Plasmids shape the diverse accessory resistomes of *Escherichia coli* ST131

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Abstract: The global problem of antimicrobial resistance (AMR) is complicated by the mix of beneficial, commensal and pathogenic bacteria with AMR genes that exchange them most commonly by plasmid conjugation. *Escherichia coli* is a significant component of the gastrointestinal microbiome, but is typically non-pathogenic in this niche. In contrast, extra-intestinal pathogenic *E. coli* including ST131 may occupy other environments like the urinary tract or bloodstream where they express genes enabling AMR and host adhesion. The extent to which uropathogenic *E. coli* ST131 Clade C shares AMR genes remains understudied at a genomic level, and here we examined this using a preterm infant resistome. Individual ST131 isolates had extensive AMR gene diversity even though there was a large shared core resistome. Comparisons with a range of reference plasmids common in ST131 showed this AMR gene composition was driven by conjugation, recombination and mobile genetic elements, highlighting that analyses restricted to the core genome could miss AMR gene transfer patterns. We found that a large portion of plasmid pEK499 were ancestral to most ST131 Clade C isolates, and had evidence of a co-evolutionary signal with chromosomal genes, as did plasmid pEK204. A type IV fimbrial operon encoded on pEK204 was rare in Clade C but illustrated the scope for plasmids to mediate host adhesion abilities in addition to AMR gene content. More generally, ST131 possessed extensive genetic diversity at key type 1, type IV, P and F17-like fimbriae genes, and that this variability in fimbrial operons was highest in subclade C2. The type, structure and composition of AMR genes, plasmids and fimbriae vary widely in ST131 Clade C and this may mediate pathogenicity and infection outcomes.

Data Summary:
The following files are available on the FigShare project "Plasmids_ST131_resistome_2020":
1. The set of 794 AMR genes derived from [74] are (with their protein sequence translation) at FigShare doi: 10.6084/m9.figshare.11961402.
2. The AMR gene profiles per sample determined by their BLAST results against CARD are at FigShare (doi: 10.6084/m9.figshare.11961612). This includes the PlasmidFinder results, and other AMR database comparisons (ARG-ANNOT, ResFinder, MegaRes, VFDB and VirulenceFinder).
3. The BLAST sequence similarity results for the *fim*, *pil*, *pap* and *ucl* operons' genes versus 4,071 *E. coli* ST131 assemblies from Decano & Downing (2019) are at FigShare doi: 10.6084/m9.figshare.11961711.
4. The genome sequences and annotation files for reference genomes NCTC13441, EC958 and SE15, and the assembled contigs for 83972 are at FigShare doi: 10.6084/m9.figshare.11961813.
5. The 4,071 *E. coli* ST131 genome assemblies from Decano & Downing (2019) are at FigShare doi: 10.6084/m9.figshare.11962278 (the first 1,680 assemblies) and doi: 10.6084/m9.figshare.11962557 (the second 2,391 assemblies).

Introduction

Extra-intestinal pathogenic *E. coli* (ExPEC) cause extensive infections outside the gut, from which they can originate. ExPEC have a wide range of virulence factor (VF) [1-4] and antimicrobial resistance (AMR) genes [5], especially sequence type (ST) 131 from serotypes O25b:H4 and O16:H5 from *E. coli* phylogroup B2 [6]. ST131 causes a substantial minority of ExPEC cases, and possibly a majority of extended-spectrum beta-lactamase (ESBL)-producing ones [7]. ST131's acquisition of key AMR genes encoded on plasmids [4,8] has coincided with its adaptation to new environments [9,10].

E. coli colonises host niches using adherence factors that are mainly produced by the Chaperone-Usher secretion pathway: type 1, P, F1C/S and AFA fimbriae [11-14]. ST131's fluoroquinolone-resistant Clade C is the main cause of human infection globally [15-16], and its D-mannose-specific type 1 fimbriae encoded by the *fim* operon can bind the host mucosal epithelium to cause urinary tract infections (UTIs) and kidney infections [18-21]. Although the genetic diversity of *fim* in Clade C has been examined extensively, fimbriae beyond *fim* has not been examined in large collections of isolates.

E. coli is the most common initial coloniser of infant intestines [22] and most *E. coli* are commensal [23] such that some even protect against pathogen invasion [24]. However, *E. coli* infection can be associated with an imbalance in microbiome composition [25] and because within-host gene exchange between commensal and pathogenic bacteria may be common [26], AMR in neonatal units is a significant concern [27]. ST131 spreads between mother-infant pairs [28] via an oro-faecal route [29], and colonisation of infants can last for long periods [30], just like many long-term carriage in adult UTIs . Consequently, AMR gene profiling can inform on pathogen treatment strategies [31] and the effect of antibiotics on microbiome compositions [32].

AMR in ST131 is chiefly driven by ESBL genes [33-34] allowing third-generation cephalosporin-resistance [35], of which the most common are cefotaximase (CTX-M) resistance alleles $bla_{\text{CTX-M-14/15/27}}$ [36]. Like many AMR genes, these are usually on plasmids and sandwiched by mobile genetic elements (MGEs) and may be moved, amplified [37-39] or lost [40-41] by horizontal gene transfer (HGT) [37-39]. Among common bacterial pathogens, *E. coli* has the highest MGE numbers and high levels of transposases and phage-associated MGEs that elevate HGT rates [42]. Diverse pathogenic bacteria can share MGEs [43-45] and HGT makes nosocomial outbreaks worse [46-48]. Most plasmids common to ST131 have IS*Ecp1*, IS*903D* and IS*26* elements [49] typically adjacent to ESBL genes that can be transferred within plasmids, to other plasmids, or to the chromosome [50].

Fluctuating antibiotic exposure, host type, anatomical niche and immune responses mean that the resistome within isolates varies and this is shaped by conjugation, recombination and MGE-mediated rearrangements [1,10,51-55]. ST131's plasmids typically are from incompatibility (Inc) groups F, I1, N and A/C [56-58] and undergo extensive HGT and mixing with other plasmids [58-60] to the extent that plasmids from ST131 vary extensively within a clonal radiation [61-62]. Plasmids can also impair cell reproduction due to energetic costs, so conjugation and rearrangements could allow for optimisation of gene dosage and coordinated gene expression.

There is extensive evidence of AMR gene conjugation in the gut [63-66] including between *E. coli* STs: transfer of a $bla_{\text{CTX-M-1}}$-positive IncI1 plasmid among ST1640, ST2144 and ST6331 in a patient's gut [67]; a $bla_{\text{OXA-48}}$ gene on a *Klebsiella pneumoniae* IncL/M-type plasmid from ST14 to ST666 [68] and from ST648 to ST866 [69]; an IncF plasmid with $bla_{\text{KPC-2}}$, $bla_{\text{OXA-9}}$ and $bla_{\text{TEM-1A}}$ genes from ST69 to ST131 [70]; and transfer of a range of sulphonamide- (*sul2*) and ampicillin-resistance genes ($bla_{\text{TEM-1b}}$) on a pNK29 plasmid [71-72]. Moreover, the frequency of conjugation was ten times higher and more stable in $bla_{\text{CTX-M-15}}$-producing ST131 than in *K. pneumoniae* [73].

This study focused on uropathogenic ST131 Clade C (O25b:H4, *fimH30*) because recent work has found high plasmid and AMR gene diversity in this Clade [50,61]. We differentiated between a large shared core resistome from a smaller accessory one that varied between closely related isolates. This was caused by distinct events of plasmid turnover and rearrangement, affecting fimbrial adhesion

operons as well. We hypothesise that certain plasmids are more compatible with ST131 genomes, and applied topological data analysis (TDA) to understand plasmid-chromosome co-evolution.

Results

A shared core and diverse accessory preterm infant resistome in *E. coli*

We examined the resistome and plasmid composition of seven *E. coli* ST131 Clade C genome assemblies from UTIs in the context of a reference resistome of 794 genes associated with antibiotic resistance in preterm infants [74] to overcome inconsistencies in existing AMR gene databases (see Methods). This resistome came from 401 stool samples longitudinally collected from 84 infants undergoing antibiotic treatment, and was assembled as 2,004 redundant contigs experimentally tested *in vitro* for resistance to 16 antibiotics (Table S1) [74]. The seven Clade C assemblies and subclade C2 reference genomes EC958 and NCTC13441 had 262 to 291 AMR contigs from this resistome [74] (Table 1). Commensal ST131 Clade A reference SE15 (O150:H5, *fimH41*) was here had 251 AMR contigs. In contrast, asymptomatic urinary tract sample 83972 from ST73 [75] had just 213 unique AMR contigs. This indicated a distinct subset of AMR genes were associated with ST131 Clade C that cause symptomatic UTIs.

ID	SRA ID	Clade	$bla_{\text{CTX-M}}$ allele(s)	Country & year	AMR contigs	
					Total	Unique
8289_1#91	ERR191724	C1	14	Ireland, 2007	268	0
8289_1#35	ERR191668	C1	14	Ireland, 2008	270	0
8289_1#3	ERR191636	C2	15	Ireland, 2005	274	0
8289_1#34	ERR191667	C2	15	Ireland, 2010	274	9
8289_1#24	ERR191657	C2	14 & 15	Ireland, 2009	283	0
8289_1#60	ERR191693	C2	15	Ireland, 2010	262	0
8289_1#27	ERR191660	C2	15	Ireland, 2010	262	0
SE15	AP009378	A	none	Japan, 2010	251	0
NCTC13441	ERS530440	C2	15	UK, 2003	280	0
EC958	HG941718	C2	15	UK, 2005	291	17
83972	-	ST73	none	Sweden, 1978	213	2

Table 1. *E. coli* genome assembly collection and metadata. The table shows the sample ID, SRA accession ID, ST131 Clade, $bla_{\text{CTX-M}}$ allele(s), year of isolation, and numbers of total and unique AMR contigs (Figure 1). All seven ST131 assemblies, EC958 and NCTC13441 were isolated from pathogenic UTIs. The seven ST131 and 83972 were assembled from short Illumina reads. EC958, NCTC13441 and SE15 were assembled from long PacBio reads.

Comparing the AMR contigs' gene product functions identified a core (intrinsic) resistome of 210 AMR contigs shared by all (Figure 1). This core resistome differed from the accessory (acquired) resistome of 109 contigs, of which 50 corresponding to 19 unique AMR genes in ST131 Clade C only (Figure 1). These accessory AMR genes were initially identified with ARIBA v2.11.1 [76] and similarity searches against the Comprehensive Antibiotic Resistance Database (CARD) [77], and candidate matches were verified by mapping the reads to the reference resistome using GROOT [78].

Ten AMR contigs in all C2 isolates corresponded to a *marR* gene (Multiple Antibiotic Resistance Regulator) that regulates antibiotic-stress activated transcription factor (TF) MarA and was associated with resistance to multiple antibiotics in the reference resistome [74]. All subclade C2 isolates except 8289_1#27 had five bla_{TEM} alleles (TEM-57, 104, 116, 215, 220) encoding an Ambler class A β-lactamase (Figure 1). Nine bla_{CMY} variants encoding ESBL AmpC (alleles CMY-37, 51, 66, 67, 85,

98, 101, 105) associated with cephalosporin-resistance [79] were resolved from 17 initial AMR contig matches unique to EC958 (Figure 1). The sole $bla_{CTX\text{-}M\text{-}14}$- and $bla_{CTX\text{-}M\text{-}15}$-positive isolate (8289_1#24) had nine contigs corresponding to unique two aminoglycoside resistance genes and two tetracycline resistance genes (Table S2), a combination that may neutralise the effect of aminoglycoside susceptibility associated with tetracycline efflux pump TetA expression [80-81]. 41 contigs in all ST131 included 28 copies of *mdfA* encoding a multi-drug efflux pump linked to broad spectrum AMR [82]. 83972 shared with the Clade C isolates only a *ftsI* gene encoding a transpeptidase catalysing peptidoglycan crosslinking [83] that is also called penicillin-binding protein 3 (PBP3) [84-85] that can be inactivated by ESBLs [86].

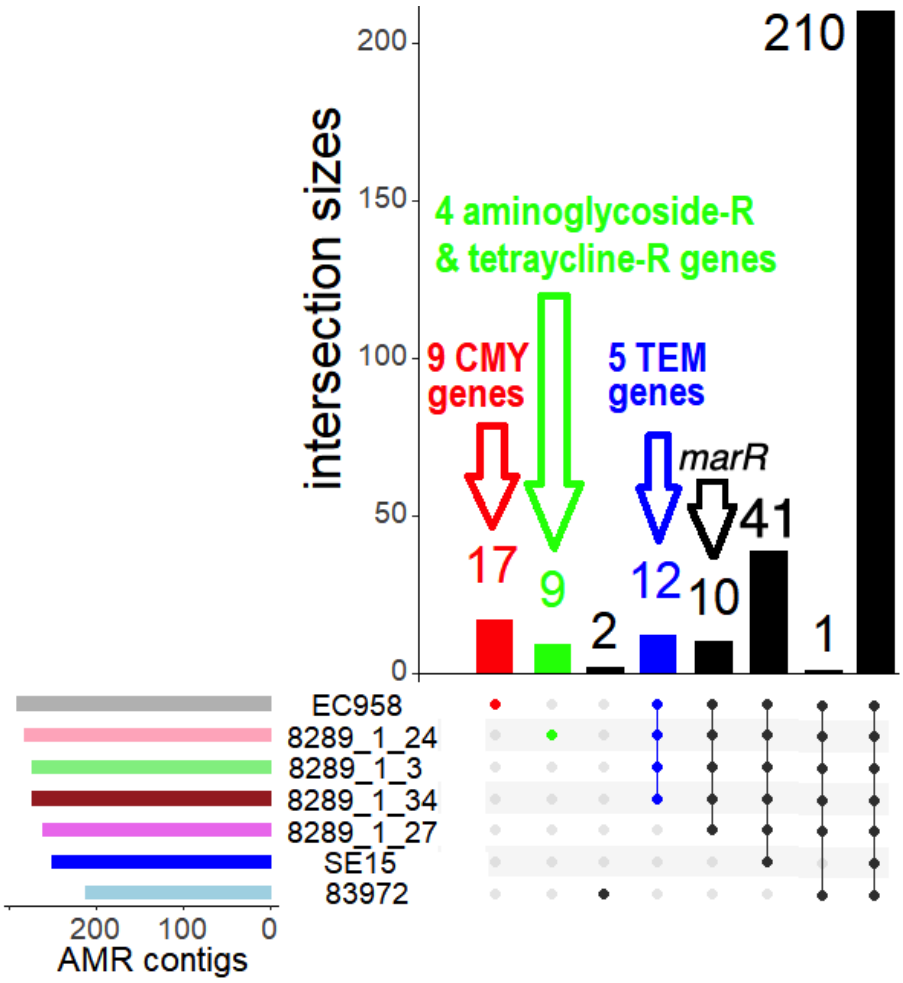

Figure 1. The overlap of preterm infant AMR contigs sharing across four ST131 subclade C2 genome assemblies (8289_1#3, 8289_1#24, 8289_1#27, 8289_1#34), reference EC958 from C2, reference SE15 from Clade A, and asymptomatic sample 83972. Top: The intersection sizes (y-axis) and the numbers of AMR contigs per set showed 210 AMR contigs were shared by all. The AMR contigs were redundant, so EC958's 17 AMR contigs (red) corresponded to nine unique bla_{CMY} genes, and 8289_1#24's nine contigs (green) had two aminoglycoside and two tetracycline resistance genes. All C2 had ten contigs with *marR*, and all C2 bar 8289_1#27 had 12 contigs (blue) denoting five bla_{TEM} genes. Bottom: The numbers of AMR contigs per sample with the corresponding sets shown by black circles. 8289_1#60 (not shown) had the same results as 8289_1#27.

Extensive plasmid rearrangements in closely related ST131 Clade C genomes

Initial tests of Inc groups in these ST131 Clade C assemblies using PlasmidFinder [94] showed that all

(Table S3). To explore relationships of the AMR genes in these Clade C samples (Table 1), we aligned their assemblies and mapped their reads to plasmids common to ST131 (pEK499, pEK516, pEK204, pCA14, pV130). Plasmids pEK499 (isolated in England), pEK516 (England) and pEK204 (Belfast) were geographically near the seven ST131 from Ireland. We used SE15's plasmid with no AMR genes as a control [87]. 8289_1#35 and 8289_1#91 (both from C1) had identical results, and *vice versa* for 8289_1#60 and 8289_1#27 (both from C2), so they are not shown below.

Clade C had more regions similar to IncF2/F1A $bla_{\text{CTX-M-15}}$-positive plasmid pEK499 than SE15, but with extensive variation (Figure 2). This plasmid lacks *traX* for conjugation but has stable inheritance (Table S4), and was gained early in the emergence of Clade C [88]. Nine of its ten key AMR genes including bla_{TEM}, $bla_{\text{OXA-1}}$ and $bla_{\text{CTX-M-15}}$ are in a 25 Kb segment at 39-64 Kb. In C2, 8289_1#3 and 8289_1#24 had all three genes, 8289_1#27, 8289_1#60 and 8289_1#34 had $bla_{\text{OXA-1}}$ and $bla_{\text{CTX-M-15}}$ but not bla_{TEM}, whereas the C1 genomes (8289_1#35 and 8289_1#91) were bla_{TEM}- and $bla_{\text{CTX-M-14}}$-positive. Conjugation (*tra*) genes were in all ST131 except 8289_1#34. This result was verified by read mapping to IncF2A plasmid pEK516 (Figure S1) that has ~75% similarity to pEK499 [59], is usually non-conjugative [89] and (unlike pEK499) has a type I partitioning locus (Table S4). The ST131 plasmid-matching regions also showed variable similarity when aligned to conjugative plasmid pCA14, where all subclade C2 bar 8289_1#3 had genes associated with macrolide resistance (Figure S2), and 8289_1#27 and 8289_1#60 had similarity spanning all of the non-conjugative plasmid pV130a contigs from sewage treatment wastewater (Figure S3) [93].

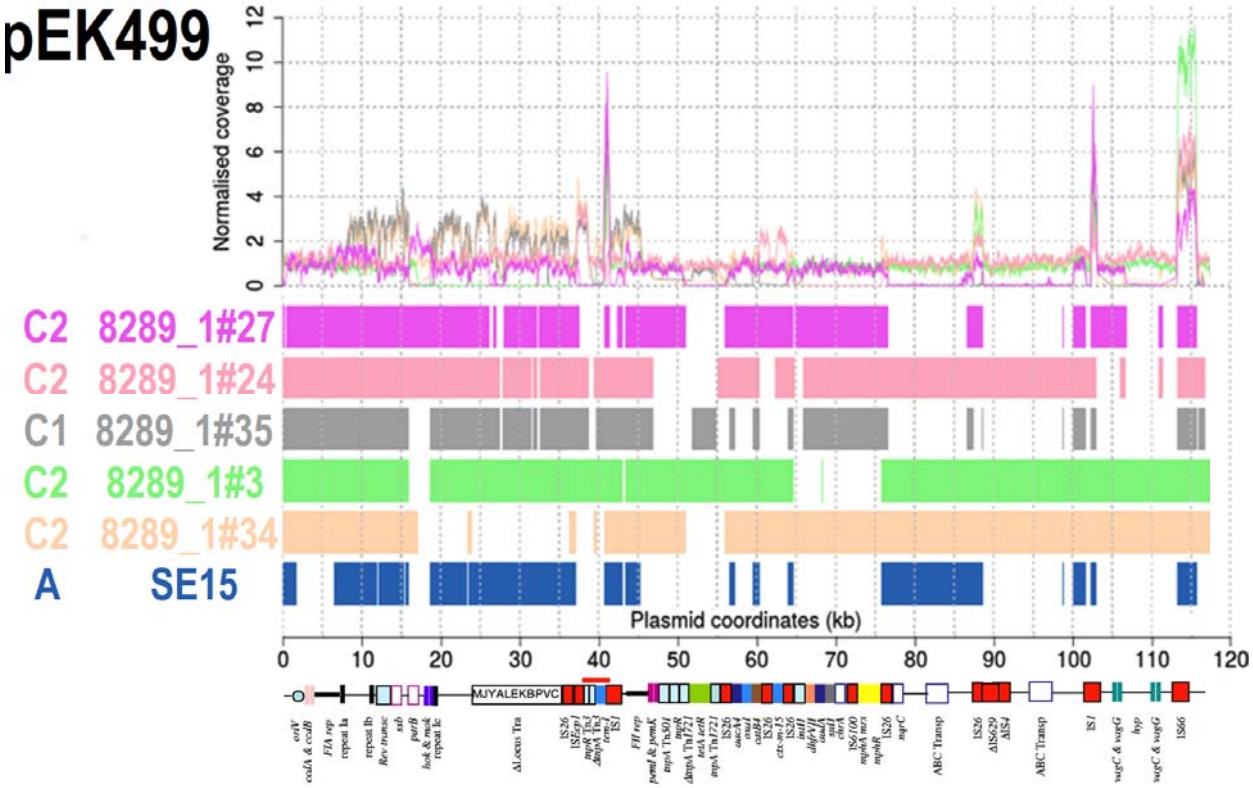

Figure 2. Comparison of pEK499 (117,536 bp) with five ST131 subclade C1 and C2 isolates and Clade A reference SE15 (navy). Top: Normalised read coverage showed high copy numbers of IS*1* (at 41 and 103 Kb) and IS*66* (at 113 Kb) elements. Bottom: BLAST alignments showed limited similarity for SE15 relative to high levels for Clade C assemblies: 8289_1#27 (C2, mauve), 8289_1#24 (C2, pink), 8289_1#35 (C1, grey), 8289_1#3 (C2, light green) and 8289_1#34 (C2, beige). Genes encoding bla_{TEM}, $bla_{\text{OXA-1}}$ and $bla_{\text{CTX-M-15}}$ were at 40, 58 and 63 Kb (respectively). The annotation was modified from [59]. Matches spanning >300 bp are shown.

Contrasting sharply with pEK499 and pEK516, reads mapped to conjugative IncI1 plasmid pEK204 found 72.8 Kb regions of similarity in 8289_1#27 (C2) where the other isolates had <10 Kb of total matching regions (Figure 3). Plasmid pEK204 is similar to IncI1 plasmid R64 and has a complete *tra* region (Table S4) [59]. 8289_1#27 had a complete I1 replicon, *oriT*, *tra* and a *pil* cluster encoding a type IV fimbriae associated with enhanced cell adherence and biofilm formation in entero-aggregative and Shiga toxin-producing *E. coli* [90]. *ImpB* and *impA* encode an error-prone DNA repair protein [91]: an additional IRR at *impB* can mobilise a 9.3 Kb unit containing $bla_{\text{TEM-1b}}$ and $bla_{\text{CTX-M}}$ [92].

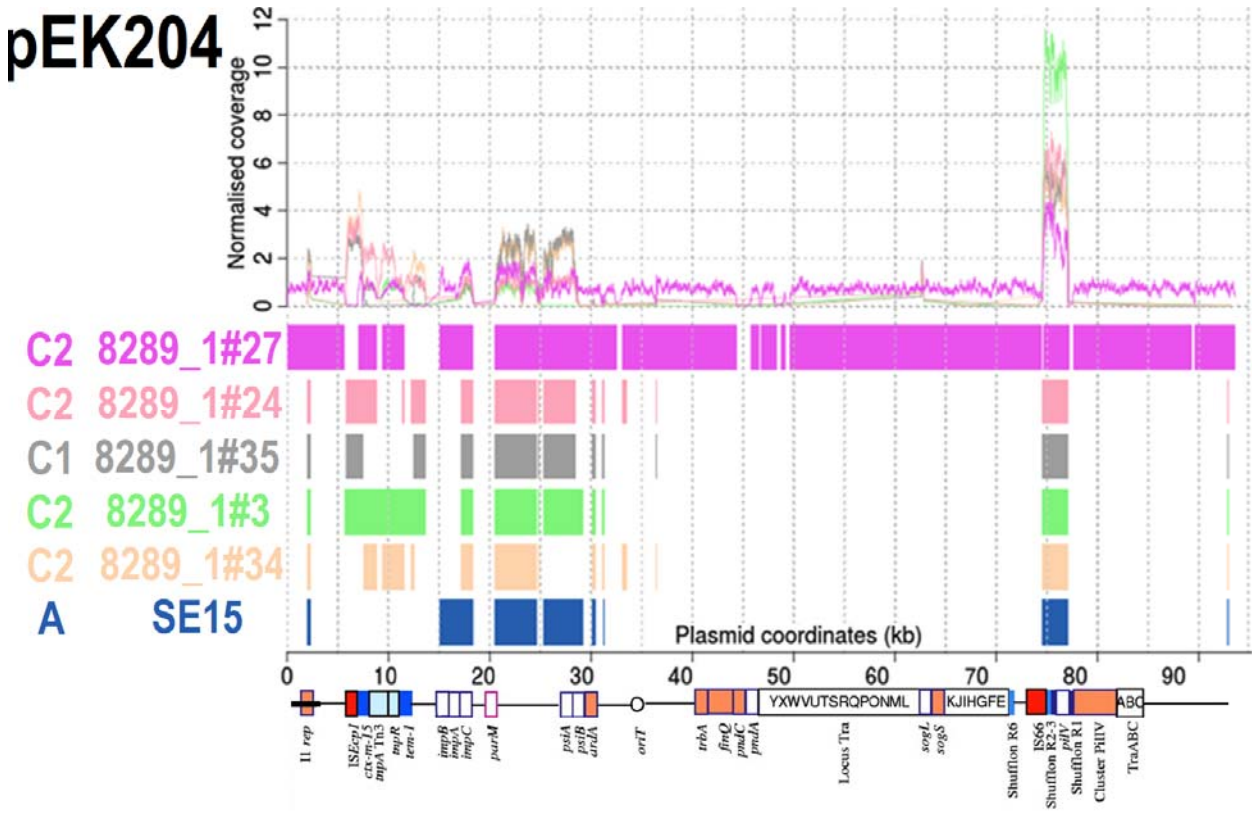

Figure 3. Comparison of pEK204 (93,732 bp) with five ST131 subclade C1 and C2 isolates and Clade A reference SE15 (navy). Top: Normalised read coverage showed high copy numbers at 75-77 Kb encoding an IS*66* and unannotated genes. Bottom: BLAST alignments showed limited similarity for SE15 and Clade C assemblies: 8289_1#24 (C2, pink), 8289_1#35 (C1, grey), 8289_1#3 (C2, light green) and 8289_1#34 (C2, beige). In contrast, 8289_1#27 (C2, mauve) had many regions similar to pEK204. The $bla_{\text{CTX-M-3}}$ gene typically associated with pEK204 differs from $bla_{\text{CTX-M-15}}$ by a R240G substitution, and so the $bla_{\text{CTX-M}}$ genes detected for samples here were $bla_{\text{CTX-M-15}}$ (not $bla_{\text{CTX-M-3}}$). Matches spanning >300 bp are shown. The annotation was modified from [59].

High rates of pEK499 and pEK204 protein-protein interactions with chromosomal *E. coli* proteins

The extensive diversity of Clade C AMR genes and plasmids raised the question of how their plasmids' gene products interact with chromosomally encoded ones. Protein-protein interaction (PPI) networks can examine plasmid-chromosome coevolution based on gene products' topological proximity [95-96], which should be higher for plasmid and chromosomal proteins that have co-existed for longer and so interact more often [97]. We used topological data analysis (TDA) to measure the number of non-trivial (indirect) loops where a loop is a chain of at least four PPIs ending at the same protein where it has started. We focus on a type of loops called "non-trivial" (see Methods). The number of non-trivial loops was scaled by the number of PPIs per dataset (Table 2).

PPI data for 4,146 *E. coli* protein-coding genes with 105,457 PPIs (all combined scores >400) from the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database v10 [98] was processed to get the numbers of non-redundant PPIs and loops per PPI for a plasmid's proteins among themselves, and then between the plasmid's proteins and all 4,146 chromosomal *E. coli* proteins. This included assessing the numbers of PPIs up to a combined score threshold of 900 with a step size of 25. We tested our approach using a published *E. coli* clustering of 60 genes [99] that had a rate of 0.244 non-trivial loops per PPI, which was lower than the rate of 0.518 obtained for all 4,146 chromosomal proteins (Table S5, Figure S4). There were lower rates of non-trivial loops per PPI for pEK499 (0.117) and pEK204 (0.096) compared to the chromosomal rate (0.518), but the plasmids' rates were almost constant across the range of combined score thresholds used (Figure S5). This held even though the number of PPIs per protein was negatively correlated with the combined score (Figure S6), suggesting that the rate of non-trivial loops per PPI was a robust measure of connectivity. Whereas pEK499 and pEK204 interacted with chromosomally encoded proteins, pCA14, pEC958A and pECSF1 did not (Table S6). Plasmid pJIE186-2 did have a small number of PPIs (Table 2) that may be VFs [100].

Plasmid	Number of genes	Number of unique genes	Length (Kb)	Number of unique PPIs		Non-trivial loops per PPI
				Within plasmid	With chromosome	
pEK204	112	87	93.7	21	548	0.096
pEK499	185	87	117.5	8	758	0.117
pEK516	103	55	64.6	8	758	0.117
pJIE186-2	138	78	137.7	2	32	0.015
pCA14	181	93	155.4	5	0	0
pEC958A	142	75	135.6	5	0	0
pECSF1	150	70	122.3	0	0	0

Table 2. The numbers of protein-coding genes per plasmid, unique genes per plasmid, length, and numbers of non-redundant PPIs within the plasmid and with the chromosome, and the rate of indirect interactions (non-trivial loops) per PPI. Plasmid pEK516 had the same PPI results as pEK499.

ST131 genomes had an ancestral pEK499-like plasmid but some gained a pEK204-like one

The seven ST131 Clade C genome assemblies above were from a set of 4,071 assemblies [61], of which 837 (20.6%) had >10 Kb of regions similar to pEK204 (Figure S7) based on BLAST alignments, but 3,108 (76.3%) with >10 Kb matching pEK499 (Figure S8). 193 assemblies had >40 Kb of pEK204-like segments, all from Clade C: 17 from C0 (out of 52, 33%), 82 from C1 (out of 1,119, 7%) and 94 from C2 (out of 2,051, 5%) (Figure 4). All 17 from C0 had an I1 replicon, $bla_{\text{CTX-M}}$ gene, bla_{TEM} gene (bar one) and at least a partial *tra* region, and 13 had the *pil* operon (76%). The 82 from C1 and 94 from C2 had lower rates of I1 replicon presence (C1 77%, C2 81%), partial *tra* regions (C1 94%, C2 86%) and *pil* clusters (C1 n=60 or 73%, C2 n=71 or 76%) but differed in the rates of gene presence for $bla_{\text{CTX-M}}$ (C1 28% vs C2 83%, odds ratio = 12.5, 95% CI 6.1-25.8, p=e-11) and bla_{TEM} (C1 59% vs C2 30%, odds ratio = 3.3, 95% CI 1.8-6.2, p=2.2e-5). This implied $bla_{\text{CTX-M}}$ (via *orf477*) was common in C2 and bla_{TEM} (via *impB*) in C1 due to different ancestral transposition of the 9.3 Kb region on pEK204.

To resolve the origin of pEK204-related *pil* HGT, we searched individually for the 14 *pil* operon genes *pilIJKMNOPQRSTUV* in the 4,071 ST131 assemblies [61]. These genes were conserved as a unit in 376 isolates (9%), including 61 from A (out of 414, 15%), 97 from B (out of 433, 22%), 17 from C0 (out of 52, 33%), 95 from C1 (out of 1,121, 8.5%) and 106 from C2 (out of 2,051, 5.2%) (Table 3) (see Data Access). Tallying this with the pEK204 matching patterns indicated potential for non-pEK204 *pil* gain in Clades A and B, the putative Clade C ancestor may have had a pEK204-like plasmid with no *pil* region, which was sometimes subsequently gained by recombination.

Given the *fim* operon's key role in ST131 evolution, we verified that *fim* was present intact in >99% of the 4,071. As expected, the IS*Ec55* insertion at *fimB* [101] that delays *fim* expression in Clade C isolates like EC958 [20] was nearly fixed in C1 (1,117, 99.6%) and C2 (2,041, 99.5%), but was rarer in A (236, 57%), B (93, 21%) and C0 (18, 35%) - the remainder had intact genes.

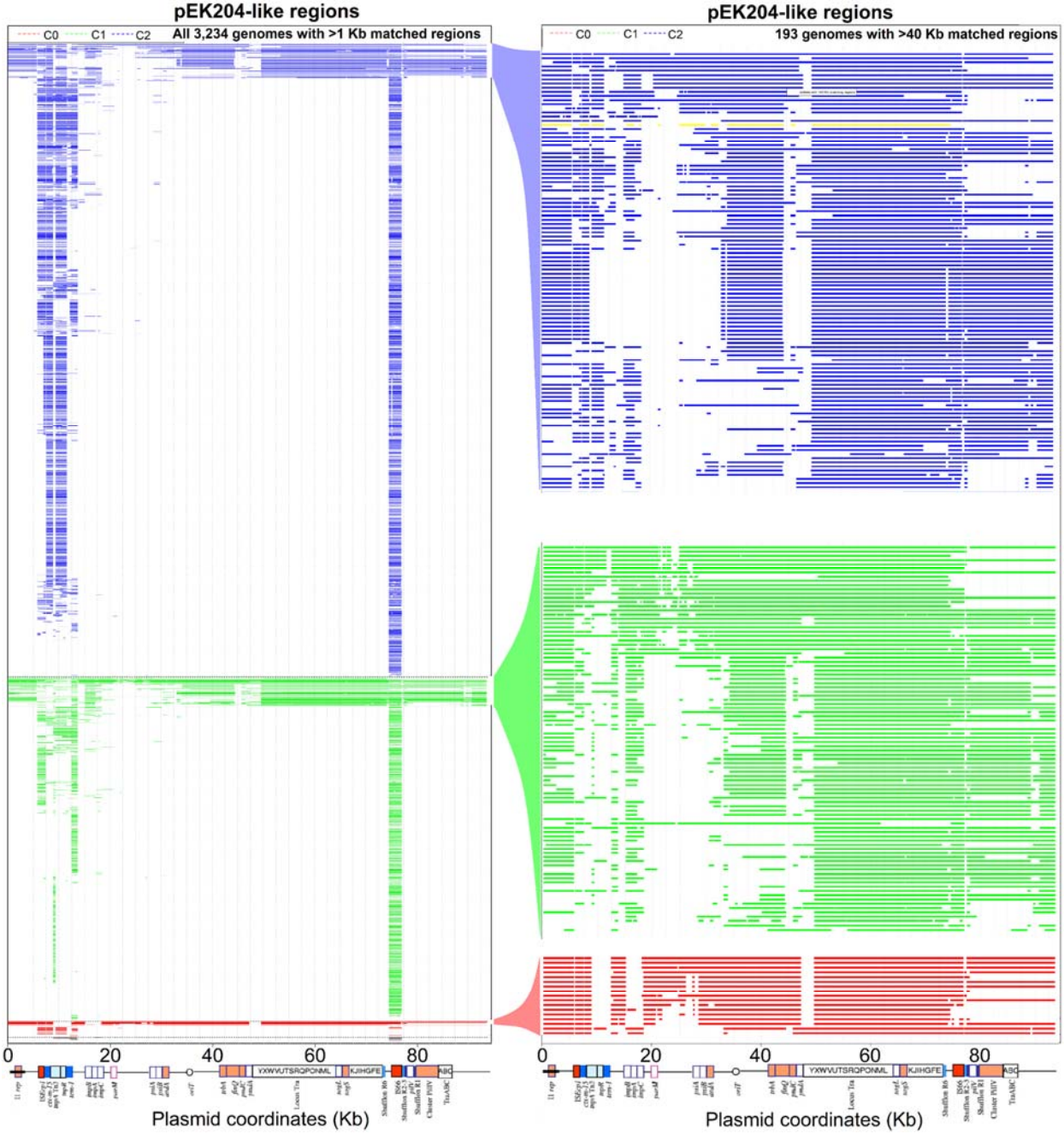

Figure 4. The distributions of pEK204-like regions in ST131 Clade C genome assemblies with >1 Kb (left, n=3,234) and >40 Kb (right, n=193) of matching segments. The regions of similarity were examined in (left) Subclades B0 (n=14 in black), C0 (n=51 in red), C1 (n=1,119 in green) and C2 (n=2,051 in blue), and (right) C0 (n=17 in red), C1 (n=82 in green) and C2 (n=94 in blue). 8289_1#27 (C2) is highlighted in yellow. Similarity across pEK204 (93,732 bp) was based on BLAST alignment. The $bla_{\text{CTX-M}}$ gene was at 8 Kb, bla_{TEM} was at 13 Kb, followed by a mix of conjugation and segregation genes at 36-70 Kb. This suggested independent integrations and rearrangements of pEK204-like plasmids across the subclades. The annotation was modified from [59].

Operon			Subclade										
pil	***pap***	***ucl***	**A**	**%**	**B**	**%**	**C0**	**%**	**C1**	**%**	**C2**	**%**	**Totals**
-	*papIBA*	-	279	67%	107	25%	3	6%	772	69%	1,192	58%	2,353
-	all	all	21	5%	13	3%	23	44%	82	7%	481	24%	620
all	*papIBA*	-	57	14%	2	<1%			75	7%	66	3%	200
-	all	-	1	<1%	94	22%			8	<1%	14	<1%	117
all	-	-	3	<1%	71	16%			5	<1%	3	<1%	82
all	all	all			16	4%	1	2%	10	<1%	33	2%	60
all	all	-	1	<1%	6	1%	16	31%			4	<1%	27
all	-	all							1	<1%			1
-	*papIBA*	all									1	<1%	1
all	*papIBA*	all											0
-	-	all											0
Partial matches			52	13%	124	29%	9	17%	168	15%	257	13%	610
Totals			414		433		52		1,121		2,051		4,071

Table 3. *Fim*, *pil*, *pap* and *ucl* operon genes detected in 4,071 ST131 genome assemblies. The numbers and percentages of isolates per subclade with complete or absent (-) *pil* (*pilK-pilV*) or *pap* or *ucl* (*uclABCD*) operon genes where a subset also had three *pap* genes (*papIBA*). This focused on isolates allocated to the eleven main allelic options, isolates with ssome level of partial operon presence or absence were too sparse to be informative. *UclBCD* but not *uclA* was in an additional 2% (n=77), including seven from A (2%), 28 from B (6%), zero from C0, 24 from C1 (2%) and 18 from C2 (1%).

We extended this to two other important fimbrial operons: *pap* (pyelonephritis-associated pili) and *ucl* (uroepithelial cell adhesin-like). All four *ucl* genes were in 17.5% (n=712) of isolates, including 21 from A (5%), 30 from B (7%), 25 from C0 (48%), 94 from C1 (8%) and notably 542 from C2 (26%) (Table 3). All the *ucl*-positive C2 genomes had a complete *pap* operon (with a single exception). The *pap* operon was in a minority (26%, n=1,049) of isolates, including 28 from A (7%), 163 from B (38%), 49 from C0 (94%), 127 from C1 (11%) and 682 from C2 (33%) (Table 3). Most isolates had *papIBA* and not the other genes 3' of them (*papHCDJKEF*) (65%, n=2,661): 359 from A (87%), 118 from B (27%), 3 from C0 (6%), 888 from C1 (79%) and 1,293 from C2 (63%). *PapI* (234 bp), which is associated with more P fimbriae expression in *E. coli* CFT073 [102], was amplified to two, three or four copies in 22% of these ST131: 26 in Clade A (6%), 51 in B (12%), 45 in C0 (87%), 119 in C1 (11%) and 658 in C2 (32%). Only 45 isolates across the clades had an amplified *papB*, and 265 assemblies had a duplicated or triplicated *papA*. These two genes (*papBA*) share a promoter *pBA*, whereas *papI* has a separate one (*pI*).

Most isolates from A, C1 and C2 and a minority of B had no *pil* nor *ucl* operons and lost the *papH-F* segment (Table 3). Clade B differed from A because some from this heterogeneous clade have either *pil* or *pap*, but not both. 83 isolates with no detected *pap* genes all had a *pil* operon, and most (71) were from Clade B. Clades A and B have *pil* independently of pEK204, so Clade C isolates may have gained *pil* on multiple occasions. During the lengthy period prior to the divergence of the rarer C0 subclade from the C1/C2 ancestor [50], the Clade C ancestor likely had a complete *pap* operon and gained the *fimB* IS*Ec55* insertion. Subsequently, some C1 gained either *pil* (80, 7%) or *ucl* (82, 7%) or both (11, 1%), and some C2 gained *ucl* (482, 24%), rather than *pil* alone (73, 4%) or both together (33, 2%).

Unlike UclA-D that are functionally independent from *fim* [103], regulatory protein PapB reduces *fim* operon expression by inhibiting FimB and activating FimE, both tyrosine site-specific recombinases that invert *fimS*, including *E. coli* CFT073 and 536 [104-106]. PapI and PapB regulate *pap* expression [105-107] depending on their concentrations and protein binding at the 416 bp regulatory region between *papI* and *papB*. Thus, retention of *papIBA* in >65% of ST131 could be linked to *fim* regulation via *pap*B. Using the PPI network data above for *E. coli* 536 and CFT073 that have the *fim*, *pap* and *ucl* operons [108-109], there were ten *fim-pap* inter-operon PPIs in both 536 and CFT073,

including pilus rod subunit PapA with FimD and FimF (as well as FimC), potentially matching the PPIs of FimA [110]. If only *papIBA* was present, the functional effects (if any) of *papI* and *papA* were uncertain: it is not clear that at the inner cell membrane FimC (replacing PapD) could mediate periplasmic transport of PapA (like FimA) subunits via the chaperone FimI (instead of PapH) to usher FimD (rather than PapC) for pilus rod assembly at the outer cell membrane, extended later by FimF (in lieu of PapK) for the base of the tip. Nonetheless, presence of these genes can inform pilicide and coilicide design, such as antibodies targeting VF PapA [110].

Discussion

AMR genes in commensal or environmental bacteria are a major reservoir for pathogens [111]. This gene flow is driven by HGT and recombination in bacteria [112], including in *E. coli* [113-116], and the microbiome [117-118]. Previous work functionally screened contigs from preterm infants for resistance to 16 antibiotics [74], and here we compared these with ST131 Clade C accessory genomes that have AMR gene differences relevant to treatment [50,52,61]. This study found a large core resistome of in ST131 Clade C including a highly amplified *marR* gene was coupled with an accessory resistome of 19 genes [119]. Most core AMR genes were shared with commensal SE15 from ST131 Clade A and with asymptomatic UTI sample *E. coli* 83972, highlighting the role of accessory AMR genes in pathogenic infection. 83972 is used for therapeutic colonisation to protect against super-infection by more virulent strains [120-123], but here had AMR genes like PBP3. This retention of certain AMR genes in asymptomatic specimens is important when examining VF and AMR gene HGT in ST131 [124-125].

By combining the seven Clade C genomes with other 4,064 assemblies, we found that most 78% ST131 had >10 Kb like plasmid pEK499 and 9% had regions with similarity to IncI1 plasmid pEK204, suggesting either discrete gains of pEK204-like plasmids in each C subclade or its presence in the Clade C ancestor. IncI1 plasmids are common in ExPEC and are associated with different ESBL genes [126-128]. This reinforced the view that ST131's accessory resistome is shaped by plasmids in the local environment, rather than population structure or geographic factors [129]. Accessory genome diagnostics tracking plasmids and MGEs in addition to AMR genes [42,130] could assist clinical treatment [131].

Given that the mosaic array of backbone plasmid genes may determine fitness effects more than individual ESBL genes [132], and evidence of long-term IncF plasmid persistence in ST131 [62], plasmids pEK204 and pEK499 proteins' higher numbers of interactions with chromosomally-encoded proteins relative to other relevant plasmids could be a signature of co-evolution. Measuring the indirect connectivity as PPI network loops per interaction showed evidence of this long-term pattern, and may help identify plasmids compatible with *E. coli* chromosomes.

The type IV pilus gene cluster (*pil*) can allow different epithelial cell adhesion via a thinner pilus and allow biofilm formation [90], and the functionally distinct F17-like *ucl* operon is associated with uroepithelial cell adhesion, biofilm formation and mobilisation by its flanking MGEs [133]. Here, the minority of ST131 with *pil* had >40 Kb of regions similar to a pEK204-like plasmid if they were from Clade C, whereas those form Clades A and B did not have pEK204-like tracts. The *ucl* operon was in 24% of subclade C2 samples: UclD (like FimH) is a two-domain tip adhesin that binds intestinal epithelial cells via O-glycans (whereas FimH uses N-glycans) [103]. ST131's fitness advantage is tightly correlated with type 1 fimbriae variant *fimH30* and delayed *fim* operon expression due to an insertion at *fimB* [16,134-135]. The *pap* operon encodes a P fimbriae with high specificity for kidney epithelial cell and erythrocyte receptor glycolipids (α-D-galactopyranosol(1-4)-α-D-galactopyranoside) [136]. Delayed *fim* expression in Clade C [20,101] may be reduced further by PapB: *papIBA* alone was retained in most Clade C isolates. Delayed *fim* expression [137] and having multiple P fimbrial gene clusters [107] associates with pyelonephritis more so than cystitis. Given that individual *E. coli* express single fimbriae at the cell surface [104,138] and isogenic cell populations express distinct fimbrial types [139], type 1 fimbriae may allow initial bladder colonisation followed

by reduced expression in favour of P fimbriae in the kidney [138]. This study's results on plasmid-mediated dynamism across the ST131 resistome and extensive diversity in fimbrial gene composition could inform on potential infection [140] and biofilm formation patterns [20].

Methods

E. coli genome isolate collection

Seven of the 11 *E. coli* genome assemblies examined were ST131 isolated from Ireland in 2005-2010 (Table 1): two from subclade C1 were $bla_{\text{CTX-M-14}}$-positive, as was 8289_1#24 from subclade C2, and all five from C2 were $bla_{\text{CTX-M-15}}$-positive. All seven were isolated from urine except 8289_1#34, which was a rectal swab. All seven belonged to rST1503 except 8289_1#24 from rST1850 [50]. The four other samples were well-studied ST131 references: SE15, NCTC13441, EC958 and Human Microbiome Project (HMP) assembly 83972. ST131 subclade C2 genomes NCTC13441 and EC958 were from UTIs in the UK, and were assembled from long PacBio reads that had $bla_{\text{CTX-M-15}}$-positive plasmids [9,59,133]. EC958 has numerous virulence-associated genes that encode adhesins, autotransporter proteins and siderophore receptors, and can cause impairment of uterine contractility in mice [141]. Clade A reference SE15 was examined as a genetic outgroup to C2 and as a commensal control that lacks many virulence-associated genes (like α-hemolysin and cytotoxic necrotizing factor). 83972 was from the urine of a Swedish girl with a three-year history of asymptomatic bacteriuria and stable renal function [142-143] and shares common ancestry with virulent pyelonephritis-causing ExPEC CFT073 [144].

Illumina library quality control and read mapping

The paired-end Illumina HiSeq libraries for each assembly were screened for low quality (Phred score <30) and short (<50 bp) reads using Trimmomatic v0.36 [145] and were corrected with BayesHammer from SPAdes v3.9 [145] (Table 4). These corrected read libraries were mapped to reference sequences with SMALT v7.6 (www.sanger.ac.uk/resources/software/smalt/), and the resulting SAM files were converted to BAM format, sorted and PCR duplicates removed using SAMtools v1.19 [146].

Variable resistome overlaps complicate a clear reference gene set

The reference resistome outlined above was a set of 794 AMR genes assembled as 2,004 redundant AMR contigs tested *in vitro* for resistance to 16 antibiotics (Table S1) [74]. We compared this set with existing AMR databases to test the robustness of resistome comparisons by aligning the 794 contigs with the CARD [77] and mapping the reads for each of the seven ST131 libraries to the CARD, ARG-ANNOT [147] and ResFinder [148] databases using GROOT [78] where the reads for each isolate were indexed using the median read length (Table 4). This showed considerable variation in the numbers and types of AMR genes determined by each database and extensive AMR gene diversity within Clade C (Table 4). For context, the read library for S250 isolated from faeces and from ST131 Clade B [149] had zero AMR genes in ARG-ANNOT and ResFinder, and five in CARD.

Homology-based resistome screening and comparison

Contig annotation and protein domain recognition were completed using the Pfam v27.0 and ProSite databases with InterProScan v5.22-61 [150]. The protein homolog dataset in CARD (2,239 genes) [77] was aligned with the genomes to annotate them using BLAST v2.2.31, where matches with a bit score >500 and >99% homology were considered valid. Alignments were visualised using R's VennDiagram v1.6.1, Seqinr v3.4-5, UpSetR v1.4.0 and WriteXLS v5.0.0 packages. Detected genes and elements were verified and visualised with Artemis and the Artemis Comparison Tool (ACT) [151].

Assessment of plasmids prevalent in ST131

Sequence and annotation files for pEK499 (NC_013122.1, EU935739), pEK516 (NC_013121.1, EU935738), pEK204 (NC_013120.1, EU935740) and all pV130 contigs (LC056314.1 to LC056328.1) [93] were downloaded. The pV130 contigs were aligned to one another to determine their uniqueness with Clustal Omega v1.2.1 [152]. Replicon typing was completed using PlasmidFinder [56]. Each plasmid was compared to the CARD. Each E. coli read library was mapped to each plasmid to verify local genetic features and quantify copy number levels, visualised with Artemis [151] and R v3.4.2's reshape2 v1.4.3, ggridges v0.5.1, ggplot2 v2.3.2.1, readr v1.3.1, dplyr v0.8.3 and ape v5.3 packages. Plasmid and gene sequence similarity was calculated using the Sequence Identity and Similarity (SIAS) tool (http://imed.med.ucm.es/Tools/sias.html). Homology searches for contigs with high similarity to pEK204 and pEK499 in 4,071 assembled ST131 genomes [60] examined matches >300 bp length with >98% sequence identity.

Library	Clade	Number of reads	Read length			ARG-ANNOT	CARD	ResFinder
			Median	Mean	SD			
8289_1#34_1	C2	2,145,896	101	99.3	6.5	9	17	15
8289_1#34_2			100	97.7	8.0			
8289_1#3_1	C2	1,824,030	101	99.4	6.5	17	39	22
8289_1#3_2			100	97.6	8.2			
8289_1#35_1	C1	2,297,624	101	99.4	6.5	44	52	45
8289_1#35_2			100	97.7	8.0			
8289_1#91_1	C1	2,227,251	101	99.3	6.6	44	57	42
8289_1#91_2			100	97.7	8.1			
8289_1#24_1	C2	2,014,250	101	99.3	6.5	56	71	55
8289_1#24_2			100	97.7	8.1			
8289_1#27_1	C2	5,756,848	101	99.4	6.5	6	9	6
8289_1#27_2			100	97.7	8.1			
8289_1#60_1	C2	1,800,070	101	99.3	6.5	14	26	21
8289_1#60_2			100	97.6	8.1			

Table 4. Read library summary statistics for each main ST131 sample and the three main reference genomes. For GROOT usage, paired-end read library files were mapped individually. SD stands for standard deviation. The numbers of AMR genes from ST131 genome assemblies identified in AMR databases ARG-ANNOT, CARD and ResFinder are shown.

Protein-protein interaction network construction and topological data analysis

TDA has been used to investigate complex and high-dimensional biological datasets, like breast cancer genomes [153], and here we applied it to PPI networks with a focus on direct and indirect connectivity across different network topologies by quantifying rates of absent PPIs (non-trivial loops). The numbers of non-trivial loops per PPI was used as a metric for indirect connectivity because it was consistent across parameters and was independent of network size. We assessed pCA14, pEK204, pEK499, pEK516 and pEC958A given the immediate relevance to ST131 genome composition, along with two plasmids with no known AMR genes as negative controls: pECSF1 (aka pSE15), and non-conjugative plasmid pJIE186-2 that has numerous VF genes that are chromosomal in some *E. coli* [100]. We examined unique genes with PPI network information only: here, results for pEK516 were identical to those for pEK499.

We extracted *E. coli* K12 MG1655 PPI data from the STRING database v10 [98] with R v3.5.2 packages BiocManager v1.30.4, dplyr v0.8.0.1, genbankr v1.10.0, rentrez v1.2.1, STRINGdb v1.22.0, tidyverse v1.2.1 and VennDiagram v1.6.20. STRING's combined scores were used because they

integrate multiple types of evidence while controlling for random PPIs [98]. Using a score threshold of 400+, the K12 dataset had 4,146 distinct protein-coding genes, of which 4,121 had interaction information, resulting in 105,457 PPIs (14,555 PPIs were present for a score threshold of 900). For each plasmid or set of genes, we obtained the unique genes and for these determined the number of pairwise PPIs within that set and with the chromosome. We tested our TDA-based approach using the results of relevant previous work [99]. For *E. coli* 536, FooB was used as an equivalent for PapB, and likewise for protein F7-2 that matched VF PapA in CFT073, and FooG for PapG.

We constructed the Vietoris-Rips complex [154] where the proteins were the vertices with PPIs as edges, so that proteins A and B with an PPI would be joined by a single edge (two proteins in one dimension, 1-D). Similarly, proteins A, B and C that are joined by three PPIs would get their PPI triangle filled in with a solid 2-D triangle. For four proteins A, B, C and D joined by all six possible pairwise PPIs, their four filled 2-D triangles A/B/C, B/C/D, A/C/D and A/B/D constitute a tetrahedral surface which gets filled in with a solid 3-D tetrahedron. This can be extended to *m+1* pairwise connected proteins, which get a solid *m-D* polytope filled into their skeleton of edges, triangles, tetrahedra, and so on. A loop connects *m* proteins, where *m* is 4, 5, 6 or more, by precisely *m* PPIs, such that each protein is involved in precisely two of these PPIs. For instance, four proteins A, B, C, D can be joined to a loop by the four PPIs A-B, B-C, C-D, D-A, where A-B is a PPI between the proteins A and B (and so on). Any loop which can be filled with triangles, along existing PPIs, is called trivial. So if there is an interaction between A and C, the loop is filled by the two triangles AB-BC-CA and CD-DA-CA and hence trivial. If there are no interactions between A and C, and none between B and D, then the loop is non-trivial.

Due to the large K12 dataset size (4,146 proteins with 105,457 interactions), instead of using general purpose implementations of the Vietoris-Rips complex (such as SAGE), specialised software [155] to optimise the Betti number computations was used. This software used the sparsity of the boundary matrices to process the rank computations efficiently in LinBox [156], with a hard-coded dimensional truncation of the Vietoris-Rips complex to avoid the large number of high-dimension simplices that could be obtained for the full dataset. For each analysis across STRING combined scores 400 to 900 with a step of 25, we computed the first Betti number of the Vietoris-Rips complex that counted the numbers of non-trivial loops (with missing PPIs inside), which was adjusted for the numbers of PPIs above the score threshold (ie, loops per PPI). The number of PPIs for the complete K12 chromosomal dataset was negatively correlated with the combined score threshold (r^2=0.964), as was the loops per PPI (r^2=0.905), consequently the chromosomal loops per PPI rate was used as a baseline metric across different score thresholds.

Operon gene homology search approach

Homology searches for each *fim* operon gene were implemented using the NCTC13441 genome coordinates to extract the corresponding sequence with SAMtools v1.9 and align each gene to the genome assemblies [60] with BLAST v2.2.31 as above, processed with R packages tidyverse v1.2.1 and dplyr v0.8.3. For all operons, minor individual gene partial matches or losses were not examined due to the large number of samples and lack of consistent patterns for rare combinations. Results on amplified genes were restricted to those with prevalence >1%. The *fimB* CDS is 600 bp, but with the IS*Ec55* insertion the region typically spanned 2,493 bp here. NCTC13441 has three *pap* genes (*papIBA*) and lacks any *ucl* or *pil* genes, so the *pil* genes used were from pEK204. *PilI* and *pilJ* encoding IncI1 conjugal transfer proteins were mainly absent with no clear association with the other *pil* genes, and so were not examined here. *PilV* encoding a pilus tip adhesin had more than one copy in 292 assemblies, as expected for a locus rearranged to change pilus binding specificity [157]. The 5 Kb *ucl* operon has four genes, a major subunit (*uclA*), chaperone (*uclB*), usher (*uclC*) and adhesin (*uclD*), whose reference sequences were determined from 83972 (CP001671). *E. coli* UTI89's chromosome (CP000243) and plasmid (CP000244) [112] were used to obtain the *pap* operon: this has regulatory protein gene *papI*, structural protein genes *papA/B* and seven structural subunit genes (*papHCDJKEF*).

<u>Supplementary Data</u>

Table S1 - The 16 antibiotics used to identify 794 AMR genes on contigs originally from preterm infants.
Table S2 - The five AMR genes unique to all ST131 Clade C genomes except 8289_1#27 encoded bla_{TEM} genes (an Ambler class A β-lactamase).
Table S3 - PlasmidFinder Inc group alignments against pV130 and seven ST131 isolates.
Table S4 - Key known AMR, plasmid persistence and conjugation genes for pEK204, pEK499, pEK516, pCA14, pV130a and pV130b.
Figure S1 - Comparison of pEK516 (64,471 bp) with ST131 and 83972.
Figure S2 - Read mapping distributions for seven ST131 to pV130 contigs.
Figure S3 - Comparison of plasmid pCA14 (155 Kb) with seven ST131 C1/C2 isolates.
Table S5 - The PPIN characteristics of the protein-coding genes from Miryala et al (2018).
Table S6 - The seven plasmids' accessions, numbers of genes with interactions given a combined score of 400+, Inc groups, conjugative ability and AMR genes.
Figure S4 - The ratio of non-trivial loops per PPI (y-axis) versus the STRING combined score for all 60 genes from Miryala et al (2018) Table 1 [99].
Figure S5 - The ratio of non-trivial loops per interaction versus the STRING combined score for all chromosomal genes, all genes on pEK499 and all those on pEK204.
Figure S6 - The ratio of interactions per protein for genes encoded on the chromosome or the pEK499 and pEK204 plasmids across the STRING combined score.
Figure S7 - The distributions of pEK204-like and pEK499-like regions in ST131 assemblies.
Figure S8 - The distributions of pEK204-like regions in ST131 Clade C genome assemblies.

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