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

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ABSTRACT

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

INTRODUCTION

The homeostasis of the intestinal microbiome is crucial to health, as shown by the ever-growing list of chronic conditions linked to microbiota dysbiosis, including obesity, diabetes, asthma, inflammatory bowel disease (IBD) and central nervous system disorders[1-4]. The antagonistic coevolution between the two most abundant components of the microbiome, bacteria and their viruses, bacteriophages, is a key candidate player in the maintenance of this microbial equilibrium [5].

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

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

Metagenomic analyses of intestinal bacterial populations have revealed that these organisms are diverse and differently abundant in healthy humans and diseased patients. Fewer studies have focused on viral populations (virome), but those that have been performed have revealed an unprecedented complexity of relationships between bacteriophages, bacteria and the mammalian host [12]. A recent comparative study showed that healthy humans share a pool of conserved intestinal bacteriophages that differs significantly from the viruses found in patients with inflammatory bowel disease (IBD) [13]. Also, in these patients, lower bacterial diversity is associated with a significantly larger number and diversity of bacteriophages[14]. Similarly, a recent microbiome study conducted on malnourished pediatric patients hospitalized with acute diarrhea showed an increase in *Escherichia coli* bacteriophages compared to healthy individuals, that negatively correlated with the abundance of the bacterial host[15]. Other studies suggest that bacteriophages play a key role in regulating intestinal bacterial populations by showing that filtered (bacteria-free) faecal microbiota transplantation (FMT) yields curative results comparable to those obtained with traditional FMT, and that viral transfer correlates with the resolution of gut infections caused by *Clostridium difficile* [16, 17]. Nonetheless, exploitation of this genomic information at the molecular level remains limited, because most of the sequences obtained do not match to a known function. Another major hurdle is the lack of association between bacteriophage sequences and those of their specific bacterial hosts. There is, therefore, a considerable gap between studies of interactions between bacteriophages and bacteria in laboratory conditions and the complexity of these interactions in the gut[13, 18].

In the environment, bacteria and bacteriophages coexist in intricate, structured interaction networks[19, 20]. Bacterial species are represented by distinct genetic lineages (strains) and

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

MICROBIOTA-DRIVEN BACTERIOPHAGE ADAPTATION

Reductionist approaches using *E. coli* and its bacteriophages have successfully deciphered major mechanisms of molecular biology [21-23]. By lifting the reductionist approach to the next level of complexity, namely the study of the intestinal microbiota, we recently described the coevolution of one bacteriophage with multiple host strains within the mouse gut [24]. We studied P10, a virulent bacteriophage from the Myoviridae family, infecting the *E coli* strain LF82, and we assessed its ability to adapt to *E. coli* strain MG1655, to which it was initially unable to bind and therefore could not infect. Such host-range expansion was observed, but only occurred during coevolution in the gut of conventional mice hosting *E. coli* strains LF82 and MG1655 within their microbiota. In planktonic *in vitro* cultures or in the gut of dixenic mice colonized solely by the two *E. coli* strains, this event was never detected. Based on these findings, we hypothesized that the mouse microbiota played a crucial role in promoting adaptation. Indeed, we showed that this adaptation was initiated by the infection of an intermediate host, *E. coli* strain MEc1, which we isolated from the murine microbiota. Mixing bacteriophage P10 *in vitro* with the three *E. coli* strains also promoted viral host-range expansion. This adaptation was accompanied by genomic differentiation in the bacteriophage population: a single point mutation in a tail fibre-encoding gene was found to be sufficient to promote host adaptation, but additional mutations were required to optimise the infectious cycle.

The spatial and temporal dynamics of the acquisition of these mutations in the structured intestinal environment remain unclear. However, our data are consistent with the hypothesis that the genomic differentiation of bacteriophage subpopulations depends on the diversity of the bacteria encountered, making the microbiota an ideal site to generate viral diversity. In addition to bacterial diversity, the spatial distribution of bacterial populations along the gut may also influence the dynamics of bacteriophage evolution [25, 26]. The colonisation of macro-environments, such as the small versus the large intestine and the their compartments (luminal and mucosal), and the occupation of specific niches within these contexts (nutrient-niche hypothesis [27]), give rise to structured networks of single or mixed bacterial populations [28] likely to promote the diversification of bacteriophages into multiple subpopulations with diverging infectivity profiles.

GENETIC BACTERIAL RESISTANCE IN THE GUT

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

Another gene, *waaY*, flanking *waaZ*, was also targeted by IS elements in three of the coevolved MG1655 clones. The occurrence of these mutations, coupled to the high degree of sequence identity between bacteriophage P10 and the LPS-binding WV8 and Felix-O1 bacteriophages [30, 31], suggests a bacterial resistance strategy based on the masking of the bacteriophage receptor. Interestingly, natural populations of *Vibro cholerae* isolated from patients with diarrhoea have also been shown to consist of heterogeneous mixtures of unique mutants resistant to bacteriophage predation [32]. However, these mutants were subject to fitness and virulence costs that might arguably affect their infection potential. Similarly, experimental phage therapy studies revealed that bacterial pathogens can develop bacteriophage resistance at the expenses of their major virulence factors, as shown in bovine enteropathogenic *E. coli* [33] or during experimental endocarditis due to *Pseudomonas aeruginosa* [34].

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

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

highlighted in our population genomics study in *in vitro* conditions [24]. A second, larger region encompasses several structural genes, including the tail fibre genes. The *gp55* and *gp57* genes, which are predicted to encode two subunits of the class I ribonucleotide reductase, were also affected, together with *gp108*, the function of which is unknown.

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

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

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

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

TRANSIENT BACTERIAL RESISTANCE IN THE GUT

Bacterial resistance to bacteriophages has long been studied and characterised *in vitro*[37], and is known to involve several mechanisms. These include the prevention of adsorption, superinfection exclusion, restriction modifications, CRISPR-Cas systems, bacteriophage exclusion (BREX), and many new recently discovered systems revealing the extreme versatility of bacterial resources for defence [38-40]. Nonetheless, little is known about the mechanisms activated *in vivo*, and their relevance and impact in natural communities. In our study, the resistance of strain MG1655 to the newly adapted bacteriophage P10 seemed to depend on preventing adsorption by modifying the bacteriophage receptor. However, this may simply reflect part of the process of bacteriophage adaptation to a new bacterial host, as the bacteriophage could rapidly fine-tune its mechanism of infection to overcome this resistance. This hypothesis is supported by the lack of emergence of resistant clones of the original bacterial host, strain LF82, in mouse faeces (data not shown), despite the presence of large numbers of both the bacteriophage and the bacterium during the course of the experiment.

We have already reported similar observations for a different *E. coli* strain, 55989, coevolving in mouse gut with either a cocktail of three virulent bacteriophages or with each bacteriophage separately. No resistance was ever detected when 20 bacterial isolates were tested against the individual bacteriophages [41, 42]. However, two to six hours of co-incubation with the same bacteriophages *in vitro* was sufficient to trigger the development of bacterial resistance [43]. We also previously tested the ability of each bacteriophage to replicate in the intestinal environment *ex vivo*, both in homogenates of the small and the large intestines and in the faeces of mice colonised with *E. coli* strain LF82 or strain 55989 [41, 44]. We found that

all bacteriophages were infectious in the ileal sections, but that replication in colonic or faecal samples was significantly impaired for some of them [41, 44].

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

MODEL OF BACTERIOPHAGE – BACTERIA COEVOLUTION IN THE GUT

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

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

This is likely to occur at the expense of the most abundant and available bacterial populations, providing a major contribution to microbiome homeostasis and to bacterial differentiation. Bacterial hosts also have opportunities to escape bacteriophage predation, resulting in genomic differentiation between microbial populations. In addition, some of these populations are likely to be protected against bacteriophage predation due to their physical inaccessibility in the environment, their limited density and/or the development of transient resistance due to their metabolic and phenotypic states. An example of such viral diversification in the human gut can be found with the expanding population of Crassphage [48].

CONCLUDING REMARKS

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

Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interest.

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Legends

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

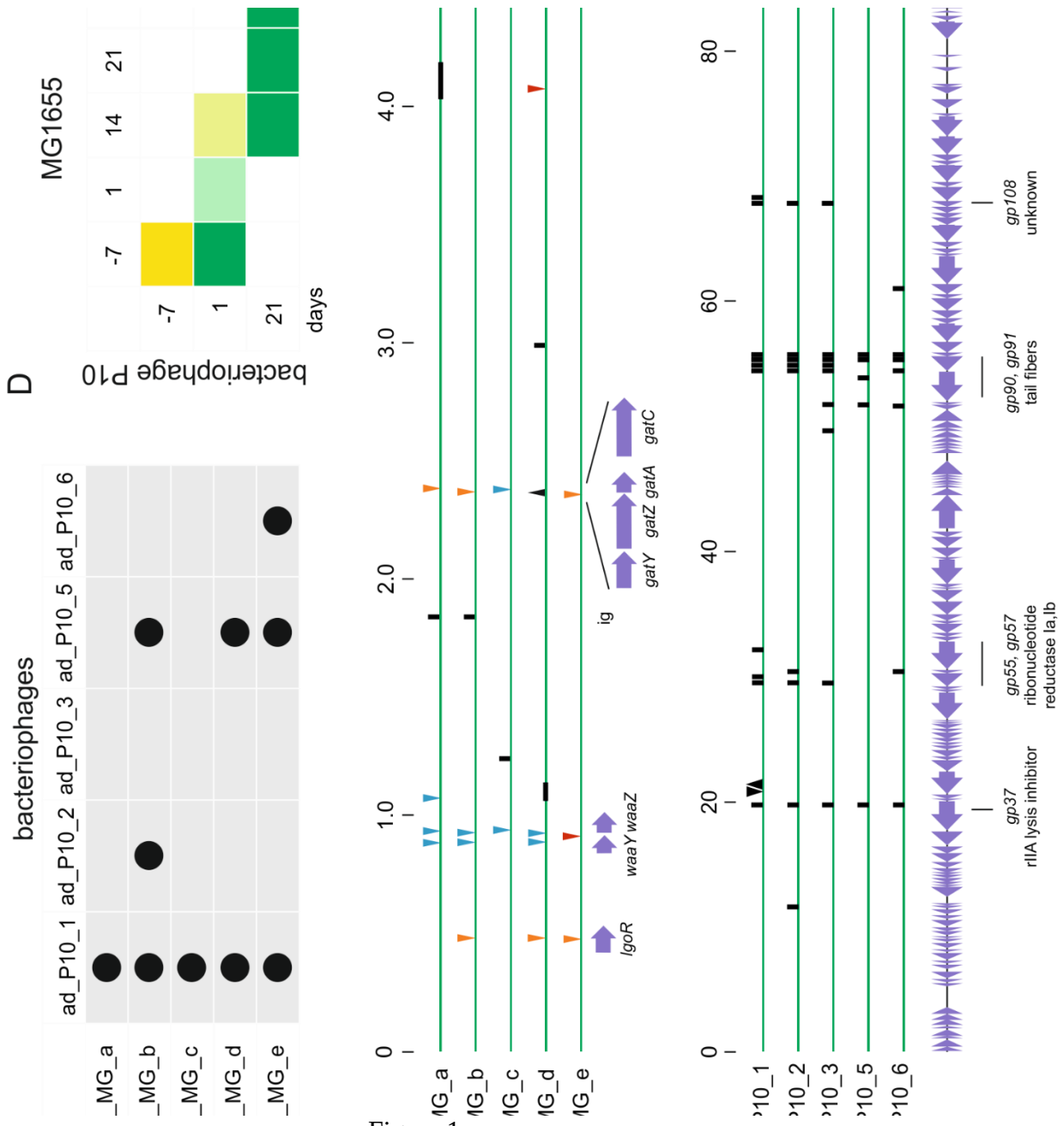
A) Adapted (ad_) P10 bacteriophages show differential infectivity towards coevolved (ev_) clones of *E. coli* strain MG1655 (MG) isolated at the same time point and that have developed bacteriophage resistance. Infectivity of five P10 bacteriophages (1-3,5-6) was tested against five MG1655 clones (a-e) by double spot technique [49] with two amounts of bacteriophages (10^6 and 10^4 pfu) in three replicates. Positive results of infection were determined by recording bacterial lysis and are shown as black dots. B) Bacterial genomic mutations under bacteriophage selective pressure in the mouse gut: ev_MG clones a-to-e were sequenced by Illumina technology and mutations were called using the Breseq variant report software v0.26 [50]. Mutations (orange, red and blue triangle - IS1, IS2 and IS5 respectively, black triangle pointing down - 1-5bp insertion, black triangle pointing up - 1-5bp deletion, vertical black rectangle – SNP and black horizontal rectangle - >1kb deletion) are reported relative to their positions in the genome. For mutation hotspots, the relative targeted genes are reported as purple arrows. For a complete list of bacterial genomic mutations see Table S1. The corresponding sequences are deposited at ENA under project PRJEB24878. C) Bacteriophage genomic mutations accumulated during coevolution with strain MG1655 in the mouse gut. Sequences of five adapted P10 bacteriophages (ad_P10_1-3,5-6) were analysed as described for bacterial clones. Mutations are relative to their positions in the bacteriophage genome (ORFs are shown as purple arrows) and mutation hotspots are indicated (same legend as for panel B). For a complete list of viral genomic mutations, see Table S2. The corresponding sequences are deposited at ENA under project PRJEB18073. D) Bacteriophages overcome genetic bacterial resistance. A time-shift experiment shows the percentage infectivity of 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 assay [49].

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.

402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ad_P10_2
ad_P10_3
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ad_P10_6
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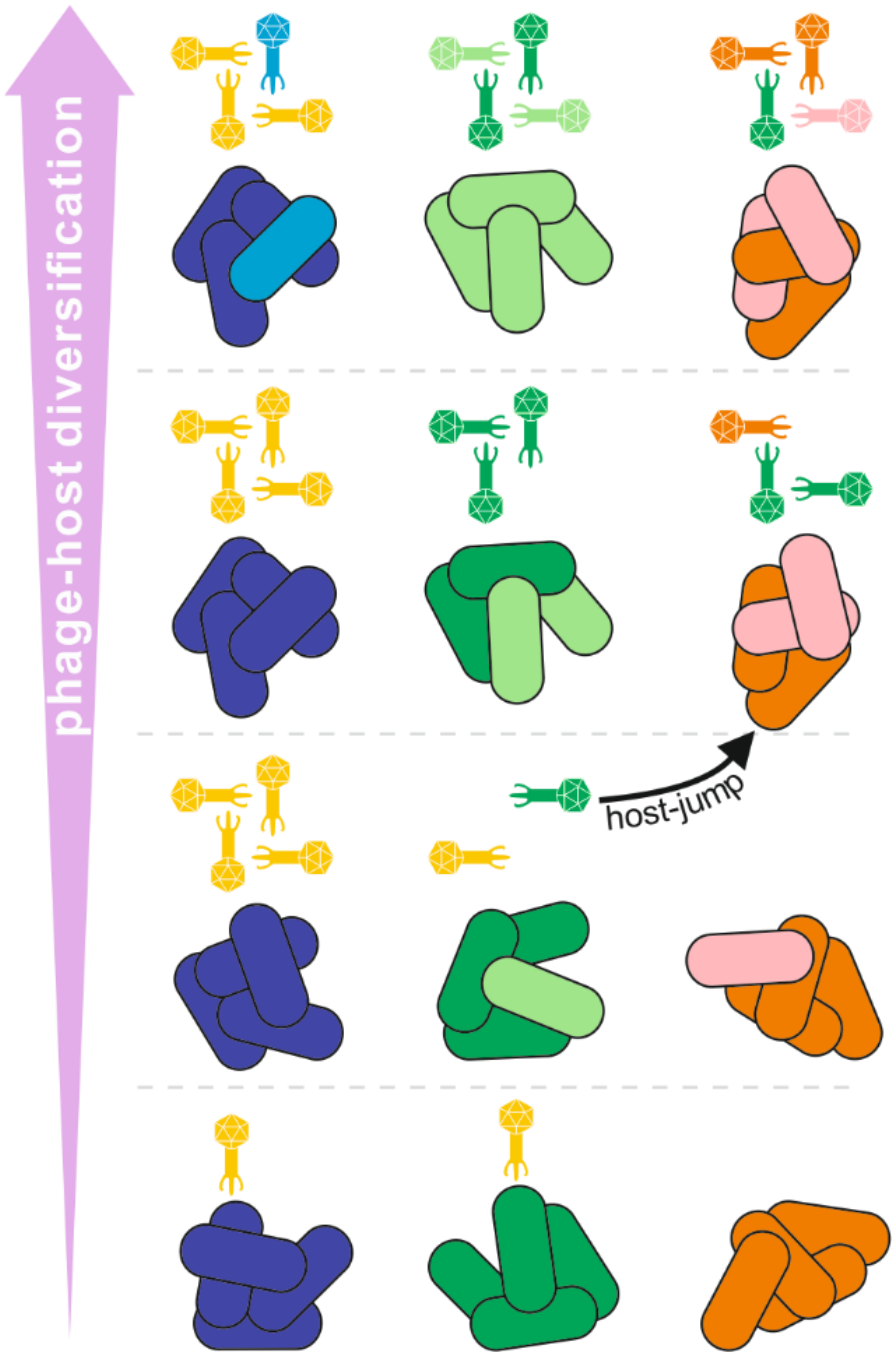
4.0
lgoR
waaY waaZ
ig
gatY gatZ gatA gatC
0
20
40
60
80
gp37
rIIA lysis inhibitor
gp55, gp57
ribonucleotide
reductase Ia,Ib
gp90, gp91
tail fibers
gp108
unknown

Figure 1



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407 Figure 2