Niche and local geography shape the pangenome of wastewater- and livestock-associated *Enterobacteriaceae*

Liam P. Shaw[1], Kevin K. Chau[1], James Kavanagh[1], Manal AbuOun[2], Emma Stubberfield[2], H. Soon Gweon[3,4], Leanne Barker[1,5], Gillian Rodger[1,5], Mike J. Bowes[3], Alasdair T. M. Hubbard[1,6], Hayleah Pickford[1,5], Jeremy Swann[1,7], Daniel Gilson[8], Richard P. Smith[8], Sarah J. Hoosdally[1], Robert Sebra[9], Howard Brett[10], Tim E. A. Peto[1,5,7], Mark J. Bailey[3], Derrick W. Crook[1,5,7], Daniel S. Read[3], Muna F. Anjum[2], A. Sarah Walker[1,5,7], & Nicole Stoesser[1,5] on behalf of the REHAB consortium.

[1] Nuffield Department of Medicine, John Radcliffe Hospital, University of Oxford, Oxford, OX3 9DU, UK

[2] Department of Bacteriology, The Animal and Plant Health Agency (APHA), Woodham Lane, Addlestone, Surrey, KT15 3NB, UK

[3] UK Centre for Ecology & Hydrology (UKCEH), Benson Lane, Crowmarsh Gifford, Wallingford, OX10 8BB, UK

[4] School of Biological Sciences, University of Reading, RG6 6AS, UK

[5] NIHR Oxford Biomedical Research Centre

[6] Department of Tropical Disease Biology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, L3 5QA, UK

[7] NIHR Health Protection Research Unit in Healthcare Associated Infections and Antimicrobial Resistance at University of Oxford in partnership with Public Health England, Oxford, OX4 9DU, UK

[8] Department of Epidemiological Sciences, The Animal and Plant Health Agency (APHA), Woodham Lane, Addlestone, Surrey, KT15 3NB, UK

[9] Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, 10029, USA

[10] Thames Water Utilities, Clearwater Court, Vastern Road, Reading, RG1 8DB, UK

Correspondence: Liam P. Shaw (liam.philip.shaw@gmail.com) and Nicole Stoesser (nicole.stoesser@ndm.ox.ac.uk)

Keywords. antimicrobial resistance (AMR), bacterial genomics, *Enterobacteriaceae*, *Escherichia coli,* pangenomes, environmental bacteria.

Escherichia coli and other _Enterobacteriaceae_ are highly diverse species with 'open' pangenomes[1,2], where genes move intra- and inter-species via horizontal gene transfer[3]. These species can cause clinical infections[4,5] as well as persist environmentally[6,7]. Environmental populations have been suggested as important reservoirs of antimicrobial resistance (AMR) genes. However, as most analyses focus on clinical isolates[8,9], the pangenome dynamics of natural populations remain understudied, particularly the role of plasmids. Here, we reconstructed near-complete genomes for 828 _Enterobacteriaceae_, including 553 _Escherichia_ spp. and 275 non-_Escherichia_ species with 2,293 circularised plasmids in total, collected from nineteen locations (livestock farms and wastewater treatment works in the United Kingdom) within a 30km radius at three timepoints over the course of a year. We find different dynamics for the chromosomal and plasmid-borne components of the pangenome, showing that plasmids have a higher burden of both AMR genes and insertion sequences, and AMR plasmids show evidence of being under stronger selective pressure. Focusing on _E. coli_, we observe that plasmid dynamics are more strongly dominated by niche and local geography, rather than phylogeny or season. Our results highlight the diversity of the AMR reservoir in these species and niches, and the importance of local strategies for controlling the emergence and spread of AMR.

Enterobacteriaceae can persist across diverse environmental niches[10] and also cause clinical infections, with AMR in *Enterobacteriaceae* emerging as a major problem in the last decade[11,12]. Dissemination of AMR genes often occurs via mobile genetic elements (MGEs) which can transfer intra- and inter-species, both locally[13] and globally[14]. Freshwater, wastewater and livestock-associated strains of *Enterobacteriaceae* have been proposed as reservoirs for AMR genes in clinical isolates[15–18], but the links between these remain cryptic[19]. Current understanding of the ecology and evolution of pangenomes is incomplete[20], with ongoing debate about the roles of niche adaptation and selection[21–24]. Published

50 *Enterobacteriaceae* genomes are biased towards clinical isolates, sampling frames reflecting
51 truly interlinked communities are limited, and much remains unknown about the population
52 genetics of *Enterobacteriaceae*[25] and the role of plasmids in non-clinical contexts[26].

53 Genomic studies of *Enterobacteriaceae* have predominantly used short-read whole genome
54 sequencing (WGS). AMR genes and their flanking regions are frequently fragmented in short-
55 read assemblies due to repetitive elements and structural rearrangements[13,27]. Combining short-
56 and long-reads ('hybrid assembly') produces complete, high-quality genomes[28], allowing
57 accurate structural resolution. Here, we used hybrid assembly of 828 sympatric
58 *Enterobacteriaceae* (*Citrobacter*, *Enterobacter*, *Escherichia* spp., and *Klebsiella*) to
59 characterise the pangenome of these genera considering both niche (cattle, pig, sheep, or
60 wastewater treatment works (WwTW)-associated) and geography (sampling location).

61 **A diverse collection of complete genomes from livestock and water-borne niches**

62 We collected samples from nineteen locations ≥5 km apart (maximum distance: 60km) in
63 South-central England (United Kingdom) in 2017, namely: fourteen livestock farms (four pig,
64 five cattle, five sheep) and water sources around five WwTWs over three seasonal timepoints
65 (Fig. 1a). A subset of 832/2098 cultured isolates from pooled samples from each sampling
66 location underwent short- and long-read sequencing and hybrid genome assembly (Fig. 1b, see
67 Methods), resulting in 828 high-quality genomes (Table S1: *n*=496 from livestock farms,
68 *n*=332 from WwTWs), from four genera: *Citrobacter* (*n*=128), *Enterobacter* (*n*=71),
69 *Escherichia* (*n*=553), and *Klebsiella* (*n*=76). Most farm isolates were *Escherichia* spp.
70 (451/496, 90.9%), with WwTW isolates having roughly even proportions of genera (Fig. S1).
71 Isolates contained a median of 1 AMR gene (range: 0-23); *Klebsiella* isolates carried a median
72 of 4 (range: 1-18).

73 Isolates were highly diverse, containing novel diversity not present in published genomes (Fig.
74 S2). *Escherichia* diversity included all main *E. coli* phylogroups, 53 *E. fergusonii*, and 13
75 isolates from clades I, II, III, and V (Fig. 1c). Phylogroup B2 was strongly associated with
76 WwTWs compared to livestock (34.3% vs. 5.1% of *Escherichia* isolates) particularly in
77 influent and effluent samples (Fig. 1c). Pigs had a greater proportion of phylogroup A isolates
78 (Fig. 1c). Of 187 identified *E. coli* multilocus sequence types (STs), 56.1% (105/187) were
79 seen only once, similar to the 61% observed by Touchon et al. in a study of non-clinical *E. coli*
80 [29]. Only 12 *Escherichia* STs were seen in both livestock and WwTW isolates, with phylogroup
81 B1 the most represented (5/12 STs). ST10 was the most prevalent ST (n=45), seen in 10/14
82 farms and 3/5 WwTWs.

83 Considering only livestock *E. coli* isolates, over time, there was a persistent phylogroup
84 signature of both livestock host and farm, with individual farm explaining more variance than
85 livestock type (R^2=28.1% vs. 25.5%, Fig. S3). However, livestock type explained less variance
86 for STs than phylogroups (R^2=8.5%), with only 39/131 STs (29.8%) seen on more than one
87 farm. There were only 26 instances where an *E. coli* ST was observed over time on the same
88 farm (involving 16 STs) and the majority of these (22/26) were STs also seen across farms (Fig.
89 S4). Considering *E. coli* strain clusters using a core genome distance of <100 single-nucleotide
90 variants (SNVs) (maximal diversity observed across sampled *E. coli*: 211,251 SNVs; median
91 pairwise distance 46,144 SNVs), there were 280 isolate pairs with <100 SNVs, of which 181
92 (64.6%) were isolates cultured from the same pooled sample (i.e. same farm, same timepoint)
93 (Fig. S5a). Overall, 10.5% of all isolate pairs from the same pooled sample had <100 SNVs
94 between them, compared to 1.4% (n=52) of isolate pairs from different timepoints on the same
95 farm and 0.2% (n=44) between different farms of the same animal (Fig. S5b). Notably, of the
96 latter, 41/44 were between cattle farms, and 36 involved a single cattle farm (RH06). There
97 were only three isolate pairs with <100 SNVs between farms of different animals (Fig. S5a).

98 Notably, all of these were between farms in close geographic proximity (two instances from
99 pig farm RH03 and cattle farm RH10, one instance from cattle farm RH07 and sheep farm
100 RH12; see Fig. 1a for distances), suggesting local strain movement. Taken together, this
101 indicates that different livestock hosts have a stable balance of *E. coli* phylogroups but each
102 farm harbours substantial strain-level diversity. There were no isolate pairs with <100 SNVs
103 between WwTW and livestock niches, and only three isolate pairs occurred across timepoints
104 at WwTWs (all at a single WwTW).

105 **Plasmid gene repertoires are linked to genus and niche**

106 We recovered 2,293 circularised plasmids across all *Enterobacteriaceae*, ranging in size from
107 1,240-824 kbp (median: 43 kbp; Table S2). There were 298/2,293 (13.0%) with no identifiable
108 plasmid replicon and the majority of these were from WwTW isolates (192/298, 64.4%).
109 Multiple replicons were carried by 723/2,293 (31.5%) and these plasmids tended to be larger
110 (median length: 106,811 bp vs. 6,275 bp for single replicon plasmids). Of *E. coli* isolates with
111 complete genomes, over two thirds (70.4%, 245/348) carried a plasmid with an IncFII replicon.
112 43.0% of circularised plasmids (986/2,293) had at least one match with >99% identity to other
113 publicly available plasmid sequences (Fig. S2b). However, 12.3% (282 of 2,293) had a top
114 identity score of <95% to a previous known sequence (Fig. S2b), and 17 plasmids with no
115 match were identified, suggesting novel plasmid diversity in our setting. We grouped
116 circularised plasmids into 611 distinct plasmid clusters, which closely matched gene content
117 (Fig. S6a). The synteny of shared genes was strongly conserved, supporting the concept of
118 plasmid 'backbones' (Fig. S6b).

119 A median of 3.3% of genes were on plasmids (range: 0-16.5%), with substantial variation by
120 genus and niche (Fig. S7a). Accounting for plasmid copy number, *E. coli* isolates had a median
121 of 5.7% of DNA present on plasmids, which was substantially higher in pig farm isolates

122 (median: 10.1%; Fig. S7b). Chromosomal genes were highly genus-specific (R^2=55.0%); the
123 plasmid-borne pangenome was far more variable but still genus-specific (R^2=6.5%) (Fig. 2).
124 Within *E. coli*, plasmid gene content was linked to niche (R^2=5.6%) and phylogroup
125 (R^2=5.2%), with a stronger interaction between niche and phylogroup (R^2=7.9%) (Fig. 2). Non-
126 mobilizable plasmid clusters were less commonly shared between different phylogroups within
127 farms compared to mobilizable or conjugative plasmids (Fig. S8). Although AMR genes were
128 predominantly found in conjugative/mobilizable plasmid clusters, plasmid clusters with AMR
129 genes were not more commonly distributed across multiple phylogroups (Chi-squared test
130 χ^2=0.64, p=0.42; Fig. S8). On pig farms however, the majority of conjugative plasmid clusters
131 across multiple phylogroups carried AMR genes, suggesting an important role within this
132 niche.

133 Positive epistasis between large (>10 kbp) and small plasmids has been suggested to promote
134 plasmid stability in *Enterobacteriaceae*[30]. In *E. coli* isolates with complete genomes (n=348),
135 we observed a significant association between small and large plasmid presence (Chi-squared
136 test χ^2=4.44, p=0.035), with 45.7% carrying at least one large (>10 kbp) and one small plasmid
137 and only 3.7% carrying a small plasmid without a large plasmid. We also found evidence of
138 specific plasmid-plasmid associations. For example, cattle *E. coli* isolates showed co-
139 occurrence of a ColRNA plasmid (cluster 37: median length 4.6 kbp) and an IncFII plasmid
140 cluster (cluster 279: median length 106 kbp), with 14/16 isolates with the ColRNA plasmid
141 also carrying the larger IncFII plasmid. Isolates were from three phylogroups (A: n=2, B1: n=5,
142 E: n=9) and four farms, suggesting a robust association which reflects plasmid epistasis
143 independent of chromosomal background.

144 **Plasmids carry an over-representation of AMR genes and insertion sequences**

145 Plasmids carried more diverse and less genus-restricted genes. Despite carrying just 3.3% of
146 total gene content, plasmid-borne genes accounted for 11.5% of unique genes (8.9-17.0%
147 considering each genus; Fig. S9) and 40.1% were seen in more than one genus (19.6-55.6%
148 considering each genus; Table S3). Plasmids also had a much greater burden of AMR genes:
149 considering isolates with circularised chromosomes (see Methods), 901/1,876 AMR genes
150 (48.0%) were found on plasmids i.e. a 14.5x relative burden in plasmids. Of 26,565 insertion
151 sequences (ISs), 3,695 (21.7%) were found on plasmids (6.6x relative burden). There was a
152 weak correlation between the number of plasmid- and chromosome-associated AMR genes
153 within an isolate (Spearman's ρ=0.11, p=0.004) but a strong positive correlation for the number
154 of ISs (Spearman's ρ=0.40, p<0.001) (Fig. S10a), seen across genera (Fig. S10b).

155 We observed different patterns of ISs across chromosomes and plasmids (Fig. S11). Some ISs
156 were strongly associated with plasmids, the strongest association being for IS*26*. However,
157 27.5% of isolates carrying IS*26* on a plasmid also carried it on their chromosome, consistent
158 with its extremely active behaviour[31]. The most prevalent IS on both chromosomes and
159 plasmids was IS*Kpn26*, with 50.2% of IS*Kpn26*-positive isolates having it both chromosomally
160 and plasmid-borne. Considering *Escherichia*, WwTW isolates showed a greater diversity of
161 ISs, with 65% of ISs found in a higher proportion of WwTW isolates compared to those from
162 farms (Fig. S12), including IS*30* which has been proposed as a marker for naturalized
163 wastewater populations of *E. coli*[32]. Overall, ISs had random levels of co-occurrence on
164 *Escherichia* plasmids (upper-tail p=0.85 from null model simulations of checkerboard score,
165 see Methods; Fig. S13a), suggesting that ISs frequently move independently between plasmid
166 backgrounds. Contrastingly, AMR genes significantly co-occurred (upper-tail p=0.02; Fig.
167 S13b), suggesting co-selection on plasmids.

168 **Plasmids carrying AMR genes show features suggestive of selection**

169 Plasmids fell into two broad classes across genera: small multicopy plasmids (<10 kbp, 10-
170 100X copy number) and large low-copy plasmids (>10 kbp, <10X) (Fig. 3a). AMR plasmids
171 were almost all large low-copy plasmids (173/184, 94.0%). Overall, plasmids had a lower
172 relative GC-content than their host chromosomes (median difference -2.5%, Fig. 3b), and
173 plasmids predicted to be mobile had a smaller relative difference. However, this difference was
174 less marked for AMR plasmids (median -0.3%) across mobility categories (Fig. 3b). Nearly
175 half had a higher GC-content than their host chromosome (46.7% vs. 17.7% of non-AMR
176 plasmids), suggesting AMR plasmids are under selective pressure to maintain their function.

177 **Evidence for recent horizontal gene transfer across genera and within isolates**

178 We identified 2,364 potential horizontal gene transfer (HGT) events involving transfers of
179 sequence >5,000 bp between isolates of different genera (see Methods). Isolates from the same
180 farm were ~10x more likely to show evidence of cross-genera HGT than would be expected
181 (Chi-squared test χ^2=1159, p<0.001; Fig. S14), and 12.3% of these cross-genera HGT events
182 involved at least one AMR gene, with most of these AMR HGT events between pig isolates
183 (37/48, 77.0%). Movement of genes can also occur within genomes. We therefore also
184 investigated occurrences where the same gene was present on both the chromosome and
185 plasmid(s) within an *E. coli* genome. We observed distinct differences between niches, with
186 increased amounts of chromosome-plasmid sharing in pig and WwTW isolates compared to
187 cattle and sheep (Fig. S15).

188 **Quantifying the roles of phylogeny, niche and geography in the *E. coli* pangenome**

189 To understand the strength of different factors shaping the pangenome, we analysed the
190 pangenome of *E. coli* in more detail. Isolates recovered from the same location spanned total
191 *E. coli* diversity (Fig. 4a). Inter-isolate core genome distances were strongly correlated with
192 chromosomal gene repertoire relatedness (GRR) (Fig. 4a). Core genome distance explained the

193 majority of variance in chromosomal GRR (Fig. 4b), but there was a consistent contribution
194 from geography and time: isolates from the same pooled sample sharing more genes than would
195 be expected (+1.2%), as did isolates from the same farm at different timepoints (+0.5%) (Fig.
196 4b). There was no such effect for isolates from different farms of the same livestock, suggesting
197 this reflects local geography rather than adaptation to livestock host. Although the variance
198 explained was much lower, local geography effects were also observed for plasmid GRR (Fig.
199 4c), but core genome distance was uncorrelated with plasmid GRR apart from for near-identical
200 strains (Fig. 4d). Isolates from different STs from different farms of the same livstock could
201 still have high plasmid GRR (Fig. 4e), suggesting that host-specific plasmids may facilitate
202 niche adaptation.

203 **Conclusions**

204 We have investigated the pangenome of major genera of sympatric *Enterobacteriaceae* from
205 locations within a 30km radius, using a diverse set of non-clinical isolates cultured from the
206 same samples, and focusing in detail on *E. coli*. Despite high overall diversity, with the majority
207 of strains only observed once in the dataset, we observed the persistence of strains and plasmids
208 on farms over the course of a year. Our results highlight the combination of persistence and
209 dynamism that characterises *Enterobacteriaceae* genomes at multiple scales, with relevance
210 both for understanding the population structure of species within *Enterobacteriaceae* and for
211 managing AMR. The existence of farm-level differences in *E. coli* populations which persist
212 over time, with a small number of possible inter-farm transfers, suggests that livestock farms
213 function as distinct but linked niches. It could be that "everything is everywhere" (frequent
214 movement of strains and genes between farms) but "the environment selects" (different farms
215 have different selective pressures). However, the observation of persistent strains over the
216 course of a year on farms, despite presumably varying selective conditions, and the

217 overrepresentation of putative cross-genera HGT events in isolates at the same location
218 suggests that geographical effects or intrinsic properties of certain bacterial/MGE lineages
219 could affect the evolution of AMR on such timescales. Future modelling work and investigation
220 will be required to distinguish these hypotheses. Overall, our findings underline the importance
221 of local control strategies for the emergence and spread of AMR beyond clinical settings.

222 Resource limitations meant that we were unable to sequence and genetically evaluate all
223 isolates that were cultured, and despite our detailed sampling we will not have captured all the
224 persistence, HGT and strain sharing events across niches. Although this study is unprecented
225 in evaluating four genera in such detail, AMR gene dissemination and important structural
226 associations of AMR genes and MGEs may also be occurring within other genera not studied
227 here. Furthermore, we did not investigate the relationship between isolates in this study and
228 clinical human compartments in the same study area; this is ongoing work.

229 In conclusion, our study highlights the plastic and dynamic nature of AMR gene dissemination
230 within the pangenome of major *Enterobacteriaceae* in several important non-clinical niches. It
231 also demonstrates how robustly evaluating the flow of AMR genes and MGEs across highly
232 diverse and dynamic niches is challenging even with extensive sampling. The implications of
233 this for adequately understanding dissemination and selection of AMR genes in a 'One Health'
234 context should not be under-estimated.

Figure 1. Overview of the diverse *Escherichia coli* isolates in this study. **(a)** Relative sampling locations of the farms (cattle, pig, sheep) and wastewater treamtent plants (WwTWs) in this study, sampled at three different timepoints. **(b)** Schematic illustration of the sampling, culture and sequencing workflow, resulting in high-quality genome assemblies with a median of 1 circularised chromosome and 2 circularised plasmids per assembly. **(c)** Mid-point rooted core genome phylogeny of *E. coli* isolates (*n*=488) based on, with tips coloured by phylogroup and ring colours showing sampling niche. Inset panel at centre of phylogeny shows phylogroup abundances (as proportion of isolates) from different sampling niches.

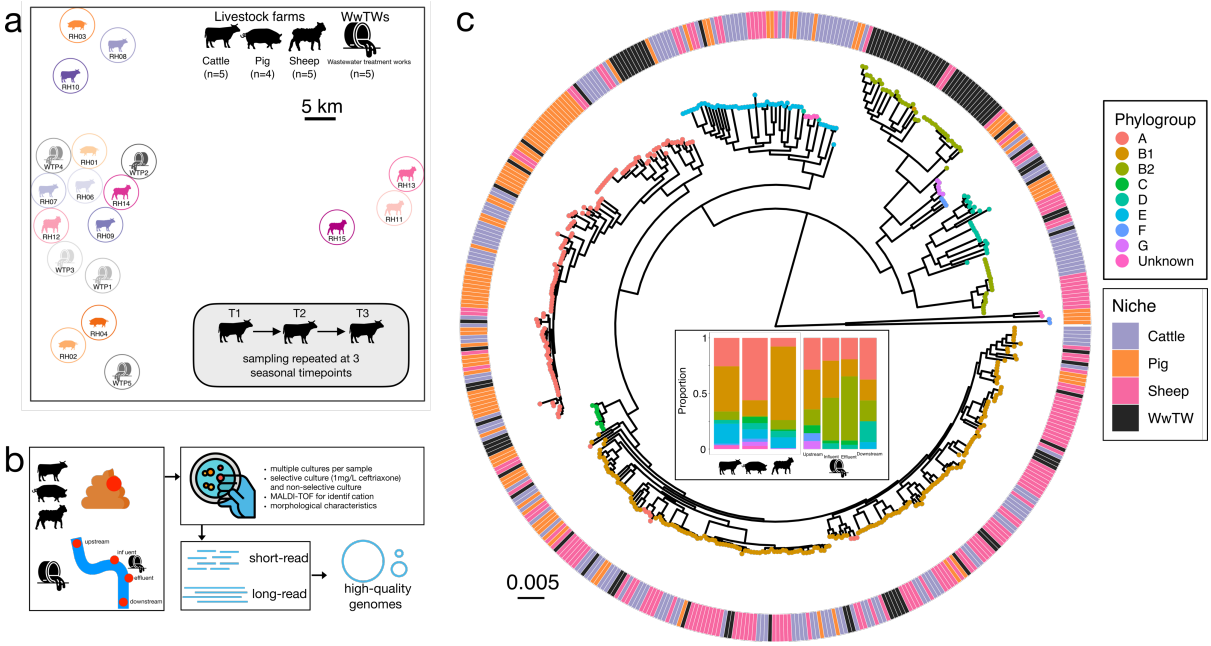


Figure 2. The plasmid-borne component of the pangenome is structured by niche and phylogeny, with greater variation than in the chromosomal component. Plots are shown for all isolates in four genera across *Enterobacteriaceae* (top row) and for *E. coli* (bottom row), for both the chromosomal component of the pangenome (left column) and the plasmid-borne component (right column). Stacked bar charts show the variance in gene content explained by niche, phylogeny (genus or phylogroup) and their interaction. The plasmid-borne component has greater residual variance than the chromosomal component, with a comparatively stronger niche-phylogeny interaction (darkest shaded bar).

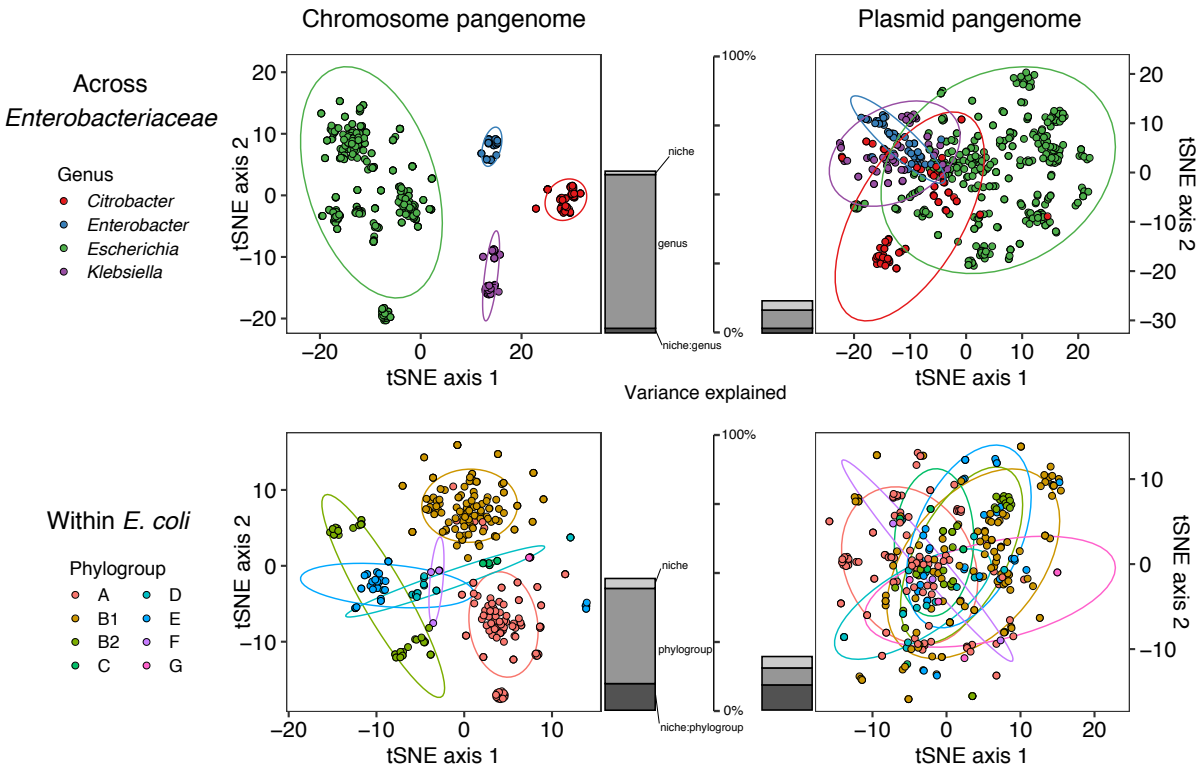


Figure 3. Distinct plasmid lifestyles between AMR and non-AMR plasmids. (a) Plasmid length (x-axis) and inferred copy number (y-axis) of all circularised plasmids (*n*=2,293), faceted by genus. Plasmids with ≥1 AMR gene (coloured points) tended to be larger and present in lower copy numbers. **(b)** Relative GC-content of all plasmids to their host chromosome for all circularised plasmids present in an assembly with a circularised chromosome (*n*=1,753 plasmids across 616 isolates), split by predicted plasmid mobility. Boxplots are shown for plasmids with ≥1 AMR gene (red) or no AMR genes (black). Comparisons with *p*-values are shown for all plasmids within a predicted mobility class. **(c)** Length distributions of plasmid clusters (see Methods).

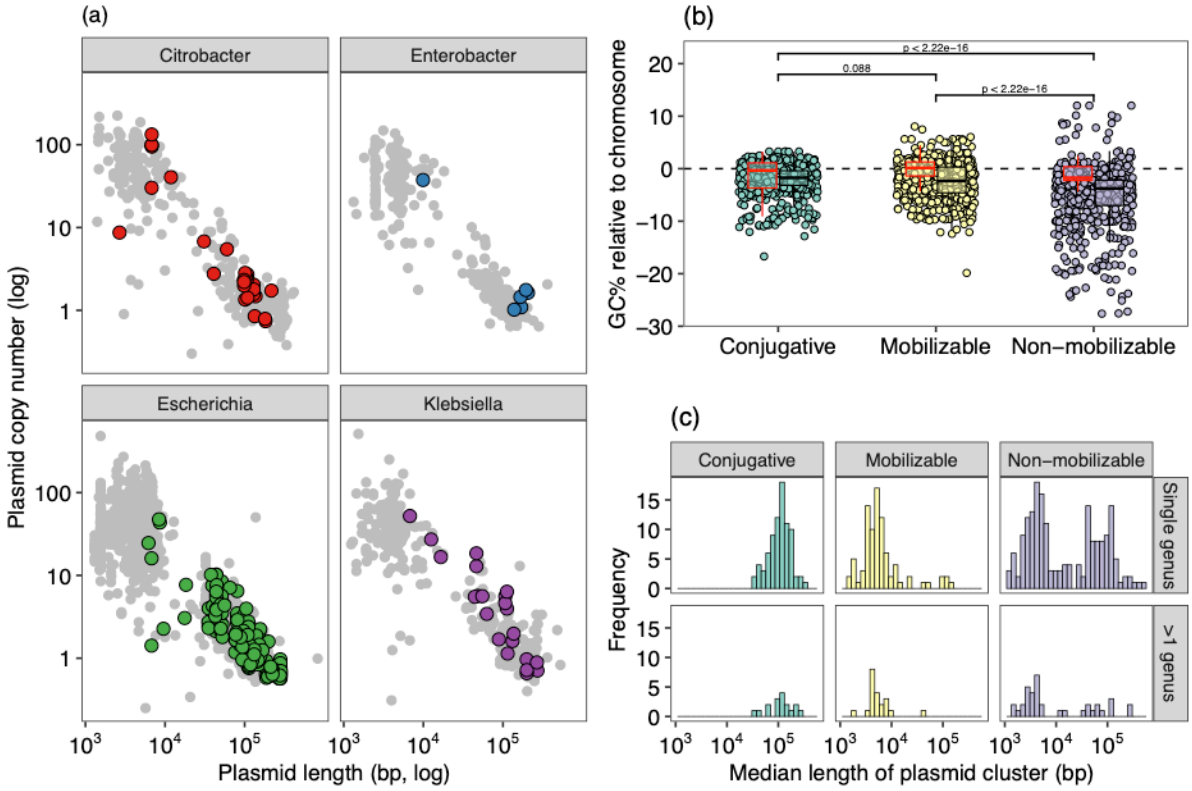
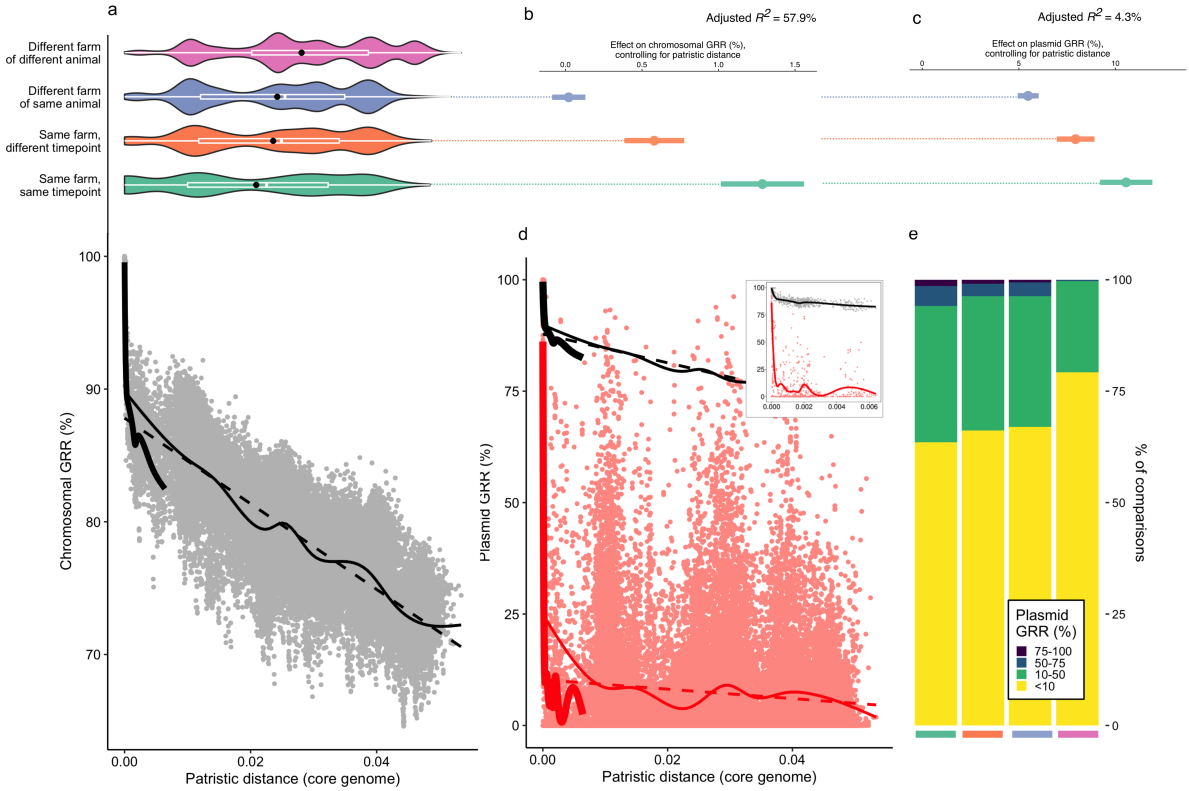


Figure 4. The interplay of phylogeny and niche in the *E. coli* pangenome. **(a)** Pairwise comparisons of gene repertoire relatedness (GRR) for chromosomal genes show that chromosomal GRR falls off rapidly at small patristic distances, followed by an approximately linear decrease. Fits show intra-ST comparisons (thick black line), all comparisons (thin black line), and a linear model (dashed black line). Violin plots above show the distribution of patristic distances depending on the relative sample source of the two isolates in the pairwise comparison (white boxplot: median and IQR; black point: mean), showing that even isolates cultured from the same sample (same farm, same timepoint) span equivalent diversity to isolates cultured from different locations. **(b)** Coefficients from a linear model for chromosomal GRR with an interaction term with patristic distance (excluding intra-ST comparisons). **(c)** Variance explained by phylogeny and geography for chromosomal and plasmid GRR. **(d)** GRR for plasmid-borne genes with patristic distance. Fits show intra-ST comparisons (thick red line), all comparisons (thin red line), and a linear model (dashed red line). Inset panel shows left-hand region of the plot with only intra-ST comparisons, with chromosomal GRR relationship also shown (grey points, black line). **(e)** Plasmid GRR comparisons shown by isolate sources, excluding intra-ST comparisons. Colours on x-axis are the same as in (a). Plots include all *E. coli* isolates with a circularised chromosome (*n*=363).



Methods

Isolates were sequenced from samples collected as part of the REHAB project in 2017, which aimed to characterise non-clinical *Enterobacteriaceae* populations in four different niches within a defined study area of South-central England: cattle farms, pig farms, sheep farms, and water environments linked to wastewater treatment works (WwTWs). Sampling occurred at each location at three separate timepoints (TPs): January-April 2017 (TP1), June-July 2017 (TP2) , October-November 2017 (TP3).

Farms. Five cattle farms, five sheep farms and four pig farms were recruited from the study area following a defined recruitment process (described in more detail in AbuOun et al.[33]). Briefly, we aimed to recruit the five largest farms for each livestock type within the area using local APHA databases, progressively inviting the next largest farm if a farm declined. All participating farmers provided written consent for farm sampling for research purposes and farm samples were taken between January and November 2017 on three separate visits ('timepoints') for each farm. Each farm was divided in five or fewer 'epidemiological groups', defined as a group of animals expected to share similar characteristics and managed in the same way. Ten pooled samples were collected from each of these groups, with each sample composed of small pinches of fresh faeces from the floor combined into a small composite sample around 5cm in diameter. Each group's ten samples were pooled, diluted up to 10^{-5} in phosphate buffer solution (PBS) (pH 7.2) and plated on to CHROMagar™ ECC (CHROMagar Microbiology, Paris, France) and CHROMagar™ ECC plates containing 1mg/L cefotaxime as a marker for multi-drug resistance. Up to ten colonies were collected from 1mg/L cefotaxime-supplemented plates and fourteen colonies from CHROMagar™ ECC plates; where ten colonies were not recovered, additional colonies were taken from the CHROMagar™ ECC plates, resulting in 24 isolates per farm. Pure isolate sub-cultures were subsequently stored at -80˚C in MicroBank beads (Pro-Lab Diagnostics, Neston, Cheshire, UK), and the bacterial species confirmed using MALDI-TOF (Bruker, Coventry, UK) or 16S rRNA sequencing[34]. The median number of sequenced isolates for a farm-timepoint combination was twelve (range: 9-14), with 496 farm isolates in total: cattle (n=178), pig (n=144), sheep (n=174).

Wastewater treatment works (WwTWs). Five WwTWs were selected based on a defined recruitment process (described in more detail in Read et al.[35]) including; geographic location within the study area, wastewater treatment configuration, wastewater population equivalent served, consented flow, and the accessibility of the effluent receiving river for sampling both upstream and downstream. Sampling took place in 2017 over three sampling rounds: February–March (TP1), June–July (TP2), and October–November (TP3). Sewage influent

312 samples were collected after WwTW coarse screens and effluent samples were collected at the last sampling point
313 before entering the river. For each sampling round, ~6 repeated 200 ml samples of influent and effluent were
314 collected between 9 am and 12 pm, using a sampling pole and sterile Whirl-Pak collection bags. Repeat samples
315 in each round were pooled prior to processing, to reduce the impact of temporal variability in wastewater flows
316 and composition. Sediment samples were collected from 100 m upstream and 250 m downstream of the effluent
317 entry point into the river. Sediment samples were collected using a custom sampling pole that held a removable
318 50 ml plastic centrifuge tube (Sigma, UK). Using a fresh sterile 50 ml tube each time, sediment from the riverbed
319 was collected from the surface layer at three points at each sampling site; near bank, the centre of the river, and
320 the far bank. These samples were pooled prior to analysis to account for spatial variability in sediment
321 composition. Influent, effluent and sediment samples were stored in an insulated box at ~4 °C until getting back
322 to the laboratory (<6 h). Influent, effluent, 100 m upstream and 250 m downstream environmental samples
323 collected from each sewage treatment works were transferred to the laboratory on ice and processed within 24
324 hours of collection. Each sample was vortexed briefly, serial diluted to 10^{-3} in nutrient broth containing 10%
325 glycerol (Oxoid, Basingstoke, UK) and plated on to CHROMagar™ Orientation agar (Chromagar, Paris, France)
326 and CHROMagar™ Orientation agar supplemented with 1 µg/ml cefotaxime (Cambridge Biosciences,
327 Cambridge, UK). Colonies with putative morphology for species of interest were subcultured from dilution plates
328 with suitably isolated growth. A total of up to 20 colonies was picked per sample: up to ten colonies were picked
329 from the 1mg/L cefotaxime-supplemented plates and the remainder picked from the non-supplemented plates.
330 Pure isolates subcultured on Columbia blood agar (CBA) (Oxoid, Basingstoke, UK) were subsequently stored at
331 -80˚C in nutrient broth containing 10% glycerol, and bacterial species confirmed using MALDI-TOF (Bruker,
332 Coventry, UK).

333 **DNA sequencing.** A subset of isolates were selected for sequencing to represent diversity within the four major
334 genera within each niche, including the use of third-generation cephalosporin resistance as a selective marker to
335 identify a sub-group of multi-drug resistant isolates within each genus. 832 isolates were each sequenced with
336 both a short-read (Illumina HiSeq 4000) and a long-read sequencing approach (four isolates selected for
337 sequencing failed subsequent hybrid assembly and were not included in further analyses). For the first timepoint,
338 the latter involved sequencing using either PacBio SMRT (*n*=192) or Oxford Nanopore Technologies (ONT)
339 methodologies (*n*=127). The results of a pilot study comparing sequencing and assembly approaches using a
340 subset of REHAB isolates[28] were used to inform the choice of ONT as the long-read sequencing approach for all
341 isolates from the second (*n*=255) and third (*n*=254) timepoints.

342 Isolate stocks from -80˚C storage were cultured on to CBA and supplemented with cefpodoxime (Fisher Scientific,
343 USA) 10 µg discs for isolates not sensitive to cefotaxime during original sample isolation. DNA was extracted
344 using the Qiagen Genomic tip/100G (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions.
345 DNA concentration was quantified by Qubit® 2.0 fluorimeter (Invitrogen, UK), and quality and fragment size
346 distribution assessed by TapeStation 2200 (Agilent, Santa Clara, USA). ONT sequencing libraries were prepared
347 by multiplexing 6-8 DNA extracts per flow cell using kits SQK-RBK004, SQK-LSK108 and EXP-NBD103
348 according to the manufacturer's protocol. Libraries were loaded onto flow cell versions FLO-MIN106 R9.4(.1)
349 SpotON and sequenced for 48 h on a GridION (ONT, Oxford, UK).

350 **Genome assembly.** We used the hybrid assembly and sequencing methods described in our pilot study[28] to
351 produce high-quality *Enterobacteriaceae* genomes from short and long reads. In brief, we used Unicycler
352 (v0.4.7)[36] with 'normal' mode, --min_component_size 500, --min_dead_end_size 500, and otherwise default
353 parameters. Final assemblies had a median of four contigs (IQR: 3-8, range: 1-391), with a median of two
354 circularised plasmids (IQR: 1-4, range: 0-14). The majority (616/828, 74.4%) of assemblies had a circularised
355 chromosome, and 558/828 (67.3%) were complete i.e. chromosome and all plasmids circularised (Table S1).

356 **Genome assignment and typing.** We assigned species and sequence type (ST) from assembled genomes using
357 mlst v2.16.4[37]. We also validated species assignments by downloading all NCBI Refseq complete genomes for
358 the four genera under study as of June 4 2020 and using fastANI (v1.3)[38] to compute average nucleotide identity
359 scores against reference genomes for each assembled genome. We took the species assignment of the top hit for
360 each assembled genome. Furthermore, we manually checked genus assignments using a tSNE plot of isolate
361 genomes against a collection of reference genomes (not shown) and made corrections to the assignment if
362 necessary. We used ClermonTyping (v1.4.1)[39] to assign phylogroup to *n*=553 *Escherichia* isolates. Considering
363 the genus *Escherichia*, there were 553 isolates, 410 with circularised chromosomes, and of these 379 were
364 complete genomes containing 961 complete plasmids in total. Considering only *E. coli*, there were 502 *E. coli*
365 isolates, 372 with circularised chromosomes, and of these 348 were complete genomes containing 878 complete
366 plasmids in total. A minority of genomes were *E. fergusonii* (n=51), from clades I-V (*n*=14), or could not be typed
367 (*n*=7), with *n*=481 genomes from within the principal *E. coli* phylogroups (A: *n*=131, B1: *n*=193, B2: *n*=59, C:
368 *n*=11, D: *n*=25, E: *n*=50, F: *n*=6, G: *n*=6).

369 Sequenced isolates from three other *Enterobacteriaceae* genera included: *Citrobacter* (*n*=128: 82 *C. freundii* and
370 46 unassigned *Citrobacter sp.*), *Enterobacter* (*n*=71: 59 *E. cloacae* and 12 unassigned *Enterobacter sp.*); and
371 *Klebsiella* (*n*=76: 40 *K. pneumoniae*, 30 *K. oxytoca,* 2 *K. aerogenes*, and 4 unassigned *Klebsiella sp*.). The majority

372 of farm-associated isolates were *E. coli*, whereas WwTW-associated isolates had roughly equal numbers of genera
373 (Fig. S1). This reflects both the diversity present in each niche and the selection strategy to sequence equal
374 numbers across genera where feasible.

375 **Pangenome analysis.** All genomes were annotated with Prokka (v1.14.0)[40]. Genes were clustered into gene
376 groups using Roary (v3.12.0)[41] across all isolates at various sequence identity thresholds with the maximum
377 number of clusters set to 300,000 (-g 300000) and without splitting paralogs (-s). At a 95% identity for blastp,
378 there were 139,788 gene groups across all genera. Further to this analysis, genes were also clustered at a higher
379 sequence identity (>99% identity threshold) in order to identify recent HGT events, which gave 214,743 gene
380 groups across all genera. For n=616 isolates with circularised chromosomes, we split the genome into
381 chromosomal and plasmid-borne components (i.e. all other contigs) to analyse the genomic location of genes. We
382 excluded isolates without circularised chromosomes from this analysis. For n=488 E. *coli* isolates (excluding *E.*
383 *fergusonii* and clades I-V), we used Panaroo (v0.1.0)[42] to extract a core genome alignment based on 2,915
384 concatenated core genes (Fig. 1c). The phylogeny was produced using iqtree (v1.6.11)[43], with branch lengths not
385 corrected for recombination, and plotted with ggtree (v2.0.1).

386 **Plasmid annotation and clustering.** We searched all plasmids against PLSDB (version: 2020-03-04)[44] which
387 contains 20,668 complete published plasmids, using 'screen' in mash (v2.0)[45] and keeping the top hit. All plasmids
388 had a match apart from 17 small plasmids predicted to be non-mobilizable (median length 4.8 kbp, range 2.9-20.7
389 kbp), from *Escherichia* (n=11), *Enterobacter* (n=2) and *Citrobacter* (n=4). We clustered plasmids using mob
390 cluster and assigned replicon types with mob typer, both part of the MOB suite[46]. Mob cluster uses single linkage
391 clustering with a cutoff of a mash distance of 0.05 (corresponding to 95% ANI), resulting in 611 clusters (Table
392 S2). In total, there were 134 different combinations of replicons observed on plasmids ('replicon haplotypes'). The
393 most abundant replicon was IncFIB (n=460) which was seen across all niches (pig [n=81], cattle [n=113], sheep
394 [n=78], and WwTWs [n=188]). Only nine small multicopy plasmids (~6 kbp) carried AMR genes, all of which
395 had a ColRNAI replicon; such ColRNAI plasmids have been proposed to be sources of evolutionary
396 innovation[47,48].

397 We considered the relationship between such 'distance-free' clustering and plasmid gene content. Based on gene
398 clustering with Roary (see above), we compared the structure of circularised plasmids using all connecting edges
399 between two genes. We defined the resemblance for both gene content (gene presence/absence) and gene structure.
400 The gene content resemblance between two plasmids with n_1 and n_2 genes respectively, with N genes in common,
401 was defined as $r_{content}=2N/(n_1+n_2)$. The edge structure resemblance between two plasmids with g gene-gene edges

402 in common, was defined as $r_{edge}=2g/(n_1+n_2)$. Typically $r_{edge}<r_{content}$ but this definition does allow for the case where
403 repeated genetic elements produce $r_{edge}>r_{content}$ (e.g. Fig. S6b).

404 **Comparison of plasmid-borne and chromosomal pangenome components.** To visualize cross-genera
405 pangenomes (e.g. Fig. 2), we used t-distributed Stochastic Neighbor Embedding (t-SNE). We used the Rtsne
406 function with a perplexity of 30 on gene presence/absence matrices using the Rtsne R package. To conduct
407 permutational analyses of variance, we used the adonis function from the vegan R package on the matrix of
408 pairwise Jaccard distances, which was calculated using the vegdist function. For between-genera analyses, we
409 used the formula *dist~niche*genus*. For within-*Escherichia* analyses, we used the formula *dist~niche*phylogroup*.

410 **Detection of antimicrobial resistance genes and insertion sequences.** We searched assemblies using ABRicate
411 (v0.9.8)[49] for acquired resistance genes (i.e. excluding mutational resistance) in the the NCBI AMRFinder Plus
412 database (PRJNA313047). We used a minimum identity threshold of 90% and a minimum coverage threshold of
413 90% (Table S4). Isolates cultured selectively from cefotaxime-supplemented plates carried more AMR genes than
414 non-selectively cultured isolates (median of 7.5 vs. 1.0), as expected. We also searched for insertion sequences
415 (ISs) using the ISFinder database[50] as a database in ABRicate with the same identity and coverage thresholds
416 (Table S5).

417 **Detection of recent horizontal gene transfer events.** We performed an all-against-all comparison of assemblies
418 with mummer (v3.23-2)[51] using the -maxmatch option to identify shared sequences of length >5,000 bp between
419 genomes of different genera (these could include both transfer of whole plasmids or partial sequences). For
420 comparing the observed distribution of cross-genera HGT events to the expected, we assumed a random
421 distribution drawn from all possible cross-genera comparisons from livestock isolates.

422 **Distribution of insertion sequences.** We constructed the bipartite presence/absence network of ISs and replicon
423 haplotypes for the 34 replicon haplotypes which were observed on 10 or more plasmids. We simulated null models
424 of co-occurrence patterns using the cooc_null_model with null model sim9, which fixes the row and column sums
425 of the presence/absence matrix, in the R package EcoSimR (v0.1.0)[52]. Simulations used n=10,000 iterations with
426 a burn-in of 500 iterations.

427 **Modelling of gene repertoire relatedness (GRR).** We selected a subset of *E. coli* genomes with a circularised
428 chromosome (n=363) and used the core genome tree constructed with iqtree (Fig. 1c, dropping other *E. coli*
429 isolates) to calculate patristic distances between isolates. We calculated chromosomal and plasmid GRR for all

430 pairwise comparisons using output from roary (95% identity threshold, as above) and fit linear models for GRR
431 (Fig. 4).

432 **Data availability.** Sequencing data and assemblies have been uploaded to NCBI under BioProject accession
433 PRJNA605147. Biosample accessions for all isolates are provided in Table S1.

434 **Image credits.** The following images are used in Figure 1: Petri dish icon made by monkik; pig, cow, sheep and
435 faeces icons made by Freepik; WwTW symbol made by Smashicons (all sourced from flaticon.com).

436 **Acknowledgements**

437 The REHAB consortium is represented by the following: Manal Abuoun, Muna F. Anjum, Mark J. Bailey, Howard
438 Brett, Mike J. Bowes, Kevin K. Chau, Derrick W. Crook, Nicola de Maio, Daniel Gilson, Sophie George, H. Soon
439 Gweon, Alasdair T. M. Hubbard, Sarah J. Hoosdally, James Kavanagh, Hannah Jones, William Matlock, Tim E.
440 A. Peto, Daniel S. Read, Robert Sebra, Liam P. Shaw, Anna E. Sheppard, Richard P. Smith, Emma Stubberfield,
441 Nicole Stoesser, Jeremy Swann, A. Sarah Walker, Neil Woodford.

442 **Author contributions**

443 Using the CRediT system, author contributions were as follows: conceptualization (LPS, MJB, DWC, DSR, MFA,
444 ASW, NS), methodology (LPS, ASW, NS), software (LPS, JS), validation (LPS, KKC, JK, MA, ES, LB, GR,
445 ATMH, HP, RS, NS), formal analysis (LPS), investigation (KKC, JK, MA, ES, HSG, LB, GR, MJBo, ATMH, HP,
446 JS, DG, RPS, RS, NS), resources (all authors), data curation (LPS, KKC, JK, MA, ES, LB, GR, ATMH, HP, JS,
447 DG, RPS, RS, NS), writing - original draft (LPS, ASW, NS), writing - review & editing (all authors), visualization
448 LPS), supervision (TEAP, MJBa, DWC, DSR, MFA, ASW, NS), project administration (MA, RPS, SJH, DSR,
449 MFA, ASW, NS), funding acquisition (MJB, DWC, DSR, MFA, NS).

450 **Competing interest declarations**

451 The authors declare no competing interests.

452 **Funding**

453 This work was funded by the Antimicrobial Resistance Cross-council Initiative supported by the seven research
454 councils [grant NE/N019989/1]. Work was also supported by the National Institute for Health Research Health
455 Protection Research Unit (NIHR HPRU) in Healthcare Associated Infections and Antimicrobial Resistance at the
456 University of Oxford (NIHR200915) in partnership with Public Health England (PHE), and by the NIHR Oxford
457 Biomedical Research Centre (BRC). Computation used the Oxford Biomedical Research Computing (BMRC)

facility, a joint development between the Wellcome Centre for Human Genetics and the Big Data Institute supported by Health Data Research UK and the NIHR Oxford Biomedical Research Centre. The computational aspects of this research were funded from the NIHR Oxford BRC with additional support from a Wellcome Trust Core Award Grant [grant 203141/Z/16/Z]. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR, the Department of Health or Public Health England. KCC is Medical Research Foundation-funded. DWC, TEAP and ASW are NIHR Senior Investigators.

References

1. Rasko, D. A. *et al.* The pangenome structure of Escherichia coli: comparative genomic analysis of E. coli commensal and pathogenic isolates. *J. Bacteriol.* **190**, 6881–6893 (2008).
2. Gordienko, E. N., Kazanov, M. D. & Gelfand, M. S. Evolution of pan-genomes of Escherichia coli, Shigella spp., and Salmonella enterica. *J. Bacteriol.* **195**, 2786–2792 (2013).
3. Juhas, M. Horizontal gene transfer in human pathogens. *Crit. Rev. Microbiol.* **41**, 101–108 (2015).
4. Stoesser, N. *et al.* Extensive Within-Host Diversity in Fecally Carried Extended-Spectrum-Beta-Lactamase-Producing Escherichia coli Isolates: Implications for Transmission Analyses. *J. Clin. Microbiol.* **53**, 2122–2131 (2015).
5. Poirel, L. *et al.* Antimicrobial Resistance in Escherichia coli. *Microbiol. Spectr.* **6**, (2018).
6. Jang, J. *et al.* Environmental Escherichia coli: ecology and public health implications-a review. *J. Appl. Microbiol.* **123**, 570–581 (2017).
7. Mahfouz, N. *et al.* High genomic diversity of multi-drug resistant wastewater Escherichia coli. *Sci. Rep.* **8**, 8928 (2018).
8. Decano, A. G. & Downing, T. An Escherichia coli ST131 pangenome atlas reveals population structure and evolution across 4,071 isolates. *Sci. Rep.* **9**, 17394 (2019).
9. Petty, N. K. *et al.* Global dissemination of a multidrug resistant Escherichia coli clone. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 5694–5699 (2014).
10. Leimbach, A., Hacker, J. & Dobrindt, U. E. coli as an all-rounder: the thin line between commensalism and pathogenicity. *Curr. Top. Microbiol. Immunol.* **358**, 3–32 (2013).
11. Logan, L. K. & Weinstein, R. A. The Epidemiology of Carbapenem-Resistant Enterobacteriaceae: The Impact and Evolution of a Global Menace. *J. Infect. Dis.* **215**, S28–S36 (2017).
12. Iredell, J., Brown, J. & Tagg, K. Antibiotic resistance in Enterobacteriaceae: mechanisms and clinical implications. *BMJ* **352**, h6420 (2016).
13. Sheppard, A. E. *et al.* Nested Russian doll-like genetic mobility drives rapid dissemination of the carbapenem resistance gene blaKPC. *Antimicrob. Agents Chemother.* **60**, 3767–3778 (2016).
14. Wang, R. *et al.* The global distribution and spread of the mobilized colistin resistance gene mcr-1. *Nat. Commun.* **9**, 1179 (2018).
15. Skurnik, D. *et al.* Effect of human vicinity on antimicrobial resistance and integrons in animal faecal Escherichia coli. *J. Antimicrob. Chemother.* **57**, 1215–1219 (2006).
16. Nnadozie, C. F. & Odume, O. N. Freshwater environments as reservoirs of antibiotic resistant bacteria and their role in the dissemination of antibiotic resistance genes. *Environ. Pollut. Barking Essex 1987* **254**, 113067 (2019).
17. Woolhouse, M., Ward, M., van Bunnik, B. & Farrar, J. Antimicrobial resistance in humans, livestock and the wider environment. *Philos. Trans. R. Soc. B Biol. Sci.* **370**, 20140083–20140083 (2015).
18. AbuOun, M. *et al.* Characterizing Antimicrobial Resistant Escherichia coli and Associated Risk Factors in a Cross-Sectional Study of Pig Farms in Great Britain. *Front. Microbiol.* **11**, 861 (2020).
19. Wyres, K. L., Lam, M. M. C. & Holt, K. E. Population genomics of Klebsiella pneumoniae. *Nat. Rev. Microbiol.* **18**, 344–359 (2020).
20. Brockhurst, M. A. *et al.* The Ecology and Evolution of Pangenomes. *Curr. Biol.* **29**, R1094–R1103 (2019).

21. Shapiro, B. J. The population genetics of pangenomes. *Nat. Microbiol.* **2**, 1574 (2017).
22. McInerney, J. O., McNally, A. & O'Connell, M. J. Why prokaryotes have pangenomes. *Nat. Microbiol.* **2**, 17040 (2017).
23. Sela, I., Wolf, Y. I. & Koonin, E. V. Theory of prokaryotic genome evolution. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 11399–11407 (2016).
24. Andreani, N. A., Hesse, E. & Vos, M. Prokaryote genome fluidity is dependent on effective population size. *ISME J.* **11**, 1719–1721 (2017).
25. Tenaillon, O., Skurnik, D., Picard, B. & Denamur, E. The population genetics of commensal Escherichia coli. *Nat. Rev. Microbiol.* **8**, 207–217 (2010).
26. MacLean, R. C. & San Millan, A. Microbial Evolution: Towards Resolving the Plasmid Paradox. *Curr. Biol. CB* **25**, R764-767 (2015).
27. George, S. *et al.* Resolving plasmid structures in Enterobacteriaceae using the MinION nanopore sequencer: assessment of MinION and MinION/Illumina hybrid data assembly approaches. *Microb. Genomics* **3**, e000118 (2017).
28. De Maio, N. *et al.* Comparison of long-read sequencing technologies in the hybrid assembly of complex bacterial genomes. *Microb. Genomics* **5**, (2019).
29. Touchon, M. *et al.* Phylogenetic background and habitat drive the genetic diversification of Escherichia coli. *bioRxiv* 2020.02.12.945709 (2020) doi:10.1101/2020.02.12.945709.
30. San Millan, A., Heilbron, K. & MacLean, R. C. Positive epistasis between co-infecting plasmids promotes plasmid survival in bacterial populations. *ISME J.* **8**, 601–612 (2014).
31. Harmer, C. J. & Hall, R. M. IS26 Family Members IS257 and IS1216 Also Form Cointegrates by Copy-In and Targeted Conservative Routes. *mSphere* **5**, (2020).
32. Zhi, S. *et al.* Evidence of Naturalized Stress-Tolerant Strains of Escherichia coli in Municipal Wastewater Treatment Plants. *Appl. Environ. Microbiol.* **82**, 5505–5518 (2016).
33. AbuOun, M. *et al.* Genomic epidemiology of antimicrobial resistance genes and gene contexts in Enterobacteriaceae in livestock farms in Central England: associations with host species and antimicrobial usage. *Forthcom. Prep.*
34. Edwards, K. J., Logan, J. M. J., Langham, S., Swift, C. & Gharbia, S. E. Utility of real-time amplification of selected 16S rRNA gene sequences as a tool for detection and identification of microbial signatures directly from clinical samples. *J. Med. Microbiol.* **61**, 645–652 (2012).
35. Daniel S. Read *et al.* Wastewater treatment, season and effluent receiving freshwaters drive the environmental dissemination of antimicrobial resistance. *Forthcom. Prep.*
36. Wick, R. R., Judd, L. M., Gorrie, C. L. & Holt, K. E. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput. Biol.* **13**, e1005595 (2017).
37. Seemann, T. *mlst*.
38. Jain, C., Rodriguez-R, L. M., Phillippy, A. M., Konstantinidis, K. T. & Aluru, S. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat. Commun.* **9**, 5114 (2018).
39. Beghain, J., Bridier-Nahmias, A., Le Nagard, H., Denamur, E. & Clermont, O. ClermonTyping: an easy-to-use and accurate in silico method for Escherichia genus strain phylotyping. *Microb. Genomics* **4**, (2018).
40. Seemann, T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **30**, 2068–9 (2014).
41. Page, A. J. *et al.* Roary: Rapid large-scale prokaryote pan genome analysis. *Bioinformatics* **31**, btv421 (2015).
42. Tonkin-Hill, G. *et al.* Producing Polished Prokaryotic Pangenomes with the Panaroo Pipeline. *bioRxiv* 2020.01.28.922989 (2020) doi:10.1101/2020.01.28.922989.
43. Nguyen, L.-T., Schmidt, H. A., von Haeseler, A. & Minh, B. Q. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* **32**, 268–274 (2015).
44. Galata, V., Fehlmann, T., Backes, C. & Keller, A. PLSDB: a resource of complete bacterial plasmids. *Nucleic Acids Res.* **47**, D195–D202 (2019).
45. Ondov, B. D. *et al.* Mash Screen: High-throughput sequence containment estimation for genome discovery. *bioRxiv* 557314 (2019) doi:10.1101/557314.
46. Robertson, J. & Nash, J. H. E. MOB-suite: software tools for clustering, reconstruction and typing of plasmids from draft assemblies. *Microb. Genomics* **4**, (2018).

47. Rodriguez-Beltran, J. *et al.* Multicopy plasmids allow bacteria to escape from fitness trade-offs during evolutionary innovation. *Nat. Ecol. Evol.* **2**, 873–881 (2018).
48. San Millan, A., Escudero, J. A., Gifford, D. R., Mazel, D. & MacLean, R. C. Multicopy plasmids potentiate the evolution of antibiotic resistance in bacteria. *Nat. Ecol. Evol.* **1**, 0010 (2017).
49. Seemann, T. *Abricate*. (2020).
50. Siguier, P., Perochon, J., Lestrade, L., Mahillon, J. & Chandler, M. ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res.* **34**, D32–D36 (2006).
51. Kurtz, S. *et al.* Versatile and open software for comparing large genomes. *Genome Biol.* **5**, R12 (2004).
52. Nicholas J. Gotelli, Edmund M. Hart & Aaron M. Ellison. *EcoSimR: Null model analysis for ecological data. R package version 0.1.0.* (2015).