA inside the immune cell takes place. This results in the stimulation of type-I
233 interferon production and suppression of tumor necrosis factor, which consequently impairs
234 phagocytosis [69].
235
236 Second, phages have also been found to encode several factors which provide advantages to
237 bacterial colonization and invasion. A number of prophage proteins are able to enhance the
238 binding of host cells to human platelets. For instance, PblA and PblB encoded by ϕSM1 in
239 *Staphylococcus mitis,* function as phage coat proteins. However, these proteins can also be
240 secreted by the bacterial cell and mediate its interaction with platelets. By interaction of PblA
241 and PblB with choline residues of the bacterial cell wall, they fulfill a function as platelet
242 adhesins, enhancing the platelet binding activities by the bacterial cells [70]. In similar fashion,
243 prophages pp1, pp4 and pp6 of *Enterococcus faecalis* produce phage tail proteins, which
244 contribute to human platelet adherence [71]. Yet another example is the STM2699 protein,
245 encoded by prophage Fels-2 in *S. enterica* serovar Typhimurium. STM2699 crosslinks the
246 *Salmonella* cells to the eukaryotic spectrin receptor, but expression is only induced after a
247 cold shock [72]. On the other hand, some bacteriophages of *V. cholera* and *S. aureus* encode
248 colonization factors that are displayed on the bacterial surface. In *V. cholera,* besides
249 encoding for the cholera toxin, the CTXϕ phage also provides a gene for the expression of
250 toxin-coregulated pili (TCP). These pili structures are essential for both bacterial attachment

to epithelial cells as well as serving as adherence receptors for CTXϕ [36]. Similarly, a ϕSPβ- like bacteriophage in *S. aureus* harbors the *sasX* gene, coding for a surface protein that promotes the adhesion to nasal epithelial cells [73,74]. Furthermore, prophage-derived genes have been found to encode for effector proteins, delivered by the injectosome to the eukaryotic cell and which are able to interact with the human defense system. For example, *S. enterica* cells that have acquired the prophage Gifsy-1 express two anti-inflammatory effectors, GogB and SarA, during late systemic infection stages. GogB-deficient cells show an increased inflammatory response, enhanced tissue damage and higher bacterial colonization rate. After injection, GogB interacts with the human proteins Skp1 and FBXO22. This interaction results in a downstream inhibition of the NFKB pathway and subsequently a limited tissue damage [75]. Recently, the working mechanism of SarA was revealed. After injection, the effector induces the production of the cytokine IL-10 by immune cells, provoking a lower production of reactive oxygen species (ROS) by macrophages. Hence, both GogB and SarA are attenuating inflammatory responses [76].

Third, bacteriophages can impact bacterial adhesion by the regulation of surface expressed proteins. For example, the conserved cell wall protein CwpV in *C. difficile* is upregulated after integration of the temperate phage ϕCD38-2. The increased CwpV expression results in an enhanced cell aggregation and biofilm formation by the host. Moreover, CwpV has anti-phage actions and thereby serves as superinfection exclusion mechanism in *C. difficile* [77,78]. Similarly, Laumay *et al.* discovered an increased expression of fibronectin-binding proteins by *S. aureus* CC398 harboring specific prophages. In this study, the prophages present in clinical *S. aureus* strains were induced and subsequently introduced in a prophage-free *S. aureus* CC398 laboratory strain. An increase in the production of virulence factors was observed, which

could not be attributed to insertional effects. Hence, it is tempting to hypothesize that those phages carry uncharacterized regulators influencing the expression of bacterial adhesins and so increase the ability to adhere to human extracellular matrix proteins [79].

Prophages as suppliers of bacterial virulence regulators

In a bacterial cell, the expression of proteins, including virulence factors, is tightly regulated. Bacteria can achieve this by the expression of transcription regulator proteins and post-transcriptional regulators [80]. Interestingly, also bacteriophages have evolved a wide range of regulators and in this manner seem able to modify the regulation of bacterial virulence [81]. The bacterial phenotype can be controlled by entire prophages as well as by prophage-encoded regulatory proteins and regulatory RNAs [2].

By integration in the bacterial genome in a site-specific manner, viruses can passively interrupt functions of their host. Canonically, the integration of temperate phages occurs in important loci, resulting in the disruption of a bacterial gene or regulating region [15]. For instance, the prophage ϕ13 integrates within the *hlb* gene of *S. aureus*, leading to the loss of β-toxin production by the pathogen [82]. Likewise, an attenuated virulence of *B. bronchiseptica* is observed by integration of phage PHB09 in its genome, due to disruption of the *pilB* gene [61]. Another sophisticated relationship that has been observed between a phage and pathogen in this context, is the phage-regulatory switch (phage-RS). In response to a specific trigger, this switch induces the excision of the prophage, without the production of new virions or lysis of the host cell [15]. The first observation of such a switch was made in the Gram-positive pathogen *Listeria monocytogenes.* In this bacterium, the *comK* gene is interrupted by the

299 temperate prophage ϕ10403S. During pathogenesis, *L. monocytogenes* is phagocytosed by
300 macrophages, which triggers the temperate phage to excise from the host chromosome. This
301 in turn generates an intact bacterial *comK* gene, enabling the production of the ComK protein
302 and leading to the activation of the Com system. As a result, the bacterium is able to escape
303 from the phagosome into the cytosol of the macrophage for replication. Next, the temperate
304 phage ϕ10403S reintroduces itself within the *comK* gene [83]. Similarly, the prophage ϕSa3mw
305 can excise from the *hlb* gene in *S. aureus* in response to oxidative stress and during biofilm
306 growth. Excision of the prophage reconstitutes the gene encoding for the β-toxin. This toxin
307 promotes host tissue colonization, modulates the immune response and increases the
308 severity of the *S. aureus* infection [84].
309
310 In addition to the regulation by entire prophages, bacteriophages can also actively regulate
311 bacterial behavior via the expression of phage-encoded, regulatory proteins. For instance,
312 some phages have shown to regulate the expression of alternative sigma factors. Those sigma
313 factors are typically induced by the host cell under environmental stress and control the
314 expression of other virulence associated bacterial genes [85]. The phage-derived gp70.1 of
315 phage PaP3 has shown to interact with RpoS of *P. aeruginosa*, causing the direct inhibition of
316 the stress regulator. As a consequence, a decline in production of pyocyanin, polysaccharides
317 and extracellular proteases and a downregulation of the global bacterial motility is observed.
318 Consequently, gp70.1 attenuates the overall virulence of the cell [86]. On the other hand, the
319 phages ϕ11 and ϕ80α in *S. aureus* induce the SigB regulon, probably due to actions of the
320 phage CI protein. This provokes an increased biofilm formation and staphyloxantin secretion,
321 which both positively impact the development of the chronic *S. aureus* infection [87].
322

Besides regulating the expression of alternative sigma factors, phages can actively control quorum sensing (QS) and thus cell-cell communication and virulence factor regulation [88]. Hargraeves *et al.* showed that the bacteriophage PhiCDHM1 of *C. difficile* harbors three genes, *agrB, agrC* and *agrD*, from the accessory gene regulator (*agr*) quorum sensing system. By expression of the phage-encoded quorum sensing genes, the prophage can interfere with the levels of secreted signaling molecules. The consequence of the phage interaction remains unclear, but it may enhance its host's fitness by toxin production or provide protection against secondary phage infections by downregulation of surface molecules [89]. In *V. cholera*, the bacteriophage VP882 encodes for a quorum sensing associated receptor, named VqmA. Upon expression, the phage receptor binds the bacterial auto-inducer 3,5-dimethylpyrazin-2-ol (DPO). Subsequently, the receptor can bind to either phage promotors or host target promotors and tune as well as respond to the host QS system [90].

Apart from tuning bacterial regulators, phages can also directly control the expression of host-encoded virulence factors. This is observed for *C. difficile,* where production of the major exotoxins TcdA and TcdB is shown to be regulated by different temperate phages. *C. difficile* strains harboring the prophage ϕCD119 show a decreased toxin expression from the PaLoc locus. Presumably this is the result of interaction of the promotor of *tcdR,* the regulator of PaLoc, with the prophage-encoded RepR repressor [91]. Likewise, a reduction in toxin production was reported by prophage ϕCD27 [92]. However, the underlying mechanism remains unclear. In contrast, prophages of *C. difficile* can also enhance toxin production. This was observed by both Sekulovic *et al*. and Goh *et al*., where respectively ϕCD38-2 and ϕC2, ϕC6 and ϕC8 resulted in an increased toxin B secretion due to unknown bacteriophage-host interactions [93,94]. In host cells harboring ϕCD38-2, this ensues from an increased transcription

of *tcdA*, *tcdB* and the associated toxin transporter *tcdE*. In contrast, the integration of ϕC2, ϕC6 and ϕC8 resulted in a positive effect on toxin B production, although without an apparent the increase of the transcription of the *toxB* gene. However, observed effects were linked to the investigated parental host strain and thus could not be directly correlated to the prophage [94]. The influence of prophages on toxin regulation was also revealed in Enterohemorrhagic *E. coli* (EHEC), where the transcription factor Cro, derived from lamboid bacteriophages encoding the Shiga toxin, activated the transcription of the type three secretion system (T3SS) [95]. The T3SS is important for the delivery of several toxins to the eukaryotic cytoplasm, for instance the translocated intimin receptor (Tir) and the *E. coli* secreted protein F-like protein from prophage U (EspFu) [96].

It is well-established that RNA molecules exert post-transcriptional regulation functions in prokaryotes [97]. Remarkably, using small non-coding RNAs (sRNAs), prophages can also regulate bacterial translation. Most phage-encoded sRNAs are anti-sense transcripts, i.e. located on the opposite strand of their target gene [98]. As a result from base-pairing close to the Shine-Dalgarno (SD) ribosome binding site between the two RNA strands, a stable inhibitory complex is formed and, in some cases, may even lead to the degradation of the targeted mRNA [99]. For instance in *E. coli,* the sRNA Esr41 encoded on the Sakai prophage-like element (SplE1) is responsible for an increased production of FliC and, consequently, an increase in cell motility. Most likely the overexpression results from the base-pairing between Esr41 and the 5'UTR of *flhDC* mRNA, which encodes for a central flagella activator [100]. In a more recent study, Esr41 was identified in one of the sRNA-mRNA pairs interacting with the endonuclease RNaseE. Expression of Esr41 by the host cell provided resistance against colicin via repression of CirA, a catecholate siderophore receptor. Moreover, the phage sRNA limited

371 the uptake of iron by specific inhibition of CirA, haem receptor ChuA and bacterioferritin Bfr
372 [99]. The sRNA Esr41 thus proved to be highly versatile, interacting with multiple mRNAs. Phage
373 encoded sRNAs that indirectly regulate mRNA translation, by modulating the activity of host-
374 encoded sRNAs, are called anti-sRNAs. As result of the sRNA-sRNA pairing, the bacterial sRNA
375 is sequestered and its function is inhibited [101]. For example, in *E. coli,* the interaction of the
376 anti-sRNA AgxR of Sp5 and the core sRNA FnrS increases the translation of mRNA *chuS*. FnrS
377 naturally represses the translation of *chuS*, which encodes for a heme oxygenase. By
378 interaction of the two sRNAs, this repression is alleviated and a higher bacterial iron uptake
379 is observed. Besides AgxR, the prophage Sp5 also encodes for a second anti-sRNA, AgvB. This
380 small RNA can pair with GcvB, a global regulator of amino acid and peptide transport in the
381 cell. Interaction of the two RNAs relieves the repression of GcvB on its targets and enhances
382 cell growth [102]. In another example, the bacteriophage BTP1 of *S. enterica* serovar
383 Typhimurium encodes an antisense sRNA, STnc6030, providing superinfection exclusion of
384 phage BTP1. The anti-sRNA pairs with the transcript *STMMW_03891* of the DNA-injection
385 gene, destabilizing the mRNA molecule. As this polycistronic mRNA molecule also includes
386 transcripts of structural BTP1 genes, the sRNA-mRNA binding effectively protects the host cell
387 from a secondary infection [103].
388
389 **Filling the gap: applications in biotechnology**
390
391 The improper and excessive use of antibiotics in the past decades did contribute to the
392 development of antibiotic resistance in clinically relevant pathogens [104]. In fact, infectious
393 diseases and the rise of multidrug resistant (MDR) pathogens are considered to be one of the
394 key societal challenges of the 21st century [105]. Therefore, we are heading towards a post-

395 antibiotic era, where new and inventive antibacterial strategies are required [106]. In the hunt
396 for those novel strategies, researchers are aspiring a more sustainable and efficient way to
397 combat pathogens that is more refractory to resistance development and therefore permits
398 a long-term application of the antibacterial strategy [107]. Bacteriophages have proven to be an
399 interesting source for this, since they are evolutionary adapted to bacteria and therefore
400 equipped with specific and diverse host subversion mechanisms [108]. Strict lytic
401 bacteriophages and phage-derived enzymes, like endolysins, are already being exploited for
402 therapeutic purposes [109,110]. Anti-virulence agents have shown to be promising to combat
403 pathogens as well and bypass several unwanted side-effects of classical antibiotics [111–113].
404 Despite the increasing interests in the contribution of temperate phages to bacterial
405 virulence, a huge gap exists between this knowledge and its exploitation for biotechnological
406 purposes. Temperate bacteriophages could be an inspiration for the development of novel
407 vaccine strategies, anti-virulence compounds and alternative phage therapies (Fig. 2).
408

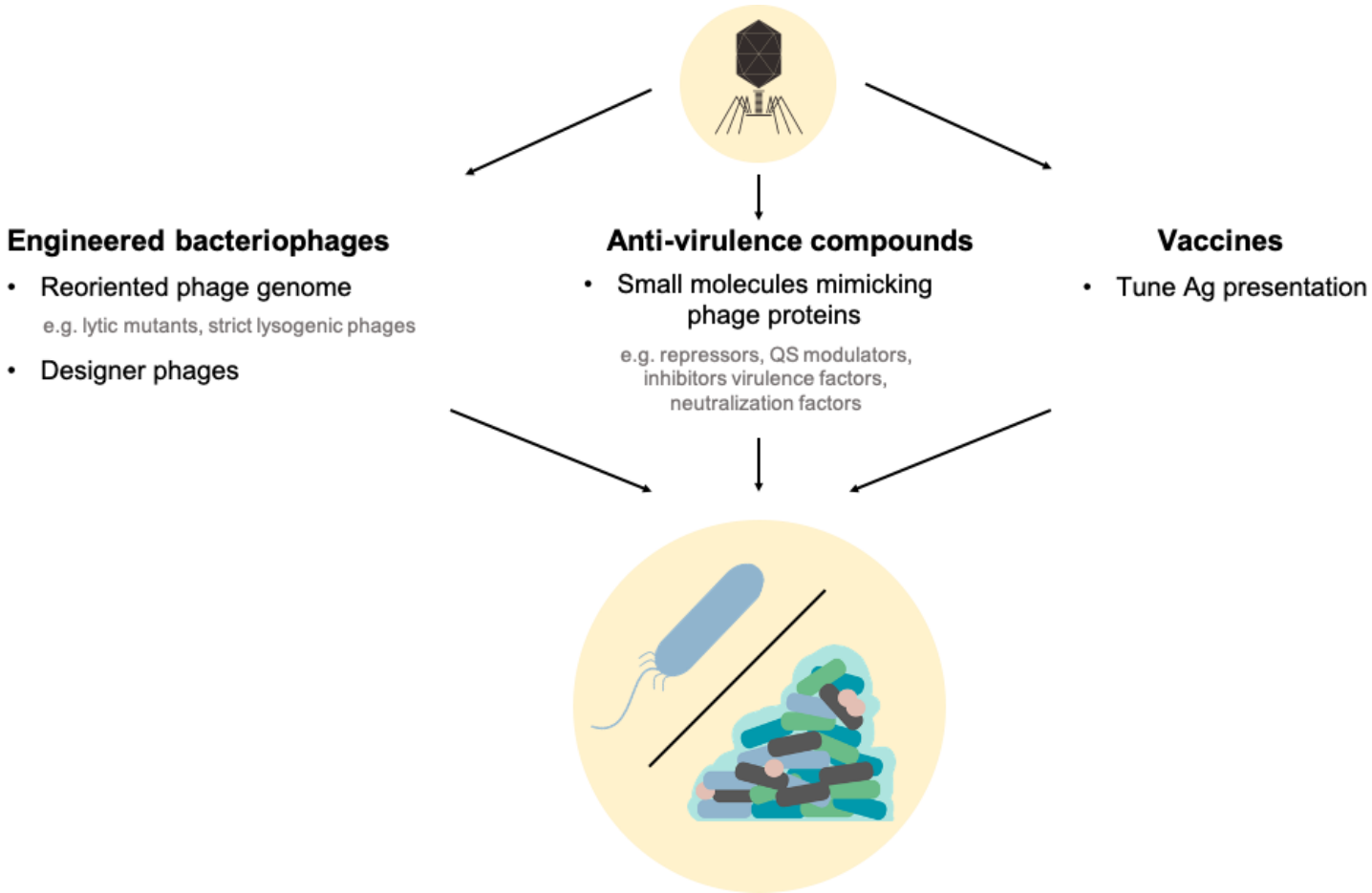

Figure 2: Temperate phages as inspiration for novel antibacterial strategies. Exploitation of temperate bacteriophages for the development antibacterial strategies in three distinct ways: engineered bacteriophages, anti-virulence compounds and vaccines. First, temperate phages could be deployed for the development of alternative phage therapy strategies. By reorienting the phage genome towards strict lytic or lysogenic phages, temperate phages might be used for therapeutic phage therapy. In addition, via synthetic biology, designer phages may be obtained, creating phages with the desired properties. Second, prophage-encoded factors affecting bacterial virulence may be an inspiration for the development of anti-virulence compounds. Third, temperate phages might fulfill a role in vaccine development via the modulation of antigen (Ag) presentation by the vaccine strains.

Engineering phages for therapeutic purposes

The concept of using strictly lytic phages to tackle multidrug resistant pathogenic strains, already dates from the first discovery of bacteriophages by Felix D'Herelle [114]. For a long time, temperate phages were deemed not suitable for phage therapy, due to the risk of horizontally transferring antibiotic resistance genes and virulence factors [115]. However, the synthetic biology field creates opportunities to use temperate phages to combat bacterial infections [116]. By the deletion of specific genes, the viral genome can be reoriented and the desired phage type can be obtained [117]. For instance, the genomes of temperate phages can be adjusted in a way that strictly lytic phages are obtained, called lytic mutants [118,119]. The novel obtained lytic phages could so extend the existing pool of lytic phages available for traditional phage therapy purposes [117]. Furthermore, additional genes could be introduced in the lytic mutant, so that it results in the acquisition of a more deadly phage [119]. Although individual patients have been treated in this way [120], it remains unclear whether this approach would be feasible at a broader scale, as unexpected and undesired interactions between the therapeutic phage and natural prophages could occur. In addition, regulatory hurdles might be present when making use of engineered bacteriophages for therapeutic purposes. For instance in Europe, the regulations concerning phage therapy are currently practiced under 'Unproven Clinical Practice Interventions' (Article 37) of the Helsinki declaration, as well as National government legislations (e.g. in Belgium), as reviewed extensively [121]. In another approach, temperate phages could be created that can integrate within the bacterial genome, yet remain unable to excise from it, for instance by deletion of the phage-encoded excisionase [122]. The most obvious application of this, would be the inhibition of virulence associated genes by phage integration, resulting in the disrupting of the bacterial encoded virulence factor. The

previously mentioned *S. aureus* bacteriophage ϕSa3int and the *B. bronchiseptica* phage PBH09 could be applied to inactivate toxin B and PilB expression, respectively [61,84]. However, the stability of such an inactivation as well as the efficiency of phage infection and bacterial virulence reduction would need to be thoroughly assessed.

Synthetic biology now allows the introduction of specific payloads into the phage genome, leading to the generation of designer phages [119]. These parts can be built in a synthetic gene circuit, which allows the expression regulation of the payloads. The delivery of exogenous genes to the desired bacterial strain occurs by transduction and a stable genomic integration of the temperate phage. Phage-encoded repressors would constitute interesting payloads, since they interfere with the expression of bacterial virulence factors. For instance, the repressors encoded by the phages ϕCD119 and ϕCD27 significantly reduce the secreted amount of toxins by *C. difficile* [91,92]. In addition, phage-repressors could be modified in such a way that a desired effect is obtained. In a recent study, the expression of the Shiga toxin by *E. coli* cells was inhibited using engineered temperate phages. Here, the temperate phage λ was utilized to create an 'anti-virulence' phage, expressing the engineered, non-degradable Shiga toxin-repressor 933W-CI^{Ind-}. A site-specific mutation was introduced in the CI protein of phage 933W, so that induction of the lytic cycle and thus expression of the Shiga toxin was prevented. Upon infection of the *E. coli* cells by the engineered phage, Shiga toxin production was inhibited both *in vitro* and in the mouse gut [123]. Furthermore, non-phage derived genes could be used as payloads. For instance, the temperate phage M13 of *E. coli* was engineered to overexpress the bacterial SOS response repressor *lexA*. Host cell infection by this bacteriophage resulted in an increased killing of the *E. coli* cells by DNA damaging quinolones [124]. Similarly, Edgar *et al.* generated engineered λ phages carrying the genes *rpsL* and *gyrA*.

When infected by those temperate phages, *E. coli* cells with resistance mutations in the antibiotic target genes, became more sensitive towards the antibiotics streptomycin and nalidixic acid, respectively [125]. This proof-of-concept study shows an intriguing example on how temperate phages can potentially increase the lifetime of antibiotics by exploiting horizontal transfer of specific genes and might resensitize MDR pathogens to antibiotic treatments.

Phage-derived anti-virulence compounds

Compounds arresting critical processes which are necessary for bacterial cell survival are traditionally used as antibacterial factors [126]. In this regard, toxic phage proteins have shown to be potential candidates for this since they are often targeting crucial protein complexes in the host cell [127]. Nevertheless, these phage components still place a burden on the cell and therefore remain susceptible to the development of bacterial resistance [128]. In contrast, by targeting important virulence factors of pathogenic bacteria, attenuated cells emerge without directly killing the pathogen [129]. By exerting limited selective pressure on the bacterial cell, this strategy has the advantage of bypassing the development of resistance. Either as stand-alone therapy or in combination with traditional antibacterial components, virulence-targeting compounds could also eradicate persistent chronical infections [126]. The option of using small molecules mimicking phage-derived proteins that impact bacterial virulence is an appealing approach to tackle pathogens. One possible strategy is to make use of phage proteins that target central regulators of bacterial virulence. In the prophage φSa3 of *S. aureus,* the transcription regulator SA1804 was discovered, which acts as a global regulator of virulence factors' expression. This phage repressor reduces the expression levels of 17 of

the 18 known virulence factors in *S. aureus* [130]. Likewise, gp70.1 of *P. aeruginosa* phage φPap3 interacts with the RNA polymerase sigma factor RpoS of both *P. aeruginosa* and *E. coli.* At the onset of the interaction, direct and indirect inhibitory effects on the expression of numerous virulence factors are observed [86].

Besides the traditional cell regulators, it is known that also quorum sensing can play a key role in the regulation of virulence factor expression [131]. It thus may be of interest to target the bacterial quorum sensing system and thereby modulate the bacterial group behavior [132]. Leblanc *et al.* demonstrated that the aquatic phage φPLPE of the non-human pathogen genus *Iodobacter*, encodes a putative acylhydrolase. This enzyme degrades N-acyl-homoserine lactone (AHL) molecules and so interferes with the QS system [133]. AHL is the main QS signaling molecule used by Gram-negative bacteria, including the opportunistic pathogen *P. aeruginosa*. Previous studies revealed that by breaking down QS autoinducers, like AHL, the QS regulation system is interrupted without affecting bacterial growth [134]. Similarly, our lab recently discovered the quorum sensing targeting protein (Qst) encoded by *P. aeruginosa* phage LUZ19, which modulates the *Pseudomonas* quorum sensing (PQS) pathway. As a result decreased levels of the PQS precursor, 2-heptyl-4(1H)-quinolone are measured [135]. In similar fashion, phage ϕDMS3 of *P. aeruginosa* contains the anti-quorum sensing protein 1 (Aqs) and inhibits both LasR, a major QS regulator, and PilB by direct interaction. Thereby, an individual phage protein showed to be able of dampening the quorum sensing mechanism of *P. aeruginosa*, as well as prevent the assembly of T4P on its surface [136].

While some phage proteins target multiple virulence factors by interacting with a global regulator, others may only target a single virulence factor. The anti-virulence factor GvrA of

513 the phage Gifsy-2, is able to reduce the virulence of *S. enterica* cells. However, this decrease
514 is only observed when the cell harbors another phage encoded factor, SodCI, which enables
515 the bacterium to survive within macrophages. Presumably, the enzymatic activity of SodCI,
516 coding for a superoxide dismutase, is either directly or indirectly affected by GvrA, resulting
517 in an overall diminished virulence [137]. Other phage proteins have been shown to affect the
518 motility of *P. aeruginosa.* Chung *et al.* discovered that the phage twitching inhibitory protein
519 (TIP) interacts with PilB and prevents pili assembly as a consequence [60]. In similar fashion,
520 multiple phage morons were identified by Tsao *et al.* that inhibit twitching or swimming
521 motility [50]. The development of small molecules which mimic those phage proteins, might be
522 an interesting anti-virulence approach. This approach still has a lot of unexplored potential,
523 since many prophages inhibit cell motility in *P. aeruginosa*. For instance, the lytic phage
524 PA5Oct and the temperate phages DMS3 and D3112 have all been proven to reduce twitching
525 motility. The observed effects could not be attributed to an effect of integration, which might
526 indicate the presence of inhibitory phage proteins [138–140].
527
528 Phage proteins might also be exploited because of their potential to modulate the eukaryotic
529 immune system via the neutralization of the cell surface. Apart from causing direct lysis of
530 cells, bacteriophage lysins have also shown to be able to attach to cell wall of Gram-positives,
531 and in this way reduce their toxicity. For example V12CBD, the cell wall binding domain (CBD)
532 derived from of the prophage-encoded PlyV12 enzyme, showed to harbor anti-virulence
533 capacities. After binding to the cell wall carbohydrates by the engineered protein, the
534 virulence of *S. aureus* was attenuated. Moreover, the expression of multiple virulence factors
535 was reduced twentyfold and cells were more susceptible for macrophage uptake [141]. In Gram-
536 negative pathogens, the key virulence factor LPS may provoke harmful events in the human

host and LPS-neutralization by antimicrobial peptides did already show promising results [107]. Moreover, tail-associated phage proteins may be of interest for LPS neutralization. Olszak *et al.* showed the binding and cleavage of the O-Ag of *P. aeruginosa* cells by gp49 of bacteriophage LKA1. Administration of the phage protein resulted in *an in vivo* decreased virulence in the model organism *Galleria melanogaster* [142]. Similarly, the tail adhesin gp12 of bacteriophage T4 has the ability to absorb to the LPS layer of *E. coli* cells. When recombinantly expressed, a decrease in the inflammatory response in mice was observed, provoking lower levels of the pro-inflammatory cytokines IL-1α and IL-6 [143]. In similar fashion, Oliviera *et al.* recently proposed a tail spike treatment against *Providencia stuartii* infections, consisting of the phage protein gp52. This tail spike protein, which degrades specifically the protective EPS capsule surrounding *P. stuartii* cells and thereby exposes the cells to the complement system, is in this manner able to enhance the serum bactericidal activity against the pathogen [144].

Phage-inspired vaccine development

In addition to the development of novel therapeutic strategies against MDR pathogens, vaccination could be a powerful tool to impede infection initiation [145]. Indeed, the induction of a protective immune response by vaccines will ultimately result in a diminished use of antibiotics [146]. Unfortunately, despite numerous attempts, there are currently no vaccines available against *S. aureus* and *P. aeruginosa*, two major MDR pathogens [147]. The principal biological hurdle for the development of vaccines against these nosocomial pathogens, is their large genomic versatility. Both bacterial strains harbor a diverse set of virulence factors, which are all regulated in a complex and intricate manner [148,149]. In addition, the expression of virulence factors and their regulation may not be conserved across different serotypes,

making vaccine development even more challenging [150]. For instance, most *P. aeruginosa* vaccines primarily result in the generation of LPS-specific antibodies. However, LPS epitopes are highly variable between, and even within, serotypes and those vaccines thereby fail to provide a broad-spectrum immune protection against all *P. aeruginosa* strains [151]. Traditional vaccine development approaches are therefore inherently flawed and can never encompass these unique traits of these MDR pathogens. To date, most vaccines being developed are subunit, killed or attenuated vaccines [152,153]. The former has the limitation of only containing individual or few antigens and thereby fails in the generation of a broad protection against all serotypes. Conversely, killed or attenuated vaccines may provoke weak protective immune responses or be potentially toxic. Altogether, innovative solutions in vaccinology are required and the conventional approaches need to be challenged. Synthetic biology may provide opportunities to overcome the challenges concerning vaccine development against those important, versatile pathogens [154].

From this perspective, we would like to postulate that bacterial viruses could be an important part of the solution here. Since temperate phages have been identified in almost all human pathogens, an increasing interest in how they could be exploited for vaccine development has been put forward [155]. Temperate phages influence immunogenic bacterial virulence factors in various ways. By exploiting this interesting characteristic, we might be able to tune the expression of a selection of virulence factors, by introducing specific phage genes into the bacterial vaccine strain, and thereby steer the protective immune responses of patients. For instance, several papers hint at the potential of outer membrane proteins (OMPs) as targets for the generation of a protective immune response against *P. aeruginosa, S. enterica* serovar Typhimurium and *Acinetobacter baumanii*, based on their conservation among different

585 clinical isolates [156–158]. In similar fashion, the expression of the cell wall protein CwpV in *C.*
586 *difficile* strain R20291 is enhanced by the prophage ϕCD38-2 [159]. By tracking down the
587 underlying mechanisms, a potentially interesting phage-derived synthetic circuit could be
588 created, which enables the upregulation of these specific bacterial proteins. However, as
589 CwpV also contributes to cell-cell aggregation and is important for tissue colonization, potent
590 safety measures would be required [78]. In addition, isolated clinical *C. difficile* strains revealed
591 the antigenic variability of this surface protein, making it a less attractive target for vaccine
592 development. Future studies might reveal similar interesting phage-encoded mechanisms in
593 other bacterial pathogens, which are enhancing the expression of immunogenic antigens and
594 could be exploited for prophylactic therapeutics.
595
596 **Conclusion and perspectives**
597
598 A straightforward predator-prey relationship between bacteriophages and bacteria does not
599 exist, as intricate and subtle interaction strategies are observed. Whereas lytic phages drive
600 bacterial evolution by the diversification of phage receptors, temperate phages display a
601 rather symbiotic relation with their hosts [12]. The huge diversity of bacteriophages and their
602 characteristics, and the already described effects of prophages on bacterial cells suggest that
603 many more interesting phage-encoded mechanisms will be revealed in the future [160,161].
604
605 The current antibiotic crisis places the need for development of novel antibacterial strategies
606 front and center. In this regard phage therapy, phage-derived enzymes and phage-based
607 nanoparticles are proving promising complements, as extensively reviewed [162–165]. Within this
608 context, temperate phages also show potential as an alternative antibacterial [117,128].

609 However, this potential remains largely unexplored and more research is needed to elucidate
610 the intricate mutualistic relationship between clinically relevant pathogens and their
611 parasites [166]. This will ensure a better understanding of the bacterial pathogen as well as an
612 increased insight on how we might exploit temperate phages for biotechnological purposes.
613 In addition, the "viral dark matter", a term coined for the unknown phage proteins, has only
614 been studied within a narrow scope, focusing on the elucidation of toxic phage proteins and
615 their direct antibacterial application [127]. However, this viral dark matter is likely to also hold
616 valuable tools for the development of -among others- anti-virulence drugs and phage-based
617 vaccines [128]. Furthermore, the synthetic biology field provides opportunities for new phage-
618 based applications, for instance by the engineering of phage parts or by the creation of
619 synthetic circuits. The former approach may be used to tailor the mechanisms of phage-
620 derived parts towards desirable properties [116]. In addition, by carefully selecting phage-borne
621 elements for circuit development, the virulence of the target cell could be tuned. Phage genes
622 coding for virulence modulation factors and phage-encoded sRNAs both could serve as
623 potential payloads. In addition, these regulating sRNAs take up limited coding space, making
624 them ideal payloads for the engineering of therapeutic, temperate phages [81]. Future
625 bacteriophage-related studies will enlarge the pool of available payloads, both at protein and
626 RNA level, and reveal alternative ways to regulate virulence factor expression.
627
628 The implementation of anti-virulence strategies involving engineered phage or designer
629 bacteria, are intrinsically linked to typical concerns for SynBio applications, including
630 regulatory hurdles, bio-containment and dual use. By incorporating safety measurements,
631 inactivation of the modified phages occurs and environmental spread is prevented when the
632 particles are released in nature [167]. Such, bio-containment strategies will likely form the crux

of accepting designer phage in therapeutic settings [168], although individual examples on the use of tailored temperate bacteriophages exist [169]. Other characteristics, including phage propagation, penetration or neutralization by the immune system remain intrinsically linked to the use of phage [170,171]. As such, expanding our fundamental understanding on the interplay between phage, bacteria and the immune system will be required to fully unlock the biotechnological potential of bacteriophages.

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Conflict of interest

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