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# 1 Horizontal gene transfer among host-associated 2 microbes

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13

## 14 In brief

15 Bacterial evolution is highly influenced by events of horizontal gene transfer. We review recent  
16 studies on mechanisms of transfer and the complex interactions that these may entail in  
17 driving eco-evolutionary dynamics of microbiomes. Integrated approaches are needed to  
18 overcome the challenge of quantifying rates and effects of transfer within hosts.

19

## 20 Abstract

21 Horizontal gene transfer is an important evolutionary force, facilitating bacterial diversity. It is  
22 thought to be pervasive in host-associated microbiomes, where bacterial densities are high  
23 and mobile elements are frequent. These genetic exchanges are also key for the rapid  
24 dissemination of antibiotic resistance. Here, we review recent studies that have greatly  
25 extended our knowledge of the mechanisms underlying horizontal gene transfer, the  
26 ecological complexities of a network of interactions involving bacteria and their mobile  
27 elements, and the effect of host physiology on the rates of genetic exchanges. Furthermore,  
28 we discuss other, fundamental challenges in detecting and quantifying genetic exchanges *in vivo*,  
29 and how studies have contributed to start overcoming these challenges. We highlight the  
30 importance of integrating novel computational approaches and theoretical models with  
31 experimental methods where multiple strains and transfer elements are studied, both *in vivo*  
32 and in controlled conditions that mimic the intricacies of host associated environments.

## 33 Introduction

34

35 All animals and plants on the planet are colonized by complex microbial communities, also  
36 called microbiomes. The characterization of host-associated microbiomes, and in particular  
37 those that are human-associated, has seen an increase in research focus. This is partially due  
38 to the realization that the expression and evolution of host traits can be affected by the  
39 composition and stability of their microbiomes<sup>1,2</sup>. Thus, human, animal and plant health can  
40 be affected both negatively and positively by the disruption of their associated microbial  
41 communities.

42

43 Microbes colonize many different niches within a host. In the human body, for instance,  
44 microbes are found in many areas, including the oral cavity, the gastrointestinal tract, the skin  
45 and even the lungs. Each of these niches comprises a distinct environment, that may be  
46 suitable for certain microbial types but not others. Furthermore, microbiomes are not static.  
47 Besides the invasion or extinction of certain microbial species, both the composition and  
48 functionality of microbiomes can change as bacteria adapt. Both bacterial evolution and  
49 migration to new host-associated niches can change their relationship with the host, even  
50 turning commensals into pathogens<sup>3</sup>. Evolutionary change in microorganisms can occur either  
51 by random mutations or by horizontal gene transfer (HGT), a major process that shapes within  
52 and between species diversity in bacterial populations. Unlike mutation, which typically  
53 involves small genetic changes in the bacterial genome that are vertically inherited, HGT  
54 transfers genetic material between different bacterial clones. This is predominantly driven by  
55 numerous mobile elements that are ubiquitous in the bacterial genomes<sup>4</sup>.

56

57 Most of our knowledge about HGT comes from *in silico* (i.e., comparative genomics) and *in*  
58 *vitro* studies, for which the latter typically represent experimental simplifications of more  
59 complex biological systems. While there is evidence that point mutations, small deletions or  
60 transposable element insertions can occur at an appreciable rate *in vivo*<sup>5</sup>, quantifying, or even  
61 detecting HGT in host-associated microbiomes is hindered by several challenges. First, the  
62 interactions that occur within and between species in microbiomes entangle ecology, evolution  
63 and epidemiology. These interactions complicate the identification of the relevant evolutionary  
64 parameters that underly each of the individual processes. Second, the intense activity of a  
65 network of selfish mobile genetic elements (MGEs), affects not only bacterial survival, but also  
66 hinder and promote genetic exchanges between cells. Finally, the host environment where  
67 microbiomes evolve pose a challenge on its own. Its complex physiology, biotic and abiotic

68 factors, including antibiotics, result in environmental conditions that are a far-cry from the  
69 simplifications typically studied *in vitro*.

70

71 Recently, multidisciplinary approaches have contributed to understanding the importance and  
72 prevalence of HGT *in vivo*. Yet, a large part of the puzzle is still missing. In this review, we will  
73 discuss the advances, complexities and challenges in both detecting and quantifying HGT in  
74 microbial communities from host-associated environments, as well as the host factors that  
75 may modulate the evolutionary dynamics. We highlight potential integrative strategies that will  
76 play an important role in uncovering the impact of HGT within hosts and, consequently, in  
77 global health.

78

# 79 The elements and mechanisms that drive horizontal 80 gene transfer

81

82 The main mechanisms of HGT are transformation, conjugation, transduction and lysogeny  
83 (Figure 1). Transformation involves the uptake of external DNA by a cell, and its genomic  
84 integration by homologous recombination. Transformation has multiple effects. Although the  
85 probability of integrating a novel sequence through homologous recombination declines  
86 exponentially with the level of sequence divergence<sup>6</sup>, transformation can contribute to genetic  
87 exchanges between bacteria<sup>7</sup>. On the other hand, because transformed DNA originates from  
88 dead bacteria, it has been argued that transformation events can be deleterious for the cell<sup>8</sup>.  
89 It is possible that one of the main roles of transformation is to act as a mechanism of genomic  
90 resilience. Uptaken sequences can be used as templates to repair deleterious mutations that  
91 occur during the replication of bacterial chromosomes, or as a way to inactivate costly MGEs<sup>9</sup>.  
92 It is difficult to accurately track transformation events in bacterial chromosomes, and its role is  
93 still poorly studied *in vivo*. This is partially due to the technical limitations in actually detecting  
94 such genetic exchanges, due to the strong homology required for recombination with uptaken  
95 DNA. Nevertheless, transformation occurs at high rates in some bacterial species like  
96 *Neisseria*<sup>10</sup> and *Hemophilus*<sup>11</sup>, and many species have competence genes<sup>12</sup>. Hence,  
97 transformation might have an important evolutionary role for bacteria in particular  
98 environmental conditions.

99

100 Transformation is limited by the short time that DNA remains intact outside the cell. Some  
101 bacteria can produce different types of membrane vesicles (MV) or outer membrane vesicles  
102 (OMV), and these nanostructures can package proteins, metabolites, and toxins present in  
103 the bacterial cell<sup>13</sup>. Both DNA and RNA can also be packaged within these extracellular  
104 vesicles, and are thus much better protected from environmental degradation. MGEs can also  
105 be packaged within MVs and OMVs, as observed for *K. pneumoniae*<sup>14</sup> and *Ruminococcus*<sup>15</sup>.  
106 This suggests that such structures might play an important, yet still understudied role during  
107 HGT *in vivo*.

108

109 Other HGT mechanisms rely on the different types of MGEs<sup>16</sup> (see Figure 1) that populate  
110 bacterial cells<sup>17</sup>, and that are relatively easier to detect. Conjugation involves the movement  
111 of plasmids or integrative conjugative elements (ICEs, that contrary to plasmids integrate  
112 bacterial genomes) through close contacts between cells of the same or of different species.

113 Transduction and lysogeny mainly involve bacteriophages (phages), viruses that infect  
114 bacteria. Contrary to conjugation, phage-mediated HGT allows genetic exchange to occur at  
115 larger physical distances between bacteria, as the DNA travels within viral capsids that protect  
116 it. Despite the role of MGEs in driving the expansion of gene repertoires in bacteria, the  
117 relationship with their bacterial hosts is not always peaceful. Both plasmids and phages have  
118 their own lifecycles, and because they are replicating entities with evolutionary interests, they  
119 can be bacterial antagonists.

120

121 The burden of MGEs for the fitness of bacteria is more evident in the case of phages, as in  
122 order to replicate, they often kill their host bacteria. The two mechanisms of HGT associated  
123 with phages are a consequence of their lifecycle. Phages can be virulent or temperate, but  
124 only the latter are capable of lysogeny, as they can integrate the bacterial chromosome. When  
125 integrated as prophages, they are transmitted vertically upon host replication. The genes they  
126 encode can be used by their bacterial hosts (lysogenic conversion). For instance, cholera  
127 outbreaks can be caused by toxins encoded in prophages<sup>18</sup>, and many other virulence factors  
128 are encoded by prophages (e.g.) in marine *Vibrio* communities<sup>19</sup>. For certain taxonomic  
129 groups, it has been estimated that ca. 50% of all bacterial genomes have at least one  
130 prophage<sup>20</sup> and this number rises to almost 80% in bacteria from the human gastrointestinal  
131 tract<sup>21</sup>. When integrated, prophages may eventually enter a lytic cycle that results in the death  
132 of their hosts. During excision and replication, some prophages can include segments of the  
133 bacterial chromosome within their viral particles, resulting in generalized, specialized or lateral  
134 transduction. In generalized transduction, bacterial DNA from anywhere in the chromosome  
135 can fully package viral particles, while specialized transduction packages genes that are in the  
136 neighbourhood of integrated prophages, in addition to the prophage itself<sup>22</sup>. Lateral  
137 transduction relies in the replication of phages *in situ*, whilst still integrated, and can result in  
138 the transfer of large segments of bacterial DNA that neighbour the prophage<sup>23</sup>. Specialized  
139 and lateral transduction are thought to be exclusive of temperate phages, while generalized  
140 transduction can occur with either temperate or virulent phages. Transduction is thought to  
141 arise due to mistakes in the packaging of the viral DNA, although it has been proposed that  
142 temperate phages can benefit from such mistakes<sup>24</sup>. Since the rates of generalized  
143 transduction vary between some phages<sup>25</sup>, transduction may be a trait under selection.

144

145 The induction of prophages in a small fraction of a lysogenic population can lead to the  
146 predation of ecological competitors of the lysogens, contributing to the successful invasion of  
147 the former<sup>26</sup>. For many host-associated species, including the pathogenic *Pseudomonas*  
148 *aeruginosa*<sup>27</sup> or *Klebsiella pneumoniae*<sup>28</sup>, this may be an important competitive strategy. When  
149 the induced phages are also capable of transduction, they can encapsidate DNA from the

150 targeted bacteria and transfer it back to the lysogen population – “autotransduction”. This  
151 process allowed a population of antibiotic sensitive lysogens of *Staphylococcus aureus* to  
152 invade and acquire antibiotic resistance (AR) genes from a resident (phage sensitive)  
153 population both *in vitro* and *in vivo*, within *Galleria mellonella* larvae<sup>29</sup>. Infection by phages  
154 depends on specific cellular receptors (e.g., capsules or lipopolysaccharides), as well as on  
155 cellular defenses<sup>30</sup>. Hence, most phages are thought to have a limited host range (e.g.,<sup>31</sup>),  
156 which also limits the reach of phage-mediated HGT.

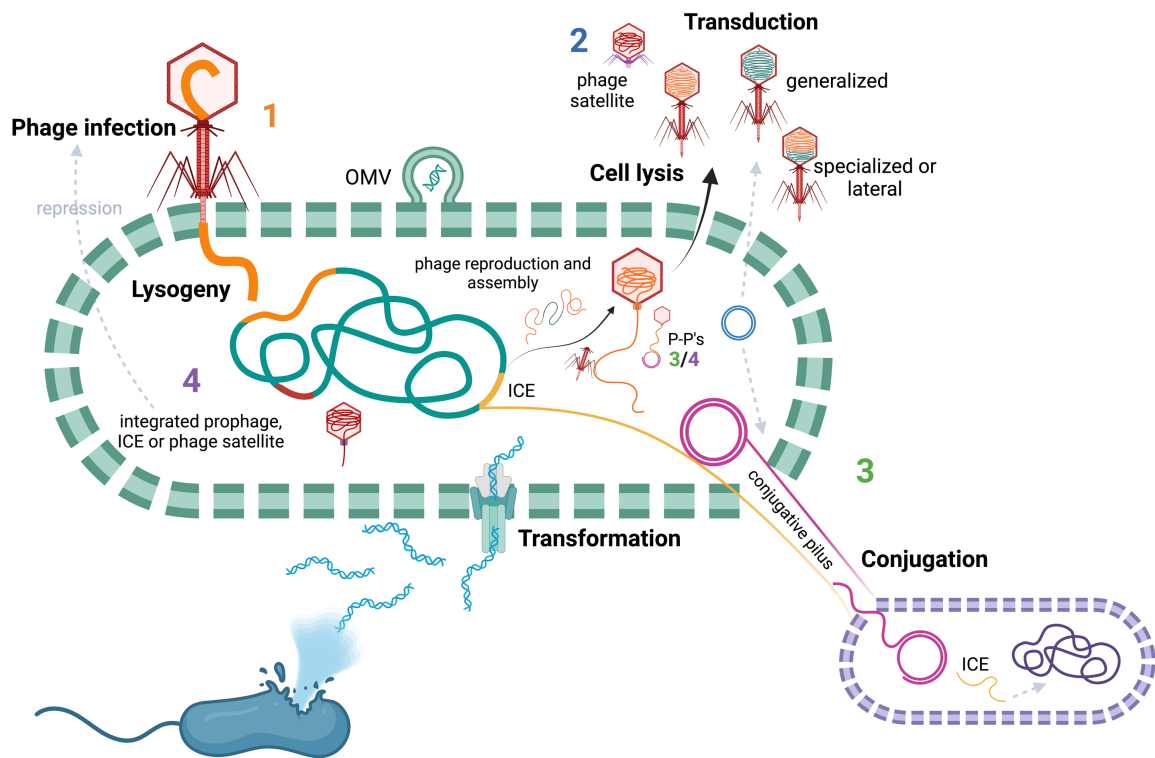
157

158 Conjugative plasmids are extrachromosomal sequences that control their own mobilization.  
159 Plasmids encode mating pair formation (MPF) genes responsible for pilus biogenesis and  
160 mating junctions, allowing the transfer of the plasmid from the bacterial donor to a recipient  
161 one. ICEs also encode these genes, but are integrated in bacterial genomes and can also be  
162 stably propagated by vertical transmission. Like prophages, both ICEs and plasmids can  
163 encode genes not directly related to their reproduction or transmission, and that increase the  
164 fitness of their bacterial host. Conjugation requires close cell-to-cell contact, and it can occur  
165 at very high rates, across very distant species (since conjugation does not require specific cell  
166 receptors)<sup>32</sup>, and can transfer large segments of bacterial DNA<sup>33</sup>. However, plasmids can  
167 impose an important fitness cost to bacteria<sup>34</sup>, due to the replication of extra DNA, genetic  
168 incompatibilities between the genes expressed by the host and those from the plasmid or  
169 plasmid-encoded addiction systems that guarantee its stability upon cell division. This means  
170 that, like phages, plasmids have a complicated relationship of dependency, parasitism and in  
171 some cases, mutual benefits with their hosts.

172

173 Phages and plasmids can themselves be exploited by other mobile elements. Phages are  
174 parasitized by phage satellites, small mobile elements that integrate bacterial genomes.  
175 Phage satellites differ from phages because they lack the genes to form viral particles. In order  
176 to mobilize, phage satellites steal viral particles from “helper” phages that co-infect the same  
177 bacterial host<sup>35</sup>. Phage satellites were recently shown to be very abundant and diverse<sup>36</sup>. Four  
178 main families of phage satellites have been identified, that besides having a common general  
179 strategy for mobilization (phage exploitation), are genetically distinct, found in different  
180 bacterial hosts, and rely on different specific hijacking mechanisms. P4-like phage satellites  
181 are found in many Enterobacteria, and particularly in *E. coli* genomes, which can encode up  
182 to three of these satellites<sup>36,37</sup>. Phage-inducible chromosomal islands (PICI)<sup>38</sup> and capsid-  
183 forming PICI (cf-PICI, phage satellites that encode their own capsid but still hijack the tails  
184 from helper phages)<sup>39</sup> are widespread both in Proteobacteria and Firmicutes. On the other  
185 hand phage-inducible chromosomal island-like elements (PLE) are exclusively found in *Vibrio*  
186 *cholerae* genomes<sup>40</sup>. Phage satellites have an important role in bacteria and can limit, or even

187 completely abolish, phage amplification (see below). They can also play a direct role in  
 188 expanding the bacterial gene repertoires, since they can encode virulence and AR genes<sup>41</sup>,  
 189 or anti-phage defense systems<sup>42,43</sup>.  
 190  
 191



	Main-type	Self-mobilizable	Main involvement in HGT processes	Localization in bacteria
1 - Bacteriophages	Virulent, temperate	Yes	Lysogeny (temperate), Generalized transduction (virulent and temperate), Specialized and lateral transduction (temperate)	Lytic infection/cytoplasm (virulent, temperate), chromosome (temperate)
2 - Phage satellites	P4-like, PLE, PICl, cf-PICl	No	Lysogeny, anti-MGE defenses	Chromosome
3 - Plasmids	pConj, pMOB, pOriT	Yes (pConj), No (pMOB, pOriT)	Conjugation, anti-MGE defenses	Extrachromosomal/episomal
4 - Integrative elements	ICE, IME	Yes (ICE), No (IME)	Conjugation, anti-MGE defenses	Chromosome

192  
 193 **Figure 1. Mobile genetic elements and the main mechanisms of horizontal gene transfer.** Schematic  
 194 representation of the different MGEs and how they drive HGT. Phages can be temperate (i.e., lysogenize bacteria  
 195 and become integrated as prophages) or virulent (only perform lytic infections). They infect bacteria and can  
 196 perform generalized, specialized or lateral transduction. Phages can be hijacked by phage satellites, that steal viral  
 197 capsids from fully functional phages. Satellites can belong to different families (the most well-known are P4-like,  
 198 PICl, cf-PICl and PLE). Conjugation occurs when bacteria transfer DNA by direct contact via a conjugative pilus.  
 199 It involves the transfer of extrachromosomal sequences typically known as plasmids. Some plasmids are  
 200 autonomous transferred (conjugative plasmids, or pConj) whilst others (mobilizable plasmids – pMob – and oriT  
 201 plasmids – pOriT) need to co-occur with other conjugative plasmids in order to mobilize. Integrative conjugative  
 202 elements (ICEs) are mobile elements that transfer by conjugation and integrate the bacterial chromosome.  
 203 Integrative mobilizable elements (IMEs) use the conjugation machinery of ICEs and/or conjugative plasmids to  
 204 mobilize. Phage-plasmids (P-Ps) are temperate phages capable of an episomal lifestyle, like plasmids, but that are  
 205 transferred within viral particles. Finally, HGT can also occur due to transformation of external DNA, and  
 206 extracellular vesicles (like membrane vesicles – MV – or outer membrane vesicles – OMV), nanostructures that  
 207 can export both transformable DNA, or even other MGEs.



208

209

210 Plasmids also have their own parasites. The conjugation apparatus of conjugative plasmids  
211 and ICEs can be exploited by mobilizable plasmids (pMob)<sup>44</sup>, that encode only an oriT (a small  
212 DNA sequence that signals the initiation of the transfer of the plasmid sequence) and a  
213 relaxase (responsible for the DNA nick required for initiating the transfer process). pMob are  
214 transferred by the conjugative pilus formed by other co-occurring plasmid(s). Both pMob and  
215 conjugative plasmids can also be exploited by plasmids that encode only an oriT (pOriT).  
216 These were recently shown to be very abundant, corresponding to 49% and 18% of the all the  
217 plasmids in *Staphylococcus aureus* and *E. coli*, respectively<sup>45</sup>. Integrative mobilizable  
218 elements (IMEs) also use the machinery from other conjugative elements to transfer across  
219 cells. IMEs encode only their excision and integration functions, but can have other genes that  
220 can increase the fitness of their hosts, including antibiotic resistance genes, toxins, and  
221 bacterial transporters and regulators<sup>46</sup>.

222

223 A fraction of non-conjugative plasmids were also recently reclassified as phage-plasmids (P-  
224 Ps)<sup>47</sup>, which are an example of the fluidity between MGEs, that defies strict classifications<sup>17</sup>.  
225 P-Ps are considered temperate phages capable of an episomal, plasmid-like lifestyle. They  
226 have the typical replication and segregation functions associated with plasmids, but they are  
227 transferred within viral particles that they encode themselves. P-Ps can thus produce virions,  
228 lyse cells and infect other hosts that are farther away, unlike the close proximity that is required  
229 for conjugation. This flexibility makes them potentially powerful drivers of HGT. For instance,  
230 a P1-like P-P in *E. coli* was shown to transfer an extended spectrum beta-lactamase through  
231 lysogenic conversion<sup>48</sup>, highlighting the potential of P-Ps in affecting global health.

232

233 The distribution of MGEs in bacterial genomes is shaped by many cellular defense systems  
234 that protect the bacterial populations from their invasion or establishment. Knowledge on the  
235 abundance, diversity and function of these anti-MGE defense systems has increased  
236 significantly (see<sup>49</sup> for a recent review), and new computational methods systematically  
237 identify many known defense systems in bacterial genomes<sup>50</sup>. This will contribute to  
238 characterize the distribution of anti-MGE defenses in host associated microbiomes. For some  
239 defense systems, their mechanistic understanding is still lacking. Other anti-MGE systems are  
240 part of the cell machinery, and are better described. For instance, RecBCD, an exonuclease  
241 that repairs double-strand breaks by homologous recombination, can also protect against  
242 invasion of some phages<sup>51</sup>. Another example are Restriction-modification (R-M) systems, that  
243 likely evolved as broad bacterial immune systems. By imprinting bacterial genomes with  
244 epigenetic marks, R-M systems defend the bacteria against foreign DNA (e.g., MGEs that lack

245 these epigenetic marks)<sup>52</sup>. Importantly, MGEs can counter these defenses by acquiring them  
246 from bacterial genomes (e.g., through homologous recombination). Thus, many MGEs encode  
247 anti-MGE defenses that protect the bacteria from other, competitor MGEs<sup>42,53</sup>. The MGEs can  
248 also themselves *be* the defense systems, as is the case for some phage satellites<sup>40</sup>. In either  
249 case, this means that systems that protect bacteria against MGEs can be mobilizable, and  
250 consequently, HGT itself can modify the frequency of subsequent HGT events.

251

252 Despite the defense mechanisms of bacteria, MGEs are pervasive in the genomes of many  
253 species. For instance, in ca. 1300 strains of *Escherichia* from environmental, human and non-  
254 human associated hosts' samples, MGEs account for more than a third of the pangenome  
255 (the collection of genes) of this species<sup>54</sup>. Given that many of these MGEs code for the  
256 information that controls for how (viral particles or conjugation) and when (induction or pilus  
257 formation) they are mobilized, it is important to consider the MGEs' interests and evolution  
258 when studying HGT in the bacterial hosts. Furthermore, in a similar way to how competition  
259 and cooperation between bacteria contribute to the diversity of bacterial communities,  
260 interactions between MGEs can also impact the diversity of host-associated microbiomes.

261

## 262 Competition and cooperation between mobile 263 genetic elements

264

265 Given the abundance and diversity of MGEs, one could easily think that “the more the merrier”  
266 for HGT. However, it is becoming increasingly evident that this is not the case, as often there  
267 are strong conflicts not only between bacteria and their MGEs, but also between the MGEs  
268 themselves<sup>4</sup>. This has consequences for the rates of HGT in bacteria<sup>55</sup>, either directly, or  
269 indirectly, by shaping the distribution of MGEs in bacteria.

270

271 An MGE can prevent or diminish the infection, replication or mobilization of other mobile  
272 elements, and thus can influence the latter's stability within populations. The fitness costs of  
273 mobile parasites for the MGEs they hijack can change with environmental, genetic and  
274 epidemiological circumstances<sup>38,56</sup>. For instance, in *V. cholerae*, PLEs completely abolish the  
275 replication of their helper phage, the virulent phage ICP1, by aborting the infection process  
276 and killing the bacterial host, effectively halting the propagation of the phage<sup>40</sup>. For this reason,  
277 PLE is considered an anti-phage defense system that plays a key role in the ICP1-*V. cholerae*  
278 interactions. Some P4-like satellites and PICIs encode specific anti-phage defense systems  
279 such as R-M<sup>42</sup> or abortive infection<sup>43</sup>. These satellite-encoded defense systems typically do

280 not target their helper phages, but other infecting phages, promoting the survival of the cell  
281 and its prophages. Likewise, many plasmids often encode CRISPR-Cas systems, that target  
282 other MGEs, particularly phages<sup>53</sup>. Further studies on these and other anti-MGE mechanisms  
283 will likely lead to new insights on the networks of exclusion between MGEs.

284

285 There is also evidence that some MGEs can cooperate, which can result in a more efficient  
286 mobilization of the MGEs and increase the rates of HGT. For instance, PIC1 in *S. aureus* can  
287 direct the transfer of unlinked chromosomal regions that contain sequences similar to the  
288 packaging (*pac*) sites of the PIC1, suggesting a direct role of phage satellites in transduction  
289 of bacterial genes through the hijacking of helper phages<sup>57</sup>. Additionally, in *S. aureus*, small  
290 non-mobilizable plasmids can be transferred by generalized transduction of both phages and  
291 PICs, suggesting that some plasmids evolved according to the size constraints of phage  
292 capsids<sup>58</sup>. Indeed, evidence is mounting that the evolution of MGEs has been shaped by the  
293 presence of other elements. A striking example is the peptide-based communication system  
294 encoded by some prophages of *Bacillus subtilis*, allowing different phages to coordinate and  
295 control their induction<sup>59</sup> or lysis-lysogeny decision<sup>60</sup>. Hence, HGT through the generation of  
296 new lysogens can also be controlled by communication between phages.

297

298 Given the plethora of MGEs, both those known and those yet to be discovered, many  
299 interactions are likely to be unknown. The different scales at which interactions can occur,  
300 combined with the different MGEs that can engage in these interactions, likely results in  
301 complex events. As an example, the phage satellite SaPI3 is induced not by a phage, but  
302 instead by another phage satellite, after which they both hijack the viral particles of the helper  
303 phage of the latter<sup>61</sup>. Furthermore, the interactions between MGEs can be affected by  
304 compensatory evolution, when these elements are costly for the host cell. Compensatory  
305 mutations might lead to more stable MGEs in bacterial populations, impacting their distribution  
306 in microbial communities. Hence, both bacteria-MGE interactions and MGE-MGE interactions  
307 might contribute to the frequency and outcomes of HGT *in vivo*, and consequently to the  
308 diversity of microbiomes.

309

310

311 **Factors in host-associated environments that shape**

312 **HGT**

313

314 Host-associated environments are the physical niches in an animal or plant, each with different  
315 sub-sections, host cell types and physical properties. Despite the control that MGEs exert  
316 over their own mobilization, animal/plant host-specific factors can also act as cues that impact  
317 certain modes of transfer. Both biotic and abiotic conditions can influence the rate and  
318 efficiency of HGT (**Figure 2**). For instance, the gastrointestinal microbiomes from humans in  
319 certain industrialized countries show higher rates of gene transfer<sup>62</sup>. The particular cause, or  
320 causes, underlying such differences are yet to be determined, but many factors intrinsic to  
321 within-host environments factors have the potential to shape HGT processes.

322

323 The spatial structure of host-associated environments impacts bacteria-bacteria and bacteria-  
324 MGE interactions<sup>63</sup>. Relative to the typically studied *in vitro* homogeneous environments,  
325 spatial structure brings physical characteristics that can affect the rates of HGT. In many host-  
326 associated environments, like plant roots or the mammalian gastrointestinal tract, microbial  
327 communities are densely packed and the close cell proximity might lead to high rates of  
328 conjugation. Indeed, in the mammalian intestine, *E. coli* can transfer the plasmid TP114 at  
329 estimated rates of  $0.1 \text{ day}^{-1}$ <sup>64</sup> and *Salmonella enterica* conjugates the plasmid p2kan with  
330 100% efficiency after 4 days of gut colonization<sup>65</sup>. Importantly, plasmid conjugation rates *in*  
331 *vivo*, or even their ability to conjugate at all, do not necessarily correlate with *in vitro*  
332 measures<sup>64</sup>. This suggests a strong interplay between the type of plasmid, the donor and  
333 recipient bacteria strains and even the genetic background of the animal host<sup>66</sup>. Additionally,  
334 the spatial structure *in vivo* is expected to affect phage-driven HGT, since the ecological and  
335 evolutionary dynamics of phage in structured environments differ from those in homogeneous  
336 contexts<sup>67</sup>. Phages are scarcely found in certain niches of host-associated environments, such  
337 as the mucosal areas of the mammalian gut, which can serve as refuges for bacteria<sup>68,69</sup>. This  
338 spatial segregation can lead to source-sink dynamics different from the typical co-evolutionary  
339 arms-races between phages and bacteria observed *in vitro*<sup>69</sup>.

340

341 The turn-over of bacterial species within microbiomes, due to adaptation, competition, novel  
342 colonizations or abiotic factors can lead to an asymmetry on the phage population infectivity  
343 at both spatial and temporal scales<sup>70</sup>. It has been suggested that this turn-over might favour a  
344 temperate phage lifecycle over a virulent one<sup>71</sup>, with the latter favoured in environments with  
345 a steadier supply of naïve hosts (e.g., in marine environments). This might contribute to  
346 explain the high prevalence of lysogens in gastrointestinal microbiomes, which can drive high  
347 rates of transduction and lysogeny in these communities<sup>21,72</sup>. Interestingly, several bacteria  
348 typically found in host-associated environments encode phage satellites<sup>36,41</sup>, but the  
349 prevalence and exchange of these elements *in vivo* has yet to be elucidated. One might

350 speculate that the intense viral dynamics in the gut environment<sup>72</sup> also potentiate the active  
351 exchange of phage satellites.

352

353 The phylogenetic diversity of host-associated microbiomes can vary across niches. For  
354 example, gut microbiomes typically contain a higher diversity than vaginal microbiomes<sup>73</sup>, and  
355 the skin microbiome shows a compartmentalization of different sub-communities in spatially  
356 separated pores, each with low diversity<sup>74</sup>. This means that even if (e.g.) the rates of  
357 conjugation might be high, the potential for gene diversification through conjugation should  
358 vary across different sites. Conjugation in diverse communities is an opportunity for bacteria  
359 to acquire truly novel functions. Yet, phylogenetic dissimilarity is often a barrier for HGT.  
360 Phylogenetic distant species are less likely to share the homology necessary for processes  
361 that rely in homologous recombination, phages typically cannot infect dissimilar hosts, and  
362 genetic networks are not always capable of accommodating novel genes. For instance,  
363 resistance to antimicrobial peptides (an ancient immune defense in animals and plants) is  
364 pervasive in gut microbiomes, but its transfer across bacterial species is limited due to  
365 functional incompatibilities between phylogenetic distant bacteria<sup>75</sup>. On the other hand, highly  
366 diverse communities can lead to adaptation of the MGEs to different strains, which in turn  
367 could lead to modified rates of HGT in their (new, or old) bacterial strains. Phages were  
368 observed to expand the range of bacteria they can infect to novel species through low-efficient  
369 infection of intermediary bacterial species in the gastrointestinal environment<sup>76</sup>. Such phage  
370 host-range expansion can lead to phage-mediated genetic exchange across more divergent  
371 bacterial strains. Hence, even if phylogenetic diversity can be a barrier to genetic exchange,  
372 some MGEs are capable to overcome (or adapt to profit from) such differences in host-  
373 associated communities.

374

375 Another potential major influence of host-associated environments in HGT is the heterogeneity  
376 of certain abiotic factors, such as pH, oxygen, nutrient levels or temperature. These factors  
377 can vary both spatially (e.g. along the intestinal tract) and temporally, and they affect the  
378 physiological state of bacteria. Hence, they can directly impact the efficiency of genetic  
379 exchange. For instance, the activation of the competence apparatus necessary for  
380 transformation is upregulated at stationary phase in *B. subtilis*, but inhibited in *Streptococcus*  
381 *pneumoniae* under the same physiological conditions<sup>77</sup>. The mammalian gut environment has  
382 also been shown to interfere with the expression of bacterial genes that mediate the efficiency  
383 of phage infection<sup>78</sup>. Induction of prophages in the gastrointestinal environment can also be  
384 driven by host-specific factors, such as bile salts or ethanolamine, a byproduct of eukaryotic  
385 cell death<sup>79</sup>. Yet, in most cases, it is not always clear how MGEs are affected by host-  
386 associated abiotic factors. For instance, out of 13 plasmids tested *in vivo* and *in vitro*, the

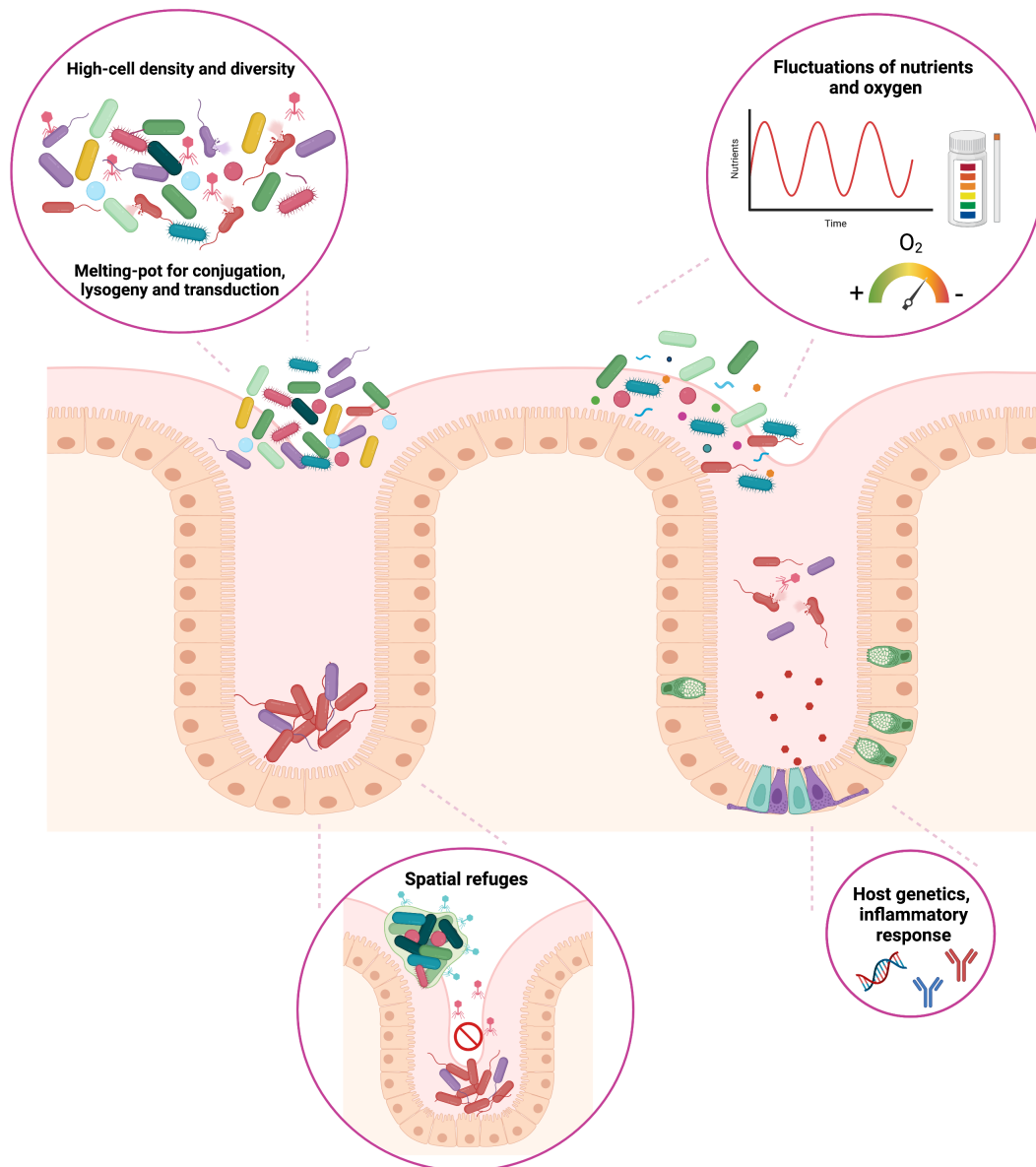
387 conjugative plasmid TP114, was the only one with increased activity at low oxygen levels<sup>64</sup>.  
388 Low oxygen is a characteristic of anaerobic conditions in the mammalian gut and in other host-  
389 associated environments, which suggests that host-specific physiological conditions can be  
390 more favourable to certain plasmids. Fluctuations in the type and availability of nutrients can  
391 also lead to an enrichment of different species over time. This has the potential to increase  
392 the activity of MGEs encoded by the favored bacteria. The scarcity of nutrients *in vitro* was  
393 shown to favor the scavenging of exogenous DNA as a source of energy, thus increasing  
394 rates of transformation<sup>80</sup>. Furthermore, several food additives were shown to increase  
395 conjugation rates of plasmids carrying antibiotic resistant genes<sup>81</sup>. This suggests that HGT in  
396 microbiomes can be partially modulated by the dietary intake of their animal hosts.

397

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401  
 402 **Figure 2. The gut environment as an example of the host-associated factors that can influence horizontal**  
 403 **gene transfer.** Schematic representation of different factors that can influence HGT in the mammalian gut. The  
 404 high bacterial cell density potentiates close contacts between phylogenetically diverse species, which can drive  
 405 high rates of conjugation and multiple contacts with viral (phage) particles. However, the spatially structured gut  
 406 can also generate niches that serve as refuges against phage infection, and consequently transduction and  
 407 lysogenization. Fluctuations in several abiotic factors, such as nutrients, temperature or pH can have an impact in  
 408 the physiology of bacteria, and thus modulate the rates of genetic transfers. Moreover, the host itself, including its  
 409 immune system, can shape both microbiome composition and the frequency and types of HGT.

410  
 411 Within a host, bacteria are also exposed to many different molecules, either produced or  
 412 ingested by the host, or produced by other members of the microbial community. These  
 413 molecules can function as inducers of SOS-responses, which can, in turn, potentiate HGT<sup>82</sup>.  
 414 Some studies have shown that the inflammatory response induced by the gut colonization of  
 415 a pathogenic *Salmonella enterica* serovar Typhimurium may lead to blooms of other

416 enterobacterial strains, and to trafficking of conjugative plasmids between pathogenic and  
417 commensal bacteria<sup>65</sup>. However, other studies have proposed that the genetics of the animal  
418 host and the composition of the microbiota itself have a higher impact on conjugation rates  
419 than the inflammatory process<sup>66</sup>. Host inflammatory responses, e.g., colitis also alters phage  
420 communities in the gut<sup>83</sup>, and promotes phage transfer between different *Salmonella*  
421 species<sup>84</sup>. Finally, HGT can also be affected by molecules produced by other bacteria present  
422 within the microbiomes. This was recently shown to be the case of colibactin, a genotoxic  
423 molecule produced by several *Enterobacteriaceae* in the mammalian gut. Colibactin can  
424 induce SOS-responses, and consequently induce the excision of prophages from both gram-  
425 positive and gram-negative bacteria that are in close proximity to the colibactin-producing  
426 cell<sup>85</sup>. These molecules impact the activity of their MGEs but also affect the composition of  
427 microbial communities as whole. Hence, the overall consequences of toxins and stress-  
428 inducing agents in HGT processes requires further study.

429

430 The host-associated environmental factors described here are both diverse and temporally  
431 dynamic, and possess distinct features that might strongly (albeit differently) impact both the  
432 mechanisms and the rates of HGT. This poses an important challenge when comparing HGT  
433 processes between *in vivo* and *in vitro* experimental conditions. Critically, it also raises  
434 difficulties in identifying the different factors involved in HGT, which is key to truly grasp the  
435 impact of this mode of microbial evolution within hosts. Some studies, which we discuss below,  
436 have made important leaps towards disentangling the factors of HGT *in vivo*.

437

438

## 439 Detecting and quantifying within-host HGT in the act

440

441 The ecological and evolutionary dynamics of multiple interacting, co-evolving MGEs pose a  
442 challenge to understand the role of HGT within complex host environments. Yet, other, more  
443 fundamental questions pose significant challenges too. In particular, the “simple” matter of  
444 how to quantify the rates of the different HGT mechanisms is crucial. This is generally not an  
445 easy task. Besides the frequency of transfer of a gene, or an MGE, other parameters need to  
446 be considered, such as the impact on fitness of the transferred DNA for both the bacteria and  
447 their mobile elements. Indeed, it is likely that many HGT events are deleterious, neutral or only  
448 slightly beneficial, and never increase sufficiently in frequency to be detected by current  
449 methods<sup>86</sup>. Other HGT events may reach detectable frequencies, but might be transient (e.g.,  
450 advantageous only in an ephemeral environment) and only remain in the population for a

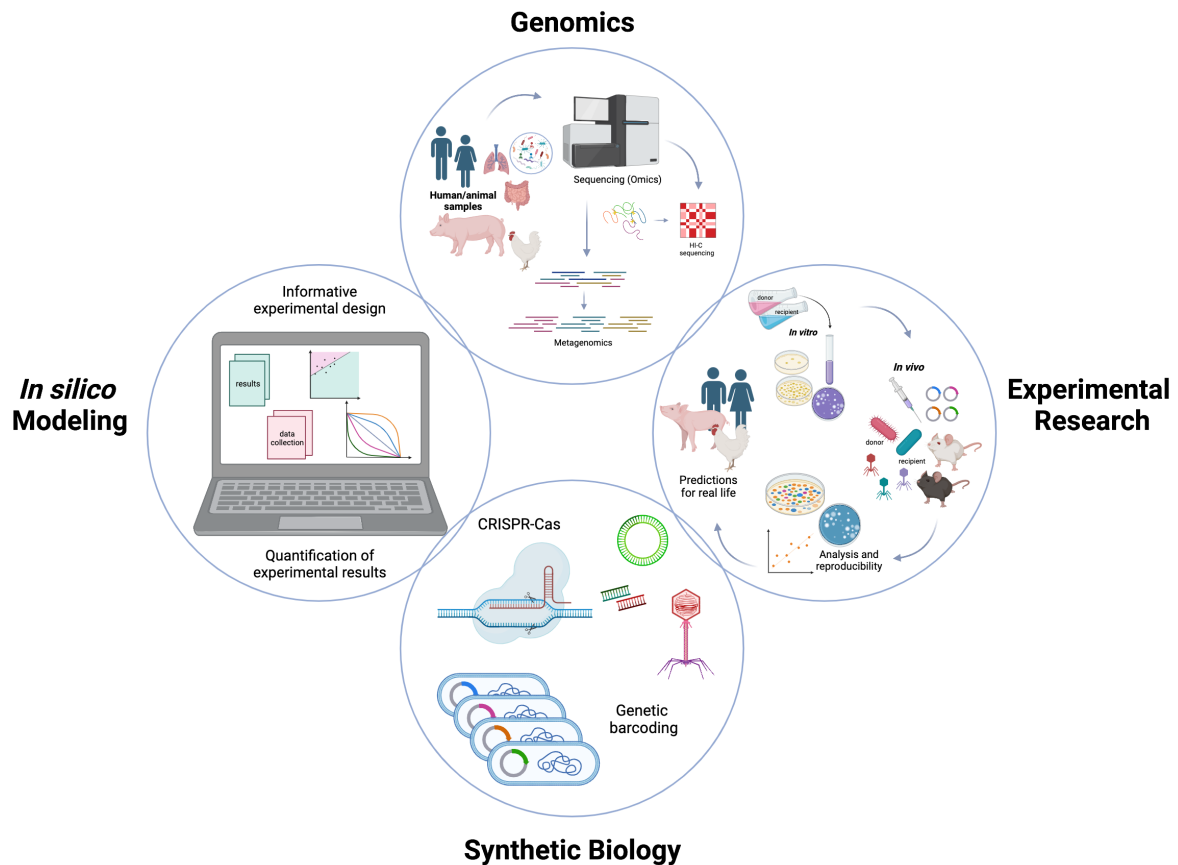


451 limited number of generations, thus remaining unaccounted for. In addition, the observation of  
452 a highly successful HGT event (i.e., that quickly spreads in a population) can be caused either  
453 by a high intrinsic rate of transfer or by a high fitness advantage of what is transferred, even if  
454 the actual rate of transfer might be low. Disentangling these factors *in vitro* is already not trivial;  
455 to do so within the ecological and physiological complexity of within-host environments is  
456 remarkably challenging. But there are different strategies that have been used to isolate these  
457 evolutionary components and to track the dynamics of HGT (**Figure 3**), either by itself, or in  
458 relation to mutational processes.

459

460 Several methods have now been developed, each with different strengths and weakness, that  
461 allow to identify MGEs, to determine their genomic context and to quantify HGT events in  
462 metagenomes (reviewed in<sup>16,87</sup>). Furthermore, specific experimental designs both *in vitro* and  
463 *in vivo*, coupled with the use of mathematical models to fit the data, have facilitated the  
464 dissociation of the two main components involved in HGT, fitness and transmission. In the  
465 case of conjugation, the integration of mathematical models and experiments led to the  
466 estimation of plasmid transfer rates between a donor and a recipient *E. coli* strain, both in the  
467 guts of mice and also in continuous-flow cultures (that simulated the gut abiotic  
468 environment)<sup>88</sup>. Plasmids were observed to spread at a lower rate in the gut of mice, relative  
469 to *in vitro* conditions, not due to inefficient or inhibited conjugation, but instead because the  
470 donor strain had a lower growth rate *in vivo*, relative to the other competitors within the  
471 microbiome. This somehow old study exemplifies that in any event of transfer, several  
472 concurrent processes (e.g., bacterial competition, plasmid segregation and the fitness costs  
473 associated with plasmid carriage) can impact the rates and outcomes of HGT.

474



475  
 476 **Figure 3. Multidisciplinary and integrative approaches are key to understand horizontal gene transfer in**  
 477 **within-host environments.** The study of HGT has benefited from the integration of different approaches,  
 478 represented here by the four different circles. Developments in genomics (e.g., next-generation sequencing or  
 479 comparative genomics) allows the detection and analysis of HGT-mediated changes at a large genomic scale.  
 480 Novel experimental approaches allow comparison between *in vitro* and *in vivo* results, and an exploration of the  
 481 dynamics of HGT in complex host associated environments. This is also driven by novel methods in synthetic  
 482 biology, that allow experimental designs with an appreciable diversity in the genetics of bacteria. Finally, *in silico*  
 483 models have been key in exploring conditions difficult to test experimentally, provide testable hypotheses for  
 484 conditions that are amenable to experimentation, and help in the quantification of relevant evolutionary parameters  
 485 (e.g., the frequency of conjugation or the rate of adaptation of phages).

486  
 487 Experimental evolution has been a powerful method to study evolutionary dynamics of  
 488 bacterial lineages in real time as they adapt to different *in vitro* environments. More recently,  
 489 it has been used to catch HGT events in the act in host-associated microbiomes, specifically  
 490 in the mammalian gastrointestinal tract. In this methodology it is typical to have a cohort of  
 491 genetical identical animal hosts (e.g., germ-free mice, devoid of any microbes) that are  
 492 colonized with one or more specific bacterial strains (e.g., that differ in their potential for HGT).  
 493 The eco-evolutionary dynamics of these strains are then followed in real time. These models  
 494 were recently used to address the costs and benefits of prophage carriage in the mouse gut,  
 495 using a well-known bacteria/phage pair: *E. coli* and its (pro)phage  $\lambda$ . By colonizing mice with

496 two strains of *E. coli*, differing solely in the presence/absence of the prophage, it was found  
497 that there is rapid phage transfer from the lysogenic to the susceptible strain in the  
498 gastrointestinal tract<sup>89</sup>. Additional colonizations with derivatives strains (either phage resistant,  
499 or with non-inducible phages) allowed to quantify the cost of carrying the phage. This cost was  
500 found to differ substantially across environments: *E. coli* lysogens were found to have a much  
501 higher induction rate in this *in vivo* system than in classic *in vitro* growth media. This study  
502 also revealed a high probability of lysogenization in the gut (~19%) and also facilitated the  
503 detection of rare events of phage gene capture within the first days of gut colonization<sup>89</sup>. In a  
504 different study, prophage carriage was found to also incur a fitness cost in *Lactobacillus*  
505 *reuteri*, but to provide a competitive fitness advantage for lysogens when competing with non-  
506 lysogens in the gut of germ-free mice<sup>90</sup>. Moreover, phage production in a *L. reuteri* strain with  
507 two active prophages differed along the digestive tract, i.e. being higher in the cecum and  
508 colon relative to the small intestine. Importantly, phage production in the gut was additionally  
509 identified to be modulated by the amount of fructose, an abundant sugar in western diets<sup>91</sup>.  
510 This is an important example of how the dynamics of MGEs (temperate phages) differ across  
511 host-associated environments (cecum, colon and small intestine), and can be modulated by  
512 abiotic factors (nutrients).

513

514 A more recent study in mice with a rich microbiota composition, demonstrated how phage-  
515 driven HGT can shape both the short<sup>92</sup> and long-term evolution<sup>93</sup> of an *E. coli* strain colonizing  
516 the gut. The colonizing strain was the target of rapid lysogenization by two prophages present  
517 within another *E. coli* strain, a natural resident of the mouse gut microbiome. These lysogenic  
518 events, which occurred in less than two weeks, were key for the successful and stable  
519 colonization of the invader *E. coli*. Afterwards, the newly evolved lysogens accumulated  
520 several mutations over thousands of generations of evolution in the gut. Some of these  
521 mutations reduced the induction rate of the acquired prophages, i.e., it was possible to follow  
522 the process of domestication of the prophages in real time. The rate of spontaneous prophage  
523 induction in evolved clones was similar to that measured in the original, resident *E. coli* strain,  
524 for which the induction rate did not change along time<sup>93</sup>. These results are consistent with the  
525 theoretical prediction of a non-zero spontaneous induction rate leading to an optimal strategy  
526 in gut commensal bacteria<sup>94</sup>. Importantly, this study highlighted how HGT and mutational  
527 processes are intertwined, and revealed that two very distinct modes of selection can drive  
528 the evolution of an invader strain: while in one mode newly emerging mutations increase in  
529 frequency but never reach fixation in the population, in the other the successive fixation of  
530 mutations strongly interplays with the HGT promoted by phages and plasmids. Another  
531 glimpse of this type of coevolution is provided by endemic infections caused by *Vibrio*  
532 *cholerae*, whose outbreaks are modulated by their lytic phages (which are, in turn, modulated

533 by their phage satellites). Bacteria and their phages recovered from the patients' longitudinal  
534 sampling has shown that *V. cholerae* isolated from a given time point were susceptible to the  
535 phages isolated at the same time point, but resistant to phages from successive time points<sup>95</sup>.  
536 The dynamics of phages correlated with fluctuations in SXT encoded in ICEs, which besides  
537 conferring AR, also encode anti-phage defenses, providing a remarkable link of both types of  
538 resistances in a single MGE.

539

540 Some studies also attempted to discern how the genetic background of an animal host affects  
541 the dynamics of HGT in its microbiome, with somewhat different conclusions across the  
542 different studies<sup>65,66</sup>. This suggests a need for systematization in approaches, which should  
543 combine both different animal genetic backgrounds and diverse bacterial strains, to better  
544 understand how (e.g.) transconjugants can rapidly and stably colonize their hosts. The costs  
545 of carrying a plasmid for the fitness of a bacterial strain is also key to understand the  
546 maintenance of transconjugants in host-associated environments. For instance, the fitness  
547 effect of a conjugative plasmid that encodes for AR was shown to be on average negative in  
548 the absence of antibiotics, across a large collection of strains of *E. coli* and *Klebsiella* that  
549 colonize human gut microbiomes<sup>96</sup>. However, there was extensive epistasis of its fitness  
550 effects across the strains tested, and remarkably, in some strains, the plasmid was even  
551 beneficial in the absence of antibiotics. This highlights that extensive variation in the fitness  
552 effects of a plasmid across strains may help explain its maintenance within some microbiomes  
553 but not others.

554

555 Technological advances in genomic engineering, comparative genomics and metagenomics  
556 also contributed to study the diversity and distribution of MGEs, as well as the frequency of  
557 HGT, within hosts. Recent developments in sequencing, such as split-read insertion  
558 detection<sup>97</sup>, long-read sequencing (e.g., Nanopore<sup>98</sup>) or single-cell sequencing<sup>99</sup> can be  
559 harnessed to improve the detection of HGT events. Other recent approaches were developed  
560 more specifically for this purpose. For instance, a CRISPR system was engineered to detect  
561 and record plasmid and phage transfer events in an *E. coli*<sup>100</sup>. The metagenomic analysis of  
562 within-host communities has allowed comparing the microbiomes and phageomes of healthy  
563 and unhealthy patients, revealing that the phage gut diversity is typically severely decreased  
564 in the latter<sup>101</sup>. Furthermore, a massive analysis of thousands of human gut metagenomes  
565 identified new families of Caudovirales phages, as well as new potential mechanisms of  
566 phage-bacteria interactions *in vivo*, that are encoded by phages. This includes Diversity-  
567 Generating Retroelements (DGR), lipid biosynthesis, directed alterations of the bacterial cell-  
568 wall, as well as catalases and iron sequestering enzymes<sup>102</sup>, all of which can be adaptive when  
569 transferred (e.g. through lysogeny) to other bacterial hosts. Such events of capture and

570 transfer of traits by MGEs might be frequent, and it may occur not only within, but also between  
571 hosts. A large scale longitudinal genomic and metabolomics analysis of maternal and infant  
572 microbiomes identified that infant microbiomes were composed of bacteria that had unique  
573 species-metabolite relationships, absent from their mothers' microbiomes. This study  
574 suggests an inheritance of microbiome-encoded metabolic traits not due to the transfer of  
575 bacteria, but likely due to HGT promoted by inherited MGEs<sup>103</sup>. Circulating MGEs may then  
576 be prevalent within hosts, as also highlighted by a metagenomic Hi-C analysis of 10 healthy  
577 human gut samples. This study indicated that, while 75% of the detected phage-like  
578 sequences are likely dormant prophages, 5% have a high sequence coverage, suggesting  
579 that the human gut microbiota has an important fraction of actively replicating phages<sup>72</sup>. The  
580 same technology has suggested that HGT occurs frequently in the individual guts of both  
581 healthy and neutropenic patients undergoing stem cell transplantation, and that it might involve  
582 the exchange of AR genes<sup>104</sup>. Conversely, a metagenomic approach that was used to directly  
583 address the bacterial content that is carried within phage particles of phages in pig fecal  
584 microbiomes demonstrated that phages do not frequently carry or encode AR genes<sup>105</sup>.

585

586 The latter studies highlight the controversial role of phages in the transfer of antibiotic  
587 resistance genes, a trait that is typically considered to be more efficiently trafficked by  
588 plasmids. Given the growing impact of AR, the dissemination of AR genes in MGEs has been,  
589 understandably, the focus of a large part of the literature on HGT. Yet, it is important to  
590 consider the evolutionary dynamics involved in the mobilization and stability of AR genes in  
591 mobile elements, and the role of antibiotics themselves in shaping the rates of HGT.

592

## 593 HGT in the context of antibiotic resistance

594

595 Antibiotics are some of the most widely used antimicrobials not only in clinical settings but also  
596 agriculture, farming and food industry. As resistance to antibiotics became a wide societal  
597 problem, particular attention has been dedicated to how such traits disseminate rapidly within  
598 and between bacterial populations. MGEs may be key for these dynamics in some bacterial  
599 species, but not necessarily in others<sup>106</sup>. A recent study, that coupled mathematical modelling  
600 with experimental assays in soil communities, demonstrated that microbial communities that  
601 encode AR genes in conjugative plasmids are more stable when facing environmental  
602 perturbations (e.g., heavy metals or antibiotics), compared to communities where bacteria  
603 encode AR within their chromosomes<sup>107</sup>. This may partially explain the prevalence of AR

604 genes in plasmids, and why the latter are one of the main culprits for the wide and fast  
605 dissemination of AR, particularly in a clinical context<sup>108</sup>. Additionally, there is a high abundance  
606 of plasmid-encoded genes involved in cellular metabolism and stress, that might underlie the  
607 development of AR mechanisms<sup>109</sup>. In many cases, the fitness advantage in surviving  
608 exposure to antibiotics is so dramatic that even very costly plasmids can be maintained.  
609 However, those costs can be mitigated. A recent study observed the acquisition of  
610 compensatory mutations in *Enterobacter* isolates that led to the prevalence of a carbapenem-  
611 resistance carrying plasmid (pOXA-48) in the gut of hospitalised patients. These  
612 compensatory mutations occurred in plasmid-associated fitness genes, resulting in a trade-off  
613 between the fitness cost of the plasmid and the level of resistance to carbapenem<sup>110</sup>. Such  
614 compensatory adaptations stabilize AR genes in the populations, and many plasmids that  
615 encode such traits are maintained even in the absence of antibiotics<sup>111</sup>. Phages are not  
616 thought to be traffickers of AR genes *in vivo*<sup>105</sup>, but other studies argue that phages are  
617 important vehicles of such genes<sup>112</sup>. The high efficiency of phage-mediated transfer of AR  
618 genes *in vitro* suggests a possible underestimation of these events *in vivo*.

619

620 Antibiotics also have a physiological effect that can impact HGT. Some antibiotics induce  
621 SOS-responses, and *in vitro* assays have shown that antibiotics, such as aminoglycosides or  
622 fluoroquinolones can trigger competence in *S. pneumoniae* and other species<sup>113-115</sup>. The effect  
623 of antibiotics on conjugation is controversial, despite some studies showing increased rates of  
624 conjugation in their presence<sup>116</sup>. The effect on phages seems to depend on the type of  
625 antibiotics. Aminoglycoside antibiotics (such as Kanamycin or Apramycin) were shown to  
626 inhibit phage infection and replication<sup>117</sup>. On the other hand DNA damaging antibiotics (like  
627 Mitomycin C or Fluoroquinolones) are well known to stimulate prophage induction in many  
628 lysogens, and it has been suggested that some common oral medications may have a similar  
629 role<sup>118</sup>. Hence, a higher prophage induction and excision due to medical drugs might lead to  
630 increased rates of lysogeny and transduction. Another typical response from bacteria to  
631 antibiotic exposure is the increased production of exopolysaccharides or lipopolysaccharides,  
632 that can promote the formation of biofilms<sup>119</sup>. Biofilms can increase the chances of contacts  
633 between bacterial cells, and conjugation rates have been proposed to increase under biofilm  
634 formation<sup>120</sup>. On the other hand, while phage propagation and dispersion might be slowed  
635 down by sparse, but locally dense, sub-populations, the chances of lysogeny itself may  
636 increase, due to a high local multiplicity of infection<sup>67</sup>. Interestingly, conjugation itself can  
637 promote the formation of biofilms<sup>120</sup>, highlighting the complex interrelationship between  
638 antibiotics, resistance to antibiotics, and mobilization of AR genes.

639

## 640 CONCLUSION

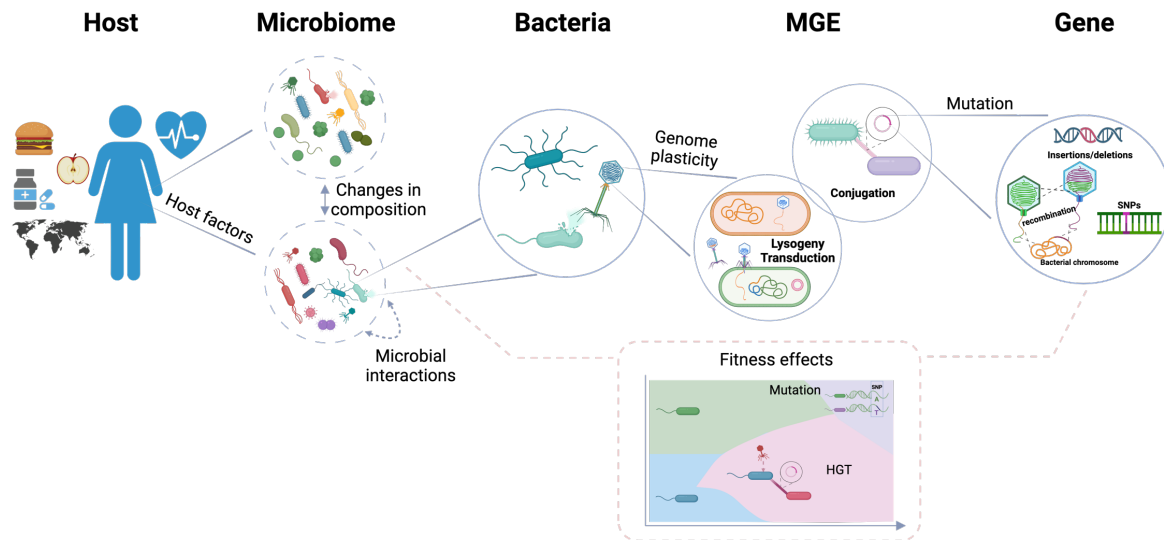
641

642 The study of microbial adaptation, and in particular of the mechanisms that involve the  
643 horizontal exchange of DNA, is at an interesting standpoint. On the one hand, the scientific  
644 community is, technologically, better suited than ever to detect all sorts of novel bacterial and  
645 mobile element variants, and how their frequencies change almost in real time. On the other  
646 hand, this deluge of information has uncovered an ever increasingly complex, rapidly evolving  
647 network of interactions, that tremendously complicates the quantification of the individual  
648 factors involved in HGT. Indeed, although there is an extensive body of highly important *in*  
649 *vitro* work in quantifying the rates of HGT, there is still a gap between those observations in  
650 simplified environments compared to those faced by bacteria *in vivo*. This is in part due to the  
651 challenges posed in measuring these complex but subtle changes across bacteria, in contexts  
652 of fluctuating conditions (including a huge variety of stresses), high bacterial diversity or even  
653 different intricate processes of adaptation occurring simultaneously.

654

655 There are different levels of selection involved and affected by HGT: from the gene, to the  
656 MGE that mobilizes the gene, to the bacterium that receives the MGE, to the microbiome of  
657 this recipient, and to the living organism (animal, or plant) that hosts this particular microbial  
658 community (**Figure 4**). This means that, in order to uncover the impact of HGT in within-host  
659 bacterial evolution, the rates and fitness effects of the mechanisms of horizontal transfer must  
660 be understood at different biological and temporal scales, that reflect both environmental and  
661 epidemiological conditions. Important care must also be given to uncoupling the relationship  
662 between the *rates of transfer* and the *effect on fitness* provided by the MGE and/or the  
663 transferred DNA. This relationship is in turn influenced by the different fluctuating conditions  
664 endured during host colonization, making a very advantageous trait in a certain ecosystem  
665 rapidly shift to deleterious in another environment. Furthermore, the accumulation of mutations  
666 subsequent to the HGT event, and how this can both influence and be influenced by HGT  
667 itself, is not yet well understood.

668



669

670 **Figure 4. A general view of the different levels, and their interactions, involved in study of within-host**  
 671 **horizontal gene transfer.** Understanding HGT *in vivo* requires the consideration of the different scales at which  
 672 ecological and evolutionary process occur. The dietary and societal conditions of the host influence the composition  
 673 and evolutionary dynamics of their microbiomes. The latter are determined by the interactions (competitive or  
 674 cooperative) and genetic exchanges that occur between the bacteria. This, in turn, is determined at the individual  
 675 bacterial level, by the combination of its rate of generating mutations, that are vertically inherited, and the rate of  
 676 horizontal transfer of multiple MGEs that increase or decrease the bacterial gene repertoires (as exemplified in the  
 677 Muller plot shown). Importantly, these same processes (competitive and cooperative interactions, vertical and  
 678 horizontal adaptation) affect the MGEs themselves. This creates a complex, multi-layered network of events that,  
 679 as a whole, determines rates and effects of HGT within and between animal hosts.

680

681 The multiverse of MGEs, mechanisms of HGT and the context dependent nature of transfer  
 682 rates and effects begs for a screening of *in vivo* measurements under conditions where  
 683 manipulation of donor-recipient densities can be controlled. In addition, given the complex  
 684 interactions between MGEs, new mathematical models with far more events and populations  
 685 need to be developed. As more events imply a larger number of parameters, more data is  
 686 required to avoid model overfitting. Thus, some ingenious tricks also need to be developed to  
 687 capitalize the most on the limited maneuverability of *in vivo* experiments. In this regard the  
 688 usage of multi-omics and synthetic biology approaches will be key. In particular, a wider  
 689 adoption of barcodes to label several donors and recipient cells, that are then introduced in  
 690 natural or synthetic ecosystems at different initial densities may aid in a better quantitative  
 691 understanding of the most dominant processes underlying successful HGT events.

692

693 Recent and exciting technological advances, coupled with a growing number of studies that  
 694 integrate computational and experimental approaches, have been key in overcoming some of  
 695 the challenges involved in detecting and quantifying within-host HGT. Given the current state



696 of affairs on such a fundamental evolutionary process with tremendous planetary-wide  
697 consequences, particularly regarding human health, we expect that other studies will follow  
698 suit. This will hopefully lead to the establishment of a substantial amount of research where  
699 disciplines such as eco-evolutionary modeling, animal experimentation and synthetic biology  
700 integrate to close the knowledge gap.

701

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706

## 707 Conflict of interest

708

709 The authors declare that they do not have any conflict of interest.

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