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1 **Antibiotic resistance genes in phage particles isolated from human feces and**
2 **induced from clinical bacterial isolates**

3

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Abstract

Phage particles have emerged as elements with the potential to mobilize antibiotic resistance genes (ARGs) in different environments, including the intestinal habitat. The aim of this study was to determine the occurrence of ARGs in phage particles present in fecal matter and induced from strains isolated from feces. Nine ARGs (*bla*$_{\text{TEM}}$, *bla*$_{\text{CTX-M-1}}$-group, *bla*$_{\text{CTX-M-9}}$-group, *bla*$_{\text{OXA-48}}$, *qnrA*, *qnrS*, *mecA*, *sul1* and *armA*) were quantified by qPCR in the phage DNA fractions of 150 fecal samples obtained from healthy individuals. These subjects had not received antibiotic treatment or travelled abroad in the three months prior to the sample collection. On the suspicion that the detected particles originated from bacterial flora, 82 *Escherichia coli* and *Klebsiella pneumoniae* isolates possessing at least one identified ARG (*bla*$_{\text{TEM}}$, *bla*$_{\text{CTX-M-1}}$-group, *bla*$_{\text{CTX-M-9}}$-group, *armA*, *qnrA*, *qnrS*, and *sul1*) were isolated and their capacity to produce phage particles carrying these ARGs after induction was evaluated. Seventy-two percent of samples were positive for at least one ARG, with *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M-9}}$-group being the most prevalent and abundant. Fifty-one isolates (62%) showed an increase in the number of copies of the respective ARG in the phage fraction after induction, with *bla*$_{\text{TEM}}$, *bla*$_{\text{CTX-M-1}}$-group, *bla*$_{\text{CTX-M-9}}$-group and *sul1* being the most abundant. Phages induced from the isolates were further purified and visualized using microscopy and their DNA showed ARG levels of up to 10^{10} gene copies/ml. This study highlights the abundance of phage particles harboring ARGs and indicates that bacterial strains in the intestinal habitat could be sources of these particles.

48 **Key words:** antibiotic resistance, bacteriophage, feces, *Escherichia coli*, *Klebsiella*
49 *pneumoniae*, horizontal genetic transfer, transduction.
50
51 ## 1. Introduction
52
53 Bacteriophages, or bacterial viruses, are the most abundant biological entities on
54 Earth and one of the keys to the evolution and potential control of bacterial populations
55 [1]. Knowledge of phages has been essential for the progress of molecular biology and
56 they have been used as models for studying different biological processes. In recent
57 decades, phages have acquired increasing relevance in molecular biology due to new
58 insights into their presence in many bacterial genomes [1,2], their role in horizontal
59 gene transfer [3], the phage-bacterium relation and bacterial defense mechanisms
60 against phage infection [4].
61
62 Meanwhile, bacterial resistance to antibiotics continues to increase and is severely
63 undermining our ability to control infectious diseases. The World Health Organization
64 (WHO) has identified antibiotic resistance as one of the most challenging problems in
65 public health care on a global scale (available at
66 http://apps.who.int/gb/ebwha/pdf_files/WHA68-REC1/A68_R1_REC1-en.pdf). The
67 causes of this increase in resistance are believed to include overuse and inconsistent
68 application of antibiotics in humans, together with the use of antibiotics in animal
69 husbandry [5,6]. The scientific community and governments have reacted by calling for
70 a better control of antibiotic usage in both humans and livestock. Researchers are trying
71 to find new generations of antibiotics to treat infections by resistant strains [7], but more

72 research into the mechanisms of resistance would also be advisable. This is the purpose
73 of the multidisciplinary "One-Health" approach [8], which aims to encourage the
74 collaborative efforts of multiple disciplines working locally, nationally, and globally.
75
76 Antibiotic resistance may be intrinsic, or conferred either by mutation or by
77 acquiring antibiotic resistance genes (ARGs) through mobile genetic elements (MGEs)
78 [5]. Clinical studies point to conjugation and transformation as the most likely
79 mechanisms of transfer [8]. However, little attention has been paid to other
80 mechanisms, such as transduction mediated by phages or phage-derived particles, which
81 have only recently emerged as potentially relevant [9–11]. Bacteriophages basically
82 consist of one nucleic acid molecule (the phage genome) surrounded by a protein
83 coating, the capsid. This packaging of the nucleic acid confers protection and hence an
84 extracellular persistence that cannot be found in naked DNA or RNA. Therefore, capsid
85 protection could be important in cases where there is no close cell-to-cell contact [12].
86
87 In preliminary studies the presence of ARGs was determined in total and phage
88 DNA in feces of individuals without any enteric disease [13]. That work is extended
89 here by the analysis of more ARGs in the phage fraction of a new collection of fecal
90 samples from 150 healthy individuals free of contact with clinical settings and who had
91 not received antibiotic therapy in the previous three months. The ARGs studied
92 consisted of four β-lactamases (*bla*$_{\text{TEM}}$, *bla*$_{\text{CTX-M-1}}$-group, *bla*$_{\text{CTX-M-9}}$-group, *bla*$_{\text{OXA-48}}$)
93 [14], two quinolone resistance genes (*qnrA* and *qnrS*)[15,16], the *mecA* gene that
94 confers resistance to methicillin in *Staphylococcus aureus* [17], the emerging *armA*
95 gene that confers resistance to aminoglycosides [18] and *sul1*, the most extended gene

96 conferring resistance to sulfonamides [19]. Another aim of the study was to gain insight
97 into the origin of the phage particles, suspected to be derived from bacterial flora.
98 Accordingly, bacterial strains (*Escherichia coli* and *Klebsiella pneumoniae*) isolated
99 from the feces that possessed a given ARG were treated to induce phage particles
100 carrying this ARG.

101

102 **2. Materials and methods**

103

104 *2.1. Fecal samples*

105

106 This study was performed with 150 human fecal samples of individuals living in
107 the city of Barcelona (Catalonia, North East Spain), collected over a period of six
108 months (from February to August 2016). All individuals were healthy, not related with
109 clinical environments nor involved in a food-borne outbreak or showing any gastro-
110 intestinal pathology or known infection. None of the subjects had consumed antibiotics
111 or travelled to foreign countries in the three months before the sampling. All samples
112 were completely anonymized. Besides ensuring the above criteria were fulfilled, no
113 individual data were collected except for age. The samples were destroyed immediately
114 after the study, which was approved by the Clinical Ethics Committee (12/065/1350).
115 Informed consent was obtained for all individuals.

116

117 *2.2. Bacterial strains*

118

119 The *E. coli* strains used as controls for the different ARGs are listed in Table 1.
120 Fecal samples were cultured on chromogenic agar chromID® CPS® Elite (bioMérieux,
121 Marcy-l'Étoile, France). After 24 h of incubation at 37ºC, all the isolates growing on the
122 plates compatible with *E. coli* or *Klebsiella* were identified by matrix-assisted laser
123 desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (MALDI
124 Autoflex IITM / TOF/TOF, Bruker, Daltonik GmbH, Germany). Antimicrobial
125 susceptibility testing was performed according to the guidelines of the Clinical and
126 Laboratory Standards Institute [20].
127 Seventy *E. coli* and 12 *K. pneumoniae* isolates were selected on the basis of the
128 presence of one of the target ARGs (one isolate per individual) to determine the
129 presence of phage particles containing the ARGs.
130
131 *2.3. Partial purification of phage DNA*
132
133 Fecal samples were homogenized 1:5 (*w:v*) in phosphate buffered saline (PBS)
134 by shaking for 15 minutes. Then, a final volume of 50 ml of the homogenate was
135 centrifuged at 3,000 *g* and the supernatant was filtered through low protein-binding 0.22
136 µm pore-size membrane filters (Millex-GP, Millipore, Bedford, MA). The suspensions
137 were treated with chloroform, centrifuged at 16,000 *g* for 10 minutes and the
138 supernatants were treated with DNAse (100 units/ml; Sigma-Aldrich, Spain) for 1 hour
139 at 37ºC. DNAse was heat-inactivated at 75ºC for 5 minutes. To rule out the presence of
140 non-packaged DNA, an aliquot of the sample was taken after DNase treatment and
141 before its desencapsidation. Using this control sample, the absence of free 16SrDNA
142 was established as well as the absence of the ARGs studied by qPCR, confirming total

143 removal of non-encapsidated DNA [13,21]. Different controls were performed to verify
144 the stability and appropriate inactivation of the DNase [10]. Packaged DNA was
145 extracted by proteinase K digestion, purified and quantified [10,13].

146

147 *2.4. Purification of bacteriophages from clinical isolates*

148

149 Twenty ml cultures of each clinical isolate in Luria Beltrani (LB) broth were
150 grown to the mid-exponential phase (optical density $(OD)_{600}$ of 0.3). Each culture was
151 aliquoted in two 10 ml tubes and one aliquot was treated with mitomycin C (mitC)
152 (final concentration 0.5 µg/ml) to induce phage particles. Both tubes were incubated for
153 6h at 37ºC by shaking in the dark and the absorbance of the culture after induction was
154 monitored by comparing the OD of the mitC-treated and non-treated cultures. Phages in
155 the supernatant lysate from both aliquot cultures were partially purified as described
156 above.

157

158 *2.5. Standard PCR and qPCR procedures*

159

160 PCRs were performed with a GeneAmp® PCR 2700 system (Applied
161 Biosystems, Barcelona, Spain). ARGs from the control strains were amplified by
162 conventional PCR, cloned in pGEM-T Easy vectors (pGEM®-T-Easy Vector, Promega,
163 Barcelona, Spain) to generate the constructs and verified by sequencing (Table 2). The
164 constructs were used to generate the standard curves.

165

166 Real-time qPCR assays for *bla*$_{TEM}$, *bla*$_{CTX-M-1}$-group, *bla*$_{CTX-M-9}$-group ,*mecA,*
167 *armA, qnrA* group*, qnrS* group and *sul1* were performed as previously described
168 [10,13,22,23]. The *bla*$_{OXA-48}$ gene qPCR assay (Table 2) was designed with the Primer
169 Express Software version 3.0 (Applied Biosystems). The gene was amplified using
170 specific primers (Table 2) from the sequence of *bla*$_{OXA-48}$ harbored in the *K.*
171 *pneumoniae* clinical isolate HSP172. The amplified *bla*$_{OXA-48}$ was sequenced and cloned
172 in pGEM-T Easy. The construct was confirmed by sequencing and used to generate the
173 standard curves [10]. The qPCR assay for *bla*$_{OXA-48}$ showed a 99.8% efficiency and a
174 quantification limit of 18.2 gene copies /µL (threshold cycle of 32.4), similar to the
175 other genes.
176
177 Primers and Taqman hydrolysis probes (Table 2) were used under standard
178 conditions in a StepOne Real-Time PCR system [10]. To further screen for PCR
179 inhibition, dilutions of known gene copy concentration of the *mecA* standard were
180 spiked with the DNA isolated from the samples, and results were compared to the
181 expected concentration. No inhibition of the PCR by the samples was detected. All the
182 samples were run in duplicate.
183
184 *2.6. Phage purification by CsCl density gradients.*
185
186 Eight isolates showing good induction and a high level of ARGs in phage DNA
187 were selected for purification by cesium chloride (CsCl) density gradients [24] and
188 electron microscopy observations. The easily visible grey bands corresponding to

bacteriophages [24,25] were collected and dialyzed. Phage DNA was extracted from the particles in the band and used to quantify the ARGs. Phage particles forming a band were visualized by electron microscopy in a JEOL 1010 transmission electron microscope (JEOL Inc. Peabody, MA USA) operating at 80 kV [26].

2.7. Statistical analysis

Computation of data and statistical tests was performed using the Statistical Package for Social Science software (SPSS). A tolerance interval with 90% confidence in 90% of the population (considering collected isolates as the population) was used to determine which isolates were considered positive for induction after mitC treatment (using OD_{600} and ARGs gene copy data).

3. Results and Discussion

3.1. ARGs in phage particles isolated from feces

Stool samples from healthy individuals in the Barcelona area were selected as described in Materials and methods. It was verified that the subjects had no contact with a clinical environment and had not received any antibiotic treatment or travelled abroad in the three months before sampling. The age of the individuals ranged from 1.3 to 85 years.

213 About 72% of the phage suspensions obtained from the stools were positive for
214 at least one ARG. From these, 29 samples (19.3%) were positive for one ARG; 40
215 samples (26.7%) for 2 ARGs; 24 samples (16.0%) for 3 ARGs; 14 samples (9.3%) for 4
216 and 2 samples (1.3%) for 5 ARGs. Among the detected ARGs, *bla*$_{TEM}$ was the most
217 prevalent, followed by *bla*$_{CTX-M-9}$-group, *sul1* and *qnrA* (Figure 1A). *bla*$_{TEM}$ was also the
218 most abundant (Fig. 1B), reaching maximum densities of 10^6 gene copies/g, although
219 the samples showed a great heterogeneity in the number of particles carrying *bla*$_{TEM}$
220 gene copies. Next in abundance were both *bla*$_{CTX-M}$ groups 1 and 9. Although not
221 among the most prevalent ARGs, *mecA* and *qnrS* showed an average abundance of 3.1
222 and 2.4 log_{10} gene copies, respectively. The average number of particles bearing *bla*$_{OXA-}$
223 $_{48}$, *qnrA* and *armA* was close to 2 log_{10} gene copies /g (although some samples showed
224 higher densities of *qnrA*), while *sul1*, despite its high prevalence, was one of the least
225 abundant genes (Fig. 1B).
226 Analyzing the distribution of ARGs among subjects in different age segments
227 (<20, 20-40, 40-60 or >60) revealed a higher prevalence of *sul1* and *bla*$_{CTX-M-1}$-group in
228 samples from older subjects, which also showed a lower level of *bla*$_{CTX-M-9}$-group, as
229 the most remarkable observations (Fig. S1A). Individuals aged 20-60 years gave a
230 higher percentage of samples without any ARGs, although this group also provided the
231 most samples. The trend line (Fig. S1B) did not show any correlation with different age
232 groups. In a previous study, correlations between age and the number of ARGs in phage
233 DNA were not observed either [13].
234
235 The ARG prevalence in the phage fraction is in agreement with previous data
236 obtained with a different set of fecal samples [13]. In the former study, 22.5% of

237 samples were negative, compared to 28% here, and the proportions of each ARG were
238 slightly higher in abundance. The most notable difference between the studies is that
239 whereas both cohorts of individuals were not affected by gastrointestinal disease, in the
240 previous study prior administration of antibiotics or travel abroad was not monitored.
241
242 *3.2. Phage particles induced from clinical bacterial isolates*
243
244 The nature of phage particles carrying ARGs detected in feces is unknown. They
245 could be free particles in the gut incorporated by ingestion of food or water, or particles
246 produced by bacterial strains present in the microbiota. To evaluate this second
247 possibility, 70 *E. coli* and 12 *K. pneumoniae* isolates from fecal samples of different
248 individuals in this study (82 individuals) were selected on the basis of the presence of
249 one or more targeted ARGs (Table 3). The isolates allowed us to analyze phage
250 particles carrying bla_{TEM}, $bla_{\text{CTX-M-1}}$-group, $bla_{\text{CTX-M-9}}$-group, *armA, qnrA, qnrS* and *sul1*.
251 Phage particles were induced from the isolates using mitC at a subinhibitory
252 concentration, a commonly used method [27]. The *Enterobacteriaceae* group does not
253 harbor *mecA,* therefore this gene was not included in this part of the study. The absence
254 of isolates with $bla_{\text{OXA-48}}$ could be expected considering that the isolation performed
255 was not specific for its detection, and in addition this gene should not be prevalent in
256 healthy carriers [28].
257
258 The effect of mitC on the isolates was determined by monitoring the reduction of
259 the OD_{600} of the treated aliquot of each culture in comparison with the untreated aliquot
260 after 6 hours of incubation at 37ºC. The OD reduction is interpreted as activation of the

261 phage lytic cycle or a similar mechanism causing cell lysis. Each isolate was mitC-
262 treated at least in duplicate, and although the OD reached differed slightly between
263 replicates, the differences between the control and the treated aliquot of the culture were
264 consistent between replicates. To statistically support which samples showed induction,
265 we considered a tolerance interval of 90% confidence in 90% of the population.
266 Therefore, we excluded isolates falling outside the tolerance range, i.e. those showing
267 an OD_{600} reduction of less than 0.2 points, which indicates a lack of cell lysis. Only
268 seven of the 82 isolates were considered non-inducible (isolates marked with an asterisk
269 in Fig. 2). In contrast, 54 isolates showed an OD_{600} decrease of > 0.5 points (Fig. 2) and
270 these were suspected of harboring prophages or phage-derived particles causing the
271 lysis of the host strain after induction.

272

273 *3.3. ARGs in phage particles induced from clinical isolates*

274

275 *bla*$_{TEM}$, *bla*$_{CTX-M-1}$-group, *bla*$_{CTX-M-9}$-group, *armA*, *qnrA*, *qnrS*, and *sul1* were
276 quantified in the phage DNA in the culture supernatant of 82 isolates treated or not with
277 mitC. ARG values were usually higher in the mitC-treated culture, attributed to the
278 generation of phage particles by the treatment. Isolates Ec7, Ec22, Ec37, Ec60 Ec70,
279 Kp2, Kp3, which did not show a reduction in the OD_{600} measurements after mitC
280 treatment (Fig. 2), accordingly did not show an increase in gene copies/ml of the
281 corresponding ARGs in phage DNA after induction (Fig. 3). *armA* is not included
282 because no differences between the induced culture and the control were detected.

283

284 After two independent induction experiments, 51 isolates (62.2%) showed an
285 increase in the ARG copy number in the phage fraction. We selected as inducible those
286 isolates with an increase of more than 0.2 log_{10} gene copies, on the basis of a tolerance
287 interval of 90% confidence in 90% of the population. In fact, for all 51 isolates that
288 showed an increase in the ARG copy number the difference was equal or greater than
289 0.5 log_{10}. Thirty-one strains (37.8%) did not show an increase in the gene copy values
290 ($\leq$ 0.2 log_{10} units). In some cases (*qnrA* and *qnrS*) (Fig. 3), particles containing an ARG
291 were only observed after induction, probably because the number of ARG-particles in
292 the untreated culture was too low and below the limit of quantification of our qPCR
293 assays. In contrast, the occurrence of ARG-particles in the uninduced culture is
294 attributed to basal, spontaneous generation of phage particles, widely reported in phages
295 [29] and phage-related particles, such as gene transfer agents (GTA)[30]. Moreover,
296 some isolates showed higher gene copy densities in the control than in the induced
297 culture, although the differences were not significant ($P > 0.05$) (Fig. 3). These results
298 could be attributed to a reduction in cell number caused by activation of the lytic cycle
299 of other prophages in the isolate chromosome, which are very commonly found in *E.*
300 *coli* or *Klebsiella spp.* [31,32]. Another possibility is that the treatment with mitC
301 reduced the growth rate of the isolate, thereby diminishing the number of particles
302 produced per cell.
303
304 Average gene copy/ml values of those samples with an increase in the number of
305 particles after mitC induction were box-plotted (Fig. 4), and differences between control
306 and mitC-treated samples were significant ($P<0.05$) for all ARGs except *sul1*. Similar
307 averaged densities were observed for all ARGs except *qnrA* and *qnrS*, which showed

308 lower values. Some ARGs (*sul1*, bla_{TEM} or $bla_{CTX-M-1}$-group) showed up to 10^9 gene
309 copies/ml after induction (Fig. 4).
310
311 *3.4. Observation of phage particles carrying ARGs*
312
313 Observation of fecal samples and the supernatant of some of the mitC-treated
314 bacterial cultures revealed tailed phage particles, corresponding to the *Myoviridae* and
315 *Siphoviridae* morphological types (like those shown in Fig. 5). Not all samples allowed
316 visualization of phage particles, either because they corresponded to samples where no
317 integral capsids were present, or very probably because they were present in
318 concentrations below those required for visualization in the electron microscope (*c.a.*
319 10^8 particles/ml) [26].
320
321 The phage particles were obtained from induced cultures of 4 *E. coli* (Ec4, Ec11,
322 Ec30, Ec69) and 4 *K. pneumoniae* (Kp4, Kp8, Kp9, Kp11) isolates selected on the basis
323 of high induction rates and an increase in the gene copies of one or more ARG after
324 mitC treatment (Fig. 3). Particles were further purified by CsCl gradients and the
325 resulting grey band corresponded to a density of 1.5 g/ml, which is in accordance with
326 what is expected for phage particles [25] (Fig. 5A). The band was recovered and used to
327 confirm the presence of phage particles. After chloroform and DNAse treatment, the
328 DNA from the phage capsids was extracted and the ARG quantified.
329
330 The eight strains showed the presence of phage particles of the *Myoviridae* and
331 *Siphoviridae* morphological types (Fig. 5B). Both groups have been reported as the

most abundant infecting *E. coli* and *Bacteroides fragilis* in fecally polluted water samples [33–35] and stool samples [36]. Analysis of fecal viromes also indicates that tailed, doubled-stranded DNA viruses of the order Caudovirales, which include *Siphoviridae*, *Myoviridae*, and *Podoviridae*, are the most abundant types in feces [37,38].

A minimal amount of 10^7 particles/ml of sample is required for electron microscopy observation, therefore the phages observed are assumed to be the most abundant in these samples. Accordingly, the packaged DNA extracted from the CsCl density gradient bands containing the phages showed densities of gene copies/ml $> 10^7$ for at least one of the ARGs (Fig. 5C) and some ARGs showed densities of up to 10^{10} gene copies/ml. It can be assumed that at least a fraction of the phage particles visualized by microscopy would carry one of the ARGs in densities in accordance with the particles observed by microscopy and at the same order of magnitude as shown in Fig. 3.

The mobilome [39] includes all the mobile genetic elements (MGEs) in a genome, while the resistome [40] refers to all the ARGs and their precursors in a bacterial genome. The two concepts are closely linked, because in general, ARGs found intrinsically in certain bacteria are mobilized to recipient cells by a range of MGEs, and their spread is the main cause of the alarming emergence of antibiotic-resistant bacteria worldwide [6]. However, the scope of the elements that comprise the mobilome has not yet been definitively defined. The role of plasmids in ARG transfer in clinical settings

355 has been widely reported [8,41–43], but it is now suspected that other elements, such as
356 phages [10,11,21] or phage-derived particles [12], could also be involved.
357
358 The intestinal habitat is a densely populated environment where phages play a
359 determinant role, either in regulation of intestinal populations, thereby influencing
360 human welfare, or as MGEs of genes related to pathogenicity [2,13,44,45]. It has been
361 suggested [12] that phages are efficient genetic vehicles due to the protection conferred
362 by the protein capsid in extracellular environments.
363
364 Some phage genomes are spontaneously induced from resistant strains by
365 environmental conditions [27,29], resulting in transcription and production of new
366 phage particles which then infect and lysogenize other uninfected host cells. Other
367 elements that can be considered as phage-related, because of their evident similarities
368 with phages, are induced in a similar way: this is the case of GTA [46,47].
369
370 The particles produced by the bacterial isolates in this study seemed to be
371 resident in our isolates as prophages and were induced by the mitC treatment. The
372 presence of ARGs in these particles opens up two possibilities. The first is that these are
373 prophages with the ARG inserted in their genome. We would then expect to be able to
374 isolate these ARG-harboring phages and plausibly to transduce the gene in relatively
375 high frequencies. This was not the case here: our transduction attempts were not
376 successful, in line with previous attempts using phage particles isolated from fecally
377 polluted samples [10]. Moreover, some sequencing studies [10,13], as in the present

378 work (data not presented), have shown a lack of phage genes flanking the targeted
379 ARGs.
380
381 The second possibility is that after induction, prophage genes in the bacterial
382 isolates have packaged bacterial DNA (including the ARG) in a sort of generalized
383 transduction or GTA-like particle. These would then be detectable by the methods used
384 and show an increase after induction, but with an absence of phage DNA genes, which
385 is more in accord with our observations. These ARG-containing phage particles would
386 theoretically be capable of attaching to new host cells and injecting their DNA [48]. In
387 fact, quite a number of phages reportedly involved in ARG transfer seem to be derived
388 from generalized transduction [49–53]. In terms of their genome, these cannot be
389 considered phages, because they contain only bacterial and not phage DNA. In line with
390 these assumptions, an interesting recent study revealed that bacterial DNA, including
391 ARGs, found in viromes was rarely encoded in phage genomes [54]. Once the
392 possibility of bacterial DNA contamination is discarded (although not completely ruled
393 out), and considering ARGs as those genes that confer real resistance, the most
394 plausible explanation for the presence of ARGs in the studied phage particles is that
395 bacterial DNA is mobilized through generalized transduction or related mechanisms
396 [54].
397
398
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411

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413

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415

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575 **Figure Legends**

576 **Figure 1.- ARGs in the phage fraction of human fecal samples**. A) Percentage of
577 positive samples for each ARG. B) Abundance of each ARG. Box plot of the average
578 values (($\log_{10}$ gene copies)/g feces) of all ARGs in the positive samples. In the box plot,
579 the cross-pieces of each box represent (from top to bottom) the maximum, upper-
580 quartile, median (black bar), lower-quartile and minimum values. The black diamond
581 shows the mean value. The upper boxes in the box plot include samples showing values
582 within the 75th percentile and lower white box samples show values within the 25th
583 percentile.

584

585 **Figure 2.- Induction of phage particles from the bacterial isolates by mitomycin C**
586 **treatment**. OD_{600} measurements for cultures of the *E. coli* (Ec) and *K. pneumoniae* (Kp)
587 isolates with (mitC) or without (control) mitomycin C (0.5 µg/ml) treatment. An
588 asterisk indicates the non-inducible strains.

589

590 **Figure 3.- ARGs in the phage fraction of each bacterial isolate from human fecal**
591 **samples.** ARG densities (gene copies/ml) in phage DNA extracted from the cultures of
592 the *E. coli* (Ec) and *K. pneumoniae* (Kp) isolates with (mitC) or without (control)
593 mitomycin C (0.5 µg/ml) treatment. Results correspond to one independent induction
594 experiment. An asterisk indicates the non-inducible strains in Figure 2.

595

596 **Figure 4.- Average ARG densities in the phage fraction of bacterial isolates.**
597 Average number of ARG copies ($\log_{10}$ gene copies/ml) in phage DNA from isolates
598 showing significant ($P<0.05$) (*) increase in the number of ARG copies after mitC

599 treatment versus uninduced controls in all ARGs except *sul1*. In the box plot, the cross-
600 pieces of each box represent (from top to bottom) the maximum, upper-quartile, median
601 (black bar), lower-quartile and minimum values. A black diamond shows the mean
602 value. The colored boxes in the box plot represent samples showing values within the
603 75th percentile; white boxes represent samples showing values within the 25th
604 percentile.

605

606 **Figure 5.- Visualization of phage particles from the induced cultures carrying**
607 **ARGs**. (A) Example of the grey band corresponding to a density of 1.5 g/ml in a tube of
608 CsCl density gradients prepared with the induced fraction of the isolate Ec4. (B).
609 Electron micrographs of phage particles purified from the eight *E. coli* and *K.*
610 *pneumoniae* induced isolates. (C) The qPCR results of the ARGs present in the phage
611 particles purified from the CsCl density bands and visualized by electron microscopy in
612 (B). Bar: 100nm.

613

614 **Figure S1.- Distribution of the ARGs in the phage DNA fraction of feces in**
615 **different ages.** A) Results for ARGs in phage DNA are presented in a stacked column
616 chart that compares the percentage of positive samples among the total number of
617 samples analyzed for each segment. B) Distribution of the number of ARGs in phage
618 DNA (0-5 ARGs) detected in each individual in relation to age. Dotted line represents
619 the trend line.

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