Plasmids link antibiotic resistance genes and phage defense systems in *E. coli*

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Abstract

Phage therapy has been proposed as an alternative to antibiotics to treat resistant infections. However, we have a limited understanding of how antibiotic resistance genes (ARGs) associate with bacterial phage defense systems (PDSs). Here, we explore the relationship between ARGs and PDSs in a sample of 2,559 plasmids originating from 1,044 *E. coli* isolates, representing a snapshot of clinical and non-clinical diversity in Oxfordshire, UK (2008-2020). In total, we identify 3,193 ARGs and 14,013 PDSs (180 unique types). We demonstrate that *E. coli* plasmids are enriched for ARGs and PDSs (both $p<0.001$), with a bias towards toxin-antitoxin, abortive infection, and restriction-modification systems (all $q<0.025$). We proceed to show that ARGs and PDSs are physically linked by plasmids ($p<0.001$). Together, our results suggest that phage therapy may inadvertently select for antibiotic resistant bacteria, and that antibiotic use may similarly drive resistance to phage.

Keywords: *Escherichia coli*; bacteriophage; anti-bacterial agent

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27 # Introduction

28 Bacterial defense systems can protect the cell against invaders, such as plasmids and phage[1].
29 Plasmids are extrachromosomal replicons often able to transfer between bacterial hosts via
30 conjugation[2]. Phage, and in particular, lytic phage, are bacterial viruses that, like plasmids, rely
31 on the host cell for replication, but unlike plasmids, ultimately kill the host during the infection
32 cycle[3]. Plasmids are often associated with antimicrobial resistance genes (ARGs), which can
33 confer resistance to the host cell, sometimes at a low fitness cost[4,5]. Moreover, plasmids can
34 also encode phage defense systems (PDSs), such as toxin-antitoxin, restriction modification,
35 and CRISPR-Cas systems, blurring the boundary between selfish genetic elements and
36 mutualistic partners[6–9].

37 One unresolved issue is the extent to which ARGs and PDSs co-occur on plasmids. This is
38 important because the physical linkage of different resistance determinants can drive co-
39 selection: selective pressure for one trait (phage resistance) may inadvertently maintain another
40 (antibiotic resistance), and vice versa. Co-selection is already well documented for ARGs and
41 other resistance genes, such as those against heavy metals or biocides, where a single stressor
42 can select for multi-resistant bacteria[10,11]. Some examples have been reported of ARGs and
43 PDSs co-localising on the same mobile genetic element, yet whether this occurs more generally
44 remains to be seen[12–14]. Understanding these patterns is particularly relevant as phage therapy
45 gains traction as a strategy to combat antibiotic resistant infections[15,16].

46 In this study, we investigated the relationship between ARGs and PDSs in a large sample of
47 2,559 plasmids from 1,044 clinical and non-clinical *E. coli* isolates[17]. We first annotate the
48 genomes for ARGs and PDSs, then explore (i) their overall enrichment on plasmids versus
49 chromosomes, (ii) the enrichment of specific PDS types on plasmids versus chromosomes, and
50 (iii) their linkage on plasmids.

52 # Methods

53 **Dataset curation**

54 Isolates and associated genome assemblies were from an existing, pre-collated dataset[17]. The
55 original dataset contained Enterobacterales species collected from within 60km in Oxfordshire
56 from 2008-2020, covering multiple clinical and non-clinical niches: human bloodstream
57 infection (BSI), livestock faeces (cows, pigs, poultry, and sheep), and wastewater (influent,
58 effluent, and rivers upstream of effluent)[18,19]. All BSI isolates and a majority of non-BSI
59 isolates were not selectively cultured for antimicrobial resistance. Species were determined
60 with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). Authors used a
61 hybrid approach (short- and long-reads) to recover reference-grade assemblies. We selected all
62 the *E. coli* genomes where the chromosome and all plasmids were fully circularised (n= 1,044).
63 Isolate and assembly metadata was also downloaded.

64 **Genome annotation**

65 We annotated for PDSs using the tool DefenseFinder (v. 2.0.0) with default paramaters, which
66 queries against a database of known PDSs[20–22]. DefenseFinder also denotes when a system is
67 "complete" ("systems" output), meaning all component genes have been annotated. We only
68 considered complete systems in this study. For ARGs, we used pre-existing annotations from
69 AMRFinderPlus (v. 3.10.18) with default parameters[23,24]. Plasmid mobility predictions
70 (conjugative, mobilisable, or non-mobilisable) also used pre-existing annotations from MOB-
71 typer from MOB-suite (v. 3.03) with default parameters. We annotated for integrons using
72 IntegronFinder 2.0 with default parameters[25]. For gene annotations we used Prodigal (v.
73 2.6.3)[26] with default parameters.

74

75 **Testing for the enrichment of ARGs and PDS genes on plasmids**

76 We first calculated the total number of plasmid genes as a fraction of the total number of genes
77 in the genome. Similarly, we calculated the fraction of just ARGs and PDSs genes on plasmids.
78 These proportions were compared using a Pearson's chi-squared test with Yates' continuity
79 correction. We conducted this across the whole dataset (see Results), but also separately for
80 BSI-associated and non-BSI-associated plasmids (Figure S5).

81 **Testing for the over-representation of PDS families on plasmids**

82 First, for each PDS type, we calculated a binary presence/absence score for each replicon. We
83 next wanted to generate a null distribution that accounted for some genomes containing more
84 plasmids than others. To do this, we performed b=10,000 label permutations of replicon type
85 (plasmid or chromosome) within each genome. After each permutation, we recorded the
86 number of plasmid-labelled contigs carrying each PDS. This generated a one-sided p-
87 value=$(n+1)/(b+1)$, where n was the number of permutations with a plasmid count at least as
88 extreme as observed (≥ for the plasmid-enrichment test; ≤ for the chromosome-enrichment test)
89 and b=10,000. Separate p-values were obtained for plasmid (H_1: observed > null) and
90 chromosome (H_1: observed < null) bias. To mitigate against multiple tests increasing the rate
91 of Type I errors, we applied both the Benjamini–Hochberg (BH) and the Benjamini–Yekutieli
92 (BY) false-discovery-rate procedures. Families with BH-adjusted $q < 0.025$ for the relevant
93 one-sided test (plasmid or chromosome) were called significant; BY-adjusted q-values are also
94 reported when $q < 0.025$ (providing a stricter, dependence-robust correction).

95 **Testing the association between ARGs and PDSs on plasmids**

96 If two plasmids are vertical descendants, or share large portions of their sequence from a
97 recombination event, they might contain many of the same genes. This can be problematic

98 when testing for the association of ARGs and PDSs, as it introduces pseudo-replicates to the
99 model. We minimised the risk of pseudo-replication by genetically dereplicating plasmids in
100 the dataset. We first took the *k*-mer plasmid clusters generated by the original authors[17]. We
101 then randomly selected a representative from each cluster. In total, we recovered n=712
102 genetically representative plasmids.

103 We next predicted the count of ARGs per plasmid with a generalised additive mixed-model
104 (GAMM) using the mgcv library[27]. Model choice was guided by Akaike Information Criterion
105 (AIC), which balances goodness-of-fit and parsimony. We found that a Tweedie family (log-
106 link) GAMM with a log-link family outperformed analogous GAMM/GLMMs with Poisson
107 and negative binomial families.

108 The model used the fixed effects (i) plasmid PDS count, (ii) plasmid GC content (mean
109 centred), and (iii) sampling niche (BSI-associated, livestock-associated, or wastewater-
110 associated). We used plasmid replicon type as a random intercept to control for plasmid
111 lineage. We also included an offset of $\log_{10}$-scaled plasmid length (bp). Originally, we also
112 included plasmid integron count and plasmid mobility (non-mobilisable, mobilisable,
113 conjugative) as fixed-effects, but these were dropped since they were insignificant (likelihood-
114 ratio test p-value>0.05). Plasmid PDS count and GC content initially used thin-plate regression
115 splines (k = 4), but by assessing the effective degrees of freedom and plots of the smooth terms,
116 we concluded they were consistent with linearity, so the smooth terms were dropped.

117 Lastly, we checked for multicollinearity and correlations among predictors. For numeric-
118 numeric fixed-effect pairs, we computed Spearman's rank correlation, and for numeric-
119 categorical fixed-effect pairs, we calculated the Kruskal-Wallis effect size. No issues were
120 identified.

121 Of the *n*=712 genetically representative plasmids, 23% (167/712) had inconclusive plasmid
122 replicon typing by PlasmidFinder (recorded as "NA"). Nonetheless, re-running the GAMM
123 with these plasmids excluded did not meaningfully change the coefficient estimates (95% CIs
124 overlapped).

125 **Code and data availability**

126 The data and metadata used in this study will be stably archived on Zenodo. The code to
127 reproduce the analysis will be made available on GitHub, and also archived on Zenodo.
128 Supplementary File 1 contains the permutation test output. Supplementary File 2 contains the
129 GAMM diagnostics.

130

131 Results

132 ***Plasmids are enriched for antimicrobial resistance genes and phage-defense system genes.***

133 We first curated a sample of *n*=1,044 *E. coli* isolates, a common human commensal and
134 pathogen, with *n*=2,559 plasmids (see Methods). Isolates were sampled within a geographically
135 (<60km in Oxfordshire, UK) and temporally (2008-2020) restricted frame, and represented
136 both clinical (human bloodstream infection [BSI]) and non-clinical (livestock-associated and
137 wastewater-associated) niches, totaling 52% (547/1,044) BSI, 42% (433/1,044) livestock-
138 associated, and 6% (64/1,044) wastewater-associated. Livestock-associated isolates were
139 further divided into 32.8% (142/433) cattle-associated, 28.9% (125/433) pig-associated, 11.8%
140 (51/433) poultry-associated, and 26.5% (115/433) sheep-associated. In total, 89% (938/1,044)
141 of isolates carried a plasmid. Isolates carried a median of 2 plasmids (range: 0-16), with the
142 most common replicon types being small colicinogenic-type plasmids (see Methods).

143 We next annotated our genomes for ARGs and PDSs (see Methods). In total, we identified
144 *n*=14,013 PDS hits with 180 distinct system types. Every chromosome carried at least one PDS,
145 contrasted with only 41% (1,051/2,559) of plasmids. Comparatively, ARGs were less common,
146 with 3,193 identified in total. Overall, 27% (286/1,044) of chromosomes and 20% (521/2,559)
147 of plasmids carried at least one ARG, respectively. Of plasmids that carried at least one ARG
148 or PDS, the three top replicon types were IncF-types: IncFIB, Col156-IncFIB-IncFII, and
149 IncFIB-IncFIC, which together accounted for 27% (325/1,225) of that plasmid subset.

150 Distributions of ARGs and PDSs were variable across *E. coli* niches (Figures S1–S4).
151 Livestock-associated plasmids (cattle, pig, poultry, and sheep) carried defense systems in 44%
152 (475/1,080) of cases but carried ARGs in only 13% (143/1,080). Within this group, pig-
153 associated plasmids were distinct: they encoded ARGs roughly four-fold more frequently: 25%
154 (101/403) of pig plasmids versus 6% (42/677) of plasmids from the other three livestock
155 groups, while their PDS prevalence remained comparable (39% [158/403] versus 47%
156 [317/677]). Wastewater-associated plasmids showed intermediate prevalence: 35% (53/150)
157 encoded PDSs and 19% (29/150) carried ARGs. BSI-associated plasmids exhibited the greatest
158 combined load outside pigs, with 39% (523/1,329) containing PDSs and 26% (349/1,329)
159 carrying ARGs.

160 Next, we tested whether ARGs and PDSs were enriched on plasmids versus chromosomes
161 (Figure 1; see Methods). Of the *n*=4,999,278 annotated coding sequences in our dataset, only
162 3.42% (170,778/4,999,278) were found on plasmids. By contrast, 8.66% of all PDS genes
163 (2,474/28,581) and 75.76% of all ARGs (2,419/3,193) were plasmid-encoded. Pearson's χ^2
164 tests (Yates-corrected) confirmed that both categories were highly over-represented on
165 plasmids relative to the genomic baseline (ARGs: $\chi^2 = 49{,}943.4$, df = 1, $p < 0.001$; PDSs: $\chi^2 =$
166 $2{,}343.8$, df = 1, $p < 0.001$). The magnitude of enrichment was therefore ~2.5-fold for PDS

167 genes and ~22-fold for ARGs. Surprisingly, this trend carried for both clinical and non-clinical

168 isolates (Figure S5).

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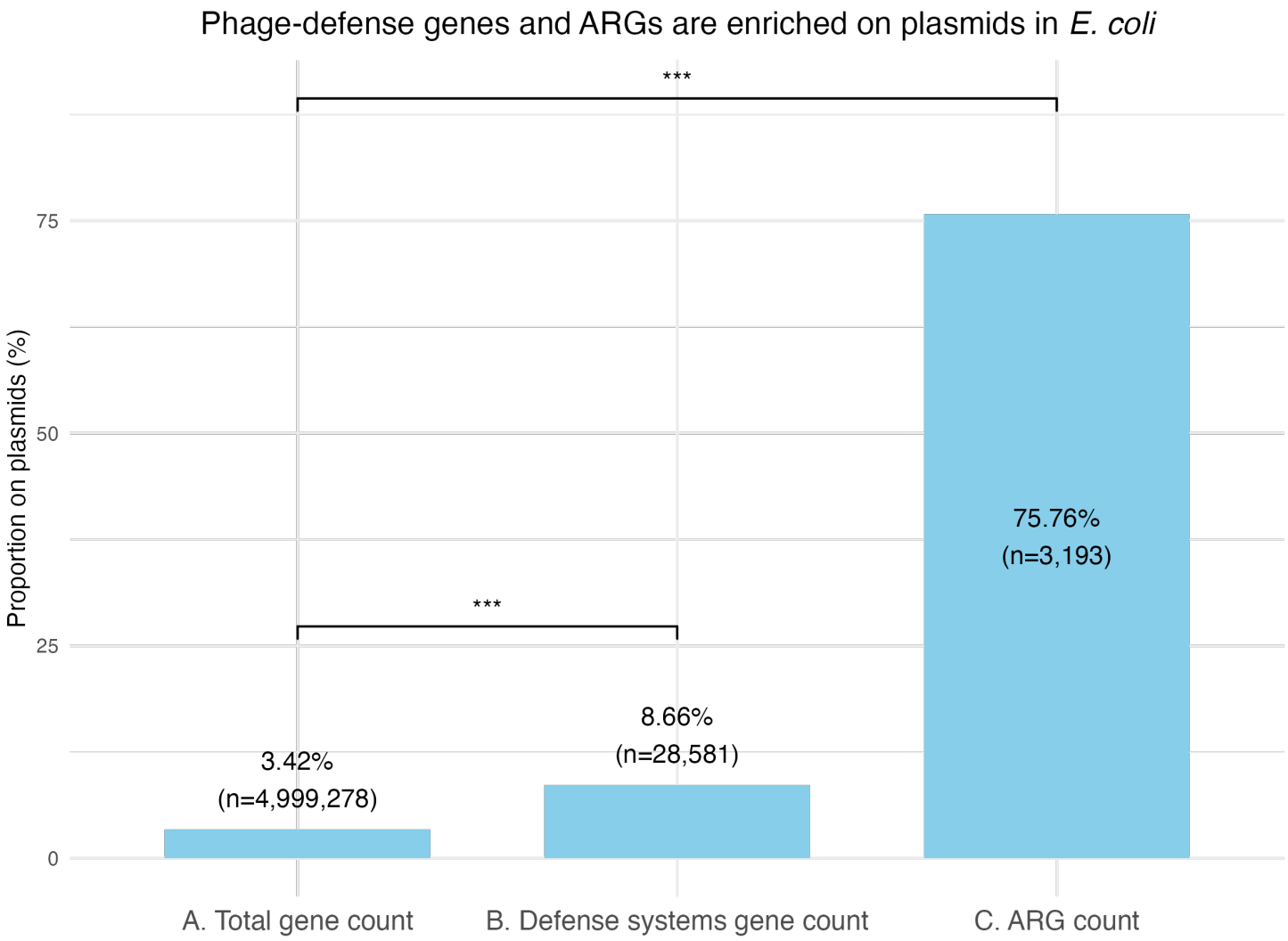

Figure 1. Plasmids are hotspots for ARGs and PDSs in *E. coli*. Bars show the percentage of genes in that category residing on plasmids. Both comparisons were significant at the $p<0.001$ level (Pearson χ^2 tests with Yates correction).

171

172 ***Plasmids and chromosomes are associated with distinct repertoires of phage-defense***
173 ***systems.***

174 We next explored whether the types of PDSs varied between plasmids and chromosomes. We
175 conducted a permutation test for each PDS (see Methods) and found 8% (15/180) of the PDS
176 families preferentially resided on plasmids (Figure 2). These 15 families made up 42.46%
177 (1,343/3,163) of defense-system occurrences on plasmids. In contrast, 20% (36/180) PDSs
178 were associated with chromosomes (Figure S6). Tests for the remaining 72% (129/180) PDSs
179 were inconclusive.

180 We found that TIR-III and Pif systems and two small uncharacterised families (HEC-09, DS-
181 28) were exclusively associated with plasmids in this dataset. Conversely, toxin-antitoxin
182 module MazEF and the cyclic-oligonucleotide producer CBASS were enriched more modestly
183 (~0.25, Figure 2A). When enrichment was quantified as absolute surplus (Figure 2B), the toxin-
184 antitoxin complex Mok-Hok-Sok was an outlier, occurring on plasmids 661 times versus the
185 approximately 100 occurrences expected by chance. Progressively smaller and significant
186 surpluses were observed for the remaining systems.

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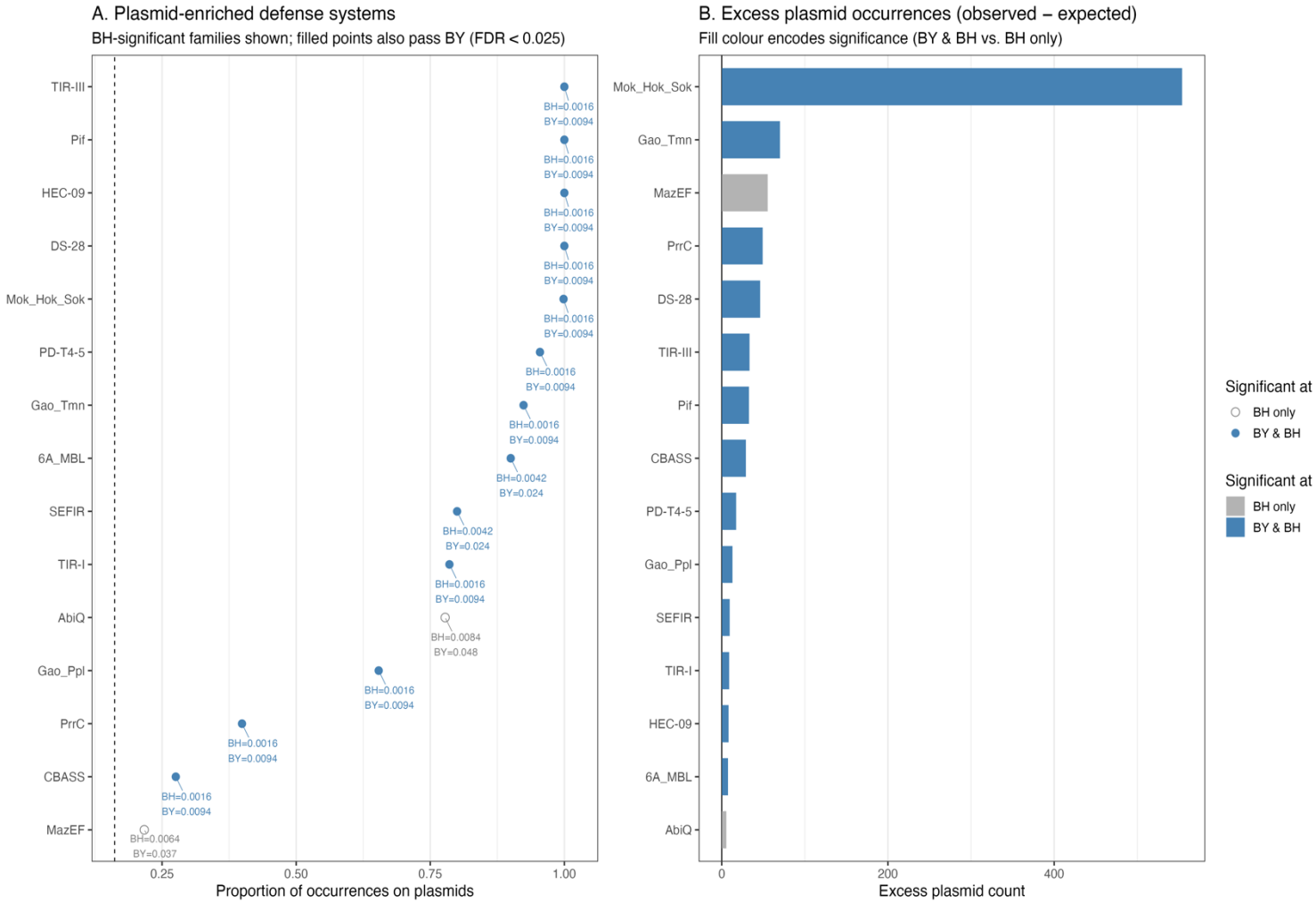

Figure 2. Defense systems associated with plasmids. (A) The x-axis shows the proportion of contigs that carried the PDS type. Each point represents a PDS type whose frequency on plasmids was greater than expected when contig labels (plasmid/chromosome) were randomly shuffled within isolates under two one-tailed permutation tests (see Methods). Open grey circles indicate $p<0.05$ significance under Benjamini-Hochberg (BH) correction only. Filled blue circles indicate significance under both BH and Benjamini-Yekutieli (BY) correction, which does not assume independence. The dashed line marks the genome-wide plasmid share of defence systems ($\bar{\pi} \approx 0.16$). **(B)** Bars count the excess number of plasmid-labelled contigs carried by each type ("observed – expected", where the expectation equals the type's total contig count $\times \bar{\pi}$). Bars are coloured the same as points in **(A)**. Only PDS families that were significantly over-represented on plasmids in the within-isolate permutation test are shown.

189

190 ***Phage-defense system count predicts ARG gene count on plasmids independent of plasmid***
191 ***size.***

192 Lastly, we explored the distribution of ARGs and PDSs within the genomes. We tested whether
193 ARGs and PDSs co-occurred on the same plasmids by constructing a generalised additive
194 mixed-model (GAMM) with a Tweedie family and log-link (see Methods). Briefly, we used
195 plasmid ARG count as a response with fixed effects (i) plasmid PDSs count, (ii) plasmid GC
196 content (mean centred), and (iii) sampling niche (BSI-associated, livestock-associated, or
197 wastewater-associated). We used plasmid replicon type as a random intercept to control for
198 plasmid lineage. To avoid pseudo-replication of plasmid sequences, we used single
199 representatives from clusters generated by genetic similarity. We also included an offset of
200 $\log_{10}$-scaled plasmid length (bp). Our total sample size was n=712 genetically representative
201 plasmids. The model output is detailed in Table 1 and visualised in Figure 3a. Further, Figure
202 3b visualises the ARG-PDS co-occurrence heatmap for all plasmids (n=2,559) in the dataset.

203 We found that the count of PDSs was a strong positive predictor of ARG count: each additional
204 phage-defense system increased the chance of an ARG being present on a plasmid by 48%
205 (95% CI=[18%, 86%]; p<0.001). Similarly, each 1% increase in plasmid GC content above
206 average increased ARG count by 37% (95% CI=[28%, 48%]; p<0.001). We found no signal
207 from isolate sampling niche. The random-effect smooth term for plasmid replicon type was
208 consistent with substantial non-linear variation (edf = 25.7, F = 1.21, p<0.001). Overall, the
209 model demonstrated good fit with adjusted R^2=0.40.

210 All isolates (1044/1044) carried a chromosomal PDS, meaning that all isolates with plasmid-
211 borne ARG (429/1,044) had the potential for dual resistance. Additionally, of the 41%
212 (429/1,044) of isolates carrying an ARG-positive plasmid, 88% (378/429) also carried at least
213 one plasmid-borne PDS. In 75% (324/429) of these cases, they were both on the same plasmid

214 (Figure 4). Further, Figure S7 shows the relative frequencies of ARGs, PDSs, and their co-
215 localisation on plasmids, and Figure S8 shows how these frequencies vary with plasmid size in
216 clinical versus non-clinical *E. coli*, consistent with clinical isolates, unlike non-clinical isolates,
217 often carrying small plasmids (<13kbp) that link ARGs and PDSs.

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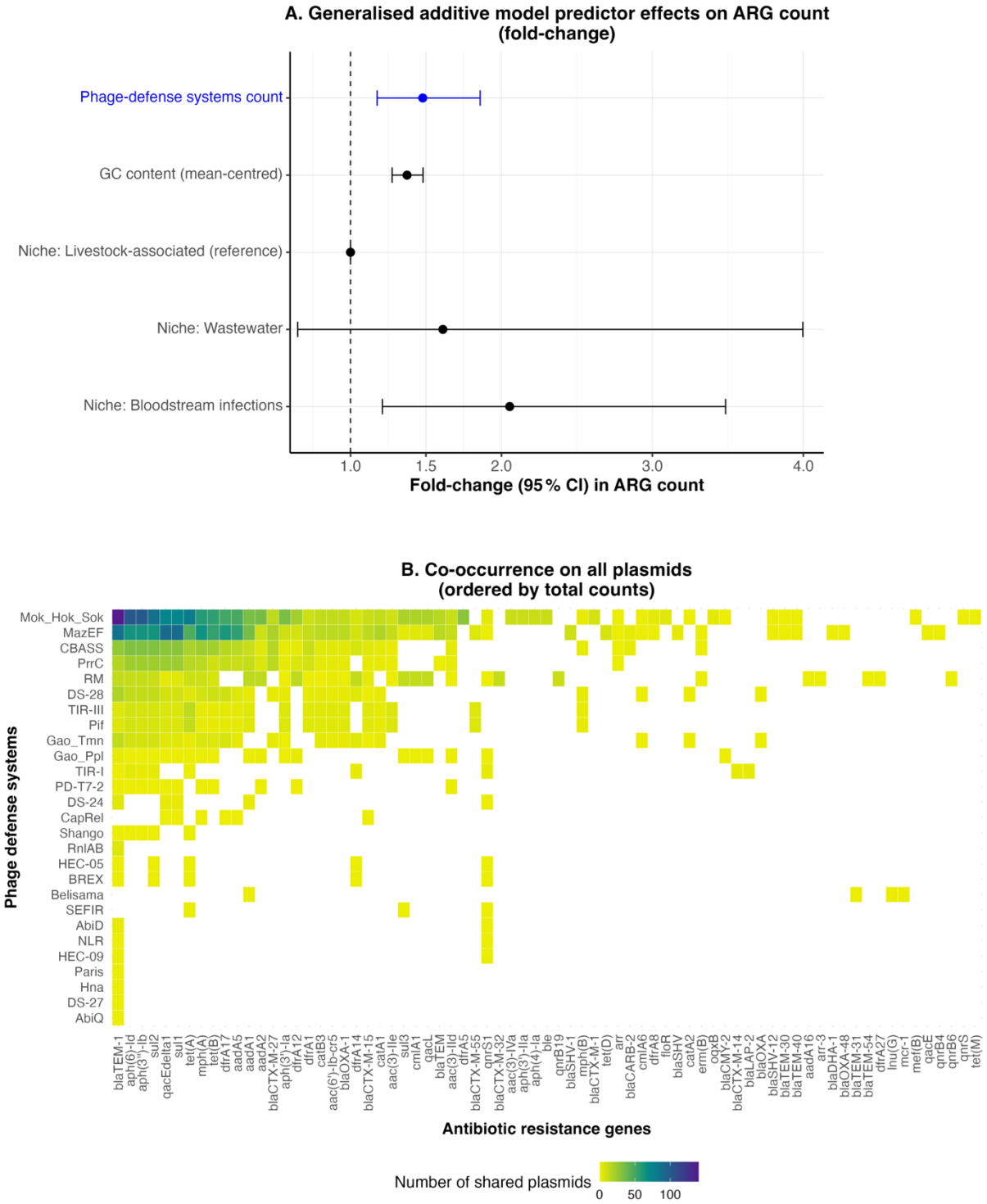

Figure 3. PDSs and ARGs co-occur on plasmids. **(A)** Forest plot of GAMM predictor coefficients as fold-changes with 95% confidence intervals ($\log_{10}$-scaled axis). The vertical dashed line at x=1 marks the reference. **(B)** Heatmap of frequency of co-localisation of ARGs and PDSs on same plasmid (n= 347). Cell colour indicates count.

220

221

Covariate		**Coefficient (95% CI)**	**Odds ratio (95% CI)**	***p*-value**
(Intercept)		-6.58 (-7.25, -5.91)	0.00 (0.00, 0.00)	<0.001
PDS count		0.39 (0.16, 0.62)	1.48 (1.18. 1.86)	<0.001
Plasmid GC content (mean-centred)		0.32 (0.24, 0.39)	1.37 (1.28, 1.48)	<0.001
Niche	BSI-associated	0.72 (0.19, 1.25)	2.05 (1.21, 3.48)	0.008
	Wastewater-associated	0.48 (-0.43, 1.39)	1.61 (0.65, 4.00)	0.3

Table 1. Parameter estimates for the GAMM. For niche, livestock-associated was the reference level. Odds ratios were calculated by taking the exponent.

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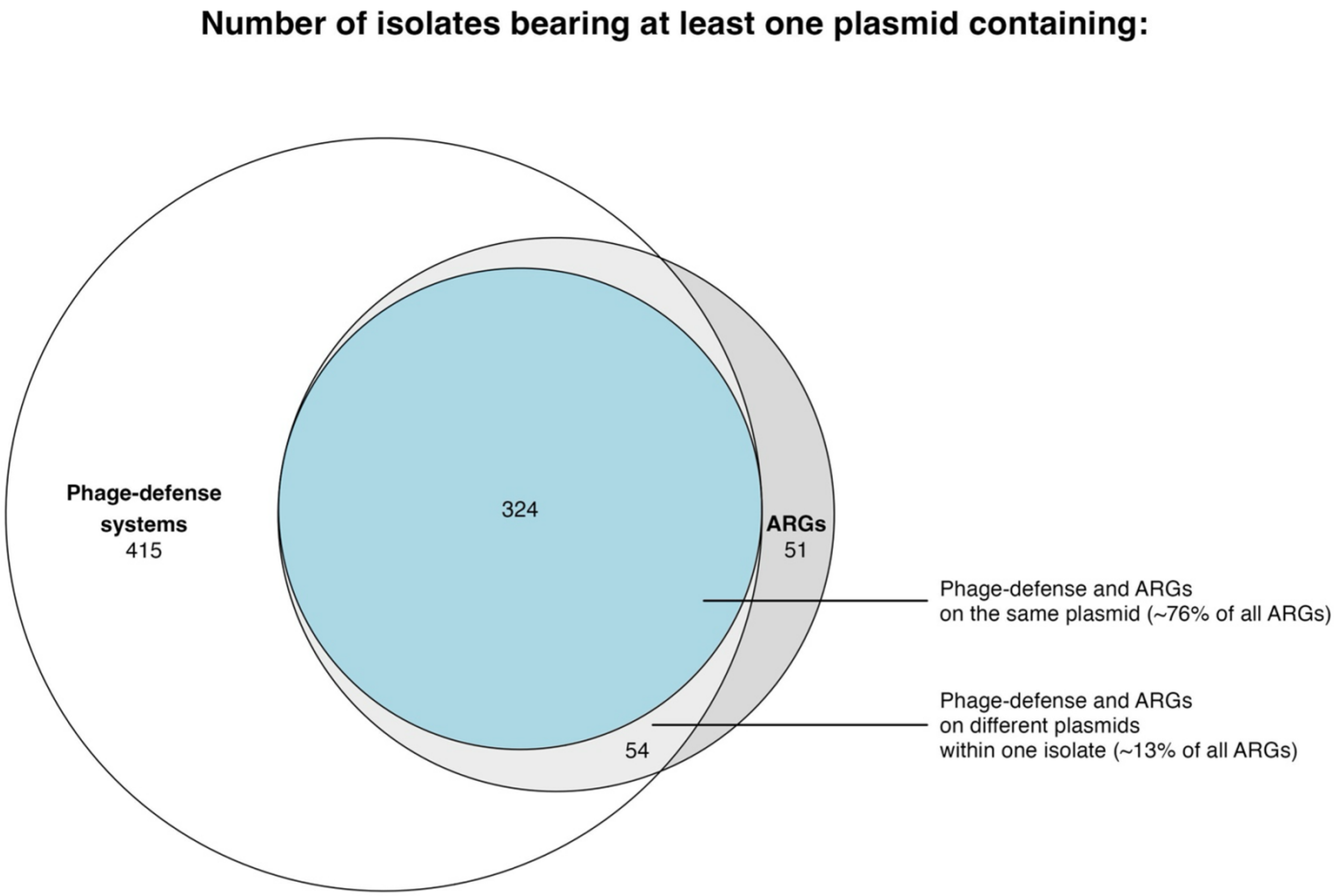

Figure 4. Overlap of ARGs and PDSs on plasmids within *E. coli* isolates. Euler diagram illustrating the intersection between sets of *E. coli* isolates that carried (i) at least one plasmid with a PDS, (ii) at least one plasmid with an ARG, and (iii) at least one plasmid with both an ARG and a PDS. The size of each ellipse is proportional to the set size. The total sample size was *n*=884 isolates.

225 # Discussion

226 We have analysed the distribution of ARGs and PDSs in a diverse sample of *E. coli* genomes.
227 We demonstrate that plasmids are enriched for both types of features, disproportionately carry
228 specific PDS families compared to the chromosome, and physically link ARGs and PDSs in
229 the genome. Overall, our findings suggest a fundamental role for plasmids in the dissemination
230 of PDSs on top of known associations with ARGs.

231 Our study has limitations. Whilst *E. coli* represents a predominant clinical pathogen, plasmids
232 are widespread across bacterial taxa and frequently cross species boundaries[28]. Future work
233 should expand these modelling approaches to incorporate a broader range of bacterial
234 hosts. We also did not model the co-occurrence of specific ARGs and PDSs. Capturing such
235 associations requires modelling frameworks that account for both vertical inheritance and
236 horizontal transfer events (such as those mediated by transposons) as well as methods capable
237 of resolving multivariate networks of association. Without such controls, pairwise tests risk
238 conflating indirect correlations with direct genetic linkage, as well as inflating Type I errors[29].

239 The co-localisation of ARGs and PDSs on plasmids can facilitate joint horizontal transfer and
240 coordinated regulation. We hypothesise that plasmids are an ideal platform for co-localising
241 ARGs and PDSs due to their variable copy number and potential for rapid evolution. In clinical
242 isolates, multicopy plasmids carrying $bla_{\text{TEM-1}}$ can amplify phenotypic resistance and
243 accelerate adaptive evolution of the gene[30,31]. In our dataset, $bla_{\text{TEM-1}}$ was often co-localised
244 with toxin-antitoxin systems such as RnlAB, PARIS, and AbiQ[32–34]on small (>13kb)
245 colicinogenic-type plasmids in the BSI-associated isolates. Analogously, plasmids carrying
246 PDSs may experience rapid and fluctuating selection in response to episodic lytic phage
247 attacks, favouring those that can quickly up or downregulate their PDSs[35,36].

248 Phage pressure alone can maintain mobile genetic elements carrying ARGs even in the absence
249 of antibiotics. For example, in *Vibrio cholerae*, episodic phage challenge increased conjugation
250 frequencies of integrative conjugative elements carrying both ARGs and PDSs[14]. In another
251 study, *E. coli* IncF plasmids carrying antibiotic resistance reduced susceptibility to coliphage
252 infection and persisted for around 10 days without antibiotic selection[37]. This suggests that
253 simply curbing antibiotic use may be insufficient to reverse the selection for antibiotic and/or
254 phage resistance. Future work should monitor plasmid gene frequencies, copy-number
255 dynamics, and host-fitness effects under isolated antibiotic, isolated phage, and combined
256 selection regimes, ideally incorporating varied dosing schedules and temporal patterns.

257

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263

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266

267 **Conflicting interests**

268 The authors declare no conflicting interests.

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