

“I will survive”: a tale of bacteriophage-bacteria coevolution in the gut

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1 **Title:**

2 “I will survive”: a tale of bacteriophage-bacteria coevolution in the gut

3

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12

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23 **ABSTRACT**

24 Viruses that infect bacteria, or bacteriophages, are among the most abundant entities in the gut
25 microbiome. However, their role and the mechanisms by which they infect bacteria in the
26 intestinal tract remain poorly understood. We recently reported that intestinal bacteria are an
27 evolutionary force, driving the expansion of the bacteriophage host range by boosting the
28 genetic variability of these viruses. Here, we expand these observations by studying
29 antagonistic bacteriophage-bacteria coevolution dynamics and revealing that bacterial genetic
30 variability is also increased under the pressure of bacteriophage predation. We propose a
31 model showing how the expansion of bacteriophage-bacteria infection networks is relative to
32 the opportunities for coevolution encountered in the intestinal tract. Our data suggest that
33 predator-prey dynamics are perpetuated and differentiated in parallel, to generate and
34 maintain intestinal microbial diversity and equilibrium.

35 **INTRODUCTION**

36 The homeostasis of the intestinal microbiome is crucial to health, as shown by the ever-
37 growing list of chronic conditions linked to microbiota dysbiosis, including obesity, diabetes,
38 asthma, inflammatory bowel disease (IBD) and central nervous system disorders¹⁻⁴. The
39 antagonistic coevolution between the two most abundant components of the microbiome,
40 bacteria and their viruses, bacteriophages, is a key candidate player in the maintenance of this
41 microbial equilibrium⁵.

42 The perpetuation of bacteriophages is intrinsically dependent on their ability to predate on the
43 bacterial populations and experimental coevolution studies have characterised the dynamics
44 of interactions between bacteria and bacteriophages^{6,7}. The development of bacterial
45 resistance, and the consequent bacteriophage adaptation towards such resistance, have been
46 identified as major forces driving their antagonistic coevolution *in vitro* and in environmental
47 samples. This arms race necessarily results in an increase in the genomic diversity of both

48 partners to ensure population survival^{8,9}, as seen in aquatic ecosystems^{10,11}. However, most
49 studies of this type are limited to single pairs of bacteria and bacteriophages and are
50 frequently performed in laboratory settings.

51 Metagenomic analyses of intestinal bacterial populations have revealed that these organisms
52 are diverse and differently abundant in healthy humans and diseased patients. Fewer studies
53 have focused on viral populations (virome), but those that have been performed have revealed
54 an unprecedented complexity of relationships between bacteriophages, bacteria and the
55 mammalian host¹². A recent comparative study showed that healthy humans share a pool of
56 conserved intestinal bacteriophages that differs significantly from the viruses found in patients
57 with inflammatory bowel disease (IBD)¹³. Also, in these patients, lower bacterial diversity is
58 associated with a significantly larger number and diversity of bacteriophages¹⁴. Similarly, a
59 recent microbiome study conducted on malnourished pediatric patients hospitalized with
60 acute diarrhea showed an increase in *Escherichia coli* bacteriophages compared to healthy
61 individuals, that negatively correlated with the abundance of the bacterial host¹⁵. Other studies
62 suggest that bacteriophages play a key role in regulating intestinal bacterial populations by
63 showing that filtered (bacteria-free) faecal microbiota transplantation (FMT) yields curative
64 results comparable to those obtained with traditional FMT, and that viral transfer correlates
65 with the resolution of gut infections caused by *Clostridium difficile*^{16,17}. Nonetheless,
66 exploitation of this genomic information at the molecular level remains limited, because most
67 of the sequences obtained do not match to a known function. Another major hurdle is the lack
68 of association between bacteriophage sequences and those of their specific bacterial hosts.
69 There is, therefore, a considerable gap between studies of interactions between bacteriophages
70 and bacteria in laboratory conditions and the complexity of these interactions in the gut^{13,18}.
71 In the environment, bacteria and bacteriophages coexist in intricate, structured interaction
72 networks^{19,20}. Bacterial species are represented by distinct genetic lineages (strains) and

73 bacteriophages are mostly strain-specific: rare are bacteriophages that infect most strains
74 within one given species and even fewer are those infecting distinct species. Thus, little is
75 known about the role of bacteriophage-bacteria infection networks in driving the
76 diversification of the gut microbial ecosystem in the context of health and disease.

77

78 **MICROBIOTA-DRIVEN BACTERIOPHAGE ADAPTATION**

79 Reductionist approaches using *E. coli* and its bacteriophages have successfully deciphered
80 major mechanisms of molecular biology²¹⁻²³. By lifting the reductionist approach to the next
81 level of complexity, namely the study of the intestinal microbiota, we recently described the
82 coevolution of one bacteriophage with multiple host strains within the mouse gut²⁴. We
83 studied P10, a virulent bacteriophage from the Myoviridae family, infecting the *E. coli* strain
84 LF82, and we assessed its ability to adapt to *E. coli* strain MG1655, to which it was initially
85 unable to bind and therefore could not infect. Such host-range expansion was observed, but
86 only occurred during coevolution in the gut of conventional mice hosting *E. coli* strains LF82
87 and MG1655 within their microbiota. In planktonic *in vitro* cultures or in the gut of dioxenic
88 mice colonized solely by the two *E. coli* strains, this event was never detected. Based on these
89 findings, we hypothesized that the mouse microbiota played a crucial role in promoting
90 adaptation. Indeed, we showed that this adaptation was initiated by the infection of an
91 intermediate host, *E. coli* strain MEc1, which we isolated from the murine microbiota. Mixing
92 bacteriophage P10 *in vitro* with the three *E. coli* strains also promoted viral host-range
93 expansion. This adaptation was accompanied by genomic differentiation in the bacteriophage
94 population: a single point mutation in a tail fibre-encoding gene was found to be sufficient to
95 promote host adaptation, but additional mutations were required to optimise the infectious
96 cycle.

97 The spatial and temporal dynamics of the acquisition of these mutations in the structured
98 intestinal environment remain unclear. However, our data are consistent with the hypothesis
99 that the genomic differentiation of bacteriophage subpopulations depends on the diversity of
100 the bacteria encountered, making the microbiota an ideal site to generate viral diversity. In
101 addition to bacterial diversity, the spatial distribution of bacterial populations along the gut
102 may also influence the dynamics of bacteriophage evolution^{25,26}. The colonisation of macro-
103 environments, such as the small versus the large intestine and their compartments (luminal
104 and mucosal), and the occupation of specific niches within these contexts (nutrient-niche
105 hypothesis²⁷), give rise to structured networks of single or mixed bacterial populations²⁸
106 likely to promote the diversification of bacteriophages into multiple subpopulations with
107 diverging infectivity profiles.

108

109 **GENETIC BACTERIAL RESISTANCE IN THE GUT**

110 Here, we analyse a second source of genomic diversity, the emergence of bacterial resistance,
111 one of the drivers of antagonistic evolution^{5,8,29}. Faecal pellets of mice in which P10
112 adaptation had occurred, yielded five MG1655 clones displaying different degrees of
113 resistance to adapted P10 bacteriophages (Fig. 1A). The genomes of these five strains
114 presented different mutations in the *waaZ* gene, which encodes a protein involved in the
115 biosynthetic pathway for the core lipopolysaccharide (LPS) (Fig. 1B; Table S1). We
116 identified four convergent paths of adaptation, characterised by gene disruption by insertion
117 sequences (ISs), IS5 and IS2, at different gene positions. We hypothesise that independent
118 convergent events leading to modifications of the LPS core biosynthesis pathway had served
119 as the first step towards adaptation of the newly targeted strain MG1655, under the selective
120 pressure of bacteriophage predation.

121 Another gene, *waaY*, flanking *waaZ*, was also targeted by IS elements in three of the
122 coevolved MG1655 clones. The occurrence of these mutations, coupled to the high degree of
123 sequence identity between bacteriophage P10 and the LPS-binding WV8 and Felix-O1
124 bacteriophages^{30,31}, suggests a bacterial resistance strategy based on the masking of the
125 bacteriophage receptor. Interestingly, natural populations of *Vibrio cholerae* isolated from
126 patients with diarrhoea have also been shown to consist of heterogeneous mixtures of unique
127 mutants resistant to bacteriophage predation³². However, these mutants were subject to
128 fitness and virulence costs that might arguably affect their infection potential. Similarly,
129 experimental phage therapy studies revealed that bacterial pathogens can develop
130 bacteriophage resistance at the expenses of their major virulence factors, as shown in bovine
131 enteropathogenic *E. coli*³³ or during experimental endocarditis due to *Pseudomonas*
132 *aeruginosa*³⁴.

133 Further genomic analysis of the MG1655 clones that had coevolved with P10 identified a
134 second hotspot for mutations in the galactitol operon, which was previously shown to be
135 pervasive in *E. coli* clones adapting to the gut environment^{35,36}. In addition, two sugar
136 metabolism pathways (maltose and galactonate) were targeted by IS insertions in genes
137 encoding the DNA-binding transcriptional regulators (*malt*, *lgoR*), with probable positive or
138 negative overall effects on pathway activation.

139 The contextual genomic variability of bacteriophages was also analysed by sequencing five
140 adapted bacteriophages differing in their ability to infect the five MG1655 clones considered
141 (Fig. 1C). The only bacteriophage able to infect all the bacterial clones had the largest number
142 of mutations (12 mutations, versus 5 to 9 in the other bacteriophages isolated; Table S1),
143 suggesting a possible faster pace of adaptation in response to bacterial resistance. The
144 mutations were clustered into four genomic regions. The first corresponds to the *rIIA* (*gp37*)
145 gene, the function of which is probably related to infection fitness, as this gene was also

146 highlighted in our population genomics study in *in vitro* conditions²⁴. A second, larger region
147 encompasses several structural genes, including the tail fibre genes. The *gp55* and *gp57*
148 genes, which are predicted to encode two subunits of the class I ribonucleotide reductase,
149 were also affected, together with *gp108*, the function of which is unknown.

150 However, the functions of the affected genes were not sufficient to associate genomic
151 mutations with differences in bacteriophage infectivity, highlighting the versatility of
152 bacteriophage infection. It remains to be determined which of these mutations accumulated
153 before and after the development of bacterial resistance.

154 We investigated these dynamics further, by performing a time-shift interaction study. We
155 isolated P10 clones ($n=40$) from three time points during coevolution: one time-point before,
156 and two after the adaptation of P10 to strain MG1655. We characterised the ability of these
157 clones to infect MG1655 clones ($n=40$) isolated at past, present and future time points in the
158 same experiment. As expected, bacteriophages isolated before the adaptation event were
159 unable to infect any of the contemporary MG1655 clones (present) (Fig. 1D). While adapted
160 bacteriophages were always able to infect bacterial clones from the past time points, those
161 isolated at the first time point after the adaptation event (day 1) showed reduced infectivity
162 towards MG1655 bacterial clones isolated at the present and, particularly, future time points.
163 However, all bacteriophages isolated subsequently (day 21) were able to infect past, present
164 and future bacterial clones, overcoming the bacterial resistance that had developed and
165 demonstrating the occurrence of continuous adaptive evolution in the mouse gut (Fig. 1D).

166 It could, therefore, be argued that bacteriophage adaptation in the gut led to a two-step
167 coevolution pathway, in which the evolutionary arms race was initially characterised by the
168 rapid development of bacterial resistance followed by a refining of bacteriophage adaptation.
169 The two populations subsequently continued to coexist, with no evidence of renewed bacterial

170 resistance, suggesting that transient resistance occurred *in situ*, protecting the bacteria against
171 bacteriophage predation, as discussed below.

172

173 **TRANSIENT BACTERIAL RESISTANCE IN THE GUT**

174 Bacterial resistance to bacteriophages has long been studied and characterised *in vitro*³⁷, and
175 is known to involve several mechanisms. These include the prevention of adsorption,
176 superinfection exclusion, restriction modifications, CRISPR-Cas systems, bacteriophage
177 exclusion (BREX), and many new recently discovered systems revealing the extreme
178 versatility of bacterial resources for defence³⁸⁻⁴⁰. Nonetheless, little is known about the
179 mechanisms activated *in vivo*, and their relevance and impact in natural communities. In our
180 study, the resistance of strain MG1655 to the newly adapted bacteriophage P10 seemed to
181 depend on preventing adsorption by modifying the bacteriophage receptor. However, this may
182 simply reflect part of the process of bacteriophage adaptation to a new bacterial host, as the
183 bacteriophage could rapidly fine-tune its mechanism of infection to overcome this resistance.
184 This hypothesis is supported by the lack of emergence of resistant clones of the original
185 bacterial host, strain LF82, in mouse faeces (data not shown), despite the presence of large
186 numbers of both the bacteriophage and the bacterium during the course of the experiment.
187 We have already reported similar observations for a different *E. coli* strain, 55989, coevolving
188 in mouse gut with either a cocktail of three virulent bacteriophages or with each
189 bacteriophage separately. No resistance was ever detected when 20 bacterial isolates were
190 tested against the individual bacteriophages^{41,42}. However, two to six hours of co-incubation
191 with the same bacteriophages *in vitro* was sufficient to trigger the development of bacterial
192 resistance⁴³. We also previously tested the ability of each bacteriophage to replicate in the
193 intestinal environment *ex vivo*, both in homogenates of the small and the large intestines and
194 in the faeces of mice colonised with *E. coli* strain LF82 or strain 55989^{41,44}. We found that

195 all bacteriophages were infectious in the ileal sections, but that replication in colonic or faecal
196 samples was significantly impaired for some of them ^{41, 44}.

197 These results support the hypothesis that the metabolic state of bacteria, which is not uniform
198 throughout the gut ⁴⁵, is the principal barrier to bacteriophage infection. Indeed, several
199 factors, such as the availability of carbon sources, oxygen, and stress responses, can have a
200 marked effect on cell surface structures, some of which are required for bacteriophage
201 infection. This physiological and structural versatility provides bacteria with opportunities for
202 transient resistance to bacteriophages without paying the cost or irreversible mutations, but
203 remaining susceptible when the physiological conditions change, such as during growth in the
204 laboratory environment. Conversely, bacteriophages can escape such resistance strategies by
205 entering into a state of pseudolysogeny or hibernation, in which the infectious cycle is halted
206 until better conditions for progeny production occur ^{46, 47}.

207

208 **MODEL OF BACTERIOPHAGE – BACTERIA COEVOLUTION IN THE GUT**

209 This dynamic picture of the coevolution of bacteria and bacteriophages serves as the basis of a
210 theoretical model describing how microbiome diversity is generated and expanded via these
211 interactions (Fig. 2). Mutations in the bacteriophage genome accumulate when they confer a
212 fitness advantage and favour perpetuation of the infection cycle. This corresponds to
213 adaptation to new host strains, and/or host strains that have acquired resistance. However, it
214 remains unclear whether bacteriophage evolution discriminates between these two bacterial
215 situations, since each adaptation event would involve specific mechanisms to overcome the
216 obstacles to predation.

217 The evolution of the microbiome results in a growing number of bacteriophage populations
218 infecting new bacterial hosts with which perpetuating the process of antagonistic coevolution.

219 This is likely to occur at the expense of the most abundant and available bacterial populations,
220 providing a major contribution to microbiome homeostasis and to bacterial differentiation.
221 Bacterial hosts also have opportunities to escape bacteriophage predation, resulting in
222 genomic differentiation between microbial populations. In addition, some of these populations
223 are likely to be protected against bacteriophage predation due to their physical inaccessibility
224 in the environment, their limited density and/or the development of transient resistance due to
225 their metabolic and phenotypic states. An example of such viral diversification in the human
226 gut can be found with the expanding population of Crassphage⁴⁸.

227 **CONCLUDING REMARKS**

228 The timing, frequency and conditions required for bacteriophage adaptation and bacterial
229 resistance during coevolution in the intestinal microbiota remain largely unpredictable.
230 However, we propose that, in healthy conditions, bacteriophage communities play a crucial
231 role in controlling bacterial populations, both by promoting heterogeneous microbial
232 differentiation and by adapting in a flexible manner to new patterns of abundance and
233 diversity in susceptible bacteria. If this fails to occur, dysbiotic conditions may arise, leading
234 to extinction or abnormal proliferation of the viral and bacterial partners, with consequences
235 for human health.

236

237 **Disclosure of potential conflicts of interest**

238 The authors declare no potential conflicts of interest.

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246

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365

366

367 Legends

368 Figure 1: Bacteriophages and bacteria coevolve in the mouse gut.

369 A) Adapted (ad_) P10 bacteriophages show differential infectivity towards coevolved (ev_)
370 clones of *E. coli* strain MG1655 (MG) isolated at the same time point and that have developed
371 bacteriophage resistance. Infectivity of five P10 bacteriophages (1-3,5-6) was tested against
372 five MG1655 clones (a-e) by double spot technique⁴⁹ with two amounts of bacteriophages
373 (10^6 and 10^4 pfu) in three replicates. Positive results of infection were determined by
374 recording bacterial lysis and are shown as black dots. B) Bacterial genomic mutations under
375 bacteriophage selective pressure in the mouse gut: ev_MG clones a-to-e were sequenced by
376 Illumina technology and mutations were called using the Breseq variant report software v0.26
377⁵⁰. Mutations (orange, red and blue triangle - IS1, IS2 and IS5 respectively, black triangle
378 pointing down - 1-5bp insertion, black triangle pointing up - 1-5bp deletion, vertical black
379 rectangle – SNP and black horizontal rectangle - >1kb deletion) are reported relative to their
380 positions in the genome. For mutation hotspots, the relative targeted genes are reported as
381 purple arrows. For a complete list of bacterial genomic mutations see Table S1. The
382 corresponding sequences are deposited at ENA under project PRJEB24878. C) Bacteriophage
383 genomic mutations accumulated during coevolution with strain MG1655 in the mouse gut.
384 Sequences of five adapted P10 bacteriophages (ad_P10_1-3,5-6) were analysed as described
385 for bacterial clones. Mutations are relative to their positions in the bacteriophage genome
386 (ORFs are shown as purple arrows) and mutation hotspots are indicated (same legend as for
387 panel B). For a complete list of viral genomic mutations, see Table S2. The corresponding
388 sequences are deposited at ENA under project PRJEB18073. D) Bacteriophages overcome
389 genetic bacterial resistance. A time-shift experiment shows the percentage infectivity of
390 fourty P10 bacteriophages from different time points tested towards fourty MG1655 clones

391 isolate from past, present and future time-points during coevolution in the mouse gut.

392 Bacterial lysis was tested by double-spot assay⁴⁹.

393

394 Figure 2: Model of bacteriophage-bacteria coevolution and differentiation in the gut.

395 From the bottom, three bacterial populations (blue, green and orange) are differentially

396 susceptible to one bacteriophage (yellow). Under bacteriophage predation, sub-populations of

397 resistant bacteria can emerge (lighter colours). These either can become dominant, leading to

398 extinction of other subpopulations, or be maintained in equilibrium. Contextually,

399 bacteriophage sub-populations diverge (represented by different colours) by adapting to

400 changes in the coevolving bacteria or to new hosts (host-jump, black arrows). The

401 consequence (top) is the progressive differentiation of both antagonistic populations.

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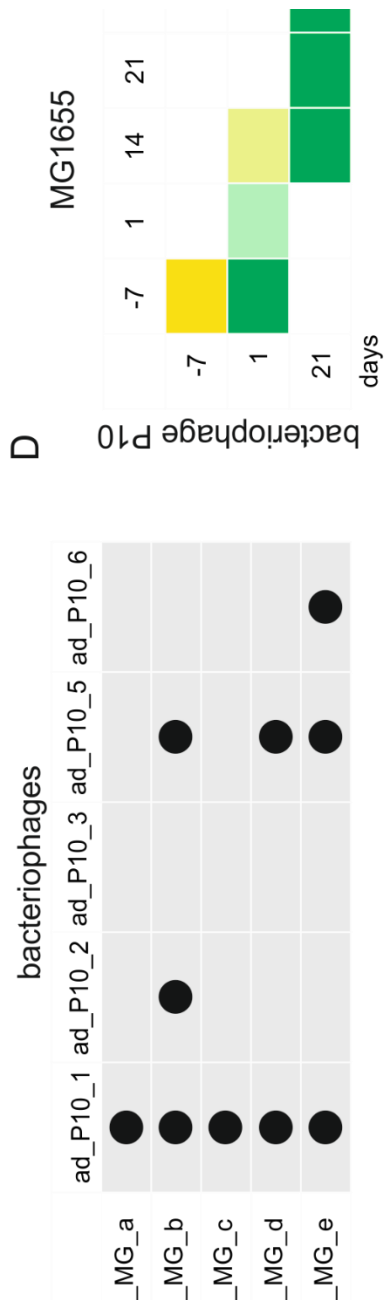
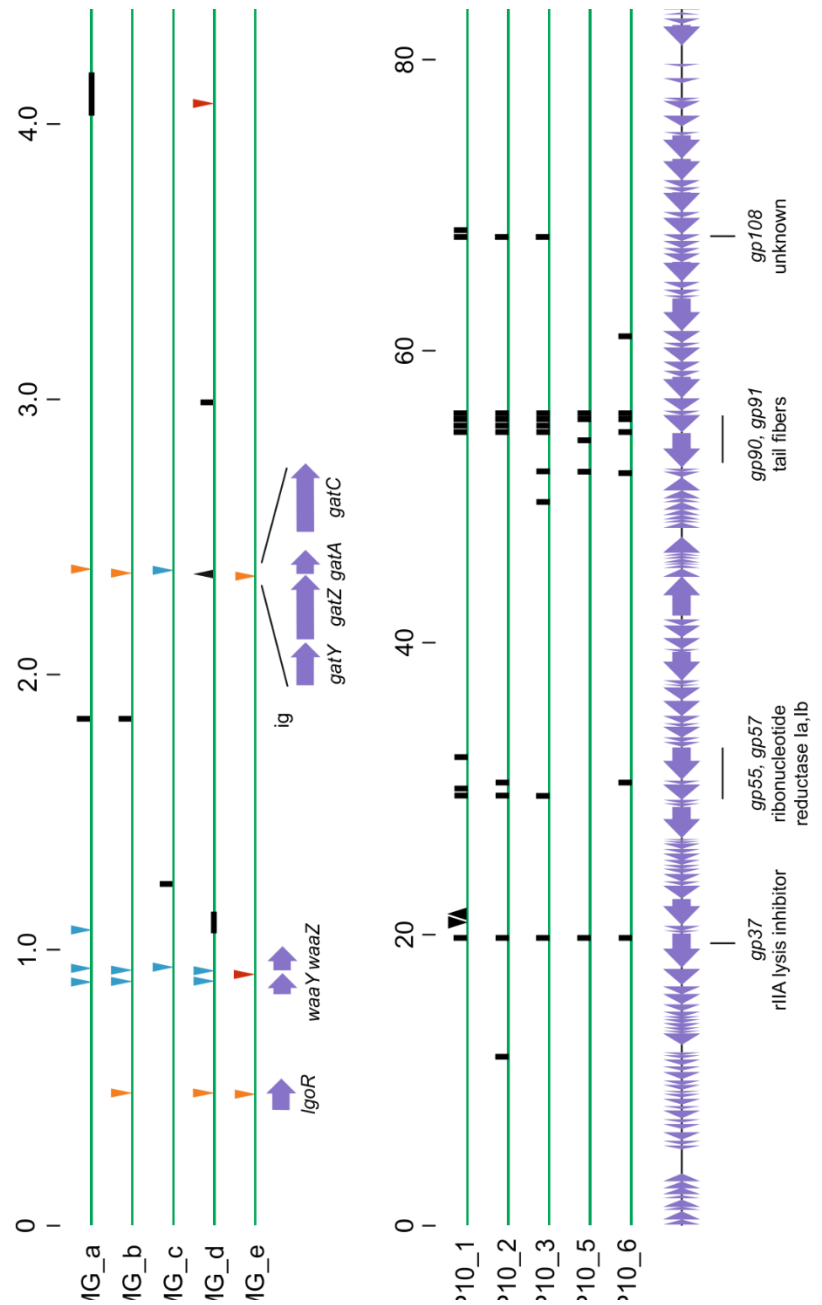
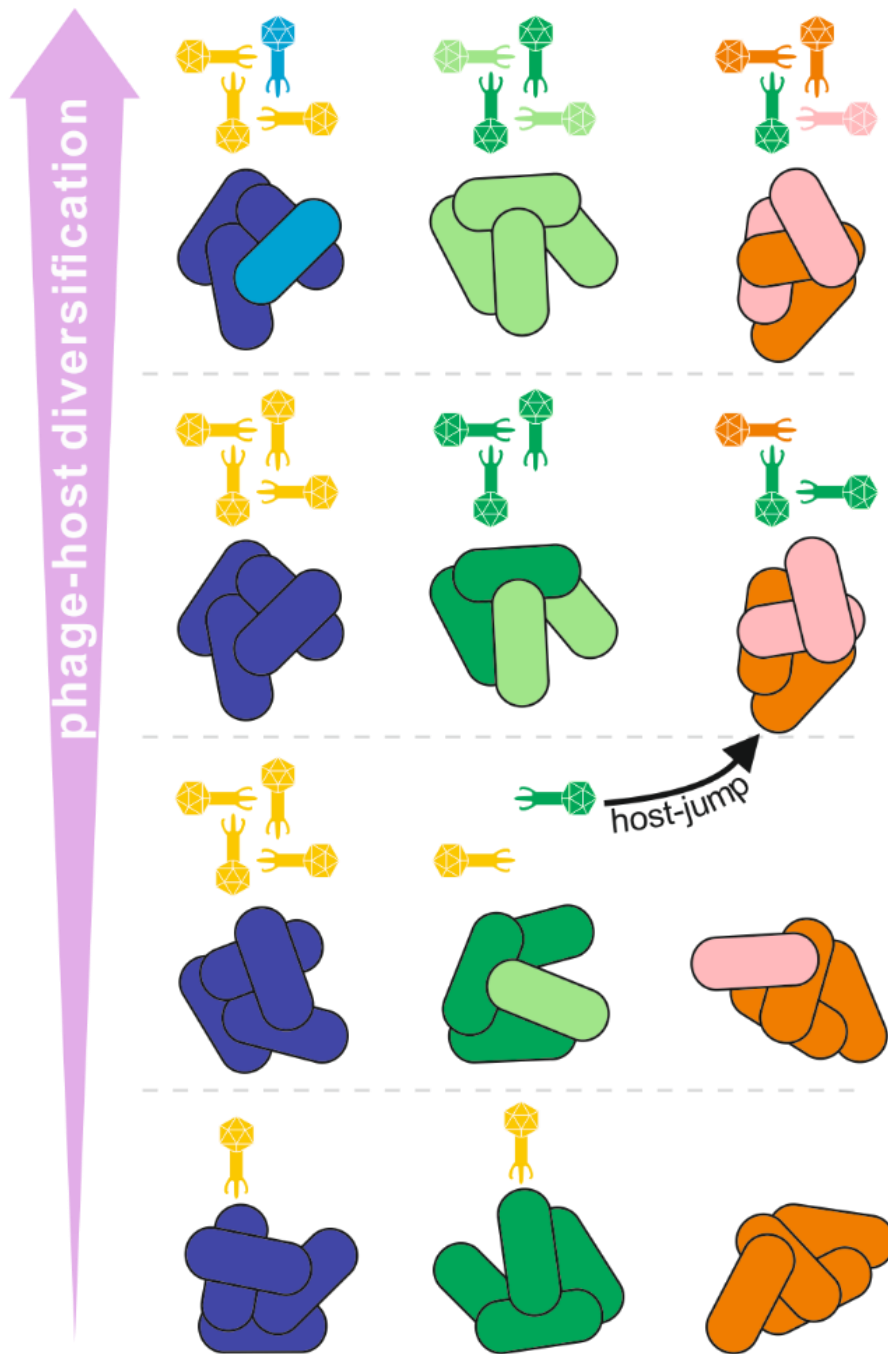


Figure 1





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Figure 2