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1 **Full title**

2 Engineering of biomolecules by bacteriophage directed evolution

3

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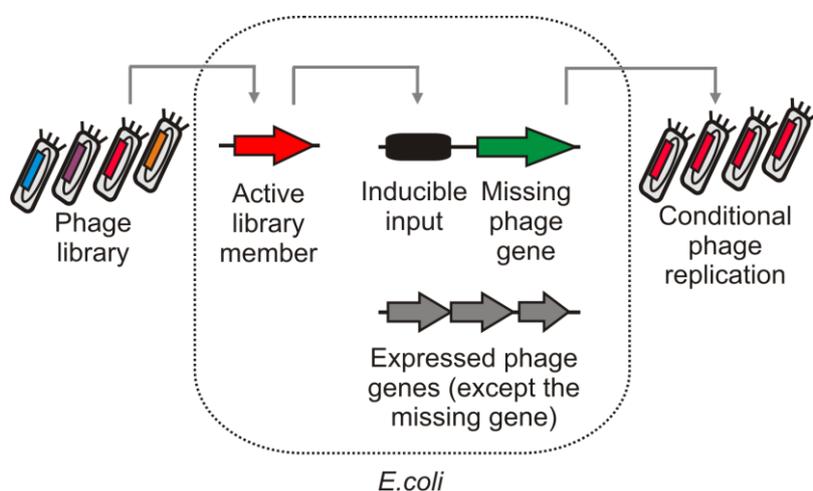
22

23 **Abstract**

24 Conventional *in vivo* directed evolution methods have primarily linked the biomolecule's
25 activity to bacterial cell growth. Recent developments instead rely on the conditional growth
26 of bacteriophages (phages), viruses that infect and replicate within bacteria. Here we review
27 recent phage-based selection systems for *in vivo* directed evolution. These approaches have
28 been applied to evolve a wide range of proteins including transcription factors, polymerases,
29 proteases, DNA-binding proteins, and protein-protein interactions. Advances in this field
30 expand the possible applications of protein and RNA engineering. This will ultimately result in
31 new biomolecules with tailor-made properties, as well as giving us a better understanding of
32 basic evolutionary processes.

33

34 **Graphical abstract (submitted in separate TIFF file)**



35

36

37 **Highlights (submitted in separate word file)**

- 38
- 39 • Directed evolution systems based on conditional phage replication (CPR) expand the potential of protein engineering.
 - 40 • CPR platforms function in both batch and continuous culture.
 - 41 • CPR systems bypass key limitations of conventional phage display.
 - 42 • Directed evolution of a wide range of proteins can be achieved by CPR.

43

44

45 **Introduction**

46 Protein engineering enables the development of valuable biomolecules for pharmaceutical
47 and biotechnological purposes. There are generally two strategies to guide protein
48 engineering: rational design or directed evolution (**Figure 1**). Rational design usually uses
49 computational tools and structural considerations to identify beneficial mutations in the
50 protein of interest [1]. Recent advances in this strategy even allow the design of proteins
51 completely *de novo* [2-6]. In comparison, directed evolution mimics natural evolution and
52 starts with a population of genotype(s) and then proceeds with the iterative generation of
53 genotype diversity and a selection based on linked phenotype activity. It is applied when too
54 little structural or biochemical information is available to guide engineering. In many cases,
55 these two strategies can be combined in a semi-rational approach to improve the activity of
56 biomolecules [7,8]. This illustrates how the method must be chosen to fit the particular
57 problem.

58
59 A variety of directed evolution techniques have been developed that employ customized
60 gene circuits [9-12]. One commonly used approach is to link the target protein's activity to
61 cell growth, which is particularly suitable when the evolving gene directly improves cellular
62 fitness [13-15]. Alternatively, the use of phage particles offers a convenient way to uncouple
63 the target protein's activity from the fitness function of a cell. Instead, an artificial genetic
64 circuit couples the evolving protein's function to increasingly efficient production of phage
65 packaging the gene of interest [16].

66
67 Directed evolution requires genotypic diversity in the gene of interest and this can either be
68 achieved *in vivo* or *in vitro*. *In vivo* mutagenesis relies on intracellular modification of the
69 target gene [17-19] whereas *in vitro* mutagenesis can be achieved extracellularly by chemical
70 modification [20], ultraviolet irradiation [21], or polymerase chain reaction (PCR) [22]. PCR-
71 based methods generally employ an error-prone polymerase or oligonucleotides that contain
72 randomized bases at the desired positions. Chemical mutagenesis and irradiation are less
73 commonly-used methods because of the lack of uniform mutational spectra [20,23]. By
74 making randomized libraries or using a progressive series of mutations, it is possible to
75 explore the 'design space' of a target gene, ultimately enabling the engineering of new
76 proteins.

77

78 In this review, we first discuss the requirements for using phages to evolve biomolecules. We
79 then focus on new directed evolution methods based on conditional phage replication that
80 have been developed thanks to advances in molecular and synthetic biology.

81

82 **Re-engineering phage-host genetic interactions to select functional** 83 **biomolecules**

84 When evolving a target gene from either a gene library or mutation system, the phenotype
85 selection can either be performed outside a living cell (*in vitro*) or inside (*in vivo*). *In vivo*
86 evolution systems allow selecting for more complex functions than *in vitro* methods (e.g.
87 phage display) which are only suitable for binary protein-molecule interactions [24] (**Figure**
88 **2a**). By contrast, intracellular evolution potentially allows selection for multi-step processes,
89 as long as they can be linked to genotype survival [25]. For example, intracellular processes
90 can facilitate the simultaneous mutation and selection of the gene of interest. Furthermore, it
91 enables the use of counterselections against an undesired biomolecule function [26]. Another
92 advantage of intracellular evolution is the subsequent compatibility of evolved genes or
93 complex gene networks with the entire host cell machinery, as these have to function in a
94 host cell context. To exploit these advantages, alternative phage-assisted directed evolution
95 platforms have been developed.

96

97 To allow enrichment of functional genes, phage selection systems require a link between the
98 desired phenotype and conditional phage replication. This can be achieved by removing an
99 essential gene required for phage replication from the phage genome and linking its
100 expression to the function of the evolving biomolecule. Alternatively, this gene (or genes)
101 could be a host co-factor required by the phage replication but dispensable to the cell (to
102 allow cell survival in the uninfected cells that are required as a host reservoir). However, the
103 only approaches developed so far rely on moving essential genes from the phage to the host
104 cell or its associated plasmids [16,27] (**Figure 2b,c**). These systems may be classified
105 according to the degree of phage engineering involved, where only a single gene may be
106 moved or practically all of them.

107

108 The evolving biomolecule has to be encoded in the phage and a genetic system has to be
109 designed to allow a functional molecule to activate the expression of the essential gene
110 (**positive selection**). When the evolving biomolecule is able to induce the expression of the
111 missing gene, infectious virions will package the DNA encoding the biomolecule, promoting
112 its survival. The conditional expression of the essential gene can be done at the

113 transcriptional or post-transcriptional levels, depending on the biomolecule to be evolved
114 (e.g. a transcription factor or a riboregulator).

115

116 Alternatively, selection may consist in designing a conditional interference with phage
117 replication if a biomolecule is functional (**negative selection**). This is used to penalize any
118 unwanted activity such as the original parental function of the biomolecule. The selection can
119 also be complex or variable, where the stringency of positive and negative selection can be
120 modulated exogenously [26].

121

122 Many alternative phage-host systems can in principle be chosen for the evolution of
123 biomolecules depending on the application. For instance, if one wanted to evolve a
124 photosynthetic protein, one might choose a cyanobacterium and one of its known phages.
125 The disadvantage of such approaches is that the phage biology is not well characterized.
126 Consequently, in this article we will focus on *E. coli* due to the lack of reported works with
127 other organisms. The *E. coli* phages M13 [28], T4 [29,30], T7 [31] or λ [32] have been used
128 to optimize protein function and stability with phage display, although M13 has been the only
129 phage vector used to evolve biomolecules *in vivo* thus far.

130

131 **Evolving biomolecules through positive selection**

132 Recently, a new method to evolve biomolecules using M13 was developed, using a redesign
133 of the host to implement a positive selection: Phage-Assisted Continuous Evolution (PACE)
134 describes a general approach for the directed evolution of proteins *in vivo* [16]. Using PACE,
135 new T7 RNA polymerase (RNAP) variants against a T3 promoter have been evolved, which
136 are not bound by the wild-type T7 RNAP. For this, the minor coat protein pIII is replaced by
137 the evolving gene of interest on the packaged M13 genome and the activity of the evolving
138 protein is linked to conditional expression of pIII on a second plasmid, named an accessory
139 plasmid (**Figure 2b**). Only phage particles assembled with pIII are infectious and propagate
140 fast enough to stay in continuous culture. Mutations only accumulate within the packaged
141 phage genome containing the target gene, and not in the *E. coli* strain, as bacteria are
142 discarded (new uninfected bacteria are continuously provided). Enhanced genetic variation is
143 obtained by a third mutagenesis plasmid (MP) that increases the mutation rate of *E. coli* cells
144 [17]. All mutator genes on this MP are under an arabinose-inducible P_{BAD} promoter allowing
145 conditional mutagenesis only at the phage replication stage. In this way, a protein with
146 desired characteristics can be evolved after dozens of reinfections within the continuous flow
147 chamber.

148

149 **Evolving biomolecules through negative selection**

150 In many cases, the requirements for evolved proteins not only include target activity but also
151 the avoidance of potential off-target effects. This can be achieved by engineering a negative
152 selection to remove variants with unwanted properties, which can be implemented by down-
153 regulating a gene required for phage replication [27,33]. Alternatively, one may exploit any of
154 the known mechanisms by which a bacterium can counteract a phage infection [34]. PACE
155 has been adapted for negative selection pressures by choosing an abortive infection
156 mechanism, where the undesired activity (activation of the original promoter) was linked to
157 the inhibition of phage propagation using a non-functional pIII variant [26].

158

159 **Modulating selection stringency for new functions**

160 An important challenge is the ability to maintain phage replication when there is a lack of
161 initial function for the biomolecule to be evolved. In the original PACE approach, an
162 intermediate selection system was used where the T7 RNAP was initially evolved to
163 transcribe a hybrid T3-T7 promoter, which had some activity, to later switch the selection to
164 the full-target T3 promoter [16]. This is actually very difficult to achieve because it requires
165 engineering a hybrid promoter that is still active with the original polymerase. Therefore this
166 cannot be easily generalised to other cases. Fortunately, an alternative method was
167 proposed that does not require re-engineering the target promoter [26] and instead relies on
168 adding a second complementary copy of the gene used for selection (here gIII). This is
169 similar to the hypothesis for the natural evolution of new functions *de novo* by gene
170 duplication, where one gene duplicate maintains the original function, while the second copy
171 is allowed to drift [35]. In the directed evolution case, the first gIII copy is under the control of
172 a T3 promoter. The second copy is under the control of a T7 promoter, but the expression of
173 this gIII is regulated (“stringency modulation”) to ensure this additional copy will cease to
174 complement the original as the evolution progresses and the T7 RNAP acquires activity for
175 the T3 promoter. Thus, the selection pressure is gradually increased over time to select the
176 new function.

177

178 **Tackling complex evolution pressures**

179 Since the initial development of PACE, the platform has been adapted for the directed
180 evolution of many different classes of proteins. For example, protease-PACE links the
181 proteolysis of a target peptide to phage replication using a protease-activated RNA
182 polymerase [36]. The system was used in the presence of two hepatitis C virus (HCV)
183 protease inhibitor drug candidates (danoprevir and asunaprevir) to evolve HCV protease
184 variants that possess up to 30-fold drug resistance. Strikingly, the predominant mutations

185 obtained in the HCV protease were consistent with the mutations observed in human
186 patients treated with danoprevir or asunaprevir. Alternatively, DNA-binding PACE is a
187 general method for the directed evolution of DNA-binding activity and specificity [25]. The
188 platform was used to engineer transcription activator-like effector nucleases (TALENs) with
189 improved DNA cleavage specificity [25]. On the other hand, protein-binding PACE enables
190 the directed evolution of protein-protein interactions [37]. The authors evolved variants of the
191 Bt toxin CryIAc against a cell receptor from the insect pest *Trichoplusia ni* with novel binding
192 affinity that can ultimately overcome insect toxin resistance. PACE was also employed to
193 continuously evolve T7 split RNA polymerases for downstream biosensor applications [38].
194 PACE has even been combined with high-throughput sequencing methods to improve
195 downstream analysis which allows the characterization of whole protein populations as they
196 adapt to selection pressures over time [39].

197

198 **Evolution using phagemids**

199 Phagemids can provide an alternative to classic full-phage selection systems. They have
200 specific advantages, such as large library sizes and avoiding the mutation of phage genes.
201 Consequently, we developed a phagemid selection system [27,33] where only the phagemid
202 (PM) containing a library member and one essential phage gene (gIII) is packaged, while all
203 the other phage components (except gVI) are provided on a modified helper phage (HP). To
204 complete the system, an accessory plasmid (AP) contains a conditional gene VI circuit
205 (**Figure 2c**). After infection, a protein with desired activity upregulates gene VI expression
206 and therefore increases phage production. In this way, a protein with desired activity can be
207 selected after several rounds of reinfection. Notably, our recently described system [27,33]
208 uses conditional production of the minor coat protein pVI instead of pIII used in PACE. This is
209 particularly useful for the directed evolution of transcription factors against basally-active
210 promoters as expressed gIII in the starter culture would otherwise cause infection resistance
211 resulting in a significantly decreased selection efficiency [40,41].

212

213 Phagemid selection has been applied for the directed evolution of a set of orthogonal
214 transcription factors based on λ cl against synthetic promoters [27]. Negative selection
215 against wild-type (WT) activity via repression has been achieved by putting the WT DNA
216 sequence between the -35 and -10 regions of each synthetic promoter. The resulting toolkit
217 contains 12 transcription factors, operating as activators, repressors, dual activator-
218 repressors or dual repressor-repressors for the use in gene network engineering. Moreover,
219 this evolution strategy functions in batch mode and therefore requires no special equipment

220 for reactor assembly, although it does rely on daily researcher interventions during selection
221 [33].

222

223 **Conclusion and perspectives**

224 Recently developed directed evolution methods based on conditional phage replication
225 further emphasize the strengths of phage-assisted protein engineering. These systems are
226 particularly useful as they bypass key limitations of the widely-used phage display technology
227 such as the simultaneous mutation and selection of complex biological functions. When
228 choosing the most suitable method, various aspects including desired protein activity,
229 available structural information, selection pressure and required selection efficiency need to
230 be considered. Intracellular phage-assisted systems can, in principle, be used for all types of
231 proteins, as long as their activity can be linked to conditional phage production (**Figure 3**).
232 Notably, this is easier to achieve for cytosolic proteins than it is for complex proteins (e.g.
233 membrane proteins). Furthermore, general limitations of bacterial expression over
234 mammalian expression such as protein solubility, posttranslational modifications and
235 disulfide bond formation have to be taken into account when using any phage-assisted
236 technology.

237

238 Phages may also be used to evolve non-coding RNAs provided that their function can be
239 linked to gene expression. This is particularly useful to complement computational designs of
240 riboregulators [42], where a cognate regulatory sequence has to be added in the 5'UTR of
241 the gene used for selection (for instance gene VI in [27,33]). Protein or RNA-based sensors
242 (activating gene expression under the presence of a target chemical inducer) may also be
243 encoded in the phage, provided one designs cycles of selections composed of two steps.
244 The first step consists of a positive selection where the sensor may activate the infectious
245 virion packaging in the presence of the chemical inducer. The second step occurs in the
246 absence of the chemical inducer, where only sensors that do not activate the negative
247 selection gene would be able to produce infectious virions. Similarly, negative selections may
248 also be used to evolve the targeted function in the case of a negative regulator of gene
249 expression (e.g., repressor). A negative selection would here act as an inverter such that
250 constitutive phage replication could be used for evolving a repressor.

251

252 Advances in the fields of DNA sequencing, gene synthesis and genome engineering will
253 likely reduce costs and improve the efficiency of current phage-assisted systems as well as
254 drive the development of new technologies based on bacteriophages other than M13 [43].

255 These advances will also impact new mutagenesis strategies, in particular ones that enable
256 targeted mutagenesis with improved mutation rates *in vivo*. The mutation of only the target
257 gene(s) while not affecting any other genetic information is desirable in order to reduce the
258 probability of selecting false positive variants in any directed evolution approach. As a
259 consequence, phage-assisted evolution technologies will continue to play a key role in
260 protein engineering efforts for basic as well as applied research.

261

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269

270 **Competing financial interests**

271 The authors declare no competing financial interests.

272

273 **References and recommended reading**

274 Papers of particular interest, published within the period of review, have been highlighted as:

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286 This study describes a general approach for the phage-assisted continuous evolution of
287 DNA-binding proteins. The authors used this method to generate transcription activator-like
288 effectors nucleases (TALENs) with improved DNA cleavage specificity.

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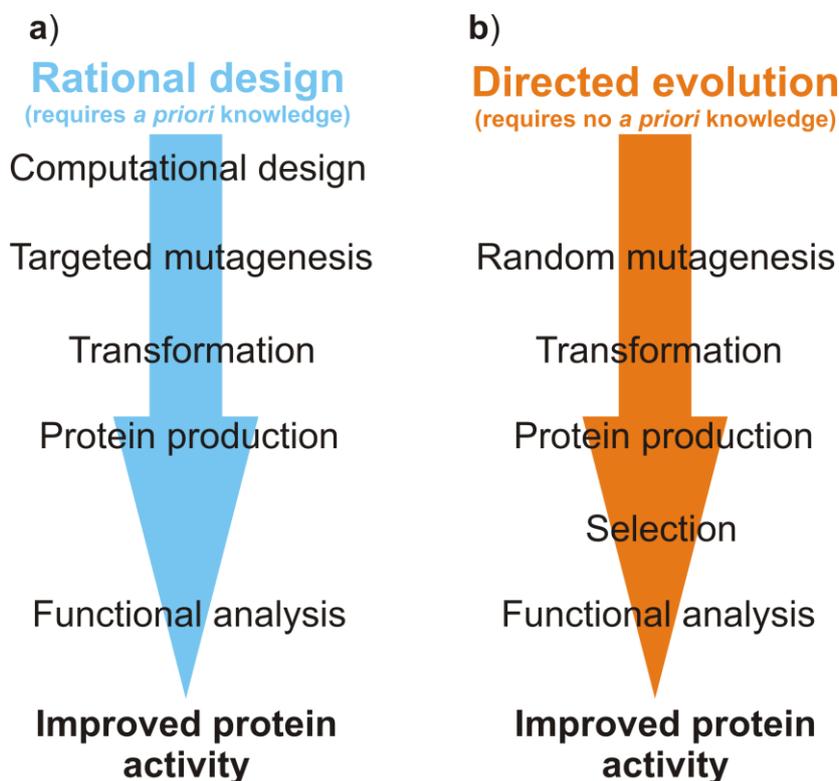
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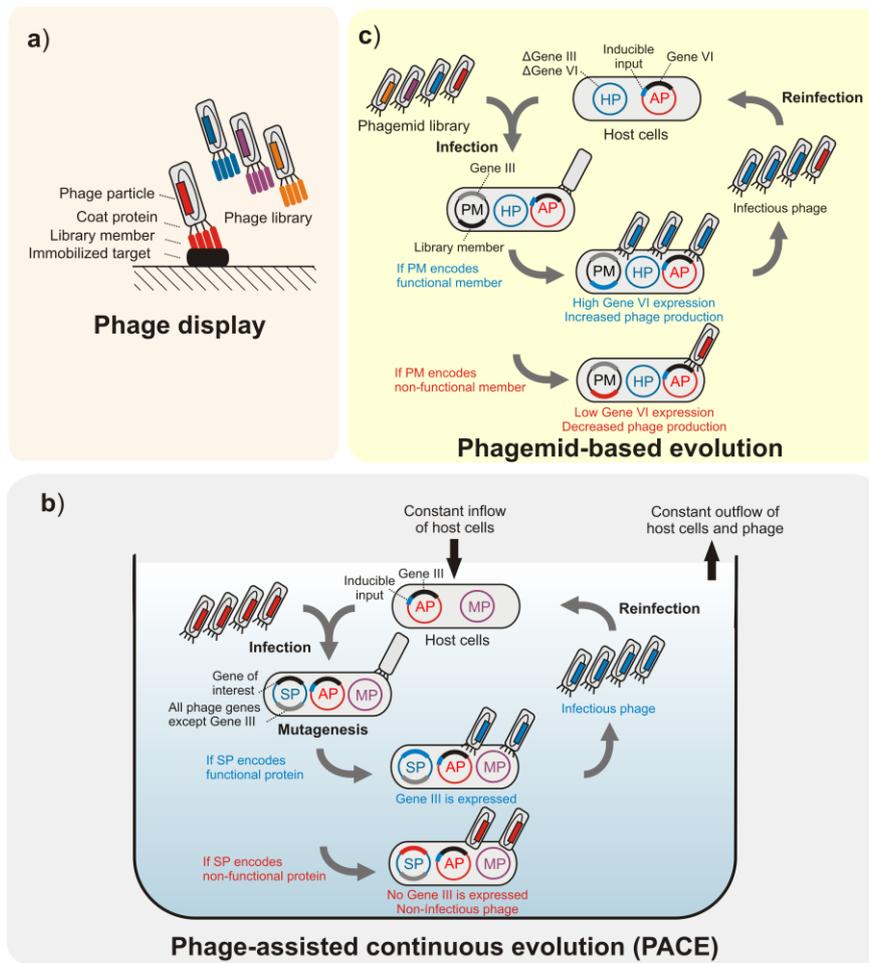
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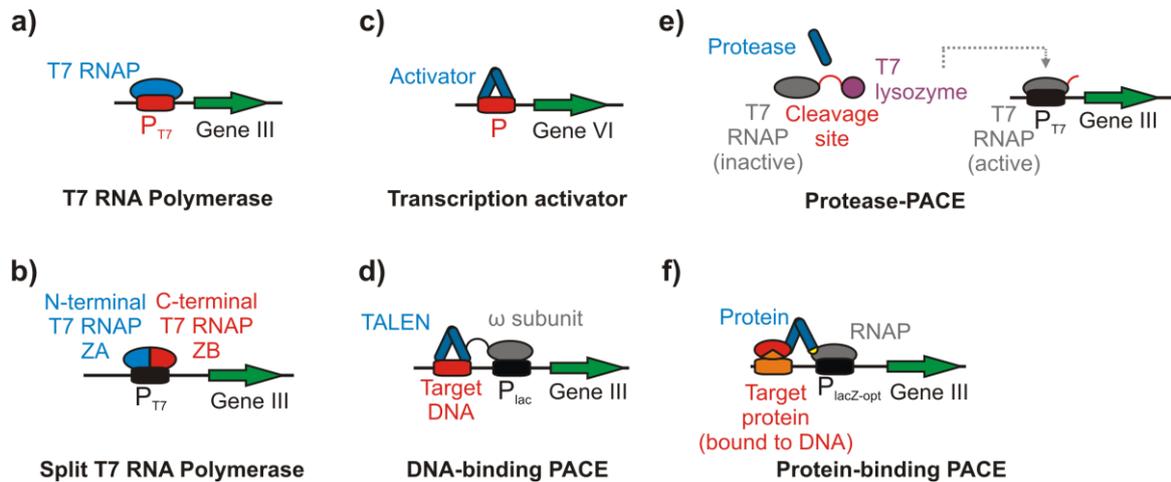
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 476 **Figure 1. Protein engineering by rational design or directed evolution. a)** Rational design uses
 477 computational tools as well as structural or other biochemical knowledge to identify beneficial
 478 mutations in the protein of interest. These mutations are inserted into the gene of interest (targeted
 479 mutagenesis) which is then expressed in host cells. Functional analysis for each protein variant is
 480 performed to confirm improved activity. **b)** Directed evolution is applied when too little structural or
 481 biochemical information is available to guide engineering. Mutations in the gene of interest are
 482 inserted randomly or by targeting specific positions in the gene sequence leading to a library of gene
 483 variants. Functional library members are then selected via a suitable selection system (e.g. phage-
 484 assisted evolution) against a target function. The activity of the selected protein is finally confirmed by
 485 functional analysis. Rational design and directed evolution are often combined to obtain the best
 486 results (semi-rational approach).

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489 **Figure 2. Phage-assisted directed evolution methods.** **a)** Affinity selection of library members by
 490 phage display. Protein variants are fused to a phage coat protein and are displayed on phage particles
 491 providing a physical connection between genotype and phenotype. **b)** Phage-assisted continuous
 492 evolution (PACE) is based on conditional M13 phage replication. The activity of the evolving protein on
 493 the selection phage (SP; contains the gene of interest and all phage genes except gene III) is linked to
 494 gIII expression on the accessory plasmid (AP; contains a conditional gene III expression circuit). Only
 495 phage particles assembled with pIII are infectious and propagate fast enough to stay in continuous
 496 culture. The system uses a mutagenesis plasmid (MP) that increases the mutation rate of *E. coli* cells
 497 to generate target gene diversity. Mutations only accumulate within the packaged phage genome
 498 containing the target gene and not in the *E. coli* strain due to the continuous nature of the system. In
 499 this way, a protein with desired characteristics can be evolved after dozens of rounds of reinfection.
 500 **c)** Phagemid-based evolution from combinatorial libraries in batch mode. The library members are
 501 located on a packaged phagemid (PM) which also contains one essential phage gene (gIII). All the
 502 other phage genes are located on a modified helper phage (HP; contains all phage genes except
 503 genes III and VI) and an accessory plasmid (AP; contains a conditional gene VI expression circuit).
 504 After infection, a protein with desired activity upregulates gene VI expression and therefore increases
 505 phage production. In this way, a protein with desired activity can be selected after several rounds of
 506 reinfection.



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Figure 3. Directed evolution of different classes of proteins based on conditional M13 phage replication. **a)** An evolving T7 RNA polymerase upregulates gene III expression in an activity-dependent manner [16]. **b)** An evolving N-terminal T7 RNA polymerase fused to a leucine zipper ZA assembles with a C-terminal T7 RNA polymerase variant fused to leucine zipper ZB leading to gene III expression in an activity-dependent manner [38]. **c)** An evolving transcription activator (e.g. λ cI) upregulates gene VI expression downstream of a specific promoter (e.g. λ P_{RM}) [27]. **d)** DNA-binding PACE enables the evolution of transcription activator-like effector nucleases (TALENs) [25]. The evolving DNA-binding protein is linked to the ω subunit of bacterial RNA polymerase III and binding to a target DNA sequence upstream of a minimal lac promoter enables gene III expression in an activity-dependent manner. **e)** Protease-PACE enables the evolution of proteases against desired cleavage sites [36]. The T7 polymerase is inhibited when bound to T7 lysozyme as it inhibits transcription initiation and the transition from initiation to elongation [44]. Proteolysis of the target cleavage site by an evolving protease activates the T7 RNA polymerase leading to gene III expression in an activity-dependent manner. **f)** Protein-binding PACE allows the evolution of protein-protein interactions [37]. The target protein is bound to the DNA upstream the promoter P_{lacZ-opt} via a fused DNA-binding domain (orange) and the RNA polymerase omega subunit (RpoZ; yellow) is fused to the evolving protein. The binding of the evolving protein to the target protein enables the transcription of gene III from the P_{lacZ-opt} promoter. The evolving protein is highlighted in blue and the target sequence is depicted in red for each individual example.