Prophages and plasmids display opposite trends in the types of accessory genes they carry

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1.4 Keyword

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2. Abstract

Prophages and plasmids often possess accessory genes encoding bacterial functions. To uncover rules governing the arsenal of accessory genes prophages and plasmids carry, we compare prophages and plasmids regarding how frequently they carry antibiotic resistance genes (ARGs) and virulence factor genes (VFGs) in 21 pathogenic bacterial species. The results show that prophages

tend to carry VFGs more frequently than ARGs in three species, whereas plasmids tend to carry ARGs more frequently than VFGs in nine species. In the other species, prophage-borne or plasmid-borne ARGs and VFGs are barely detected. We suggest that phages and plasmids differentiate in the types of accessory gene they carry because phages typically kill host cells when they transmit to other cells, whereas plasmids do not.

3. Introduction

Mobile genetic elements (MGEs), such as phages and plasmids, often possess accessory genes encoding bacterial phenotypes that are not apparently integral to the replication and transmission of MGEs. For example, plasmids frequently possess various antibiotic resistance genes (ARGs) [1-3]. Phages and plasmids also possess virulence factor genes (VFGs) required for bacterial pathogenicity [4-8]. Mediating the horizontal transfer of accessory genes between bacteria, MGEs play important roles for the evolution of bacterial genomes and phenotypes [9, 10].

MGEs are parasites of bacteria. Thus, horizontal gene transfer (HGT) mediated by MGEs can be regarded as the genetic manipulation of hosts by parasites [11]. Given that MGEs are self-interested evolving entities, MGEs are expected to possess accessory genes that advantage themselves [12, 13]. For example, plasmids are considered to gain selective advantages from ARGs by improving the survival of their bacterial hosts in heterogeneous environments [13-16]. It has also been hypothesised that phages gain selective advantages from VFGs by modifying environments in which their bacterial hosts live [17].

What evolutionary rules govern the arsenal of accessory genes carried by MGEs [12, 13]? Such rules, if they exist, might reflect the different infection strategies of MGEs. For example, phages typically lyse host cells to transmit to other cells, whereas plasmids do not. Consequently, phages might not gain much of an advantage by carrying genes that improve the survival of bacteria, such as ARGs. To understand the rules governing HGT mediated by MGEs, it is thus beneficial to investigate whether different MGEs carry different types of accessory genes.

To address the above question, we consider an ongoing debate about phage-borne ARGs. While it is well established that plasmids frequently carry ARGs [1-3], how frequently phages carry ARGs is controversial [18]. Phages mediate HGT through multiple mechanisms, among which specialised transduction is the most similar to HGT mediated by plasmid [19]. In specialised transduction, phages transfer genes carried in their genomes. Therefore, specialised transduction is strictly coupled with the infectious transmission of phages, the coupling that is also entailed in plasmid conjugation [19]. Laboratory experiments have demonstrated that phages are capable of transferring ARGs to bacteria through specialised transduction [20]. However, the specialised

transduction of ARGs in nature has been scarcely documented [3, 21]. While metagenomic studies have detected ARGs in viral fractions of environmental DNA samples [22-25], other studies provide evidence suggesting that the detection of ARGs was due to the contamination of bacterial DNA in the viral fractions [26, 27]. Genomics studies have predicted a number of prophages—i.e., phage genomes inserted into bacterial chromosomes as a consequence of specialised transduction—carrying ARGs in the genomes of *Acinetobacter baumannii* [28], *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* [29] [see also [30]]. Also, a previous study has isolated 29 phages from wastewater, of which 15 carry ARGs, suggesting that phages frequently possess ARGs [31]. However, these results appear at odds with a recent comprehensive analysis of phage genomes in public databases, which shows that ARGs are carried by only 0.3% of phages [32]. Taken together, the existing studies present mixed messages about the frequency at which phages carry ARGs.

To investigate how frequently phages carry ARGs, here we compare the distributions of ARGs and VFGs between the prophages and plasmids of pathogenic bacteria by comprehensively analysing public databases. We consider prophages instead of phages to compare different MGEs belonging to the same bacterial genomes. Our approach is designed to mitigate two issues we consider to be involved in the computational analyses of ARGs encoded in prophages, which are not taken into account in previous studies [28-30]. First, the misidentification of prophages can cause systematic biases in the number of prophage-borne ARGs. For example, non-prophage regions can be misidentified as prophages, causing overestimation in the number of prophage-borne ARGs. Contrariwise, a true prophage can be missed, which leads to underestimation in the number of prophage-borne ARGs. To avoid these biases due to prophage prediction, we compare the number of prophage-borne ARGs to that of prophage-borne VFGs, where both numbers are expected to be biased by common factors so that the biases can be cancelled out. The second issue involved in the analysis of prophage-borne ARGs is a sampling bias in bacterial genomes, which can cause overestimation in the numbers of ARGs and VFGs owing to the double-counting of orthologous genes. The degree to which this bias occurs can depend on the types of gene. To correct this bias, we cluster all genes into putative orthologous groups based on sequence similarity and synteny conservation and count the numbers of putative orthologous groups of ARGs and VFGs (OGARGs and OGVFGs, respectively). Finally, to investigate a potential differentiation between prophages and plasmids, we also analyse the distributions of ARGs and VFGs in plasmids. The results suggest that prophages are biased towards carrying VFGs, whereas plasmids are biased towards carrying ARGs. However, in many species, both ARGs and VFGs are hardly detected in prophages and plasmids.

4. Methods

Our method is sketched in Figure 1.

103 ### 4.1 Data acquisition

104 The three VFG databases, viz., VFDB (3685 genes in set A), Victors (5085 genes), and PATRIC_VF
105 (1293 genes), were downloaded from the respective websites in December 2020 [33-35]. The VFGs
106 in Victors were refined by removing those carried by non-bacterial pathogens or lacking NCBI protein
107 GIs (4575 genes remained). Some VFGs in Victors were missing protein sequences, which were
108 downloaded from Genbank based on their protein GIs [36]. All VFGs were pooled and clustered to
109 remove redundancy with CD-HIT with the protein sequence identity threshold of 1.0 [37], resulting
110 in a combined database of 7218 VFGs.

111

112 The genome assemblies with the 'Complete' status were downloaded from RefSeq in September
113 2021 with the following criteria [38]: a species had at least 60 complete genomes in RefSeq and at
114 least 70 VFGs in the combined VFG database. These criteria resulted in 21 species of bacterial
115 pathogens spanning three phyla, Actinobacteria, Firmicute, and Proteobacteria, as follows (numbers
116 in brackets indicate the number of genomes examined in this study): *Acinetobacter baumannii* (275),
117 *Bacillus anthracis* (99), *Bordetella pertussis* (562), *Brucella melitensis* (64), *Burkholderia pseudomallei*
118 (126), *Campylobacter jejuni* (220), *Enterococcus faecalis* (64), *Escherichia coli* (1444), *Haemophilus*
119 *influenzae* (92), *Helicobacter pylori* (225), *Klebsiella pneumoniae* (873), *Legionella pneumophila*
120 (102), *Listeria monocytogenes* (263), *Mycobacterium tuberculosis* (285), *Neisseria meningitidis* (122),
121 *Pseudomonas aeruginosa* (320), *Salmonella enterica* (996), *Staphylococcus aureus* (618),
122 *Streptococcus agalactiae* (91), *Streptococcus pyogenes* (235), and *Vibrio cholerae* (99). The complete
123 list of genomes analysed in this study is in Table S1.

124

125 ### 4.2 Prophage prediction

126 Prophages were predicted with VIBRANT (version 1.2.1), which was selected for three reasons. First,
127 VIBRANT has comparatively high performance as reported by a recent benchmark [39]. Second, it is
128 a standalone tool, which can be run on local computers. Third, its algorithm is based on the similarity
129 search of known phage proteins, which is suitable because our aim was to analyse known prophages
130 rather than discover novel prophages. VIBRANT was run against the genomic nucleotide sequences
131 with default parameters.

132

133 ### 4.3 Plasmids

134 Contigs were considered as plasmids or chromosomes if they were annotated as such in the RefSeq
135 assembly report files (in total, there were 9279 plasmid and 6607 chromosome contigs). Contigs
136 annotated as 'Segment', 'Genome Segment', or 'Extrachromosomal Element' were ignored (3, 1, or 5
137 contigs, respectively).

138

139 ## 4.4 ARG prediction

140 ARGs were predicted with AMRFinderPlus (version 3.10.5) and the core subset of the database
141 (version 2021-09-11.1) with the organism option if possible [40]. AMRFinderPlus was run against
142 translated coding sequences. The genes predicted by AMRFinderPlus as ARGs (i.e., "element subtype
143 AMR") and not annotated as pseudo-genes in RefSeq were considered as ARGs (those predicted as
144 "element subtype POINT", which contain point mutations associated with AR, were excluded).

145

146 ## 4.5 VFG prediction

147 Every VFG in the combined VFG database was queried against every bacterial genome with BLASTP
148 with E-value threshold of 1e-9 [41]. A gene in a bacterial genome (bacterial gene, for short) could
149 match multiple VFGs in the combined VFG database, in which case the VFG with the highest bit-
150 score was selected as the best match. A bacterial gene was considered as encoding VF if it met the
151 following additional criteria: (i) it was not annotated as a pseudo-gene in RefSeq [38]; (ii) the BLASTP
152 alignment between the bacterial gene and its best match VFG, if any, had at least 80% sequence
153 identity and covers at least 80% of both the bacterial gene and the best match VFG; (iii) the species
154 of the genome in which the bacterial gene resides was identical to the species in which the best
155 match VFG resides [33-35].

156

157 ## 4.6 Orthology prediction

158 Genes annotated in RefSeq were clustered into putative orthologous groups based on protein
159 sequence similarity and synteny conservation, as follows. First, preliminary orthologous pairs of
160 genes were identified between every pair of genomes within each species through all-against-all
161 sequence similarity searches using ProteinOrtho version 6.0.25 (with DIAMOND ver. 2.0.6 [42]; E-
162 value cut-off of 1e-5; minimum coverage of best alignments of 75%; minimum per-cent identity of
163 best alignments of 25%; minimum reciprocal similarity of 0.95) [43]. ProteinOrtho defines a
164 preliminary orthologous pair of genes as a reciprocal nearly-best hit (RNBH), as follows. A nearly-
165 best hit (NBH) of a gene queried against a target genome is defined as a hit whose bit-score is not
166 smaller by a factor f than that of the best hit. The value of f was 0.95, which is the default value of
167 ProteinOrtho. If two genes are mutually NBH of each other, they form RNBH [43].

168

169 RNBHs obtained with ProteinOrtho were pruned based on synteny conservation with an in-house
170 script, as follows. Let x and y be a pair of genes forming RNBH, and let X and Y be the genomic
171 neighbours of x and y, respectively, where the genomic neighbour of a gene (denoted by z) is
172 defined as a set of 21 genes consisting of ten genes upstream of z, ten genes downstream of z, and z
173 itself (all contigs were assumed to be circular, and the orientation of genes were ignored). Let N_x and
174 N_y be the number of genes in X and Y that form RNBHs with at least one gene in Y and X, respectively
175 (note that a single gene in one genome can form RNBHs with multiple genes in another genome
176 owing to tandem duplication). If both N_x and N_y are greater than ten (i.e., a majority of the genes in X

form RNBHs with the genes in *Y*, and *vice versa*), the RNBH formed by *x* and *y* was kept; otherwise, it was discarded [44].

Finally, the pruned RNBHs were clustered into putative orthologous groups with the spectral clustering algorithm implemented in ProteinOrtho version 6.0.25 (minimum algebraic connectivity of 0.1; exact step 3; minimum number of species of 0; purity of 1e-7) [43].

4.7 Classification of orthologous gene groups

A gene (VFG or ARG) was considered as encoded in a prophage if the entire gene is included within a genomic region predicted as a prophage.

An orthologous group of genes was considered to be encoded in a prophage residing in a chromosome (chromosomal prophage, for short), a prophage residing in a plasmid (plasmidic prophage), or a plasmid if the majority of the genes belonging to the group were in chromosomal prophages, plasmidic prophages, or plasmids, respectively (the cases of ties were ignored). Orthologous groups in plasmidic prophages were classified separately from those in chromosomal prophages or plasmids, for two reasons. First, it was ambiguous whether they should be regarded as encoded by plasmids, prophages, or both. Second, plasmidic prophages potentially represent a distinct class of mobile genetic elements called phage-plasmids [45].

An orthologous group of genes was considered as an ARG or VFG if the majority of the genes belonging to the group were predicted as ARGs or VFGs, respectively. The majority rule was used because a subset of genes in OGARG or OGVFG could be predicted as non-ARGs or non-VFGs, respectively, owing to sequence divergence. However, for most orthologous groups of ARGs and VFGs, all genes in a group were predicted as either ARGs or VFGs. Moreover, no orthologous group contained both ARGs and VFGs.

5. Results

5.1 Prophage prediction

To examine the distribution of ARGs in prophages, we computationally predicted prophages using VIBRANT [46] and ARGs using AMRFinderPlus [40] in the genomes of 21 pathogenic bacterial species downloaded from the RefSeq database [38] (Methods; Tables S1 and S2). To avoid double-counting orthologous ARGs in different genomes, we clustered all genes into putative orthologous groups based on sequence similarity and synteny conservation (Methods). We then counted the number of

211 orthologous groups of ARGs (OGARGs) encoded in the predicted prophages, distinguishing between
212 prophages residing in bacterial chromosomes and prophages residing in plasmids (chromosomal
213 prophage and plasmidic prophage, respectively, for short). This distinction was made because it was
214 ambiguous whether ARGs in plasmidic prophages should be regarded as encoded in prophages,
215 plasmids, or both [plasmidic prophages potentially represent phage-plasmids, which are a separate
216 class of MGEs from typical phages and plasmids [45]]. The result shows that a few to several
217 prophage-borne OGARGs were detected in ten of the 21 examined species (Tables 1 and S3).

218

219 To probe the precision of the above prediction, we manually examined whether predicted
220 prophages carrying ARG contained at least one phage-structure gene (e.g., phage baseplate, capsid,
221 portal, tail, tail fibre, tail sheath, tail assembly, head-tail connector, and tail tape measure) according
222 to the RefSeq annotation. Although this criterion cannot perfectly distinguish true prophages from
223 false ones, it allows us to split the predicted prophages into those enriched with true prophages and
224 those enriched with false prophages, allowing us to probe the precision of the prophage prediction.
225 The result of the examination shows that 24 out of 29 (i.e., 83% of) chromosomal prophages carrying
226 ARGs contained phage-structure genes, whereas seven out of 18 (i.e., 39% of) plasmidic prophages
227 carrying ARGs contained phage-structure genes (Table S4). This result means that 28 out of 33 (i.e.,
228 85% of) OGARGs in chromosomal prophages are in prophages carrying phage-structure genes,
229 whereas nine out of 21 (i.e., 43% of) OGARGs in plasmidic prophages are in prophages carrying
230 phage-structure genes (Table 1; note that the number of prophages carrying ARGs is smaller than
231 that of prophage-borne ARGs because one prophage can carry multiple ARGs). This result suggests
232 that the precision of VIBRANT is acceptable for chromosomal prophages.

233

234 During the manual examination, we noticed that a subset of plasmidic prophages carrying ARGs also
235 carried genes encoding integron integrases according to the RefSeq annotation (Tables S4).
236 Moreover, these prophages have a higher frequency of lacking phage-structure genes than those
237 without integron integrase genes (IIGs, for short). Specifically, seven out of ten (i.e., 70% of)
238 prophages carrying both ARGs and IIGs lack phage-structure genes, whereas nine out of 37 (i.e., 24%
239 of) prophages carrying ARGs and lacking IIGs lack phage-structure genes. VIBRANT predicted that the
240 genes annotated by RefSeq as IIGs matched phage integrase genes, which are typical components of
241 phage genomes. However, the RefSeq annotation implies that proteins encoded by these genes are
242 more similar to integron integrases than phage integrases because the RefSeq annotation considers
243 a broader set of protein families than does VIBRANT [38, 46]. Therefore, the above result suggests
244 that about a half of the prophages carrying ARGs and lacking phage-structure genes might have
245 arisen from the misidentification of integrons as prophages [see also [47]].

246

247 **5.2 Prophages carry significantly more VFGs than ARGs in multiple species**

248

249 To compare the frequencies of ARGs to those of VFGs in chromosomal prophages, we used BLASTP
250 [41] to search bacterial genomes for VFGs collected from VFDB [33], Victors [34] and PATRIC_VF [35]
251 (Methods). We counted the number of orthologous groups of VFGs (OGVFGs, for short) encoded in
252 the predicted prophages (Tables 1 and S5). The number of OGVFGs cannot directly be compared to
253 that of OGARGs because we have no *a priori* reason to expect that bacteria possess an equal number
254 of VFGs and ARGs. Thus, we instead compared the relative frequencies of OGVFGs and OGARGs
255 against the genomic background (see Figure 1 for the illustration of what we did). Specifically, we
256 performed binomial tests under the null hypothesis that the relative frequencies of OGARGs and
257 OGVFGs in the chromosomal prophages of each species are the same as those of all OGARGs and
258 OGVFGs in the genomes of the respective species. In this test, we included prophages lacking phage-
259 structure genes for fairness because prophages carrying VFGs were too numerous to be manually
260 examined (however, we found that none of the prophages carrying VFGs carried IIG, which suggests
261 that the prophages carrying VFGs do not contain false positives arising from integron
262 misidentification). Also, we corrected P values using the Holm–Bonferroni method to control the
263 family-wise error rate of all the statistical tests conducted in this study [48, 49]. The results of the
264 tests indicate that the relative frequencies of OGARGs and OGVFGs in chromosomal prophages are
265 significantly different from those of respective genomic backgrounds in the following three species
266 (Figure 2): *E. coli* (Gammaproteobacteria), *S. enterica* (Gammaproteobacteria), and *S. aureus*
267 (Firmicute). In all these species, chromosomal prophages carry VFGs more frequently than ARGs. The
268 remaining 18 species, where significant biases were not detected, can be grouped into three
269 categories: the one species where a large number of prophage-borne VFGs were detected, but a bias
270 was not significant (*S. pyogenes*), the eight species where only a small number of prophage-borne
271 ARGs or VFGs were detected (*A. baumannii*, *B. anthracis*, *H. influenzae*, *K. pneumoniae*, *N.*
272 *meningitidis*, *P. aeruginosa*, *S. agalactiae*, and *V. cholerae*), the six species where no prophage-borne
273 ARG and VFG were detected (*B. pertussis*, *B. melitensis*, *B. pseudomallei*, *C. jejuni*, *E. faecalis*, *L.*
274 *pneumophila*), and the three species where prophages were rarely or hardly detected (*H. pylori*, *L.*
275 *monocytogenes*, and *M. tuberculosis*) (Table 1 and Figure 2). The paucity or absence of prophage-
276 borne ARGs and VFGs could be due to the limited sensitivity of the prophage prediction tool.
277 However, a large number of prophages were predicted in the second and third categories of species.
278 Therefore, if the prophage prediction tool missed prophages carrying ARGs or VFGs in these species,
279 those prophages are likely to be distinct from the currently known phages. Taken together, the
280 above results suggest that phages tend to carry ARGs less frequently than VFGs if they carry a
281 sufficient number of ARGs or VFGs; however, in many bacterial species, they carry little or no ARGs
282 and VFGs.

283

284 ### 5.3 Plasmids carry more ARGs than VFGs in many of species

285 To compare prophages to plasmids, we counted the number of OGARGs and OGVFGs encoded in
286 plasmids (Table 2). We then performed binomial tests under the null hypothesis that the relative
287 frequencies of OGARGs and OGVFGs in the plasmids of each species are the same as those of all
288 OGARGs and OGVFGs in the genomes of the respective species. The results of the tests indicate that

289 the relative frequencies of OGARGs and OGVFGs in plasmids are significantly different from those of
290 the genomic background in nine out of the 21 examined species (Figure 3). In all these species,
291 plasmids are biased towards carrying ARGs more frequently than VFGs. These species include six
292 species of Gammaproteobacteria (*A. baumannii*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. enterica*,
293 and *V. cholerae*), one species of Epsilonproteobacteria (*C. jejuni*), and two species of Firmicutes (*E.*
294 *faecalis* and *S. aureus*). The remaining 12 species, where significant biases were not detected, can be
295 grouped into three categories: the four species where plasmids are hardly present (*B. pertussis*, *B.*
296 *melitensis*, *M. tuberculosis*, and *N. meningitidis*), the seven species where plasmids are rare, and
297 plasmid-borne ARGs and VFGs were barely detected (*B. pseudomallei*, *H. influenzae*, *H. pylori*, *L.*
298 *pneumophila*, *L. monocytogenes*, *S. agalactiae*, and S. pyogenes), and the one species where
299 plasmids carry more VFGs than ARGs, but a bias was not significant (*B. anthracis*). Taken together,
300 the above results suggest that plasmids tend to carry ARGs more frequently than VFGs if they carry a
301 sufficient number of VFGs or ARGs, with a possible exception of *B. anthracis*; however, in several
302 species, plasmids barely carry VFGs and ARGs.

303

304 6. Discussion

305 The results presented above indicate that prophages tend to carry VFGs more frequently than ARGs
306 in three of the 21 examined species. In most of the other species, prophage-borne ARGs and VFGs
307 were hardly detected. In contrast, plasmids carry ARGs more frequently than VFGs in nine of the 21
308 examined species. In most of the other species, plasmid-borne ARGs and VFGs were barely detected.
309 Taken together, these results indicate that prophages and plasmids display opposite trends if they
310 carry a sufficient number of VFGs or ARGs: prophages are biased towards carrying VFGs, whereas
311 plasmids toward carrying ARGs. This difference between phages and plasmids led us to formulate
312 the following hypothesis to test for future (Figure 4): Temperate phages do not gain much benefit
313 from carrying ARGs because if their hosts are in danger, they can abandon their hosts and seek new
314 ones [50-54]. In contrast, both phages and plasmids can benefit from VFGs because VFGs can
315 accelerate bacterial replication by making bacteria exploit their hosts more aggressively [17].

316

317 The results described above, however, do not mean that plasmids rarely carry VFGs or that
318 prophages always carry VFGs. For example, in *K. pneumoniae*, plasmids carry ARGs more frequently
319 than VFGs; nevertheless, plasmids carry a large number of VFGs (viz., 96 out of 372 orthologous
320 groups), whereas prophages carry none (Tables 1 and 2). A similar, yet less striking, pattern is seen in
321 *E. faecalis*. Given that many prophages are predicted in these species (Table 1, Figure 2), the absence
322 of prophage-borne VFGs is unlikely to be merely due to the limited sensitivity of the prophage
323 detection tool. By contrast, in *S. aureus* and *S. pyogenes*, prophages carry greater numbers of VFGs
324 than do plasmids (Tables 1 and 2). This heterogeneity in the distribution of VFGs suggests that
325 plasmids and prophages play variable roles in the pathogenicity of different bacterial species.

326

327 The absolute numbers of prophage-borne ARGs reported in this study need to be interpreted with
328 caution because of the limited sensitivity and precision of the prophage prediction tool. In particular,
329 these numbers do not necessary indicate that prophages are devoid of ARGs in many bacterial
330 species since prophages carrying ARGs could have been missed. However, the conclusion of this
331 study does not directly depend on the absolute numbers of prophage-borne ARGs because it is
332 based on comparison between the relative frequencies of prophage-borne ARGs and VFGs.

333

334 Regarding the limitations of prophage prediction tools, it is pertinent to discuss a discrepancy
335 between our result and the result of Kondo et al. (2021) with respect to *P. aeruginosa* [29]. While we
336 found no prophage-borne ARGs in *P. aeruginosa*, Kondo et al. (2021) reports that more than 10% of
337 *P. aeruginosa* genomes possess prophage-borne ARGs [29]. The important difference between
338 Kondo et al. (2021) and our study is that they used different tools for prophage prediction: Kondo et
339 al. (2021) uses PHASTER [55], whereas we used VIBRANT [46]. To investigate the cause of the above
340 discrepancy, we manually examined the 11 prophages described in Kondo et al. (2021) that carry
341 ARGs and are predicted as "intact" by PHASTER in *P. aeruginosa* [29]. We found that the examined
342 prophages could be grouped into two categories (Table S6). In the first group (five prophages), both
343 VIBRANT and PHASTER predicted prophages in almost the same genomic locations. However,
344 PHASTER predicts longer genomic regions including ARGs as prophages, whereas VIBRANT predicted
345 shorter regions excluding ARGs. We do not know which prophage boundaries are more accurate. In
346 the second group (6 prophages), prophages were predicted only by PHASTER. These prophages,
347 however, contained no phage-structure genes. Although they contained phage-related genes, such
348 as integrase, transposase, and protease, these genes are not exclusively associated with phages.
349 Moreover, PHASTER annotated tellurium resistance proteins, TerD, as virion structural proteins in
350 three prophages in the second group, which is likely to be erroneous. These findings suggest that the
351 second group of the prophages could be false positives. Prophages predicted as "incomplete" or
352 "questionable" by PHASTER are less likely to be true than those predicted as "intact". Taken
353 together, the above results suggest that the frequency of prophage-borne ARGs in *P. aeruginosa* is
354 potentially underestimated in our study and overestimated in Kondo et al. (2021) owing to the
355 limitations of the prophage prediction tools.

356

357 In interpreting the results obtained in this study, we assumed that ARGs and VFGs found within
358 prophages were carried by phage genomes. However, these genes could have been inserted into
359 pre-exiting inactivated prophages (i.e., inserted after lysogeny). Although this possibility cannot be
360 completely excluded, the following evidence suggests that not all ARGs and VFGs are inserted into
361 pre-existing inactivated prophages. A previous study has shown that ARGs and VFGs are found in the
362 genomes of temperate phages (which are thus not prophages) and that these genes are hardly
363 found in the genomes of virulent phages [32]. This result would not be expected if all ARGs and
364 VFGs were inserted into pre-existing inactivated prophages. More important, we do not have an *a*
365 *priori* expectation that VFGs are more likely to be inserted into pre-existing prophages than ARGs. In
366 the absence of such an expectation, our results are likely to be robust to post-hoc insertions of ARGs

367 and VFGs because they are based on comparison between the relative frequencies of ARGs and
368 VFGs.

369

370 That phages do not possess ARGs does not necessarily mean that phages do not mediate the
371 horizontal transfer of ARGs because they can mediate HGT even if their genomes do not contain
372 ARGs. Phages mediate HGT through three known mechanisms: specialised, generalised, and lateral
373 transduction [19, 56, 57]. In specialised transduction (the focus of this study), a transferred gene
374 constitutes a part of a phage genome [19]. By contrast, in generalised and lateral transduction, a
375 transferred gene is originally encoded in bacterial DNA, which is encapsulated into phage particles
376 and subsequently transferred to other cells [19, 56, 57]. Thus, phages can mediate the horizontal
377 transfer of ARGs even if their genomes do not contain ARGs.

378

379 In conclusion, the results presented above lend support to the hypothesis that MGEs differ in the
380 functional categories of accessory genes they carry depending on their infection strategies.

381

382 # 7. Author statements

383 ## 7.1 Conflicts of interest

384 The authors declare that there are no conflicts of interest.

385

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389

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393

394 # 8. Tables and figures

395 **Table 1**. Numbers of predicted prophages per genome (SD, standard deviation), and number of
396 orthologous groups of antibiotic resistance genes (OGARGs) predicted by AMRFinderPlus in genomes

397 (total), predicted plasmidic prophages, and predicted chromosomal prophages. Numbers in brackets
398 are for OGARGs in prophages containing at least one phage-structure gene.

	prophage (SD)	OGARG			OGVFG		
		total	prophage		total	prophage	
			plasmidic	chromosomal		plasmidic	chromosomal
A. baumannii	4.3 (1.7)	455	0	4 (4)	48	0	0
B. anthracis	4.1 (0.6)	17	0	1 (1)	39	0	0
B. pertussis	1.9 (0.3)	0	0	0	95	0	0
B. melitensis	1.0 (0.3)	1	0	0	183	0	0
B. pseudomallei	2.2 (1.3)	3	0	0	192	0	0
C. jejuni	1.1 (1.2)	65	0	0	480	0	0
E. faecalis	3.8 (1.8)	179	2 (0)	0	114	1	0
E. coli	7.1 (3.8)	3041	9 (7)	9 (9)	18019	6	1590
H. influenzae	3.0 (1.4)	7	0	0	161	0	1
H. pylori	0.2 (0.5)	6	0	0	285	0	0
K. pneumoniae	4.5 (2.1)	1473	7 (2)	7 (7)	372	0	0
L. pneumophila	0.1 (0.3)	5	0	0	773	0	0
L. monocytogenes	2.5 (1.3)	5	0	0	172	0	0
M. tuberculosis	0 (0.1)	4	0	0	997	0	0
N. meningitidis	1.9 (0.6)	4	0	0	123	0	1
P. aeruginosa	4.5 (2.7)	543	0	0	526	0	2
S. enterica	3.9 (1.8)	826	2 (0)	0	1844	2	64
S. aureus	3.5 (1.3)	423	0	6 (2)	217	0	28
S. agalactiae	2.0 (1.0)	46	0	1 (1)	71	0	0
S. pyogenes	3.1 (1.5)	28	0	3 (3)	103	0	48
V. cholerae	1.5 (1.2)	101	1 (0)	2 (1)	251	1	5

399

400 **Table 2**. Numbers of plasmids per genome (SD, standard deviation), and numbers of orthologous
401 groups of virulence factor genes (OGVFGs) and those of antibiotic resistance genes (OGARGs) in
402 genomes (total) and plasmids.

	Plasmid (SD)	OGARG		OGVFG	
		total	plasmid	total	plasmid
A. baumannii	1.7 (1.6)	455	195	48	0
B. anthracis	1.5 (0.9)	17	1	39	14
B. pertussis	0.0 (0.0)	0	0	95	0
B. melitensis	0.0 (0.0)	1	0	183	0
B. pseudomallei	0.1 (0.3)	3	0	192	0
C. jejuni	0.3 (0.6)	65	21	480	9
E. faecalis	1.4 (1.4)	179	127	114	24
E. coli	2.0 (1.9)	3041	2397	18019	2452
H. influenzae	0.0 (0.1)	7	1	161	0
H. pylori	0.2 (0.4)	6	0	285	0
K. pneumoniae	3.4 (2.2)	1473	1274	372	96
L. pneumophila	0.3 (0.5)	5	1	773	0
L. monocytogenes	0.2 (0.5)	5	0	172	1
M. tuberculosis	0.0 (0.0)	4	0	997	0
N. meningitidis	0.0 (0.0)	4	0	123	0
P. aeruginosa	0.2 (0.6)	543	128	526	3
S. enterica	1.1 (1.2)	826	638	1844	46
S. aureus	0.8 (1.0)	423	182	217	9
S. agalactiae	0.0 (0.1)	46	1	71	0
S. pyogenes	0.0 (0.2)	28	3	103	0
V. cholerae	0.1 (0.4)	101	36	251	0

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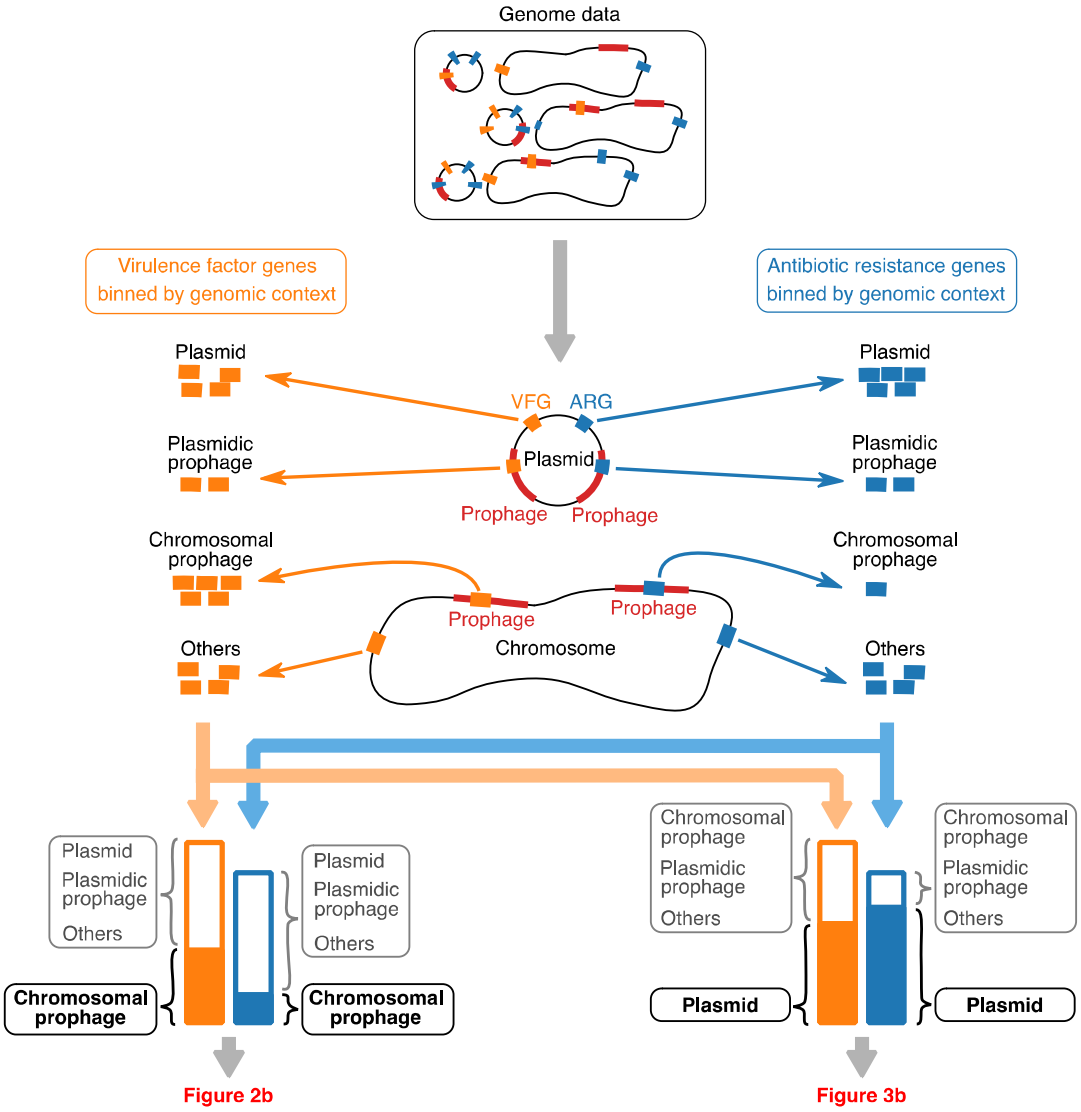

405

406 Figure 1. Schematic drawing of what we did in this study. For simplicity, clustering of orthologous
407 genes is omitted from depiction (see Methods).

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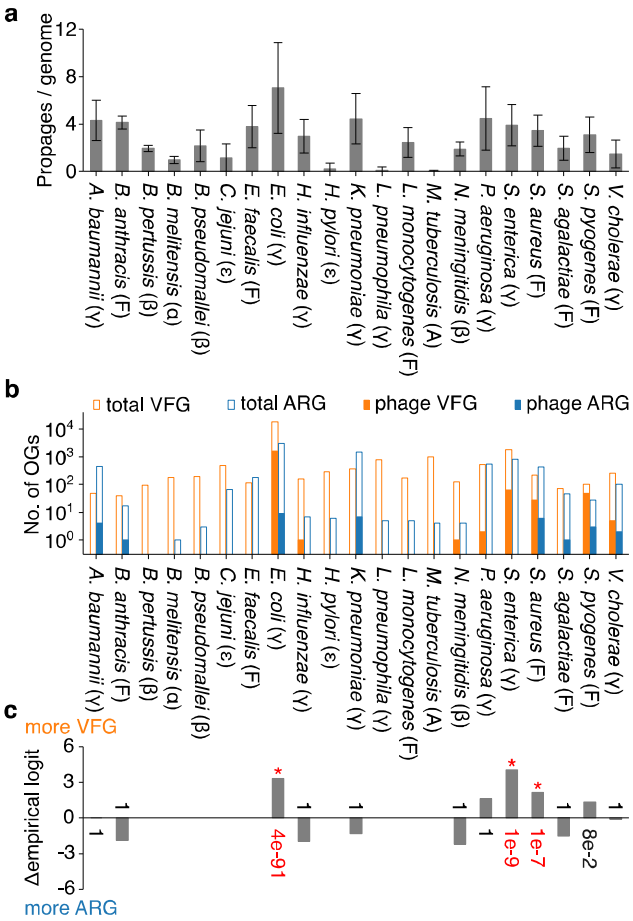

Figure 2. **a** Average number of prophages per genome and standard deviation. **b** Numbers of orthologous groups of virulence factor genes (OGVFGs) and antibiotic resistance genes (OGARGs) in prophages (filled bars) and genomes (open bars). Symbols in brackets indicate taxonomic groups: Actinobacteria (A), Firmicute (F), Alphaproteobacteria (α), Betaproteobacteria (β), Epsilonproteobacteria (ε), and Gammaproteobacteria (γ). **c** Degree of bias towards carrying VFGs more frequently than ARGs. Negative values indicate opposite bias (zero indicates no bias). Difference between empirical logits (denoted as Δempirical logit) is defined as $\log([a+0.5]/[b+0.5]) - \log([c+0.5]/[d+0.5])$, where a and b are numbers of OGVFGs and OGARGs in prophages, respectively (filled bars in a), and c and d are numbers of all OGVFGs and OGARGs in genomes, respectively (open bars in a). Numbers next to bars are P values of two-sided binomial test under null hypothesis that a and b are numbers drawn from binomial distribution with probabilities $c/(c+d)$ and $d/(c+d)$, respectively (corrected by the Holm–Bonferroni method). Asterisks and red numbers indicate that P values are less than or equal to 0.05.

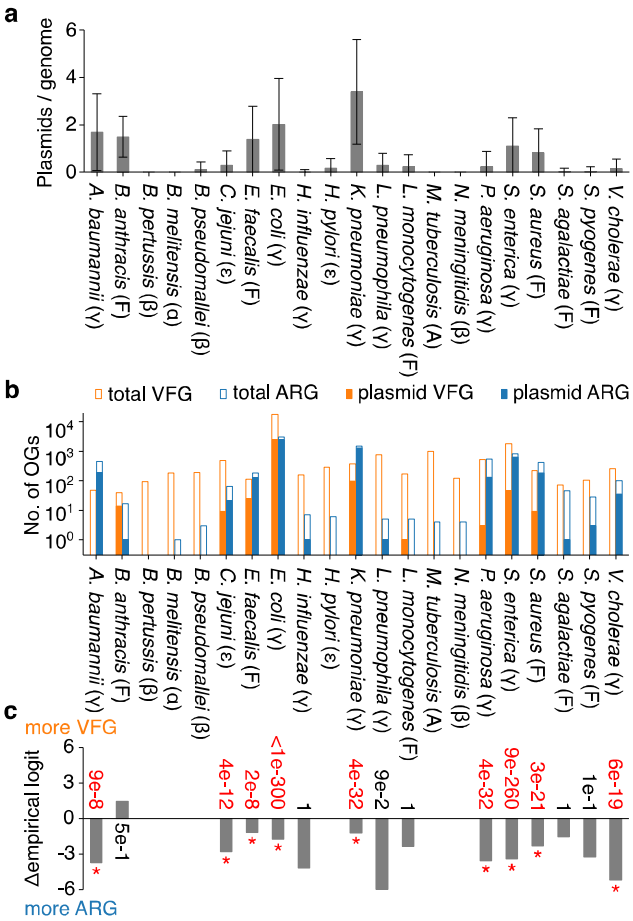

Figure 3. **a** Average number of plasmids per genome and standard deviation. **b** Numbers of orthologous groups of virulence factor genes (OGVFGs) and antibiotic resistance genes (OGARGs) in plasmids (filled bars) and genomes (open bars). Symbols in brackets indicate phyla: Actinobacteria (A), Firmicute (F), Alphaproteobacteria (α), Betaproteobacteria (β), Epsilonproteobacteria (ε), and Gammaproteobacteria (γ). **c** Degree of bias towards carrying VFGs more frequently than ARGs. Negative values indicate opposite bias (zero indicates no bias). Difference between empirical logits (denoted as Δempirical logit) is defined as $\log([a+0.5]/[b+0.5]) - \log([c+0.5]/[d+0.5])$, where a and b are numbers of OGVFGs and OGARGs in prophages, respectively (filled bars in a), and c and d are numbers of all OGVFGs and OGARGs in genomes, respectively (open bars in a). Numbers next to bars are P values of two-sided binomial test under null hypothesis that a and b are numbers drawn from binomial distribution with probabilities $c/(c+d)$ and $d/(c+d)$, respectively (corrected by the Holm–Bonferroni method). Asterisks and red numbers indicate that P values are less than or equal to 0.05.

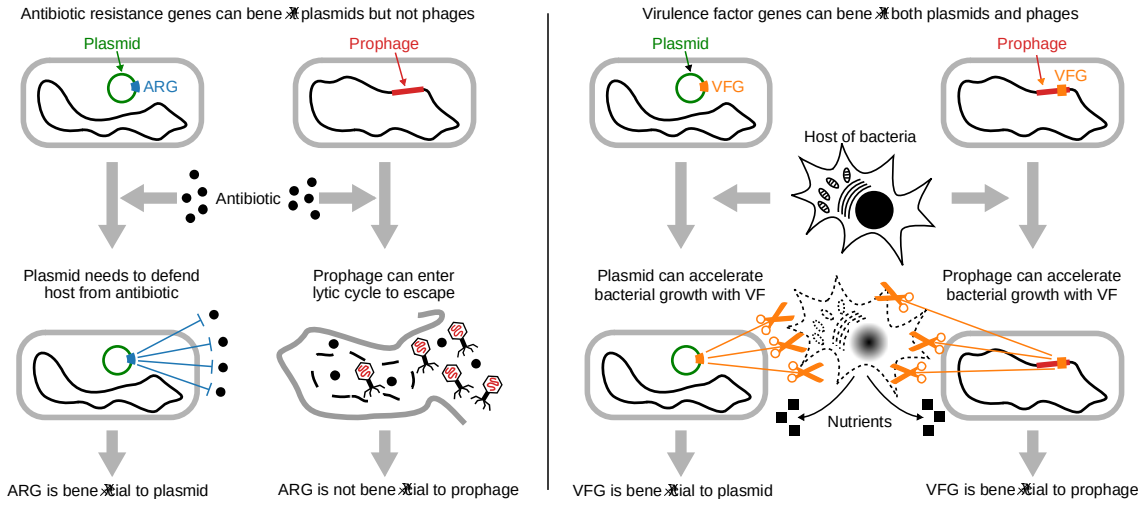

Figure 4. Schematic drawing of hypothesis formulated based on our results. Plasmids gain benefit from carrying ARG because they cannot abandon their hosts and thus need to minimise death of bacteria. However, temperate phages do not gain much benefit from carrying ARGs because if their hosts are in danger, they can abandon their hosts by entering lytic cycles and seek new hosts. In contrast, both plasmids and phages can benefit from VFGs because VFGs can enhance growth of bacteria by causing bacteria to exploit their hosts more aggressively.

9. Supplementary information

Supplementary files can be downloaded from
https://www.biorxiv.org/content/10.1101/2022.07.21.500938v1

Table S1: List of all genome assemblies used in this study.

Table S2: List of all prophages predicted by VIBRANT.

Table S3: List of all orthologous groups of antibiotic resistance genes.

Table S4: Information about all prophages manually examined.

Table S5: List of all orthologous groups of virulence factor genes.

Table S6: List of prophages that are described in Kondo et al. (2021) and manually examined in this study.

In-house scripts

10. References

1. Bennett, P.M., *Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria.* British Journal of Pharmacology, 2009. **153**(S1): p. S347-S357.

2. von Wintersdorff, C.J.H., et al., *Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer.* Frontiers in Microbiology, 2016. **7**: p. 173.

3. Partridge, S.R., et al., *Mobile Genetic Elements Associated with Antimicrobial Resistance.* Clinical microbiology reviews, 2018. **31**(4): p. e00088-17.

4. Wagner, P.L. and M.K. Waldor, *Bacteriophage control of bacterial virulence.* Infection and Immunity, 2002. **70**(8): p. 3985-3993.

5. Gyles, C. and P. Boerlin, *Horizontally Transferred Genetic Elements and Their Role in Pathogenesis of Bacterial Disease.* Veterinary Pathology, 2014. **51**(2): p. 328-340.

6. O'Neill, J., *Review on antimicrobial resistance. Tackling drug-resistant infections globally: Final report and recommendations*. 2016.

7. Pilla, G. and C.M. Tang, *Going around in circles: Virulence plasmids in enteric pathogens.* Nature Reviews Microbiology, 2018. **16**(8): p. 484-495.

8. Murray, C.J.L., et al., *Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis.* The Lancet, 2022. **399**(10325): p. 629-55.

9. Frost, L.S., et al., *Mobile genetic elements: the agents of open source evolution.* Nature Reviews Microbiology, 2005. **3**(9): p. 722-732.

10. Hall, J.P.J., M.A. Brockhurst, and E. Harrison, *Sampling the mobile gene pool: innovation via horizontal gene transfer in bacteria.* Philosophical Transactions of the Royal Society B: Biological Sciences, 2017. **372**(1735): p. 20160424.

11. Ghaly, T.M. and M.R. Gillings, *Mobile DNAs as ecologically and evolutionarily independent units of life.* Trends in Microbiology, 2018. **26**(11): p. 904-912.

12. Rankin, D.J., E.P.C. Rocha, and S.P. Brown, *What traits are carried on mobile genetic elements and why?* Heredity, 2011. **106**(1): p. 1-10.

13. Eberhard, W.G., *Evolution in bacterial plasmids and levels of selection*. Quarterly Review of Biology, 1990. **65**(1): p. 3-22.

14. Bergstrom, C.T., M. Lipsitch, and B.R. Levin, *Natural selection, infectious transfer and the existence conditions for bacterial plasmids.* Genetics, 2000. **155**(4): p. 1505-19.

15. Svara, F. and D.J. Rankin, *The evolution of plasmid-carried antibiotic resistance.* BMC Evolutionary Biology, 2011. **11**(1): p. 130-130.

16. Lehtinen, S., J.S. Huisman, and S. Bonhoeffer, *Evolutionary mechanisms that determine which bacterial genes are carried on plasmids.* Evolution Letters, 2021. **5**(3): p. 290-301.

17. Abedon, S.T. and J.T. LeJeune, *Why Bacteriophage Encode Exotoxins and other Virulence Factors.* Evolutionary Bioinformatics, 2005. **1**: p. 117693430500100-117693430500100.

18. Wallace, M.J., S.R.S. Fishbein, and G. Dantas, *Antimicrobial resistance in enteric bacteria: current state and next-generation solutions.* Gut Microbes, 2020. **12**(1).

19. Snyder, L., et al., *Molecular Genetics of Bacteria*. 4th ed ed. 2013, Washington, D.C.: ASM Press.

20. Villa, T.G., et al., *Horizontal Gene Transfer Between Bacteriophages and Bacteria: Antibiotic Resistances and Toxin Production*. 2019, Springer International Publishing. p. 97-142.

21. Giovanetti, E., et al., *Transduction of the Streptococcus pyogenes bacteriophage Φm46.1, carrying resistance genes mef(A) and tet(O), to other Streptococcus species.* Frontiers in Microbiology, 2015. **5**(DEC): p. 746.

22. Modi, S.R., et al., *Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome.* Nature, 2013. **499**(7457): p. 219-222.

23. Subirats, J., et al., *Metagenomic analysis reveals that bacteriophages are reservoirs of antibiotic resistance genes.* International Journal of Antimicrobial Agents, 2016. **48**(2): p. 163-167.

24. Debroas, D. and C. Siguret, *Viruses as key reservoirs of antibiotic resistance genes in the environment.* The ISME Journal, 2019. **13**(11): p. 2856-2867.

25. Strange, J.E.S., et al., *Metagenomics analysis of bacteriophages and antimicrobial resistance from global urban sewage.* Scientific Reports, 2021. **11**(1): p. 1600.

26. Muniesa, M., et al., *Bacteriophages and diffusion of β-lactamase genes.* Emerging Infectious Diseases, 2004. **10**(6): p. 1134-1137.

27. Enault, F., et al., *Phages rarely encode antibiotic resistance genes: a cautionary tale for virome analyses.* The ISME Journal, 2017. **11**(1): p. 237-247.

28. López-Leal, G., et al., *Prophages Encode Antibiotic Resistance Genes in Acinetobacter baumannii.* Microbial Drug Resistance, 2020.

29. Kondo, K., M. Kawano, and M. Sugai, *Distribution of Antimicrobial Resistance and Virulence Genes within the Prophage-Associated Regions in Nosocomial Pathogens.* mSphere, 2021.

30. Kleinheinz, K.A., K.G. Joensen, and M.V. Larsen, *Applying the ResFinder and VirulenceFinder web-services for easy identification of acquired antibiotic resistance and E.*

coli virulence genes in bacteriophage and prophage nucleotide sequences. Bacteriophage, 2014. **4**(2): p. e27943-e27943.

31. Gunathilaka, G.U., et al., *Phages in urban wastewater have the potential to disseminate antibiotic resistance.* International Journal of Antimicrobial Agents, 2017. **50**(5): p. 678-683.

32. Cook, R., et al., *INfrastructure for a PHAge REference Database: Identification of Large-Scale Biases in the Current Collection of Cultured Phage Genomes.* PHAGE, 2021.

33. Liu, B., et al., *VFDB 2019: a comparative pathogenomic platform with an interactive web interface.* Nucleic Acids Research, 2018. **47**(D1): p. D687-D692.

34. Sayers, S., et al., *Victors: a web-based knowledge base of virulence factors in human and animal pathogens.* Nucleic Acids Research, 2019. **47**(D1): p. D693-D700.

35. Davis, J.J., et al., *The PATRIC Bioinformatics Resource Center: expanding data and analysis capabilities.* Nucleic Acids Research, 2020. **48**(D1): p. D606-D612.

36. Sayers, E.W., et al., *GenBank.* Nucleic Acids Research, 2020. **48**(D1): p. D84-D86.

37. Fu, L., et al., *CD-HIT: Accelerated for clustering the next-generation sequencing data.* Bioinformatics, 2012. **28**(23): p. 3150-3152.

38. Li, W., et al., *RefSeq: expanding the Prokaryotic Genome Annotation Pipeline reach with protein family model curation.* Nucleic Acids Research, 2021. **49**(D1): p. D1020-D1028.

39. Roach, M.J., et al., *Philympics 2021: Prophage Predictions Perplex Programs.* F1000Research, 2021. **10**: p. 758-758.

40. Feldgarden, M., et al., *AMRFinderPlus and the Reference Gene Catalog facilitate examination of the genomic links among antimicrobial resistance, stress response, and virulence.* Scientific Reports, 2021. **11**(1): p. 12728-12728.

41. Altschul, S.F., et al., *Basic local alignment search tool.* Journal of Molecular Biology, 1990. **215**(3): p. 403-410.

42. Buchfink, B., C. Xie, and D.H. Huson, *Fast and sensitive protein alignment using DIAMOND.* Nature Methods, 2015. **12**(1): p. 59-60.

43. Lechner, M., et al., *Proteinortho: Detection of (Co-)orthologs in large-scale analysis.* BMC Bioinformatics, 2011. **12**(1): p. 124-124.

44. Novichkov, P.S., et al., *ATGC: a database of orthologous genes from closely related prokaryotic genomes and a research platform for microevolution of prokaryotes.* Nucleic Acids Research, 2008. **37**(suppl_1): p. D448-D454.

45. Pfeifer, E., et al., *Bacteria have numerous distinctive groups of phage-plasmids with conserved phage and variable plasmid gene repertoires.* Nucleic Acids Research, 2021.

46. Kieft, K., Z. Zhou, and K. Anantharaman, *VIBRANT: automated recovery, annotation and curation of microbial viruses, and evaluation of viral community function from genomic sequences.* Microbiome, 2020. **8**(1): p. 90-90.

47. Fouts, D.E., *Phage_Finder: Automated identification and classification of prophage regions in complete bacterial genome sequences.* Nucleic Acids Research, 2006. **34**(20): p. 5839–5851-5839–5851.

48. Holm, S., *A Simple Sequentially Rejective Multiple Test Procedure.* Scandinavian Journal of Statistics, 1979. **6**(2): p. 65–70-65–70.

49. Seabold, S. and J. Perktold. *statsmodels: Econometric and statistical modeling with Python.* in *9th Python in Science Conference*.

50. Matsushiro, A., et al., *Induction of Prophages of Enterohemorrhagic Escherichia coli O157:H7 with Norfloxacin.* Journal of Bacteriology, 1999. **181**(7): p. 2257–2260-2257–2260.

51. Goerke, C., J. Köller, and C. Wolz, *Ciprofloxacin and Trimethoprim Cause Phage Induction and Virulence Modulation in Staphylococcus aureus.* Antimicrobial Agents and Chemotherapy, 2006. **50**(1): p. 171–177-171–177.

52. Allen, H.K., et al., *Antibiotics in feed induce prophages in swine fecal microbiomes.* mBio, 2011. **2**(6): p. e00260–11-e00260–11.

53. López, E., et al., *Induction of Prophages by Fluoroquinolones in Streptococcus pneumoniae: Implications for Emergence of Resistance in Genetically-Related Clones.* PLOS ONE, 2014. **9**(4): p. e94358-e94358.

54. Sutcliffe, S.G., et al., *Common Oral Medications Lead to Prophage Induction in Bacterial Isolates from the Human Gut.* Viruses, 2021. **13**(3): p. 455-455.

55. Arndt, D., et al., *PHASTER: a better, faster version of the PHAST phage search tool.* Nucleic Acids Research, 2016. **44**(W1): p. W16-W21.

56. Chen, J., et al., *Genome hypermobility by lateral transduction.* Science, 2018. **362**(6411): p. 207-212.

57. Chiang, Y.N., J.R. Penadés, and J. Chen, *Genetic transduction by phages and chromosomal islands: The new and noncanonical.* PLOS Pathogens, 2019. **15**(8): p. e1007878-e1007878.