1 **Costs of antibiotic resistance genes depend on host strain and environment and can influence**
2 **community composition.**

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Abstract

Antibiotic resistance genes (ARGs) is a major contributor to increasing levels of antibiotic resistance in clinical and agricultural settings. ARGs are strongly selected in environments containing corresponding antibiotics, but it is less clear how ARGs are maintained in environments where antibiotic selection might be weak or sporadic. In particular, few studies have directly estimated the effect of ARGs on host fitness in the absence of direct selection. To the extent that ARGs impose costs, it is not clear if these are fixed or might depend on the host strain, perhaps marking some ARG-host combinations as low-cost reservoirs that can act to maintain ARGs in the absence of antibiotic selection. We quantified the fitness effects of six ARGs in each of 11 diverse *Escherichia* spp. strains in two environments. While three ARGs *(*$bla_{\text{TEM-116}}$, *cat*, and *dfrA5*, encoding resistance to β-lactam antibiotics, chloramphenicol, and trimethoprim, respectively) imposed an overall cost, all ARGs had an effect in at least one host strain, reflecting a significant ARG effect-by-strain interaction effect. A simulation model predicts that these interactions cause the ecological success of ARGs to depend on available host strains, and, to a lesser extent, for successful host strains to depend on the ARGs present in a community. Together, these results indicate the importance of considering ARG effects over different host strains, especially the potential of reservoir strains that allow resistance to persist in the absence of direct selection, in efforts to understand resistance dynamics.

Importance

Antibiotic resistance is a major and increasing public health concern. Resistance occurs through a variety of mechanisms but commonly involves bacterial strains acquiring antibiotic resistance genes (ARGs) encoded by mobile elements. It is obvious and well-documented that ARGs will be selected in bacteria that are exposed to the antibiotics they confer resistance to. ARGs can also confer costs to bacteria—in environments that do not contain antibiotics, these costs can lead to the loss of ARGs. We show that ARG costs can be significant and that they depend on the host bacterial strain and the environment in which strains are grown. This dependence creates host-environment refuges for many ARGs, allowing them to be maintained in the absence of direct selection.

Introduction

Widespread use of antibiotics has led to selection for antibiotic-resistant bacteria, which has been identified as a major global health concern [1]. Resistance can derive from spontaneous mutations that generally reduce affinity or access of antibiotics to cellular targets and from antibiotic resistance genes (ARGs) that typically act to detoxify or export antibiotics from cells [2]. The link between antibiotic exposure and selection of resistance is clear: ARGs tend to rise in frequency shortly after widespread use of the cognate antibiotics and ARGs of recent origin are associated with host bacteria that can cause infection in humans or domestic animals [3–6]. Even exposure to low levels of antibiotics can confer a significant advantage to resistant cells, potentially allowing selection in a wide range of environments [7].

The benefit conferred by ARGs to host cells during antibiotic exposure is, however, only one factor determining their overall success [8–11]. Of 22 ARGs for which a recent origin could be inferred, most were from species with human or domestic animal association [5]. This association is linked to antibiotic exposure, supporting the idea that direct selection for resistance is important for the success of ARGs [12]. However, many species that are associated with humans or domestic animals, will nevertheless transit through environments with low antibiotic concentrations. For other ARGs, no originating host species can be inferred, consistent with them originating in poorly characterized environmental species [5]. These considerations underlie the view that any costs associated with encoding ARGs will play an important role in determining their overall success in bacterial populations [10,13,14].

Costs of antibiotic resistance have been examined in some detail for spontaneously occurring resistance alleles [15]. In one experiment, costs associated with rifampicin resistance were sufficient to drive selection for frequent reversion to sensitivity in a mouse infection model [16]. In other cases, costs drive the selection of secondary mutations that compensate for the costs of resistance allowing the resistance phenotype to persist with relaxed antibiotic-driven selection (reviewed in [13,15]. Spontaneous resistance often occurs through mutations in essential genes encoding products targeted by the relevant antibiotic [17]. Changes to these products will generally alter their structure in some way that reduces the strength of antibiotic interaction and are likely to have some deleterious effect on the original function [14]. By contrast, less work has been done examining effects to host cells of expressing introduced ARGs.

64 Studies have shown fitness costs to hosts that carry ARG-encoding plasmids but estimates of the
65 effect of ARGs themselves are often indirect and differences in experimental designs make
66 comparisons difficult. One meta-analysis found that the costs associated with plasmids generally
67 increase with the number of encoded ARGs [14]. That study found some support for interactions
68 between resistance genes affecting their cost, but it was not possible to identify costs of specific
69 ARGs or to determine if those costs depend on the host strain in which they are assessed. Studies
70 estimating the effect of specific ARGs include analysis of the *tetAR* operon, encoding tetracycline
71 resistance, that was estimated to impose a cost of around 1% in rich medium [18,19]. Similarly, β-
72 lactamases, a large class of resistance genes that confer resistance to one or more of the β-lactam
73 antibiotics, have been shown to decrease host fitness, though different variants have different effects
74 in different hosts [20–23]. In at least some cases, however, ARGs, including *tetAR*, not only do not
75 confer a cost but can provide a benefit to host cells, underlining the importance of understanding
76 potential variation in costs across different hosts [24–26].

77 The possibility that ARGs have different costs in different hosts is especially important to understand
78 because of the potential for costs of ARGs to be transmitted between different hosts through an
79 association with horizontally mobile elements (reviewed in 12). Studies of mutation fitness effects,
80 including some conferring antibiotic resistance, often find significant differences depending on the
81 host background [27–32]. For example, although not isolating the effect of the ARG itself, a plasmid
82 encoding the $bla_{\text{OXA-48}}$ resistance gene conferred an overall cost across a range of host strains, but
83 with a range between ~20% cost and ~20% benefit [33]. Simulations predict that this high variation
84 can increase plasmid, and, therefore, ARG, persistence in a multi-member community because hosts
85 in which it has low or no cost can serve as reservoirs during periods when the plasmid is not directly
86 selected [33]. These results make it clear that any attempt to understand the dynamics of ARGs needs
87 to account for the possibility of differential effects in different genetic, and likely, environmental,
88 contexts.

89 We separately cloned six ARGs representing four resistance classes into a control plasmid and
90 estimated their fitness effect in each of 11 genetically diverse *Escherichia* spp.. We found that costs
91 were diverse across ARGs and strains, and that they interact so that different strains have the potential
92 to act as no-cost reservoirs for different ARGs. Simulations predict that the magnitude of ARG
93 effects are sufficient to cause both the presence of different ARGs to influence which strains can
94 persist in a community and the availability of strains to determine which ARGs can be maintained.
95 Our study makes clear that ARGs should not be simply considered as costly (or neutral). Instead,

96 effect depend on the host strain in which they are assessed. This dependence is important to consider
97 in attempts to predict the ability of ARGs to persist in antibiotic-free environments.

98 **Materials and Methods**

99 *Selection of candidate ARGs.* The ARGs used in this study were chosen based on their prevalence in
100 accessible *E. coli* genomes and their availability in our laboratory [34]. The 2022 release of the
101 CARD Prevalence, Genomes and Variants dataset gives the prevalence of our chosen ARGs among
102 sequenced *E. coli* genomes as: *aadA* (ARG type = 15.3%; specific ARG allele = 1.73%), *cat* (5.3%,
103 4.1%), *dfrA5* (19.4%, 1.8%), $bla_{TEM-116}$ (22.7%, 0.07%), $bla_{CTX-M-15}$ (17.1%, 5.83%) and bla_{SHV12}
104 (0.59%, 0.04%), identifying them as clinically important ARGs.

105 *Construction of ARG-plasmids and bacterial strains.* Focal ARGs were originally cloned into
106 pUA66, which contains a promoterless *gfp* [35]. However, we found leaky expression of GFP in this
107 vector (data not shown). To avoid any influence of GFP expression on fitness measurements, we
108 removed the GFP by amplifying the pUA66 backbone (using pUA_minusFP_F:
109 5'atgtccagacctgcaggcatg; pUA_minusFP_R: 5'ggatccatcgaggtgaagacg) and circularizing the product
110 with a bridging oligonucleotide (5'cgtcttcacctcgatggatccatgtccagacctgcaggcatg) using the
111 NEBuilder® HiFi DNA Assembly (NEB) kit. The resulting plasmid, pmFP, retains the kanamycin
112 resistance gene originally present on pUA66. The six ARGs were amplified from template sources as
113 listed in Table S1 and separately added into the pmFP vector (amplified using primers: pUA66_F:
114 aataggcgtatcacgagg and pUA66_EcoRI_R: gaattcatggtttcttagacgtcgg) using NEBuilder® HiFi DNA
115 Assembly. All PCR reactions used Q5® High-Fidelity polymerase (NEB) unless otherwise specified.
116 When sequencing cloned ARGs we found that the $bla_{TEM-116}$ gene in pTarget, which was used as a
117 template, has a non-synonymous mutation (Q274R) relative to the reference $bla_{TEM-116}$ sequence
118 (NCBI accession: U36911). To distinguish between these genes we identify the variant used here as
119 $bla_{TEM-116}$*.

120 The six constructed pmFP::ARG plasmids, and the reference pmFP backbone vector, were each used
121 to transform one *E. albertii* and 10 genetically divergent *E. coli* strains (Fig. S1) following the
122 method outlined in ref. [36]. After transformation, cells were recovered at 37 °C for one hour before
123 being plated on lysogeny broth (LB) supplemented with 50 μg/ml kanamycin to select plasmid
124 containing cells. One strain, H305, was resistant to ampicillin, chloramphenicol, and trimethoprim,
125 antibiotics. Omitting H305 makes no qualitative difference to the interpretation of any analysis we
126 present, though we note that H305 was one of two strains in which no ARG conferred a significant

127 effect on fitness and one of three strains in which no ARG was costly. We leave it in the presented
128 analyses because already resistant strains can clearly serve as hosts to ARG-encoding plasmids in
129 natural communities and because it is reasonable to think that additional copies of a given resistance
130 gene can impose some additional fitness effect.

131 *Fitness competitions.* To measure the fitness effect of each ARG, we performed a series of
132 competition assays. The fitness effect of an ARG was estimated as the fitness difference between
133 cells carrying an ARG plasmid (W_{ARG}) and cells carrying the control pmFP plasmid vector (W_{Vec}).
134 The fitness of ARG and vector plasmid-carrying cells was estimated indirectly by competing each
135 against reference cells carrying a GFP expressing plasmid (pUA66-$_{PrpsL}$GFP).

136 Cells carrying an ARG plasmid, pmFP, or pUA66-$_{PrpsL}$GFP, were inoculated in 200 µL of Davis-
137 Mignioli broth supplemented with 250 µg/ml glucose (DM250) and 50 µg/ml kanamycin from frozen
138 stocks and cultured at 37ºC overnight. The overnight cultures were diluted 1:100 in 200 µL of fresh
139 DM250 and incubated for a 24-hour growth cycle. Cultures were grown over two additional daily
140 growth cycles with 1:100 dilution between each cycle to condition them to the antibiotic-free
141 medium, then used to set up competition assays. Five microliters of culture of each competitor –
142 reference cells with pUA66-$_{PrpsL}$GFP and either cells with an ARG plasmid or with the control
143 plasmid – were mixed in 40 µL of fresh DM250. Forty microliters of the mix was added to phosphate
144 buffered saline (PBS) and placed on ice or fixed with formaldehyde before being assayed by flow
145 cytometry to determine the proportion of reference (GFP+) and ARG or control plasmid-containing
146 cells (Day 0). The remaining 10 µL of the mix was added to 190 µL of fresh DM250 and incubated
147 for 24 hours to allow strains to compete. Following this competition, cultures were diluted 20-fold in
148 PBS and assayed by flow cytometry (Day 1). This protocol was repeated to obtain ARG effect
149 estimates in a competition environment supplemented with kanamycin, to which all plasmids confer
150 resistance to. At least five independent fitness measurements were collected for each host strain-ARG
151 combination. Construction of some combinations occurred later than others due to factors such as
152 delays in constructing ARG vectors. For this reason some combinations have higher replication than
153 others.

154 The fitness effect of an ARG was estimated as W_{ARG} / W_{Vec}, where $W_{ARG} = \ln(ARG_{Day1} \times 100 /$
155 $ARG_{Day0}) / \ln(GFP_{Day1} \times 100 / GFP_{Day0})$ and $W_{Vec} = \ln(Vec_{Day1} / Vec_{Day0}) / \ln(GFP_{Day1} / GFP_{Day0})$.
156 ARG_{Day1}, ARG_{Day0}, Vec_{Day1} and Vec_{Day0} are the proportion of flow cytometry events without a GFP
157 signal, and GFP_{Day1} and GFP_{Day0} are the proportion of events with a GFP signal. Day 1 proportions

158 are multiplied by 100 to account for growth occurring during the competition. We note that a number
159 of flow cytometry runs resulted in total event counts or counts of one competitor that were
160 substantially different to mean estimates over all replicates of any given competition type. The most
161 likely reason for this is that the natural isolate strains used here are subject to clumping that
162 introduces variability in passage of cells through the cytometer flow cell. To reduce the effect of this
163 variation we excluded competitions where the starting frequency of either competitor was estimated
164 as more than 15% different than the target of 50%. This step removed a total of 810 of 4372
165 competitions. We emphasize that this filtering was performed without regard to the fitness estimated
166 considering the change in frequency over the course of the competition. We also omitted a further 16
167 fitness estimates of one host strain collected in one experimental block. These estimates were more
168 than 50% higher than a set of 205 estimates collected for the same strain in other experimental
169 blocks, indicating that they were compromised by some experimental error.

170 *Assessing plasmid loss and compensation.* In the antibiotic-free assay environment it is possible that
171 plasmid loss could occur, which could influence fitness estimates through production of relatively
172 higher fitness plasmid free sub-populations. To estimate the influence of plasmid-losing cells during
173 the competition assay, monocultures of cells with the pUA66-$_{PrpsL}$GFP plasmid were grown following
174 the competition protocol and plated on non-selective media to allow identification of the proportion
175 of plasmid containing cells. After three transfer cycles the frequency of cells that lost the plasmid was
176 0.004 (± 0.004 95%CI, n = 4). If we conservatively, consider this frequency to represent the fraction
177 of cells incorrectly identified as being the ARG-plasmid containing competitor instead of cells that
178 lost the reference pUA66-$_{PrpsL}$GFP plasmid during a single day of competition, this would represent a
179 fitness inflation of the ARG of only <0.1% over the true value. Moreover, plasmid-loss cannot easily
180 explain differences in costs of different ARGs in the same host. Finally, we repeated fitness estimates
181 in an environment supplemented with kanamycin, which ensures that only plasmid-containing cells
182 are present. The overall magnitude of fitness effects in this environment were similar to those in a
183 kanamycin-free environment, suggesting that plasmid loss is not influencing fitness estimates in the
184 conditions used in our experiment.

185 Compensatory mutations that reduce the cost of an ARG could occur during our competition assays.
186 In practice, however, they are very unlikely to significantly effect fitness estimates. For example, a
187 compensatory mutation conferring a benefit of 15% (i.e., completely compensating the largest cost
188 found in our experiment) starting at ten copies in populations of the size used in our experiment will

189 reach a frequency of ~0.0001 in 60 generations, a span that coves the growth of a freezer stock,
190 competition acclimation, and the competition itself.

191 *Determination of copy number of the* bla$_{TEM-1}$ *plasmid.* The copy number of plasmids encoding the
192 *bla*$_{TEM-116}$* ARG was measured by quantitative PCR (qPCR) using the comparative Ct (ΔΔCt)
193 method [37]. Plasmid carrying cells were cultured overnight in DM250 supplemented with 50 μg/ml
194 kanamycin and DNA isolated using a Wizard Genomic DNA Purification Kit (Promega).
195 Amplification of target genes was carried out using a SYBR Green based qPCR mix consisting of
196 Q5® High-Fidelity 2× Master Mix (NEB), SYBR Green, and relevant primers (at a final
197 concentration of 0.25 μM). Between one and two ng of template DNA was used per 10 μl reaction.
198 Reactions were performed using a Thermo Scientific PikoReal Real-Time PCR System with default
199 settings. Primers were designed using PrimerQuest (Integrated DNA Technologies, Inc.) to amplify
200 the chromosomal *dxs* gene, in order to determine the absolute Ct value of the bacterial chromosome
201 (Ct_{ch}; xs_qpcr_F1:cgagaaactggcgatcctta; dxs_qpcr_R1: cttcatcaagcggtttcaca), and the pUA66 plasmid
202 encoded *aph(3')-IIa* gene, to determine the Ct value of the plasmid (Ct_p; pUA_Kan_qpcr_F1:
203 ctcgtcaagaaggcgatagaag; pUA_Kan_qpcr_R1:cgttggctacccgtgatatt). The relative copy number of the
204 *bla*$_{TEM-116}$* plasmid to the chromosome (ΔCt) was calculated as $2^{-(Ct_p - Ct_{ch})}$. The ΔCt of different
205 bacterial strains was then normalized to the lab strain, REL606, to obtain the ΔΔCt value.

206 *Statistics, phylogeny construction and phylogenetic signal testing.* The R statistical computing
207 platform was used for all analysis and visualization [38]. The flowCore package [39] was used to
208 analyze flow cytometry data and the lme4 and lmerTest [40,41] packages were used to run and
209 evaluate mixed models. Environment, ARG and host strain were included as random effects in these
210 models as noted in the text. We isolate the fitness effect of an ARG in each ARG-strain combination
211 as the difference in fitness effect of a plasmid encoding the ARG to the fitness effect of the vector
212 pmFP plasmid. To account for the comparison of each ARG to the same vector pmFP plasmid we use
213 Dunnett's tests implemented in the multcomp package, which control for many-against-one
214 comparisons [42]. Core and accessory genomes of a collection of 96 environmental *E. coli* isolates
215 were determined and used previously to build phylogenies [43]. These phylogenies were used to test
216 for a phylogenetic signal of ARG effects using Pagel's lambda and Blomberg's K metrics as
217 implemented in the phylosig function of the phytools package 1.0-3 [44].

218 *Simulation of host-ARG communities.* Competitive fitness assays allow fitness of plasmid-containing
219 strains to be measured and compared to a corresponding ancestral strain. It is not possible to extend

220 this approach to compare the fitness of our distinct progenitor strains because competition between
221 these strains may be affected by complex interactions, for example mediated by colicins or phage,
222 that lead to non-transitivity in their relative fitness [45]. To estimate the fitness of all strains, we
223 analyzed growth curves of progenitor strains to estimate their fitness relative to one another as
224 determined only by differences in resource use [45]. The fitness of each strain-ARG combination was
225 determined by analysing the growth curve of each progenitor strain to determine its baseline fitness
226 and then altering that baseline to account for the fitness effect of each ARG measured in that strain
227 (as measured by competition assays). Resulting fitness estimates of each strain-ARG combination
228 were used to parameterize an individual based model that simulated dynamics in a community with
229 strain-ARG fitness drawn form a normal distribution with mean and standard deviation taken from
230 experimental estimates. We draw from a distribution of fitness values to account for the measurement
231 error inherent in our estimates and note that it can mean that ARGs with a higher mean relative cost,
232 but larger distribution width, can be more successful than more fit ARGs with a smaller distribution
233 width. Simulations were initiated with equal numbers of each strain-ARG combination in a
234 population of size 10^4 individuals and propagated using a Wright-Fisher model for 200 generations.
235 Strains and ARGs remaining above a threshold frequency of 0.01 in at least 20% of 10 replicate
236 simulations were considered to have persisted in the community. Simulations were repeated
237 separately omitting each strain and each ARG from the starting community to determine the effect of
238 each individual strain and ARG in determining the final community composition. The model includes
239 only fitness differences of strains integrated over a complete competition cycle. As such, feedbacks
240 are not present and a single strain-ARG combination will eventually win in each simulated
241 competition. This is not a realistic, scenario and we currently have experiments underway to examine
242 interactions directly. Nevertheless, our model does clearly demonstrate that costs of ARGs are
243 sufficient that they can be expected to effect competition outcomes.

244 **Results**

245 *ARGs can impose costs and benefits in the absence of direct selection.* We separately cloned six
246 ARGs belonging to four distinct mechanistic classes into a low-copy number plasmid vector (Table
247 1). The fitness effects of each ARG was estimated in an antibiotic-free environment in each of 11
248 divergent *Escherichia* spp. host strains (Fig. 1). We found significant differences in the mean effect
249 of the different ARGs ($\chi^2 = 40.4$, $P < 0.001$). Of the six tested ARGs, three conferred a significant
250 overall change in fitness measured across all host strains: $bla_{TEM116*}$ conferred a 2.7% cost (±1.0%
251 95CI; Dunnett's test $P < 0.001$), *cat* conferred a 1.8% cost (±1.2% 95CI; Dunnett's test $P = 0.01$) and

252 *dfrA5* conferred a 1.6% cost (±1.2% 95CI; Dunnett's test P = 0.05). This result clearly indicates that
253 ARGs can confer significant effects on fitness in the absence of direct selection for their resistance
254 phenotypes. Moreover, the three β-lactamases had significantly different effects on host fitness,
255 demonstrating that effects can differ even between ARGs with similar resistance profiles ($\chi^2 = 32.1$, P
256 < 0.001).

257 *Fitness effects of ARGs depend on the host strain.* The results presented above consider the overall
258 effect of each tested ARG over 11 host strains. To determine if effects differed between strains, we
259 extended our model to include an ARG effect-by-strain interaction term. This extended model
260 provided a significantly improved fit compared to the previous model, indicating a dependence of
261 ARG effects on host genotype ($\chi^2 = 94.9$, P < 0.001). We found that all ARGs conferred a significant
262 fitness effect in at least one strain (Dunnett's test P < 0.05: *aadA* - 4 strains; $bla_{CTX\text{-}M\text{-}15}$ - 2 strains;
263 bla_{SHV12} - 1 strain; $bla_{TEM116*}$, *cat*, *dfr*A5 - 3 strains) (Fig. 2). In 11 of 15 cases significant effects were
264 negative, with every ARG except bla_{SHV12} imposing a cost in at least one strain. From the perspective
265 of host strains, all except H305 and B354 had a significant change in fitness caused by at least one
266 ARG (Dunnett's test P < 0.05). The mean fitness difference between different ARG-strain
267 combinations was substantially larger than the mean difference between ARG effects, indicating a
268 potential for some strains to act as reservoirs for ARGs in antibiotic-free environments (ARG-Strain
269 combinations mean difference = 2.5% (1.9 – 3.3% 95CI); ARG mean difference = 1.2% (0 – 2.8%
270 95CI)). Indeed, all of the six tested ARGs were neutral or conferred a benefit in at least one host
271 strain.

272 *Fitness effects of ARGs depend on the environment.* Fitness estimates reported above were carried out
273 in a minimal medium environment in the absence of any antibiotic. The focal ARGs were cloned into
274 a vector that also included an additional ARG gene (*aph3'-II*, encoding resistance to kanamycin),
275 giving us the opportunity to test if a secondary antibiotic might influence the physiology of strains in
276 a way that alters the effect of non-selected ARGs. We note that kanamycin is an aminoglycoside
277 antibiotic with some similarity to spectinomycin and streptomycin, substrates for the *aadA* ARG, but
278 there is no detectable cross-activity [46]. When we measured the fitness effects of each ARG-strain
279 combination in the same environment as used previously, but supplemented with kanamycin, we
280 again found a significant interaction between ARG and host strain in determining fitness ($\chi^2 = 47.0$, P
281 < 0.001)(Fig. S2). The fitness of all strains were significantly affected by at least one ARG and all
282 ARGs except *aadA, cat* and bla_{SHV12} significantly affected fitness of at least one strain. There was a
283 significant effect of the environment when comparing fitness estimates made in the minimal medium

284 and kanamycin supplemented environments ($\chi^2 = 74.4$, $P < 0.001$). Evidently, a general pattern of
285 ARG fitness effects depending on strain background was common across the environments, although
286 the exact nature of that dependence is different.

287 *Fitness effects of ARGs are not explained by plasmid copy number.* A possible explanation for
288 different effects of ARGs across host strains is that the plasmid they are encoded on has a different
289 copy number in different strains. Different copy number can affect expression levels of plasmid-
290 borne genes and, thus, any phenotypic effect of gene products. One well known example is the effect
291 of bla_{TEM1} copy number on the degree of ampicillin resistance [47]. We used qPCR to determine the
292 copy number of the $bla_{TEM116*}$ plasmid across our set of 11 host strains. This ARG showed the highest
293 variation in fitness cost so represents a good test of the contribution of copy number differences to
294 differences in fitness effects. We found that the relationship between $bla_{TEM116*}$ copy number and
295 fitness cost was positive, but non-significant, in both the antibiotic-free environment and in the
296 kanamycin-supplemented environment, indicating that differences in plasmid copy number are
297 unlikely to represent a general mechanism of strain-specific ARG fitness effects (minimal: $r = 0.28$, P
298 $= 0.36$; kanamycin-supplemented: $r = 0.26$, $P = 0.41$).

299 *ARG effects are not predicted by evolutionary relationships between host strains.* Variation in ARG
300 effects across host strains must derive from differences in their genotypes. To test if the dependence
301 of ARG effect on host strain can be predicted through knowledge of the genetic relationship of strains
302 we tested for a phylogenetic signal in the effect of each ARG. We estimated Pagel's λ, a measure of
303 the phylogenetic signal in a response variable, considering the fitness effect of each ARG across
304 phylogenies derived from the core and accessory genomes of our host strains, excluding B156, which
305 we do not have a genome sequence for [48]. In no case was any significant signal found. An
306 alternative measure of phylogenetic signal, Blomberg's K [49], gave a qualitatively consistent result
307 (Table S2). With only 11 host strains, the power of individual tests is not high. However, the lack of
308 signal given that our host strains are genetically divergent and that tests were repeated for each of our
309 six ARGs, indicate, at least, that that fitness effects of the tested ARGs are not well predicted by the
310 overall genetic similarity of diverse host strains.

311 *Dependence of ARG fitness effect on host strain predicts interdependence between host strain and*
312 *ARG success.* The above results demonstrate that a range of ARGs confer different fitness effects in
313 different host strains. We predict that this interaction can cause the success of host strains and ARGs
314 to be interdependent: the overall fitness of each ARG depends on available host strains and the
315 overall fitness of each host strain depends on the ARGs it carries. A consequence of this dependence

316 is a potential for some strains to act as low-cost reservoirs of ARGs, allowing these ARGs to persist
317 when they would not otherwise be able to. It is also possible that the presence of some ARGs will
318 affect the relative fitness of some strains allowing them to be maintained in a community when they
319 would otherwise be outcompeted.

320 As a first step to examine implications of host-ARG fitness interdependence we simulated 200
321 generations of growth of a community started with equal numbers of all 66 host-ARGs combinations
322 considered in our study. At the end of the simulation strains and ARGs remaining above a threshold
323 frequency in at least 20% of replicate simulated communities were counted as having persisted (see
324 Materials and Methods for details). We found that the final community contained one host strain,
325 B354, and a single ARG, bla_{SHV12} (Fig. 3A and B, top right squares). This reflects that these strain-
326 ARG combinations had the highest fitness of any that we measured (Fig. 2 combined with baseline
327 strain fitness estimates, see Materials and Methods for details). To determine the effect of strain
328 dependent ARG costs on this result, we repeated simulations separately omitting each strain and
329 ARG. In some cases, quite different final communities were produced (Fig. 3). For example,
330 omission of the B354 strain from the starting community led to the H504 strain dominating. In this
331 strain as many as three of the six focal ARGs remained present at the end of the simulation period,
332 reflecting the relatively small and consistent effects of ARGs in the H504 host strain.

333 **Discussion**

334 We quantified the fitness effect of six different ARGs in each of 11 host strains. Selection for genes
335 depends on the net effect of positive selection for the function they encode and selection against any
336 costs of expressing that function [50,51]. ARGs provide a benefit by protecting cells in the presence
337 of a cognate antibiotic but many ARGs have been isolated from environments where antibiotics are at
338 low concentrations and where costs might be influential in determining their fate [10,12–14].
339 Supporting the need to consider the role of ARG costs in determining their dynamics, we found that
340 ARG costs were common (Figs 1, 2, S2). Considered over the two assay environments, all ARGs
341 except bla_{SHV12} were costly in at least one host. ARG effects differed over host strains, but the fitness
342 of all tested hosts was reduced by at least one ARG (Fig 2, S2). Conversely, all ARGs were neutral or
343 beneficial in at least one host strain, indicating the potential for specific strains to provide a 'selective
344 refuge' for ARGs in the absence of antibiotic selection (Fig 2, S2).

345 Co-existence theory has been developed to understand the role of specific interactions in determining
346 broader community dynamics [52–54]. This theory has recently been applied to consider ARG

347 success given the availability of a range of environmental niches in which host strains have different
348 fitness [11]. One key finding of that work was that ARGs that reduce host fitness in fewer
349 environmental niches will generally be more successful than those that impose general costs over
350 most environments. Because ARGs are frequently encoded by horizontally mobile elements that can
351 transfer between hosts, different host strains can be thought of as distinct niches with transfer
352 facilitating fluctuating selection. From the perspective of the ARG, strains in which they confer low
353 costs represent refuges allowing them to minimize niche overlap with competitors. The more refuge
354 strains that are available to an ARG, the more successful it is expected to be.

355 We have not directly tested the role of refuge strains on ARG persistence, but, even in the absence of
356 any horizontal transfer, our simulations indicate that measured strain-dependent ARG fitness effects
357 are of a magnitude that can influence the outcome of community composition (Fig. 3). We also note
358 that ARG-strain interactions depended on the assay environment, a finding consistent with other
359 studies measuring effects of antibiotic resistance [16,55–58] . This dependence increases the chance
360 that refuge strains will be available for any given ARG but may also select for transfer between
361 strains if costs change following environmental shifts.

362 We emphasise that the important result that strain-dependent ARG costs can influence strain and
363 ARG success derive from a simple model that considers fitness estimates based on integrated growth
364 over a competition cycle with a single limiting nutrient. We do not incorporate frequency
365 dependence, and do not include direct inter-strain interactions, so that each starting community must
366 eventually fix the single strain-ARG combination with the highest fitness. Consideration of more
367 complex competitive interactions will increase the likelihood of diverse communities emerging,
368 making it more likely that strain-dependent ARG effects will be relevant to determining final
369 community compositions [59]. Unfortunately, competitive interactions among the strains we use are
370 not well enough understood to be included in a model, but we are beginning community competition
371 experiments to test directly test their influence.

372 Although the influence of strain-dependent ARG costs on ARG dynamics has not, to our knowledge,
373 been examined directly, there are observations that are consistent with the idea that differential costs
374 can affect ARG success. In one study trimethoprim use was decreased by 85% for two years in one
375 Swedish county [60]. This change was correlated with a small overall decrease in frequency of the
376 *dfrA* resistance gene but this small change masked larger changes within different *E. coli*
377 subpopulations. Trimethoprim resistance was both unevenly distributed across subpopulations at the
378 beginning of the intervention and changed differently over subpopulations during the intervention,

379 decreasing from 67% to 41% in isolates from one subpopulation, but increasing from 17% to 31% in
380 isolates from another [60].The authors note that explanations other than strain-specific differential
381 fitness costs are possible, including clonal expansions of sequence types within subpopulations may
382 influence ARG frequencies, though the frequent horizontal transfer of resistance genes means that
383 they are unlikely to be the sole explanation.

384 One complication of predictions based on co-existence theory is that costs of ARGs can evolve.
385 Indeed, costs of resistance phenotypes, especially those due to spontaneous mutations, are often
386 reduced during growth in antibiotic-free environments (reviewed in 13,15). Several studies have also
387 demonstrated changes in the fitness effect of ARGs due to chromosomal mutations [30,61,62]. A
388 consequence of evolved changes in ARG costs is that ARG effects will not only differ across host
389 stains and environments, but also through time as host strains adapt and compensate for any costs
390 they confer. How this effect might influence long-term ARG fitness will be an important area for
391 future study.

392 Finally, we note one example in our findings that highlights how difficult it is likely to be to predict
393 ARG fitness costs. Within the set of six ARGs we considered, three—$bla_{\text{TEM116*}}$, $bla_{\text{CTX-M-15}}$ and
394 bla_{SHV12}—were β-lactamases. We expected the similar function of the encoded gene products to lead
395 to similar host dependent effects, but in fact these genes showed a significant host strain interaction
396 effect [63]. Indeed, one, $bla_{\text{TEM116*}}$, conferred the highest cost of the genes we considered while
397 another, $bla_{\text{CTX-M-15}}$, conferred the highest benefit (Fig. 1). While we do not focus on the molecular
398 basis of costs, we note that previous work on the effects of β-lactamases have proposed several
399 mechanisms that may be relevant to our findings. β-lactamases need to be transported to the
400 periplasmic region of cells to function and some signal peptides that direct this secretion have been
401 associated with a fitness cost [20]. β-lactamases also have similarity with the penicillin binding
402 proteins (PBPs) that process peptidoglycan components of cell walls. Residual activity of β-
403 lactamases for PBP substrates might interfere with the dynamic process of peptidoglycan processing
404 required to accommodate cell growth [21,22]. The host strains we used are genetically distinct, likely
405 leading to different metabolic, physiological, and regulatory backgrounds on which effects of ARGs
406 are tested. Whatever the mechanism of ARG costs, differences among our host strains evidently
407 provide opportunity for them to play out differently.

408 Investigation of the influence of ARGs on microbial communities has been dominated by
409 consideration of their effects in the presence of antibiotics. It was generally thought that ARGs would
410 impose costs that would select against them in environments without this selection pressure, but it

now seems that this assumption was optimistic [64]. While many ARGs can impose costs, a dependence on environment and host strain may create refuges that keep ARG frequencies high even in the absence of direct selection.

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563

564 **Table 1. ARGs used in this study.**

Gene	Enzyme type	Conferring resistance to:	Mechanism
aadA	Aminoglycoside adenyltransferase	Aminoglycosides such as spectinomycin and streptomycin	Drug-modification
$bla_{TEM-116}$*	Beta lactamase	β-lactam such as ampicillin	Drug-modification
bla_{SHV12}	Beta lactamase	β-lactam such as ampicillin and cefotaxime	Drug-modification
$bla_{CTX-M-15}$	Beta lactamase	β-lactam such as ampicillin and cefotaxime	Drug-modification
cat	Chloramphenicol acetyl transferase	Chloramphenicol	Drug-modification
dfrA5	Dihydrofolate reductase	Trimethoprim	Target replacement

565

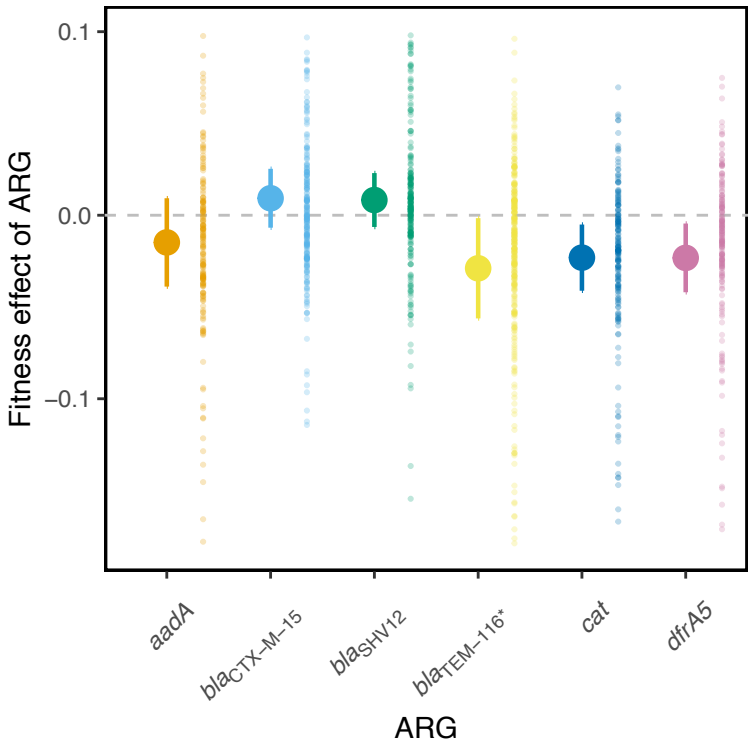

Figure 1. Fitness effect of each ARG across 11 *Escherichia* spp. host strains. For each ARG the solid symbol indicates mean fitness over all replicate estimates and error bars indicate 95% CI. Small background points indicate individual estimates used to calculate each mean. The horizontal dashed line indicates no fitness effect. n > 200 for each ARG (summed across all 11 host strains, see figure 2 for effects per host strain).

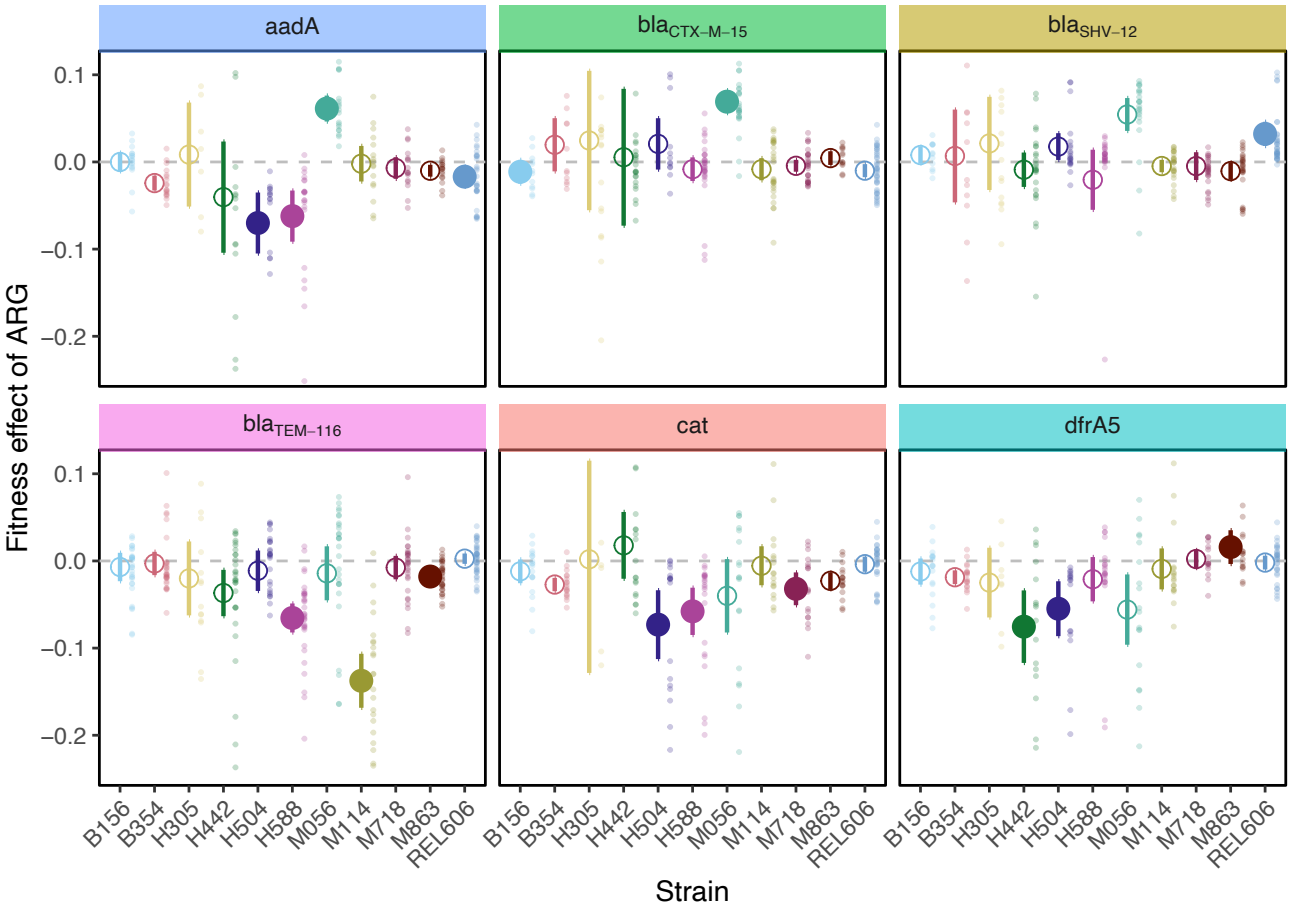

Figure 2. Fitness effect of ARGs in each host strain. For each host-ARG combination large symbols indicate mean fitness over all replicate estimates and error bars indicate 95% CI. Large symbols are filled if the mean ARG effect is different from 0 after correction from multiple comparisons using Dunnett's test (M&M for details). Background points indicate individual estimates used to calculate each mean. The horizontal dashed line indicates no fitness effect. Background label panels are coloured to match the ARG color used in figure 1. $n \geq 5$ for each host-ARG combination.

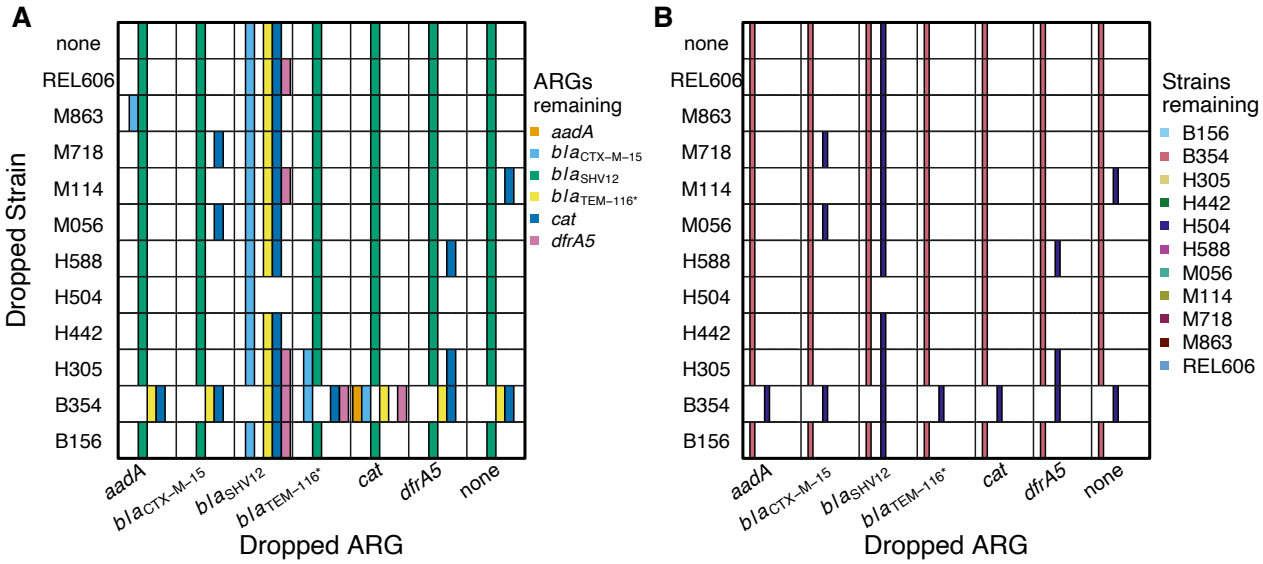

Figure 3 Host strain and ARG persistence during community growth in the absence of direct ARG selection. Communities consisting of all 66 host strain-ARG combinations (top right squares of each panel) and with omission of one strain (Dropped Strain axis) and/or one ARG (Dropped ARG axis) were simulated over 200 generations using fitness values that combined a baseline fitness of each strain with changes in fitness caused by addition of ARGs to that strain (details in Materials and Methods). Coloured bars indicate the ARGs (panel A) and host strains (panel B) remaining at greater than 1% in at least two of ten replicate communities simulated in each condition. Simulations are based mostly on fitness differences estimated over a growth cycle in an environment containing a single limiting resource.