Delft University of Technology

Exploiting Bacteriophage Proteomes
The Hidden Biotechnological Potential

Santos, Sílvio B.; Costa, Ana Rita; Carvalho, Carla; Nóbrega, Franklin L.; Azeredo, Joana

DOI
10.1016/j.tibtech.2018.04.006

Publication date
2018

Document Version
Accepted author manuscript

Published in
Trends in Biotechnology

Citation (APA)
Santos, S. B., Costa, A. R., Carvalho, C., Nóbrega, F. L., & Azeredo, J. (2018). Exploiting Bacteriophage Proteomes: The Hidden Biotechnological Potential. *Trends in Biotechnology*. https://doi.org/10.1016/j.tibtech.2018.04.006

Important note
To cite this publication, please use the final published version (if applicable).
Please check the document version above.

Copyright
Other than for strictly personal use, it is not permitted to download, forward or distribute the text or part of it, without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license such as Creative Commons.

Takedown policy
Please contact us and provide details if you believe this document breaches copyrights.
We will remove access to the work immediately and investigate your claim.

This work is downloaded from Delft University of Technology.
For technical reasons the number of authors shown on this cover page is limited to a maximum of 10.

© 2018 Manuscript version made available under CC-BY-NC-ND 4.0 license https://creativecommons.org/licenses/by-nc-nd/4.0/

1 # Exploiting bacteriophage proteomes: the hidden biotechnological
2 potential

3

4 Sílvio B. Santos, Ana Rita Costa, Carla Carvalho#, Franklin L. Nóbrega#, Joana Azeredo*

5

6 Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057,
7 Braga, Portugal

8

9 * Correspondence: jazeredo@deb.uminho.pt (J. Azeredo)

10 Tel. + 351 253 604 419 Fax. + 351 253 604 429

11 # Present addresses: Carla Carvalho, International Iberian Nanotechnology Laboratory
12 (INL), Braga, Portugal; Franklin L. Nóbrega, Department of Bionanoscience, Kavli
13 Institute of Nanoscience, Delft University of Technology, Delft, The Netherlands

14 https://www.ceb.uminho.pt/bbig; https://www.ceb.uminho.pt;
15 https://www.facebook.com/cebuminho

16

17 ## Abstract

18 Bacteriophages encode many distinct proteins for the successful infection of a
19 bacterial host. Each protein plays a specific role in the phage replication cycle, from
20 host recognition, through takeover of the host machinery, and up to cell lysis for
21 progeny release. As the roles of these proteins are being revealed, more
22 biotechnological applications can be anticipated. Phage-encoded proteins are now
23 being explored for the control, detection and typing of bacteria; as vehicles for drug
24 delivery; and for vaccine development. In this review we discuss how engineering
25 approaches can be used to improve the natural properties of these proteins, and set
26 forth the most innovative applications that demonstrate the unlimited
27 biotechnological potential held by phage-encoded proteins.

28

29 **Keywords:** Phage-encoded proteins; genetic engineering; host specificity; bacteriolytic
30 activity; bacteria control and detection; anti-CRISPR.

31

32 ## Bacteriophage research reloaded

33 **Bacteriophage** research is being driven by the global threat of antibiotic resistance,
34 resulting in an increasing wealth of knowledge on phage genes and proteins.
35 Simultaneously, recent progress in sequencing technologies, DNA manipulation and
36 **synthetic biology** approaches has been fostering phage **proteome** exploitation and
37 engineering of specific phage proteins into improved forms. Innovative research on
38 phage-encoded proteins is thus now progressing quickly.

39 Having been a central part of molecular biology for many years, phage-encoded
40 proteins are now being explored in health, industrial, food, and agricultural settings,
41 for purposes not limited to bacteria control. Many applications have been envisioned
42 and this is only considering the low percentage of phage proteins of known function.
43 Although some of the applications found for phage-encoded proteins may be
44 performed by the phage itself, the use of phage proteins instead may have strong
45 advantages in terms of regulation and public acceptance, and also in manipulating
46 them for improved properties.

47 Phages have spent billions of years evolving and developing a powerful protein
48 armamentarium to recognize, infect and kill bacteria in a very efficient way.
49 Understanding the phage replication cycle is key to identify the proteins involved (**Box
50 1**, **Figure 1, Key Figure**), to discern their specific function, and thus to unveil the
51 potential held for biotechnology. The particular applications in which phage proteins
52 can be employed are defined by their intrinsic properties, the technologies at our
53 disposal, and our creativity.

54 Here we overview the most recent progress reported on the use of phage-encoded
55 proteins and highlight their most innovative uses, showcasing the virtually unlimited
56 biotechnological opportunities hidden in bacteriophage genomes.

57

58 ## Receptor binding proteins

59 Specificity is a fundamental aspect of phage-host interaction and depends upon the
60 phage receptor binding proteins (RBPs). These highly variable structures are part of the

61 phage particle (**Figures 1A, 1C and 2A**) and make the first contact with the host, being
62 responsible for recognizing specific receptors on the cell surface. Therefore, RBPs are
63 powerful tools for specific pathogen detection (**Figure 2**), and more recently have
64 shown potential in diagnostics and therapy.

65 RBPs have been successfully employed for pathogen detection in food in a rapid,
66 sensitive and specific manner using methods as RBP-based magnetic separation
67 combined with PCR [1], Enzyme-Linked Long Tail Fiber Assay (ELLTA) employing RBP-
68 coated paramagnetic beads [2], and RBP-coated long-period gratings [3] and
69 interdigital capacitors [4]. These proteins overcome some of the limitations of
70 antibodies that hamper their use in *in situ* applications, *e.g.* pH, temperature and
71 protease sensitivity, while exhibiting comparable or even superior specificity and
72 affinity. RBP-based detection systems can already be found in the market: bioMérieux
73 commercializes the *Vidas Up* kit for the *in situ* detection of foodborne pathogens.

74 RBPs are specific enough that they can distinguish **glucosylation** variants of O-antigens
75 when classical methods fail to do so [5]. This is relevant for understanding bacterial
76 **immunogenicity** and spread of disease, and may also be employed for monitoring
77 phase variations during large scale O-antigen generation for vaccine production [5]. In
78 fact, the binding affinity and specificity of RBPs have also found application in bacteria-
79 based processes of therapeutic protein production, for the detection and removal of
80 bacterial endotoxin contaminants [6]. One product with this purpose is commercialized
81 by Hyglos GmbH (http://www.hyglos.de). Alternatively, the specificity of some RBPs
82 for the bacterial **lipopolysaccharide** (LPS) may be explored for modulating and
83 counteracting the effects of LPS-induced inflammatory response *in vivo*[7]. While
84 natural RBPs have proven useful, progress in synthetic biology has created new
85 opportunities for the design of recombinant phage-derived proteins with enhanced
86 properties and novel applications. For example, introduction of specific tags like a Cys-
87 tag [8] to the RBPs originated an oriented immobilization of the proteins onto
88 surfaces, significantly improving their capture efficiency and performance as detection
89 probes. Also, deleting the endorhamnosidase enzymatic activity of a tail spike, a phage
90 RBP (**Figure 1A and 1C**), led to improved performance of the protein as a detection
91 probe [8]. This enzymatic activity is undesirable for diagnostic (and capture) purposes

92 because it causes hydrolysis of bacterial LPS preventing “irreversible” binding of the
93 RBP to the bacterial surface. Functional analysis of RBPs identified the C-terminal
94 domain as responsible for recognition and binding to the host receptor [9]. These C-
95 terminal domains can thus also be used to develop detection tools (**Figure 2**). This can
96 be advantageous for recombinant expression and application in diagnosis providing a
97 broader host spectrum and higher specificity and sensitivity in the detection [10].
98 Furthermore, RBPs both in the intact and truncated versions can be engineered to
99 broaden, narrow or acquire new specificities for different targets, by substituting one
100 or a few amino acids allowing a wider range of application [11].

101 Because RBPs are highly diverse, *in silico* identification may be difficult. Functional
102 analysis is thus usually required which can be time consuming and limit the
103 identification of novel RBPs. Moreover, the structural nature and **multimerization** of
104 RBPs often requires alternative cloning and expression methodologies (*e.g.* inclusion of
105 chaperones) to avoid insoluble and misfolded proteins. These are still a limiting step to
106 the development of novel RBP-based biotechnological applications.

107 Due to the inherent high specificity and affinity of RBPs, most of their applications are
108 being directed toward pathogen detection, whereas not much has been published for
109 pathogen control. Still, a few studies have explored the use of RBPs with enzymatic
110 activity to reduce bacterial colonization *in vivo* [12]. Given the specificity of RBPs, it is
111 particularly worth hypothesizing their usefulness in the design of targeted and tailor-
112 made antimicrobials by fusing RBPs with unspecific drugs. Also, fusion with a peptide
113 able to induce phagocytosis and/or agglutination could improve the immune response
114 against specific problematic pathogenic bacteria targeted by the RBP.

115

116 Depolymerases

117 Some bacteria have developed a capsular structure, usually composed of
118 **polysaccharides**, intimately associated to the cell surface. The capsule provides
119 multiple advantages to bacteria, like protection against host immunity, antibiotics and
120 desiccation, and increased adherence to host cells and surfaces, thus playing an
121 important role in virulence [13]. Moreover, capsules also protect bacterial cells from

122 phage infection [14]. Nevertheless some phages have evolved to use the capsule as an
123 adsorption receptor and to degrade its capsular polysaccharides (CPS) [15].
124 Degradation of the CPS allows phages to penetrate the capsule and gain access to the
125 receptor on the outer membrane of the cell for DNA ejection [16]. These phage-
126 encoded enzymes, known as depolymerases, are typically present as part of the phage
127 structure (*e.g.* as part of RBPs) [17], but may also be in a free form diffused in the
128 medium (*i.e.* depolymerases encoded in the phage genome that are not part of the
129 phage particle, and are released during host cell lysis). Specificity is their main feature,
130 digesting only certain types of polysaccharides [15, 18].

131 The properties of depolymerases anticipate their use for multiple purposes. Since
132 depolymerases deprive bacteria of their capsule, they reduce bacterial virulence and
133 render the cells sensitive to host defenses such as **phagocytosis**. The enzymes may
134 thus be employed as **adjuvants** of the host immune system, as proven both *in vitro*
135 [15] and *in vivo* with high rates of animal survival [15, 19]. Additionally, released
136 polysaccharides can be used as **immunogens** for **glycoconjugated vaccine** production
137 [20].

138 There is also growing interest in the use of depolymerases as anti-biofilm agents with
139 applications in health and industrial sectors: most phages infecting exopolysaccharide
140 (EPS)-producing bacteria have depolymerases, and some of them are able to disrupt
141 the **biofilm** by degrading the EPS [21]. By disruption and dispersal of the biofilm matrix,
142 the enzymes can help the activity of antibiotics [22], disinfectants [23] or even other
143 phages against the typically highly resistant biofilms, facilitating the penetration of
144 these agents across the biofilm. Depolymerases were also observed to prevent biofilm
145 formation [24], suggesting the use of depolymerases for surface coating to avoid
146 bacterial colonization.

147 The main feature of depolymerases, which is their specificity, may constitute a
148 limitation to control bacteria presenting different capsule types. This can be overcome
149 by engineering approaches, or simply by using enzyme cocktails targeting different
150 CPS/EPS. Still, the extraordinary specificity of depolymerases for capsular types can be
151 harnessed for diagnosis and typing applications [15] and as a tool in the rapidly
152 growing field of **glycobiology**, *e.g.* for determining glycan profiles [25].

153

154 ## Endolysins

155 Double-stranded DNA (dsDNA) phages have evolved a lytic system for the release of
156 newly formed virions trapped inside the bacterial cell after replication, mostly based
157 on two proteins: endolysin and holin. Endolysins are peptidoglycan hydrolases that
158 accumulate in the cytoplasm of the host cell until the holin forms pores in the plasma
159 membrane, giving access to endolysins to degrade the peptidoglycan of the cell wall
160 and cause cell lysis [26].

161 The inherent bacteriolytic activity of endolysins immediately suggested their
162 antimicrobial potential, which was promptly supported by initial findings on the
163 enzyme´s ability to lyse Gram-positive bacteria when added exogenously [27]. The high
164 potential to control Gram-positive bacteria, coupled with a high specificity, lack of
165 known toxicity, and unlikeliness of development of bacterial resistance [28], has made
166 endolysins the most explored phage-encoded proteins so far. Research has proven the
167 capacity of these enzymes to control localized [29, 30] and systemic [29] infections of
168 Gram-positive pathogenic bacteria both *in vitro* and *in vivo* [31, 32]; to efficiently
169 remove recalcitrant biofilms [33]; to prevent foodborne diseases by controlling for
170 example milk [34] and fruit pathogens [35] contributing thus for food safety; and also
171 as disinfectants on surfaces and equipment [34]. Endolysins can also be used in
172 combination with other treatments, such as **high hydrostatic pressure processing** to
173 control pathogens in low processed, ready-to-eat food products [36].

174 The modular structure of endolysins (**Figure 3**) targeting Gram-positive bacteria,
175 composed of an enzymatic catalytic domain (ECD) and a cell wall binding domain (CBD)
176 connected by a linker [37], rapidly suggested the opportunity to engineer new
177 endolysins with improved properties by combining different domains. Indeed, swap or
178 combination of CBD domains has proven successful both for expanding the
179 bacteriolytic spectrum [38], improving the anti-biofilm properties [39], and for
180 increasing the activity [40], stability and solubility [41] of endolysins. It is thus
181 foreseeable that engineering approaches will allow for the creation of **chimeric**
182 endolysins targeting any and all desired Gram-positive bacteria.

183 The success of endolysin engineering is evident, with some formulations currently in 184 clinical trials or reaching the market. Staphefekt SA.100 is an engineered phage 185 endolysin to treat methicillin-sensitive and methicillin-resistant *S. aureus* skin 186 infections [30], commercialized by Micreos and available in Europe in cream or gel 187 formulations (https://www.gladskin.com/en/). Another formulation, SAL200, 188 containing the recombinant endolysin SAL-1 against methicillin-resistant *S. aureus* [42], 189 reported no adverse effects when administered intravenously in a first-in-human 190 phase 1 study [43], and was recently successful in the treatment of chronic *S. aureus*-191 related dermatoses in three patients, with no signs of resistance [30].

192 The high efficiency of endolysins against Gram-positive bacteria is not observed for 193 Gram-negative cells due to the existence of an outer membrane protecting the 194 peptidoglycan and obstructing the access of endolysins from the outside. Some 195 strategies have been designed to overcome this limitation (**Figure 3**). For example, the 196 combination of endolysins with outer membrane permeabilizers gave them access to 197 the Gram-negative cell peptidoglycan and rapidly reduced cells by several orders of 198 magnitude [44], proving that endolysins are not limited to Gram-positive bacteria. 199 Recently, genetic engineering gave rise to a new generation of lytic enzymes, known as 200 Artilysins®, with improved antibacterial activity on Gram-negative cells [45, 46]. 201 Artilysins are thus engineered proteins composed of an endolysin and an **outer** 202 **membrane permeabilizing peptide** or **cell penetrating peptide** able to give the 203 endolysin access to the peptidoglycan [45, 46].

204 Artilysins have also been created to improve the properties of endolysins targeting 205 Gram-positive bacteria, having shown an improved bactericidal activity and reduced 206 dependence on external conditions [47].

207 Furthermore, although Artilysins have not been thought for such purpose, fusions of 208 endolysins or other lytic phage proteins with cell penetrating peptides may also have 209 applications in the control of intracellular pathogenic bacteria, which are usually 210 refractory to both antibiotics and the immune system. This strategy explores the 211 capacity of cell penetrating peptides to cross epithelial cell membranes.

212 Beside their obvious application on bacteria control, endolysins have been used also as 213 tags to improve crystallization [48], solubility and purification of recombinant proteins

214 [49]. In a distinct approach, the ECD domain of endolysins was used as an alternative
215 to sonication or high pressure homogenization for the release and purification of
216 proteins expressed in *Escherichia coli* [50], a more amenable process for large scale
217 protein isolation. **Bacterial ghosts** can also be produced using endolysins for the
218 development of non-living vaccine candidates [51].

219 The recognition elements of endolysins, CBDs, are highly specific for certain
220 peptidoglycan types. This specificity has been exploited for the construction of simple,
221 rapid, and cost-effective **biosensors** for bacterial detection, *e.g.* in diagnosis, with
222 results superior to those of antibody-based approaches [52, 53]. The use of a CBD as
223 the recognition element conjugated with colloidal gold nanoparticles (that produce a
224 colorimetric signal) in a nitrocellulose-based lateral flow assays a good example of such
225 biosensors [52].Interestingly, CBDs were reported capable of detecting not only
226 bacteria but also their spores, with important applications for food industry,
227 significantly decreasing the detection time [54]. In a distinct approach, CBDs have been
228 used as the targeting element of antimicrobial nanoparticle conjugates for the specific
229 delivery of antimicrobials to pathogenic bacteria [55]. Sharing similar features, *i.e.* high
230 specificity and affinity, CBDs and RBPs have been explored with similar approaches and
231 are expected to have a comparable role in biotechnology (**Figure 2**).

232

233 Holins

234 As a part of the lytic system of dsDNA phages, holins play two fundamental roles: they
235 create holes in the inner membrane for the release of the endolysins, and determine
236 the timing for the end of the infection cycle. Holins accumulate in the inner membrane
237 of the cell with no effect on its integrity until they reach critical concentration that
238 triggers holin activation [56]. Two types of holins have been described: **canonical**
239 **holins** that form large pores and **pinholins** that form small pores [57].

240 Holins can cause cell death independent of endolysins and, unlike these, have a broad-
241 spectrum unspecific antibacterial activity against both Gram-positive and Gram-
242 negative bacteria [58]. These features have attracted interest towards the application
243 of holins in bacterial control, although perhaps limited to disinfection of surfaces or

244 foodstuff due to the lack of specificity [58]. For applications requiring specificity, it may
245 be possible to fuse holins to a peptide that specifically binds to the target bacteria,
246 since fusion has been shown not to affect holin activity [59]. For this purpose, CBDs of
247 endolysins and phage RBPs may be an option.

248 The combination of holins with endolysins was shown to be a possible approach to
249 control Gram-positive bacteria with higher efficiency than endolysins alone [60]. A
250 similar strategy, fusing holin and endolysin, may also be an option to control Gram-
251 negative pathogens with high efficiency. Holins would form pores on the outer
252 membrane allowing access of the attached endolysins to the peptidoglycan layer of
253 these bacteria. This **hybrid** protein could be a broader-spectrum alternative to the
254 previously described Artilysins, although not evaluated so far.

255 The lethality of holins is associated to loss of viability due to the holes formed on the
256 cell membrane, and not to cell burst [63]. While canonical holins form holes that can
257 be crossed by proteins or protein complexes up to 500 kDa [64], pinholins form much
258 smaller pores and may thus be an excellent option to target **endotoxin**-containing
259 bacteria, preventing the release of their toxic content.

260 The biotechnological prospecting of holins is not limited to bacterial control; they have
261 shown promise also in cancer treatment. Gene therapy using cytotoxic proteins to
262 treat cancer is being intensively studied. The ability of holins to form lesions on the
263 bacterial membrane suggested their cytotoxic activity on eukaryotic tumor cells. The
264 expression of a gene encoding the lambda holin inside eukaryotic cells under a tightly
265 controlled expression system substantially reduced cell viability *in vitro* and inhibited
266 tumor growth *in vivo* demonstrating the potential of holins as a new therapeutic
267 protein for cancer gene therapy [65]. Additionally, the combination of holin and
268 endolysin in plasmids presents a successful alternative for the creation of bacterial
269 ghosts of high immunogenicity for the development of non-living vaccine candidates
270 [61, 62, 66].

271 Holin/endolysin combinations can be used for induction-controlled delivery of antigens
272 into the cytoplasm of mammalian cells. Attenuated bacteria are engineered to possess
273 the desired antigen and invade mammalian cells due to a natural intracellular
274 parasitism. Bacteria then deliver their antigenic cargo by autolysis caused by

expression of the cloned holin/endolysin genes under the control of specific promoters that respond only to the intracellular environment of mammalian cells [67]. This approach may be used for delivering other cargoes, including bacteriophage-based proteins to control intracellular bacterial pathogens and modulate their causative infection.

The controlled expression of a holin/lysin system was also applied for the purification of **minicells**, themselves used as drug/gene-delivery systems [68]. Often minicell batches are contaminated with parent cells, a critical disadvantage for their practical application due to possible endotoxin release in human cells. Induction of the cloned holin/endolysin system will result in autolysins of the parent cells, thus reducing contamination of the final product. This method not only allows for an efficient separation of highly pure minicells but also avoids the inconvenient conventional multi-step purification approach. The holin/endolysin strategies can also be applied as an economic and simple alternative for the release of products from microbial cells in industrial production of **bio-based chemicals** [69]. The production of intracellular compounds requires cell lysis for product recovery, usually achieved by expensive chemical and mechanical cell disruption methods that add further complexity to the downstream purification processes. The use of phage holin/endolysin systems under the control of a green-light regulated promoter significantly enhances intracellular compound release due to an increased fragility of the cell membrane. This system thus provides recovery of cell compounds with minimal contaminants and energy, avoids the use of conventional chemical inducers using a photosynthetic regulated promoter, and increases safety of **genetically modified organisms** (GMOs) since the modified cells are unable to survive under sunlight if accidentally released in the environment.

Further developments on holin applications will depend on progress on the processes of cloning and high-yield expression of these proteins. The lethality of holins to expression cells and their low solubility as membrane proteins are currently a significant limitation.

Structural murein hydrolases

305 Phages of both Gram-positive and Gram-negative bacteria have been found to employ
306 virion-associated peptidoglycan hydrolases (VAPGHs) at the initial stage of phage
307 infection. These enzymes locally degrade the cell wall peptidoglycan of the host,
308 allowing the phage to eject its genome into the host cell [70]. Whether these enzymes
309 are used in every infection or only under less optimal conditions is still up to debate
310 [71].

311 Although they are structural enzymes, VAPGHs share some features with endolysins,
312 *i.e.* high substrate specificity, peptidoglycan cleavage mechanisms, and modular
313 structure. This not only suggests an antimicrobial potential similar to endolysins (**Table**
314 **1**) [72], but also supports their engineering via domain swapping for improved
315 properties and reduced likelihood of resistant strain development.

316 One interesting property of VAPGHs is that they tend to exhibit remarkably high
317 thermal stability [73]. This feature suggests potential uses in food technology, where
318 high temperatures are commonly used. This was demonstrated by the high
319 antimicrobial activity achieved in milk pasteurized at 72 ˚C by a CHAP domain
320 (enzymatic motif) of a VAPGH fused to the SH3 domain (binding motif) of lysostaphin
321 [74]. Interestingly, this fusion approach seems to be a common trend in VAPGHs
322 engineering and has consistently broadened the host range of the chimeric enzyme
323 [75] and improved the lytic activity both *in vitro* [75] and *in situ* [76]. The bacteriolytic
324 activity of CHAP-SH3 chimera can be further improved when combined with endolysins
325 [75].

326 VAPGHs have so far been scarcely explored, but their features similar to endolysins
327 anticipate comparable progress and applications.

328

329 ## Anti-CRISPR proteins

330 Studies on the mechanisms of defense of bacteria against phages led to the discovery
331 of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-
332 associated genes (Cas) system (CRISPR-Cas), a prokaryotic version of **adaptive**
333 **immunity**. In short, when an invading DNA (*e.g.* viral or plasmid) enters a cell
334 containing a CRISPR system, some DNA fragments are captured and incorporated in
335 the CRISPR repeats. When the cell is invaded a second time by the same DNA, the
336 latter is cleaved by the Cas nuclease [77] impairing phage infection.

337 Six distinct CRISPR-Cas types are currently known, possessing different sets of proteins
338 that enable function [78]. Among these, the Type II CRISPR-Cas system has the
339 advantage of relying on a single protein for function [79]. This protein, Cas9, and its
340 variants have become a potent new tool for targeted mutagenesis and genome editing
341 of all living entities [80]. There are concerns however about gene therapy with Cas9
342 causing off-target **gene editing** with unwanted side effects, and also about the
343 development of a possible immune reaction against Cas proteins [81].

344 Recently it was found that some phages are able to counterattack the bacterial
345 CRISPR-Cas system by producing proteins able to block its action: the anti-CRISPR (Acr)
346 proteins [82]. This immediately attracted attention towards the Acr proteins as
347 possible modulators of CRISPR-Cas gene therapy. Those identified so far are able to
348 target the Cas proteins of their phage hosts [83], with a few also targeting the variant
349 SpyCas9, the most used protein for genome editing applications [84]. To control
350 CRISPR-Cas9 gene therapy, the Acr proteins may be delivered a few hours after the
351 Cas9 editing tool, decreasing the off-target gene editing and its unpredicted
352 consequences within cells or tissues [85]. Research on Acr proteins has only just
353 begun, so further understanding and novel fascinating applications are expected to
354 emerge soon.

355

356 ## Other (old) phage-encoded proteins

357 Phages encode multiple proteins other than those mentioned above, some of which
358 have also found applications in biotechnology. The most notable example is seen in

359 molecular biology, in which phage-encoded proteins have played a central role for
360 many years. Now, even these old and well-known proteins are finding novel and
361 diverse uses, as summarized in **Box 2**.

362

363 Concluding Remarks and Future Perspectives

364 Here we have discussed the multiple ways in which different phage-encoded proteins
365 have been used for human benefit. From therapy, to bacteria typing and detection,
366 surface disinfection, food decontamination, drug delivery and even vaccine
367 development, the biotechnological potential held by these proteins has been widely
368 demonstrated.

369 Genetic engineering and biotechnology allowed tailoring of phage proteins for desired
370 properties, leading to further improvements. But the prospects of phage-encoded
371 proteins can be more far-reaching than those achieved so far (see **Outstanding**
372 **Questions**). Surprising applications are emerging at a fast pace; and this is just
373 considering a small part of the powerful armamentarium phages possess to parasitize
374 bacteria, since only a low percentage of phage genes have a known function.

375 With the recent available genetic and molecular tools and large datasets of raw
376 sequencing data, research should now center on bioinformatics and functional analysis
377 of phages genes to unveil all possible protein properties, even for those proteins with
378 already known function. For example, a tail tubular protein thought to have only a
379 structural function was recently found also to possess lytic activity with therapeutic
380 potential [86]. Early phage proteins are particularly interesting as they are responsible
381 for hijacking the host machinery to a phage-oriented metabolism. Identification of
382 these proteins and understanding their function is still one of the major challenges of
383 phage research, and knowledge on early phage proteins is key to metabolic
384 manipulation of bacteria with numerous potential biotechnological applications.

385 In fact, it is plausible to assume that knowledge on the function of most phage genes
386 will arise and that major discoveries are yet to come, some of which undoubtedly
387 undergoing powerful translation into medical, agricultural and industrial
388 biotechnologies.

389

390 **Box 1. The Bacteriophage Replication Cycle**

391 Bacteriophages are bacterial viruses consisting of a nucleic acid genome enclosed within a
392 proteinaceous coat. Like all viruses, phages are metabolically inert and depend upon infection
393 of a bacterial host for replication.

394 **The beginning:** Infection begins with the adsorption of the phage on the host's cell surface,
395 relying on phage proteins that specifically recognize receptor structures. This is the first step
396 defining the range of hosts that can be infected by the phage (host specificity) and may involve
397 the action of phage enzymes able to degrade host membrane structures hiding the cell
398 receptors. After adsorption, the phage ejects its genome into the cell, a process that may also
399 be aided by phage-encoded enzymes able to produce pores in the peptidoglycan layer of the
400 bacteria [87]. Once its genome is ejected into the cell, the phage can adopt distinct replication
401 strategies.

402 **The lytic cycle:** After nucleic acid ejection, ***Caudovirales*** phages can assume a lytic replication
403 cycle and expression of phage early genes immediately follows, hijacking the host cellular
404 machinery and redirecting it to phage DNA replication and protein synthesis. Some phage
405 proteins are also involved in the phage genome replication process. After taking control of the
406 cell, the phage genes encoding its structural proteins are expressed as well as all the accessory
407 proteins. The DNA is packaged into the empty heads, and the structural proteins are
408 assembled in a process called maturation [87]**.** The mature phage progeny particles are now
409 able to start a new infection cycle but are trapped inside the host cell. At this moment, late
410 phage proteins, such as holins and endolysins, are produced; these will form pores in the inner
411 membrane and degrade the peptidoglycan leading to cell lysis, and death, for progeny release
412 [88].

413 **The lysogenic cycle:** Temperate phages assume a lysogenic cycle characterized by integrating
414 the phage genome into the bacterial chromosome. The so-called prophage is stably replicated
415 in synchrony with the bacterial chromosome, being transmitted to each daughter cell and
416 remaining silent for extended periods. This "dormant" state is maintained while the bacteria
417 (called a lysogen) grows "normally". When exposed to specific stimuli (*e.g.* stressful conditions
418 that cause DNA damage) the prophage is induced, entering a lytic replication cycle and killing
419 the cell as described above [89].

420

421 **Box 2. Novel Applications for Old Phage-Encoded Proteins**

422 After ejection of a phage genome into a host cell, a fast and complex process occurs towards
423 the takeover of the cell metabolism for the production of new virions; a multitude of proteins
424 and enzymes collaborate for this purpose. Most of these proteins were crucial for the
425 development of molecular biology, and are now finding application in novel techniques. Phage
426 RNA polymerases were recently used to reconstitute *in vitro* genetic circuits for the precise
427 mathematical modelling of biological reactions [90]. The T4 DNA ligase has been employed for
428 the detection of single-nucleotide polymorphisms (SNPs) [91] and to form ligation junctions
429 between DNA segments in circular chromosome conformation capture (4C-seq). 4C-seq is a
430 powerful technique used to study the 3D genome organization in the nuclear space [92]. The
431 exonuclease Red from phage lambda has proven useful for genome modification using
432 techniques of recombineering [93] and multiplex automated genome engineering (MAGE) [94].
433 More recently it was used to construct single-chain variable fragment antibody libraries [95],
434 and to detect antibiotic residues in foodstuff [96]. Phage scaffolding proteins or procapsids can
435 be used to generate protein-based containers for carrying different cargos. In particular, the
436 procapsid of phage T4 seems to be the most attractive for allowing simultaneous packaging of
437 specific active proteins and DNAs. The *in vitro* packaging of DNA is however limited to linear
438 molecules, a limitation that may be overcome by packing the linear DNA together with a
439 recircularization enzyme, increasing the DNA's biological activity on the target [97]. Capsids of
440 phage P22 have been loaded with contrast agents to increase image contrast in magnetic
441 resonance imaging [98]. Scaffolding proteins and procapsids have also been used for vaccine
442 development [99] and for modulating insulin receptor signaling [100]. The portal protein (DNA
443 packaging motor) of phi29 was recently used for peptide fingerprinting, with suggested
444 application for detection of disease-associated peptide biomarkers [101]. Phage integrases
445 have become valuable tools for precise genome editing using the dual integrase cassette
446 exchange (DICE) system [102] and the recombinase mediated cassette exchange (RCME)
447 system [103], and for the construction of memory genetic logic gates for detecting biological
448 events [104].

449

450 ## Glossary

451 **Adjuvant**: substance that enhances the immune response of the body to an antigen;
452 common in vaccines.

453 **Adaptive immunity**: component of immunity mediated by lymphocytes, highly specific
454 and adaptable towards a pathogen or toxin, and characterized by immunological
455 memory.

456 **Bacterial ghost**: Gram-negative bacterial cell envelope, devoid of all cytoplasmic
457 content but retaining an intact membrane structure and all surface proteins of the
458 original bacteria.

459 **Bacteriophage:** virus that specifically infects and replicates within Bacteria and
460 Archaea.

461 **Bio-based chemical**: chemical made from substances derived from a biological (living)
462 or renewable source.

463 **Biofilm**: community of microorganisms enclosed in a matrix and adhered to biotic or
464 abiotic surfaces, which collaborate closely for survival and persistence.

465 **Biosensor**: analytical device that uses a biological component for the detection of a
466 specific analyte, converting a biological response into an electrical signal by a
467 transducer.

468 **Canonical holin**: phage protein that forms large pores in the inner membrane of the
469 cell through which endolysins accumulated in the cytoplasm can cross to reach the
470 bacterial peptidoglycan.

471 ***Caudovirales***: taxonomic order of Virus that consists of three families of
472 bacteriophages with a tail, and which represents 96% of the phages observed at the
473 TEM so far.

474 **Cell penetrating peptide**: short peptide able to ubiquitously cross cellular membranes
475 with low toxicity, and transport into the cell a wide variety of biologically active
476 conjugates.

477 **Chimeric**: composed of different parts (*e.g.* protein domains) from similar sources.

478 **Endotoxin**: toxic heat-stable phospholipid-polysaccharide macromolecule associated
479 with the outer membranes of Gram-negative bacteria, which is released from the cell
480 only upon lysis.

481 **Gene editing**: the use of biotechnological techniques to make insertions, deletions or
482 replacements of DNA sequences at specific sites in the genome of an organism or cell.

483 **Genetically modified organism:** organism whose genome has been altered using
484 genetic engineering techniques.

485 **Glucosylation**: controlled enzymatic modification of a protein by addition of a glucosyl
486 group.

487 **Glycobiology**: study of the structure, function, and biology of carbohydrates,
488 molecules relevant in medical, biotechnological and basic research fields.

489 **Glycoconjugated vaccine**: vaccines that use carbohydrate antigens chemically coupled
490 to a carrier protein to enhance immunogenicity.

491 **High hydrostatic pressure processing**: non-thermal technique for preserving and
492 sterilizing food by subjecting the product to a high level of hydrostatic pressure.

493 **Hybrid**: composed of different parts (*e.g.* protein domains) from different sources.

494 **Immunogenicity**: ability of a substance to provoke an immune response.

495 **Immunogen**: substance that elicits immunogenicity.

496 **Lipopolysaccharide**: large molecule consisting of a lipid and a polysaccharide joined by
497 a covalent bond, which can be found in the outer membrane of Gram-negative
498 bacteria.

499 **Lysogenic**: bacterium or archaea harboring a temperate bacteriophage as a prophage
500 or plasmid.

501 **Lytic**: relating to or causing lysis.

502 **Minicell**: small bacterial cell which contains no nuclear material and is unable to grow
503 or divide.

504 **Multimerization:** process of assembling multimers of a molecule, in which multimers
505 are aggregates of multiple molecules that are held together with non-covalent bonds.

506 **Outer membrane permeabilizing peptide:** a peptide that acts onto the outer
507 membrane of cells making them permeable to other molecules.

508 **Phagocytosis**: engulfing and often destruction of microorganisms, other cells or
509 foreign particles by phagocytic cells, *e.g.* macrophages.

510 **Pinholin**: holin that forms small pores through which ions move causing depolarization
511 of the cell membrane and consequent activation of a specific type of endolysins
512 anchored to the inner membrane.

513 **Polysaccharide**: polymeric carbohydrate molecules composed of long chains of
514 monosaccharide units bound together by glycosidic linkages.

515 **Proteome**: entire set of proteins expressed by an organism over its entire life cycle, or
516 at a certain time and under defined conditions.

517 **Synthetic biology**: artificial design and engineering of novel biological systems,
518 organisms or devices, for purposes of improving applications for industry or
519 biological/biotechnological research.

520

521 ## Acknowledgements

522 This study was supported by the Portuguese Foundation for Science and Technology
523 (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit, COMPETE
524 2020 (POCI-01-0145-FEDER-006684) and the Project PTDC/BBB-BSS/6471/2014 (POCI-
525 01-0145-FEDER-016678). Sílvio B. Santos, Ana Rita Costa and Carla Carvalho were
526 supported by FCT grants SFRH/BPD/75311/2010, SFRH/BPD/94648/2013 and
527 SFRH/BPD/79365/2011, respectively. Franklin L. Nobrega was supported by FCT grant
528 SFRH/BD/86462/2012 and by the Netherlands Organization for Scientific Research
529 (NWO) Veni grant 016.Veni.181.092.

530

References

1. Poshtiban, S., *et al.* (2013) Phage receptor binding protein-based magnetic enrichment method as an aid for real time PCR detection of foodborne bacteria. *Analyst* 138, 5619-5626
2. Denyes, J.M., *et al.* (2017) Modified bacteriophage S16 long tail fiber proteins for rapid and specific immobilization and detection of Salmonella cells. *Appl Environ Microbiol* 83, e00277-17
3. Brzozowska, E., *et al.* (2016) Label-free Gram-negative bacteria detection using bacteriophage-adhesin-coated long-period gratings. *Biomed Opt Express* 7, 829-840
4. Rydosz, A., *et al.* (2016) A broadband capacitive sensing method for label-free bacterial LPS detection. *Biosens Bioelectron* 75, 328-336
5. Schmidt, A., *et al.* (2016) Bacteriophage tailspike protein based assay to monitor phase variable glucosylations in Salmonella O-antigens. *BMC Microbiol* 16, 207
6. Miller, S., *et al.* Hyglos Invest. Method for detecting and removing endotoxin, US8822641
7. Miernikiewicz, P., *et al.* (2016) T4 Phage tail adhesin gp12 counteracts lps-induced inflammation in vivo. *Front Microbiol* 7, 1112
8. Singh, A., *et al.* (2010) Bacteriophage tailspike proteins as molecular probes for sensitive and selective bacterial detection. *Biosens Bioelectron* 26, 131-138
9. Le, S., *et al.* (2013) Mapping the tail fiber as the receptor binding protein responsible for differential host specificity of Pseudomonas aeruginosa bacteriophages PaP1 and JG004. *PLoS One* 8, e68562
10. Javed, M.A., *et al.* (2013) Bacteriophage receptor binding protein based assays for the simultaneous detection of Campylobacter jejuni and Campylobacter coli. *PLoS One* 8, e69770
11. Uchiyama, J., *et al.* (2011) Improved adsorption of an enterococcus faecalis bacteriophage ΦEF24C with a spontaneous point mutation. *PLoS One* 6, e26648
12. Waseh, S., *et al.* (2010) Orally administered P22 phage tailspike protein reduces salmonella colonization in chickens: prospects of a novel therapy against bacterial infections. *PLoS One* 5, e13904

562 13. Merino, S. and Tomás, J.M. (2015) Bacterial capsules and evasion of immune
563 responses. *eLS*, 1-10
564 14. Scholl, D.*, et al.* (2005) Escherichia coli's K1 capsule is a barrier to bacteriophage
565 T7. *Appl Environ Microbiol* 71, 34872-34874
566 15. Hsieh, P.-F.*, et al.* (2017) Two T7-like bacteriophages, K5-2 and K5-4, each encodes
567 two capsule depolymerases: isolation and functional characterization. *Sci Rep* 7,
568 4624
569 16. Rakhuba, D.*, et al.* (2010) Bacteriophage receptors, mechanisms of phage
570 adsorption and penentration into host cell. *Pol J Microbiol* 59, 145-155
571 17. Pires, D.P.*, et al.* (2016) Bacteriophage-encoded depolymerases: their diversity
572 and biotechnological applications. *Appl Microbiol Biotechnol* 100, 2141-2151
573 18. Oliveira, H.*, et al.* (2017) Ability of phages to infect Acinetobacter calcoaceticus-
574 Acinetobacter baumannii complex species through acquisition of different pectate
575 lyase depolymerase domains. *Environ Microbiol* 19, 5060-5077
576 19. Majkowska-Skrobek, G.*, et al.* (2016) Capsule-targeting depolymerase, derived
577 from Klebsiella KP36 phage, as a tool for the development of anti-virulent strategy.
578 *Viruses* 8, 324
579 20. Lee, I.M.*, et al.* (2017) Structural basis for fragmenting the exopolysaccharide of
580 Acinetobacter baumannii by bacteriophage ΦAB6 tailspike protein. *Sci Rep* 7,
581 42711
582 21. Gutiérrez, D.*, et al.* (2015) Role of the pre-neck appendage protein (Dpo7) from
583 phage vB_SepiS-phiIPLA7 as an anti-biofilm agent in Staphylococcal species. *Front*
584 *Microbiol* 6, 1315
585 22. Alkawash, M.A.*, et al.* (2006) Alginate lyase enhances antibiotic killing of mucoid
586 Pseudomonas aeruginosa in biofilms. *APMIS* 114, 131-138
587 23. Tait, K.*, et al.* (2002) The efficacy of bacteriophage as a method of biofilm
588 eradication. *Biofouling* 18, 305-311
589 24. Guo, Z.*, et al.* (2017) Identification and characterization of Dpo42, a novel
590 depolymerase derived from the Escherichia coli phage vB_EcoM_ECOO78. *Front*
591 *Microbiol* 8, 1460
592 25. Dalziel, M.*, et al.* (2014) Emerging principles for the therapeutic exploitation of
593 glycosylation. *Science* 343, 1235681

594 26. Young, R. (2014) Phage lysis: three steps, three choices, one outcome. *J Microbiol*
595 52, 243-258
596 27. Nelson, D., *et al.* (2001) Prevention and elimination of upper respiratory
597 colonization of mice by group A streptococci by using a bacteriophage lytic
598 enzyme. *Proc Natl Acad Sci USA* 98, 4107-4112
599 28. Rodríguez-Rubio, L., *et al.* (2013) The phage lytic proteins from the Staphylococcus
600 aureus bacteriophage vB_SauS-phiIPLA88 display multiple active catalytic domains
601 and do not trigger staphylococcal resistance. *PLoS ONE* 8, e64671
602 29. Chopra, S., *et al.* (2016) Potential of combination therapy of endolysin MR-10 and
603 minocycline in treating MRSA induced systemic and localized burn wound
604 infections in mice. *Int J Med Microbiol* 306, 707-716
605 30. Totté, J.E.E., *et al.* (2017) Successful treatment of chronic Staphylococcus aureus-
606 related dermatoses with the topical endolysin Staphefekt SA.100: A report of 3
607 cases. *Case Rep Dermatol* 9, 19-25
608 31. Shen, Y., *et al.* (2016) A bacteriophage endolysin that eliminates intracellular
609 streptococci. *Elife* 5, e13152
610 32. Park, S., *et al.* (2018) Characterisation of the antibacterial properties of the
611 recombinant phage endolysins AP50-31 and LysB4 as potent bactericidal agents
612 against Bacillus anthracis. *Sci Rep* 8, 18
613 33. Gutiérrez, D., *et al.* (2014) Effective removal of Staphylococcal biofilms by the
614 endolysin LysH5. *PLoS ONE* 9, e107307
615 34. Chang, Y., *et al.* (2017) Characterization of a novel endolysin LysSA11 and its utility
616 as a potent biocontrol agent against Staphylococcus aureus on food and utensils.
617 *Food Microbiol* 68, 112-120
618 35. Kim, W.-S., *et al.* (2004) Expression of bacteriophage φEa1h lysozyme in
619 Escherichia coli and its activity in growth inhibition of Erwinia amylovora.
620 *Microbiology* 150, 2707-2714
621 36. van Nassau, T.J., *et al.* (2017) Combination of endolysins and high pressure to
622 inactivate Listeria monocytogenes. *Food Microbiol* 68, 81-88
623 37. Oliveira, H., *et al.* (2013) Molecular aspects and comparative genomics of
624 bacteriophage endolysins. *J Virol* 87, 4558-4570

625 38. Dong, Q., *et al.* (2015) Construction of a chimeric lysin Ply187N-V12C with
626 extended lytic activity against staphylococci and streptococci. *Microb Biotechnol* 8,
627 210-220
628 39. Fernández, L., *et al.* (2017) Downregulation of autolysin-encoding genes by phage-
629 derived lytic proteins inhibits biofilm formation in Staphylococcus aureus.
630 *Antimicrob Agent Chemother* 61, e02724-16
631 40. Mao, J., *et al.* (2013) Chimeric Ply187 endolysin kills Staphylococcus aureus more
632 effectively than the parental enzyme. *FEMS Microbiol Lett* 342, 30-36
633 41. Haddad Kashani, H., *et al.* (2017) A novel chimeric endolysin with antibacterial
634 activity against methicillin-resistant Staphylococcus aureus. *Front Cell Infect*
635 *Microbiol* 7, 290
636 42. Jun, S.Y., *et al.* (2013) Antibacterial properties of a pre-formulated recombinant
637 phage endolysin, SAL-1. *Int J Antimicrob Agent* 41, 156-161
638 43. Jun, S.Y., *et al.* (2017) Pharmacokinetics and tolerance of the phage endolysin-
639 based candidate drug SAL200 after a single intravenous administration among
640 healthy volunteers. *Antimicrob Agents Chemother* 61, e02629-02616
641 44. Oliveira, H., *et al.* (2016) Structural and enzymatic characterization of ABgp46, a
642 novel phage endolysin with broad anti-gram-negative bacterial activity. *Front*
643 *Microbiol* 7, 208
644 45. Briers, Y., *et al.* (2014) Art-175 is a highly efficient antibacterial against multidrug-
645 resistant strains and persisters of Pseudomonas aeruginosa. *Antimicrob Agents*
646 *Chemother* 58, 3774-3784
647 46. Defraine, V., *et al.* (2016) Efficacy of artilysin Art-175 against resistant and
648 persistent Acinetobacter baumannii. *Antimicrob Agents Chemother* 60, 3480-3488
649 47. Rodríguez-Rubio, L., *et al.* (2016) 'Artilysation' of endolysin λSa2lys strongly
650 improves its enzymatic and antibacterial activity against streptococci. *Sci Rep* 6,
651 35382
652 48. Yang, H., *et al.* (2015) Structural mechanism of ergosterol regulation by fungal
653 sterol transcription factor Upc2. *Nat Commun* 6, 6129
654 49. Boura, E., *et al.* (2017) Metal ions-binding T4 lysozyme as an intramolecular
655 protein purification tag compatible with X-ray crystallography. *Prot Sci* 26, 1116-
656 1123

657 50. Joshi, H. and Jain, V. (2017) Novel method to rapidly and efficiently lyse
658 Escherichia coli for the isolation of recombinant protein. *Anal Biochem* 528, 1-6
659 51. Won, G.*, et al.* (2017) A novel method to generate Salmonella Typhi Ty21a ghosts
660 exploiting the λ phage holin-endolysin system. *Oncotarget* 8, 48186-48195
661 52. Kong, M.*, et al.* (2017) Lateral flow assay-based bacterial detection using
662 engineered cell wall binding domains of a phage endolysin. *Biosens Bioelectron* 96,
663 173-177
664 53. Yu, J.*, et al.* (2016) Sensitive and rapid detection of staphylococcus aureus in milk
665 via cell binding domain of lysin. *Biosens Bioelectron* 77, 366-371
666 54. Gómez-Torres, N.*, et al.* (2017) Development of a specific fluorescent phage
667 endolysin for in situ detection of Clostridium species associated with cheese
668 spoilage. *Microb Biotechnol* (in press)
669 55. Kim, D.*, et al.* (2017) Targeted killing of pathogenic bacteria with cell wall binding
670 domain (CBD)-antimicrobial nanoparticle conjugates. *AIChE Annual Meeting* 676a
671 56. Dewey, J.S.*, et al.* (2010) Micron-scale holes terminate the phage infection cycle.
672 *Proc Natl Acad Sci* 107, 2219-2223
673 57. Catalão, M.J.*, et al.* (2013) Diversity in bacterial lysis systems: bacteriophages show
674 the way. *FEMS Microbiol Rev* 37, 554-571
675 58. Song, J.*, et al.* (2016) Identification and characterization of HolGH15: the holin of
676 Staphylococcus aureus bacteriophage GH15. *J Gen Virol* 97, 1272-1281
677 59. White, R.*, et al.* (2011) Holin triggering in real time. *Proc Natl Acad Sci* 108, 798-
678 803
679 60. Shi, Y.*, et al.* (2012) Combined antibacterial activity of phage lytic proteins holin
680 and lysin from Streptococcus suis bacteriophage SMP. *Curr Microbiol* 65, 28-34
681 61. Won, G.*, et al.* (2017) Improved lysis efficiency and immunogenicity of Salmonella
682 ghosts mediated by co-expression of λ phage holin-endolysin and ϕX174 gene E.
683 *Sci Rep* 7, 45139
684 62. Won, G. and Lee, J.H. (2017) Salmonella Typhimurium, the major causative agent
685 of foodborne illness inactivated by a phage lysis system provides effective
686 protection against lethal challenge by induction of robust cell-mediated immune
687 responses and activation of dendritic cells. *Vet Res* 48, 66

688 63. Farkasovská, J.*, et al.* (2004) Identification of a holin encoded by the Streptomyces
689 aureofaciens phage micro1/6; functional analysis in Escherichia coli system. *Folia*
690 *Microbiol* 49, 679-684
691 64. Wang, I.-N.*, et al.* (2003) Sizing the holin lesion with an endolysin-β-galactosidase
692 fusion. *J Bacteriol* 185, 779-787
693 65. Agu, C.A.*, et al.* (2006) The cytotoxic activity of the bacteriophage λ-holin protein
694 reduces tumour growth rates in mammary cancer cell xenograft models. *J Gene*
695 *Med* 8, 229-241
696 66. Horii, T.*, et al.* (2002) Characterization of a holin (HolNU3–1) in methicillin-
697 resistant Staphylococcus aureus host. *FEMS Immunol Med Microbiol* 34, 307-310
698 67. Kuo, C.-Y.*, et al.* (2009) A stably engineered, suicidal strain of Listeria
699 monocytogenes delivers protein and/or DNA to fully differentiated intestinal
700 epithelial monolayers. *Mol Pharm* 6, 1052-1061
701 68. Park, S.-Y.*, et al.* (2011) A coupling process for improving purity of bacterial
702 minicells by holin/lysin. *J Microbiol Meth* 86, 108-110
703 69. Miyake, K.*, et al.* (2014) A green-light inducible lytic system for cyanobacterial
704 cells. *Biotechnol Biofuel* 7, 56-56
705 70. Latka, A.*, et al.* (2017) Bacteriophage-encoded virion-associated enzymes to
706 overcome the carbohydrate barriers during the infection process. *Appl Microbiol*
707 *Biotechnol* 101, 3103-3119
708 71. Rodríguez-Rubio, L.*, et al.* (2013) The peptidoglycan hydrolase of Staphylococcus
709 aureus bacteriophage φ11 plays a structural role in the viral particle. *Appl Environ*
710 *Microbiol* 79, 6187-6190
711 72. Gutiérrez, D.*, et al.* (2017) Real-time assessment of Staphylococcus aureus biofilm
712 disruption by phage-derived proteins. *Front Microbiol* 8, 1632
713 73. Lavigne, R.*, et al.* (2004) Identification and characterization of a highly
714 thermostable bacteriophage lysozyme. *Cell Mol Life Sci* 61, 2753-2759
715 74. Rodríguez-Rubio, L.*, et al.* (2013) Potential of the virion-associated peptidoglycan
716 hydrolase HydH5 and its derivative fusion proteins in milk biopreservation. *PLoS*
717 *ONE* 8, e54828
718 75. Rodríguez-Rubio, L.*, et al.* (2012) Enhanced staphylolytic activity of the
719 Staphylococcus aureus bacteriophage vB_SauS-phiIPLA88 HydH5 virion-associated

720 peptidoglycan hydrolase: Fusions, deletions, and synergy with LysH5. *Appl Environ*
721 *Microbiol* 78, 2241-2248
722 76. Vipra, A.A., *et al.* (2012) Antistaphylococcal activity of bacteriophage derived
723 chimeric protein P128. *BMC Microbiol* 12, 41
724 77. Mohanraju, P., *et al.* (2016) Diverse evolutionary roots and mechanistic variations
725 of the CRISPR-Cas systems. *Science* 353, aad5147.
726 78. Marraffini, L.A. (2015) CRISPR-Cas immunity in prokaryotes. *Nature* 526, 55-61
727 79. Barrangou, R. and Doudna, J.A. (2016) Applications of CRISPR technologies in
728 research and beyond. *Nat Biotech* 34, 933-941
729 80. Ma, H., *et al.* (2017) Correction of a pathogenic gene mutation in human embryos.
730 *Nature* 548, 413-419
731 81. Zischewski, J., *et al.* (2017) Detection of on-target and off-target mutations
732 generated by CRISPR/Cas9 and other sequence-specific nucleases. *Biotechnol Adv*
733 35, 95-104
734 82. Hynes, A.P., *et al.* (2017) An anti-CRISPR from a virulent streptococcal phage
735 inhibits Streptococcus pyogenes Cas9. *Nature Microbiol* 2, 1364-1380
736 83. Pawluk, A., *et al.* (2017) Disabling a type I-E CRISPR-Cas nuclease with a
737 bacteriophage-encoded anti-CRISPR protein. *mBio* 8, e01751-17
738 84. Rauch, B.J., *et al.* (2017) Inhibition of CRISPR-Cas9 with bacteriophage proteins.
739 *Cell* 168, 150-158.e110
740 85. Shin, J., *et al.* (2017) Disabling Cas9 by an anti-CRISPR DNA mimic. *Sci Adv* 3,
741 e1701620
742 86. Pyra, A., *et al.* (2017) Tail tubular protein A: a dual-function tail protein of
743 Klebsiella pneumoniae bacteriophage KP32. *Sci Rep* 7, 2223
744 87. Kutter, E. and Sulakvelidze, A. (2004) *Bacteriophages: Biology and Applications*
745 CRC Press
746 88 Drulis-Kawa, Z., *et al.* (2012) Learning from Bacteriophages - Advantages and
747 Limitations of Phage and Phage-Encoded Protein Applications. *Current Protein and*
748 *Peptide Science* 13, 699-722
749 89. Drulis-Kawa, Z., *et al.* (2012) Learning from bacteriophages - Advantages and
750 limitations of phage and phage-encoded protein applications. *Curr Prot Pept Sci*
751 13, 699-722

752 90. Citorik, R.J., *et al.* (2014) Bacteriophage-based synthetic biology for the study of
753 infectious diseases. *Curr Opin Microbiol* 19, 59-69
754 91. Zeng, L. and Xiao, Z. (2017) A lateral flow biosensor for the detection of single
755 nucleotide polymorphisms. In *Biosensors and Biodetection: Methods and*
756 *Protocols, Volume 2: Electrochemical, Bioelectronic, Piezoelectric, Cellular and*
757 *Molecular Biosensors* (Prickril, B. and Rasooly, A., eds), pp. 421-430, Springer New
758 York
759 92. Schwartz, M., *et al.* (2015) Comparative analysis of T4 DNA ligases and DNA
760 polymerases used in chromosome conformation capture assays. *Biotechniques* 58,
761 195-199
762 93. Czarniak, F. and Hensel, M. (2015) Red-mediated recombineering of Salmonella
763 enterica genomes. In *Salmonella: Methods and Protocols* (Schatten, H. and
764 Eisenstark, A., eds), pp. 63-79, Springer New York
765 94. Gallagher, R.R., *et al.* (2014) Rapid editing and evolution of bacterial genomes
766 using libraries of synthetic DNA. *Nat. Protocols* 9, 2301-2316
767 95. Kato, M. and Hanyu, Y. (2017) Enzymatic assembly for scFv library construction. In
768 *Synthetic Antibodies: Methods and Protocols* (Tiller, T., ed), pp. 31-44, Springer
769 New York
770 96. Wang, X., *et al.* (2016) Highly sensitive homogeneous electrochemical aptasensor
771 for antibiotic residues detection based on dual recycling amplification strategy.
772 *Biosens Bioelectron* 82, 49-54
773 97. Liu, J.L., *et al.* (2014) Viral nanoparticle-encapsidated enzyme and restructured
774 DNA for cell delivery and gene expression. *Proc Natl Acad Sci* 111, 13319-13324
775 98. Usselman, R.J., *et al.* (2015) Gadolinium-loaded viral capsids as magnetic
776 resonance imaging contrast agents. *Appl Magn Reson* 46, 349-355
777 99. Mattiacio, J.L., *et al.* (2017) Display of HIV-1 envelope protein on lambda phage
778 scaffold as a vaccine platform. In *Recombinant Virus Vaccines: Methods and*
779 *Protocols* (Ferran, M.C. and Skuse, G.R., eds), pp. 245-253, Springer New York
780 100.Chan, J.Y., *et al.* (2017) Targeting insulin receptor in breast cancer using small
781 engineered protein scaffolds. *Mol Cancer Ther* 16, 1324-1334
782 101.Ji, Z., *et al.* (2016) Fingerprinting of peptides with a large channel of bacteriophage
783 Phi29 dna packaging motor. *Small* 12, 4572-4578

784 102.Zhu, F., *et al.* (2014) DICE, an efficient system for iterative genomic editing in
785 human pluripotent stem cells. *Nucleic Acids Res* 42, e34-e34
786 103.Inniss, M.C., *et al.* (2017) A novel Bxb1 integrase RMCE system for high fidelity
787 site-specific integration of mAb expression cassette in CHO Cells. *Biotechnol*
788 *Bioeng* 114, 1837-1846
789 104.Hsiao, V., *et al.* (2016) A population-based temporal logic gate for timing and
790 recording chemical events. *Mol Sys Biol* 12, 869
791 105.Olszak, T., *et al.* (2017) The O-specific polysaccharide lyase from the phage LKA1
792 tailspike reduces Pseudomonas virulence. *Sci Rep* 7, 16302

793

794 ## Figure legends

795

796 **Figure 1, Key Figure. Proteins with biotechnological applications expressed during**
797 **the course of a bacteriophage replication cycle.** (A) Structural composition of
798 bacterium and phage, and symbols representing phage-encoded proteins. (B) Phage
799 infection begins with adsorption, the interaction of the phage with a specific receptor
800 on the bacterial surface, typically involving (C) the activity of receptor binding proteins
801 (e.g. tail fibers and tail spikes) and depolymerases. (D) This interaction leads to phage
802 genome ejection into the bacterial cytoplasm, which may (E) be aided by VAPGHs that
803 break the bacterial peptidoglycan layer. (F) Once inside, the phage genome may
804 encounter bacterial defenses that aim to degrade it, and counteract it with proteins as
805 anti-CRISPRs (Acr). At this stage (G) the phage may opt for two distinct life cycles: the
806 lysogenic life cycle (H) where the phage genome integrates the bacterial genome with
807 the help of integrases originating bacterial lysogens. The resulting prophage (I)
808 replicates together with the bacterial genome for several generations; or the lytic life
809 cycle (J) where the phage highjacks the bacterial molecular machinery for genome
810 replication and protein expression, using also (K) its own proteins. (L) The structural
811 proteins are then expressed and (M) new phage virions are assembled. To release the
812 newly formed virions (N) late phage proteins as endolysins and holins/pinholins act on
813 the cell membrane to pierce it and (O) cause cell lysis, allowing for a new round of
814 phage infection. (P) Symbol code for the biotechnological applications of phage-
815 encoded proteins indicated in the life cycle.

816

817 **Figure 2. Biotechnological applications of phage-derived cell binding proteins.** (A)
818 Phage receptor binding proteins as tail spikes and tail fibers (or domains thereof), and
819 the cell wall binding domain (CBD) of endolysins have been used as specific cell binding
820 peptides (CBP) in combination with different components for bacterial detection,
821 capture and targeting. The CBP can be combined with (B) fluorescent proteins (e.g.
822 GFP) for the detection of desired bacteria using fluorescent microscopy and/or flow
823 cytometry; (C) horseradish peroxidase (HRP) for the detection of specific bacteria by

824 enzyme-linked immunosorbent assay (ELISA) after adding an HPR substrate; (D)
825 magnetic nanoparticles for the capture of specific bacteria using a magnetic field, and
826 subsequent bacterial enrichment; (E) gold nanoparticles to create a biosensor-
827 detectable signal after binding to the target bacteria; (F) unspecific anti-microbial
828 peptides (AMP) for targeting and elimination of specific bacteria; and (G) a biosensor
829 that senses modifications on the CBP caused by its interaction with specific bacteria
830 and consequently produces a detectable signal.

831

832 **Figure 3. Strategies for endolysin engineering.** Endolysins of bacteriophages infecting
833 Gram-positive bacteria have a modular structure composed of an enzymatic catalytic
834 domain (ECD) and a cell wall binding domain (CBD) connected by a linker (L). The
835 modular structure of these enzymes led to strategies of engineering based on domain
836 swapping or combination of CBDs with other hydrolytic enzymes. By doing so,
837 endolysins with high catalytic activity and low specificity (ECD1-CBD1), or a strong
838 hydrolytic enzyme (hydrolase), may be combined with endolysins with low catalytic
839 activity and high specificity (ECD2-CBD2) to obtain a chimeric protein either with high
840 catalytic activity and specificity (ECD1-CBD2) to strongly target specific bacteria or with
841 unspecific and high catalytic activity (ECD1) for disinfection purposes. Instead,
842 endolysins of phages infecting Gram-negative bacteria typically have a globular
843 structure composed of a single ECD, although more rarely they may also have a
844 modular structure similar to endolysins targeting Gram-positive bacteria (in an
845 opposite arrangement). Engineering of these endolysins has mostly consisted on their
846 combination with extra peptides (EP-ECD and ECD-EP), such as outer membrane
847 permeabilizing peptides or cell penetrating peptides (mostly polycationic) to give the
848 endolysin access to the peptidoglycan layer of the Gram-negative bacteria.

849

850 # Tables

851

852 **Table 1.** Summary of the most recent biotechnological applications of phage-encoded proteins

Protein (source)	Application	Description	Year	Ref
RBP				
Long tail fiber (gp37-gp38) (Salmonella phage S16)	Biosensors for whole cell detection	Recombinant long tail fiber (LTF) proteins were used to coat paramagnetic beads for the efficient capture of *Salmonella* Typhimurium cells from food samples. By integrating this LTF-based enrichment method with horseradish peroxidase-conjugated LTF, a new method named Enzyme-linked LTF assay (EELTA) was created which detects as few as 10^2 CFU/mL of S. Typhimurium in 2 h.	2017	[2]
Tail spike LKA1gp49 (Pseudomonas phage LKA1)	Anti-virulence strategies	The tail spike protein (TSP) specifically binds and cleaves B-band lipopolysaccharide (LPS) of *Pseudomonas aeruginosa* PAO1. When employed in an *in vivo Galleria mellonella* model, the enzyme reduce *P. aeruginosa* virulence and sensitized the bacteria to serum complement activity.	2017	[105]
Tail fiber protein tip (gp37) (Escherichia phage T4)	Biosensors for LPS detection	The adhesin was used as the recognition element of a new highly sensitive label-free microwave sensor. The adhesin specifically recognizes the LPS of *Escherichia coli*, causing a change in the capacitance and conductance of the sensor, used as an indicator of LPS detection.	2016	[4]
Tail spikes (Salmonella phages 9NA and P22)	Serotyping	TSPs specifically distinguished glucosylation phenotypes of *Salmonella* O-antigens, when classical methods failed to do so. Variations in glucosylated O-antigens are related to immunogenicity, so the TSP can be used to monitor *Salmonella* epidemiology. It can also be useful for monitoring phase variations during large scale preparation of O-antigens for vaccine production.	2016	[5]
Short tail fiber protein (gp12) (Escherichia phage T4)	Modulator of LPS-induced inflammatory effects.	The tail fiber administered together with LPS in a murine model decrease the inflammatory response to LPS. This suggests the use of the tail fiber as a potential tool for modulating and counteracting LPS-related immune responses.	2016	[7]
Tail fiber protein tip (gp37) (Escherichia phage T4)	Biosensors for whole cell detection	The adhesin was used to create a highly sensitive sensor for the detection of Gram-negative bacteria containing OmpC. The sensor, based on long-period gratings coated with the adhesin, demonstrated sensitivity higher than reference tests.	2016	[3]
Depolymerase				
Depolymerases ORF37 and ORF38 (Klebsiella phages K5-2 and K5-4)	Anti-virulence strategies	The depolymerases were active against the capsules of *Klebsiella*, suggesting their use as adjuvants of the host immune system by decreasing capsule-associated virulence.	2017	[15]
Depolymerase (orf40) (Acinetobacter phage phiAB6)	Development of glycoconjugate vaccines	The depolymerase specifically hydrolysed the exopolysaccharides of *Acinetobacter baumannii*. The released polysaccharides may be used as immunogens for glycoconjugated vaccine production.	2017	[20]
Depolymerase Dpo42 (Escherichia phage vB_EcoM_ECOO78)	Biofilm prevention	The depolymerase degraded the capsular polysaccharides surrounding *E. coli* cells, and exhibited a dose-dependent capacity to prevent biofilm formation.	2017	[24]
Depolymerase depoKP36 (Klebsiella phage KP36)	Anti-virulence strategies	The depolymerase was active both *in vitro* and *in vivo*, significantly inhibiting the mortality of *Galleria mellonella* larvae induced by *Klebsiella pneumoniae*. The depolymerase stability over a broad range of conditions makes it suitable for the development of new treatments for *K. pneumoniae* infections.	2016	[19]
Depolymerase Dpo7 (Staphylococcal phage vB_SepiS-phiIPLA7)	Biofilm prevention and removal	The depolymerase efficiently prevented and removed biofilm-attached staphylococcal cells, although restricted to polysaccharide-producer strains.	2015	[21]

Endolysin				
Endolysin SA.100 (Staphylococcal phage) *Commercialized by Micreos*	Localized antibacterial therapy	The endolysin product Staphefekt SA.100 was successfully used to treat patients with chronic and recurrent *S. aureus*-related dermatoses. There were no signs of induction of resistance by the protein.	2017	[30]
Endolysins PlyP40, Ply511, and PlyP825 (Listeria phages P40, A511, and ProCC P825)	Combinational food preservation techniques	The endolysins were individually combined with high hydrostatic pressure processing, resulting in the synergistic killing of *Listeria monocytogenes*. The results suggest the use of combined processes for the inactivation of *L. monocytogenes* in low processed, ready-to-eat food products.	2017	[36]
Endolysin LysSA11 (Staphylococcal phage SA11)	Food biocontrol and utensil sanitization	Endolysin LysSA11 was active against methicillin-resistant *Staphylococcus aureus* contaminating both food and utensils. The endolysin was similarly active at refrigeration and room temperatures.	2017	[34]
Endolysins AP50-31 and LysB4 (Bacillus phages AP50 and B4)	Antibacterial therapy	The endolysins demonstrated rapid and broad bacteriolytic activity in vitro against strains within the Bacillus genus. Intranasal administration of LysB4 protected mice from death after infection with *Bacillus anthracis* Sterne spores.	2018	[35]
ECD of endolysin A (Mycobacterium phage D29)	Protein purification	The ECD was used as an alternative to sonication or high pressure homogenization for the efficient lysis of *E. coli* cells during protein purification. The ECD is expressed intracellularly and remains non-toxic until chloroform is added to the culture medium. This permeabilizes the bacterial cell membrane allowing diffusion of ECD to the peptidoglycan layer where it acts causing cell lysis. The method is applicable in high-throughput and large-scale protein purification.	2017	[50]
Chimeric endolysin LysK (Staphylococcal phage K)	Antibacterial therapy	The properties of primary, secondary and tertiary structure of endolysin LysK were improved using *in silico* design, and resulted in enhanced stability, solubility and antibacterial activity of the enzyme against *S. aureus*, *S. epidermidis* and *Enterococcus*.	2017	[41]
Recombinant endolysin Sal-1 (Staphylococcal phage SAP-1)	Antibacterial therapy	A recombinant form of endolysin SAL-1 was used for the development of drug SAL200, for the treatment of antibiotic-resistant staphylococcal infections. Phase 1 studies of SAL200 administered intravenously reported no serious adverse effects, supporting the progress of the drug for later phase studies.	2017	[43]
Modified lysozyme mbT4L (Escherichia phage T4)	Purification tag	A metal ions-binding mutant of phage T4 lysozyme (mbT4L) was used as a purification tag in immobilized-metal affinity chromatography (IMAC), proving advantageous over the conventional IMAC technique. The mbT4L protein is suggested to be compatible also with X-ray crystallography.	2017	[49]
Chimeric protein CHAPSH3b (Staphylococcal phage vB_SauS-phiIPLA88)	Biofilm control	The chimeric protein is a fusion of the ECD of VAPGH HydH5 with the CBD of lysostaphin. The protein was able to control biofilm-embedded *S. aureus* and decrease biofilm formation by some strains.	2017	[39]
Endolysin MR-10 (Staphylococcus phage MR-10)	Localized and systemic combinational antibacterial therapy with antibiotics	The endolysin was combined with the antibiotic minocycline, in a single dose, resulting in complete survival of mice with systemic methicillin-resistant *S. aureus* infection. Encouraging results were also obtained when applying the combined therapy to localized burn wound infections.	2016	[29]
Endolysin PlyC (Streptococcal phage C1)	Intracellular antibacterial therapy	The endolysin PlyC was able to control intracellular *Streptococcus pyogenes* by crossing epithelial cell membranes. By doing so, the endolysin creates new opportunities to avoid refractory infections caused by the internalized pathogen.	2016	[31]
Artilysin Art-175 (Pseudomonas phage varphiKZ)	Antibacterial therapy	Artilysin Art-175 instantaneously killed stationary-phase cells of multidrug-resistant *A. baumannii*, with no sign of development of resistance.	2016	[46]
Artilysin Art-240 (Streptococcal phage λSa2)	Antibacterial therapy	Fusion of endolysin λSa2lys with the polycationic peptide PCNP generated Artilysin Art-240, a hybrid protein with specificity similar to the parental enzyme, but increased stability and	2016	[47]

		bactericidal activity.		
Endolysin ABgp46 (Acinetobacter phage vB_AbaP_CEB1)	Antibacterial therapy	The endolysin ABgp46 demonstrated antibacterial activity against several multidrug resistant *A. baumannii* strains. The activity of the endolysin was broadened to other Gram-negative pathogens, including *P. aeruginosa* and *S.* Typhimurium, when combined with the outer membrane permeabilizing agents citric and malic acid.	2016	[44]
Endolysin E (Escherichia phage T4)	Crystallization	The endolysin was fused to the ligand-binding domain (LBD) of the fungal sterol transcription factor Upc2, improving expression and crystallization of Upc2 LBD.	2015	[48]
Chimeric endolysin Ply187N-V12C (Staphylococcal phage 187, enterococcal phage phi1)	Antibacterial therapy	Fusion of the ECD of staphylococcal endolysin Ply187 with the CBD of enterococcal endolysin PlyV12 extended the lytic activity of Ply187 to streptococci and enterococci.	2015	[38]
CBD				
CBD of endolysin LysB4 (Bacillus phage B4)	Biosensors for whole cell detection	The CBD was used as the recognition element of a nitrocellulose-based lateral flow assay, which employed colloidal gold nanoparticles as a colorimetric signal for bacterial detection. The method provided results superior to those of antibody-based approaches, detecting 10^4 CFU/mL of *Bacillus cereus* in 20 min.	2017	[52]
CBD of endolysin CTP1L (Clostridium phage phiCTP1)	Biosensors for whole cell detection	The CBD was fused to green fluorescent protein (GFP) for the specific detection of dairy-related *Clostridium* species by fluorescence microscopy. The GFP-CBD was also capable of binding to clostridial spores, and permitted the visualization of vegetative cells of *Clostridium tyrobutyricum* directly in the matrix of late blowing defect cheese.	2017	[54]
CBD (NA)	Targeted delivery of antimicrobials	The CBD was fused to antimicrobial silver nanoparticles (AgNPs) for their targeted delivery. The construct improved specificity and killing efficiency of the target bacteria, when compared to bare AgNPs.	2017	[55]
CBD of endolysin PlyV12 (Enterococcal phage Φ1)	Biosensors for whole cell detection	CBD of endolysin PlyV12 was coupled to immunomagnetic separation for the rapid and sensitive detection of *S. aureus* cells in spiked milk. The sensitivity of detection (detection limit of $4x10^3$ CFU/mL) is improved by the large number of binding sites available at the cell surface for CBD attachment.	2016	[53]
Holin				
Holin HolGH15 (Staphylococcal phage GH15)	Antibacterial therapy	The holin has shown efficient antibacterial activity against a broad range of species, including *S. aureus*, *L. monocytogenes*, *Bacillus subtilis*, *P. aeruginosa*, *K. pneumoniae* and E. coli.	2016	[58]
Endolysin and holin				
Endolysin R and holin S (Escherichia phage lambda)	Production of bacterial ghosts for vaccine production	The holin and endolysin of phage lambda were used to construct novel plasmids for the production of *Salmonella* ghosts of high immunogenicity. This strategy is expected to contribute to the development of novel and safe non-living vaccine candidates.	2017	[51, 61, 62]
Endolysin E and holin T (Escherichia phage T4)	Biofuel recovery	The endolysin and holin genes of phage T4 were introduced in cyanobacteria under the control of a promoter regulated by a physical signal. When the cells are exposed to the signal, the lysis proteins are expressed causing cell death. This strategy is suggested for the recovery of biofuels and related compounds without resorting to chemical inducers and mechanical disruption, with the advantage of controlling the accidental release of cyanobacteria.	2014	[69]
VAPGH				
VAPGH HydH5 (Staphylococcal phage vB_SauS-phiIPLA88)	Biocontrol in dairy	The VAPGH and its variants fused to lysostaphin (or domains thereof) were shown to have efficient lytic activity against *S. aureus* in both raw milk and milk pasteurized at 72 ˚C.	2013	[74]
Anti-CRISPR				

anti-CRISPR proteins AcrIIA2 and AcrIIA4 (Listeria prophages)	Regulation of CRISPR-Cas9 genome editing	The anti-CRISPR proteins were shown to inhibit the widely used *Streptococcus pyogenes* Cas9 when assayed in bacteria and human cells.	2017	[84]
anti-CRISPR protein AcrIIA5 (Streptococcal phage D4276)	Regulation of CRISPR-Cas9 genome editing	The first anti-CRISPR protein isolated from a virulent phage was able to completely inhibit *S. pyogenes* Cas9 (SpCas9) activity *in vitro*.	2017	[82]
anti-CRISPR protein AcrIIA4 (Listeria monocytogenes prophages)	Regulation of CRISPR-Cas9 genome editing	The anti-CRISPR protein bound only to assembled Cas9-single-guide RNA complexes and not to the Cas9 protein alone. AcrIIA4 delivered a few hours after the Cas9 editing tool in human cells allowed on-target Cas9-mediated gene editing and reduced off-target editing and its unpredicted side effects.	2017	[85]
anti-CRISPR AcrE1 (gp34) (Pseudomonas phage JBD5)	Regulation of type I-E CRISPR-Cas	The anti-CRISPR bound Cas3 and inactivated the type I-E CRISPR-Cas system in *P. aeruginosa*. AcrE1 can convert the endogenous type I-E CRISPR system into a programmable transcriptional repressor, providing a new biotechnological tool for genetic studies of bacteria encoding this CRISPR system.	2017	[83]
Other				
P22 capsid (Enterobacteria phage P22)	Improvement of contrast agents	The viral capsids were loaded with the paramagnetic gadolinium ion (positive contrast agent) to use as contrast agents in magnetic resonance imaging. The capsids efficiently enhanced the relaxivity of the contrast agent, which is expected to increase image contrast.	2017	[98]
Lambda capsid (Escherichia phage lambda)	Vaccine development	The capsid was used as a scaffold for the display of the human immunodeficiency virus envelope spike protein. This vector can be used for vaccine development, with advantages over mammalian virus vectors of genetic tractability, inexpensive production, aptness for scale-up, and stability.	2017	[99]
T4 procapsid (Escherichia phage T4)	Targeted gene/cancer therapy	The procapsid was packaged *in vitro* with a mCherry expression plasmid and *in vivo* with the active Cre recombinase. The capsid-based nanoparticles were delivered into cancer cells, in which the Cre recombinase circularized the linear expression plasmid, resulting in enhanced expression of mCherry. This strategy overcomes the main limitation of *in vitro* DNA packaging, its restriction to linear molecules, which hinders the biological activity of the DNA on the target.	2014	[97]
BxB1 integrase (Mycobacterium phage BxB1)	Cell line engineering	The integrase was used to build a novel recombinase mediated cassette exchange (RMCE) system, with fidelity of RMCE events higher than those obtained with the common Flp/FRT RMCE system. This system provides a novel tool for the engineering cell lines for biotherapeutic production.	2017	[103]
Serine integrases TP901-1 (intA) and Bxb1 (intB) (Lactococcus phage TP901-1 and Mycobacterium phage Bxb1)	Construction of memory genetic logic gates for detection of biological events	The integrases were used to create a two-input temporal logic gate capable of sensing and recording the order of inputs, the timing between inputs, and the duration of input pulses. The integrases were specifically used for unidirectional DNA recombination to detect and encode sequences of input events.	2016	[104]
PhiC31 and BxB1 integrases (Streptomyces phage phiC31, Mycobacterium phage BxB1)	Genetic modification of human stem cells	The integrases were used for the development of DICE, Dual integrase cassette exchange. The system offers rapid, efficient and precise gene insertion in stem cells, and is particularly well suited for repeated modifications of the same locus.	2014	[102]
Red recombinase (Escherichia phage lambda)	Engineering of bacterial genome	The Red recombinase of phage lambda was used for enterobacteria genome mutagenesis, such as rapid generation of genome deletions, site-directed mutagenesis, generation of reporter fusions or chimeric genes, and transplantation of regulatory elements into the cell chromosome.	2015	[93]
T7 modified RNA polymerase inhibitor	Modulation of insulin receptor	The *E. coli* RNA polymerase inhibitor was subjected to direct evolution for the development of variants able to inhibit insulin	2017	[100]

(modified gp2) (Escherichia phage T7)	signalling	receptors. The variants inhibited insulin-mediated proliferation of breast cancer cells, without downregulating the expression of the insulin receptor.		
T4 DNA ligase (Escherichia phage T4)	Single nucleotide polymorphism (SNP) analysis	The DNA ligase was used for the development of a simple and robust lateral flow biosensor method for the detection of SNPs. The method is suggested to have great potential for the detection of genetic diseases, cancer-related mutations, and drug-resistant mutations of infectious agents, and for the development of personalized medicine.	2017	[91]
T7 exonuclease (Escherichia phage T7)	Detection of antibiotic residues	The exonuclease was used for developing a highly sensitive homogeneous electrochemical strategy for the detection of ampicillin residues, which can be applied both for clinical and food safety purposes. The exonuclease provides amplification of electrochemical signals with a limit of detection superior to those reported in the literature.	2016	[96]
phi29 motor channel (gp10 portal protein) (Bacillus phage phi29)	Fingerprinting of peptides	The motor channel was used for peptide fingerprinting in single molecule electrophysiological assays. The protein is used to generate peaks of current blockage that serve as typical fingerprint for peptides with high confidence. The results demonstrate the potential of the motor channel for detection of disease-associated peptide biomarkers.	2016	[101]
Exonuclease Exo, and DNA-binding proteins Beta and Gam (Escherichia phage lambda)	Engineering of bacterial genome	The Exo, Beta, and Gam proteins were exploited for the development of MAGE, Multiplex Automated Genome Engineering. MAGE uses synthetic single-stranded DNA to introduce targeted modifications into the chromosome of *E. coli*, to generate combinatorial genetic diversity in a cell population, or for genome-wide editing.	2014	[94]

853