

Tracking key virulence loci encoding aerobactin and salmochelin siderophore synthesis in *Klebsiella pneumoniae*

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1 **ABSTRACT:**

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3 **Background:** *Klebsiella pneumoniae* is a recognised agent of multidrug-resistant
4 (MDR) healthcare-associated infections, however individual strains vary in their
5 virulence potential due to the presence of mobile accessory genes. In particular, gene
6 clusters encoding the biosynthesis of siderophores aerobactin (*iuc*) and salmochelin
7 (*iro*) are associated with invasive disease and are common amongst hypervirulent *K.*
8 *pneumoniae* clones that cause severe community-associated infections such as liver
9 abscess and pneumonia. Concerningly *iuc* has also been reported in MDR strains in
10 the hospital setting, where it was associated with increased mortality, highlighting the
11 need to understand, detect and track the mobility of these virulence loci in the *K.*
12 *pneumoniae* population.

13

14 **Methods:** Here we examined the genetic diversity, distribution and mobilisation of
15 *iuc* and *iro* loci among 2503 *K. pneumoniae* genomes using comparative genomics
16 approaches, and developed tools for tracking them via genomic surveillance.

17

18 **Results:** *Iro* and *iuc* were detected at low prevalence (<10%). Considerable genetic
19 diversity was observed, resolving into five *iro* and six *iuc* lineages that show distinct
20 patterns of mobilisation and dissemination in the *K. pneumoniae* population. The
21 major burden of *iuc* and *iro* amongst the genomes analysed was due to two linked
22 lineages (*iuc1/iro1*, 74% and *iuc2/iro2*, 14%), each carried by a distinct non-self-
23 transmissible IncFIB_K virulence plasmid type that we designate KpVP-1 and KpVP-2.
24 These dominant types also carry hypermucoidy (*rmpA*) determinants and include all
25 previously described virulence plasmids of *K. pneumoniae*. The other *iuc* and *iro*
26 lineages were associated with diverse plasmids, including some carrying FII
27 conjugative transfer regions and some imported from *E. coli*; the exceptions were *iro3*
28 (mobilised by ICEKp1), and *iuc4* (fixed in the chromosome of *K. pneumoniae*
29 subspecies *rhinoscleromatis*). *Iro/iuc* MGEs appear to be stably maintained at high
30 frequency within known hypervirulent strains (ST23, ST86, etc), but were also
31 detected at low prevalence in others such as MDR strain ST258.

32

33 **Conclusions:** *Iuc* and *iro* are mobilised in *K. pneumoniae* via a limited number of
34 MGEs. This study provides a framework for identifying and tracking these important

35 virulence loci, which will be important for genomic surveillance efforts including
36 monitoring for the emergence of hypervirulent MDR *K. pneumoniae* strains.

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39 Keywords: *Klebsiella pneumoniae*, virulence, hypervirulence, salmochelin,
40 aerobactin, virulence plasmids, plasmids, invasive disease, genomic surveillance

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44 BACKGROUND

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46 The enteric opportunistic bacterial pathogen *Klebsiella pneumoniae* imposes
 47 an increasing infection burden worldwide (1,2). These infections typically fall into
 48 one of two distinct categories; healthcare-associated (HA) infections caused by strains
 49 that are frequently multidrug-resistant (MDR), and community-associated (CA)
 50 infections arising from so-called hypervirulent strains that can cause highly invasive
 51 infections such as liver abscess but are usually drug sensitive (3,2). The antimicrobial
 52 resistance (AMR) and/or virulence determinants possessed by the associated bacteria
 53 are generally found on mobile genetic elements (MGEs) that transmit between *K.*
 54 *pneumoniae* cells via horizontal gene transfer (HGT) (4). These MGEs, most typically
 55 plasmids and integrative and conjugative elements (ICEs), are therefore important
 56 constituents of the accessory genome that imbue *K. pneumoniae* organisms with their
 57 distinct HA or CA clinical profiles.

58 It is apparent that a wide diversity of *K. pneumoniae* can cause infections in
 59 hospitalised patients (3,5,6), and that basic pathogenicity factors such as
 60 lipopolysaccharide, capsular polysaccharide, type 3 fimbriae and the siderophore
 61 enterobactin (Ent) are common to all *K. pneumoniae* and conserved in the
 62 chromosome as core genes (1,3). However enhanced virulence or ‘hypervirulence’ is
 63 associated with specific capsular serotypes (K1, K2, K5) and with MGE-encoded
 64 accessory genes that are much rarer in the *K. pneumoniae* population (3). Of
 65 particular importance are those encoding additional siderophore systems, namely
 66 yersiniabactin (Ybt) (3,7,8), aerobactin (Iuc) (9) and salmochelin (Iro)(10).

67 Synthesis of acquired siderophores contributes to *K. pneumoniae* virulence via
 68 multiple mechanisms. Iron assimilation via the conserved siderophore Ent is
 69 hampered by human neutrophils and epithelial cells through the secretion of lipocalin-
 70 2 (Lcn2), which binds and inhibits subsequent bacterial uptake of iron-loaded Ent
 71 (11). Ybt, Iro and Iuc are not subject to Lcn2 binding: Iro is a glycosylated derivative
 72 of Ent, while Ybt and Iuc possess an entirely distinct structure from Ent. The ability
 73 of salmochelin to counter Lcn2 binding is important for bacterial growth, and has
 74 been shown to correlate with enhanced virulence in a mouse sepsis model (12). The
 75 association between aerobactin and virulence has long been recognised, with multiple
 76 studies demonstrating its key role in increased iron acquisition, bacterial growth
 77 and/or virulence in various murine models, human ascites fluid and blood (9,13–15).

78 Even in strains that possess all four siderophore-encoding loci, *Iuc* appears to play the
79 most critical role in virulence both *in vitro* and *in vivo* (13), and serves as an
80 important biomarker for identifying hypervirulent isolates (16).

81 In *K. pneumoniae*, Ybt biosynthesis is encoded by the *ybt* locus, which is
82 typically located on a chromosomal ICE known as ICEKp (of which there are at least
83 14 distinct variants) and was recently also reported on plasmids (7,8,17). A screen of
84 2500 *K. pneumoniae* genomes showed *ybt* to be prevalent in one third of the
85 population, and associated with hundreds of ICEKp acquisition events across the
86 chromosomes of both hypervirulent and MDR lineages (8). In contrast, *Iuc* and *Iro*
87 synthesis is encoded by loci (*iuc* and *iro*, depicted in **Fig. 1**) that are typically co-
88 located on the so-called “virulence plasmids” of *K. pneumoniae*. The best
89 characterised virulence plasmids are the 224 kbp plasmid pK2044 from serotype K1,
90 sequence type (ST) 23 strain NTUH-K2044 (18); the 219 kbp plasmid pLVPK from
91 K2, ST86 strain CG43 (19); and the 121 kbp plasmid Kp52.145pII from serotype K2,
92 ST66 strain Kp52.145 (strain also known as 52145 or B5055; plasmid also known as
93 pKP100) (9,20). These plasmids also carry additional virulence determinants
94 including *rmpA* genes that upregulate capsule production, conferring a hypermucoid
95 phenotype that is considered a hallmark of hypervirulent strains (21); other gene
96 clusters associated with iron uptake and utilisation; and other loci encoding resistance
97 to heavy metals such as copper (*pco-pbr*), silver (*sil*) and tellurite (*ter*) (4). In addition
98 to the virulence plasmid-encoded *iro* and *rmpA* genes, the ST23 strain NTUH-K2044
99 also carries a chromosomal copy of *iro* and *rmpA* located within ICEKp1 (7);
100 however this is not a typical feature of ST23 (22).

101 The majority of *K. pneumoniae* lineages associated with liver abscess and
102 other invasive community acquired infections (e.g. clonal group (CG) 23, CG86,
103 CG380) carry virulence plasmids encoding *iro*, *iuc* and *rmpA* (3,9,23–25,16).
104 However whilst virulence and AMR genes are both transmitted within the *K.*
105 *pneumoniae* population via plasmids, until recently these plasmids have mainly been
106 segregated in non-overlapping populations such that the virulence plasmids encoding
107 *iuc* and *iro* have rarely been detected in MDR populations that cause HA infections
108 and outbreaks (4,3,26). However, the virulence plasmid Kp52.145pII has been shown
109 experimentally to be mobilisable (21), and there are emerging reports of MDR clones
110 such as ST11, ST147 and ST15 acquiring virulence plasmids (27,28). The
111 combination of hypervirulence and MDR can result in invasive infections that are

very difficult to treat. This can result in dangerous hospital outbreaks; for example an aerobactin-producing carbapenemase-producing ST11 strain recently caused a fatal outbreak of ventilator-associated pneumonia in a Chinese intensive care unit, with 100% mortality (27,29). AMR plasmids are also occasionally acquired by ST23 and other hypervirulent *K. pneumoniae* clones (25,30,31).

The ease with which virulence plasmids spread in the *K. pneumoniae* population poses a significant global health threat, highlighting the importance of understanding and monitoring the movement of these loci between different strains and clones. Here we investigate the diversity of aerobactin and salmochelin synthesis loci in 2733 *K. pneumoniae* complex genomes, aiming to understand the diversity and distribution of these virulence loci in the population, and to develop a framework for their inclusion in genomic surveillance efforts.

METHODS

Bacterial genome sequences

2733 genomes of the *K. pneumoniae* complex, including isolates collected from diverse sources and geographical locations were analysed in this study (see **Table S1**). The genomes represent a convenience sample of our own isolate collections from clinical and species diversity studies (5,8,22,32), as well as sequences that were publicly available in GenBank or via the NCTC 3000 project (<https://www.sanger.ac.uk/resources/downloads/bacteria/nctc/>) at the commencement of the study. The majority of these genomes were also included in our previous genome study screening for yersiniabactin and colibactin (8).

For n=1847 genomes (see **Table S1**), Illumina short reads were available and these were used to generate consistently optimised *de novo* assembly graphs using Unicycler v0.3.0b with SPAdes v3.8.1 (33,34). The remaining n=886 genomes were publicly available only in the form of draft genome assemblies. All genome assemblies were re-annotated using Prokka (35) to allow for standardised comparison. All genomes were assigned to species by comparison to a curated set of Enterobacteriaceae genomes, using mash (implemented in Kleborate, <https://github.com/katholt/Kleborate>); this confirmed 2503 *K. pneumoniae*, 12 *K.*

146 *quasipneumoniae* subsp. *quasipneumoniae*, 59 *K. quasipneumoniae* subsp.
147 *similipneumoniae*, 158 *K. variicola* and 1 *K. quasivariicola* (**Table S1**).

148

149 **Long read sequencing of isolates**

150 Three isolates in our own collection (INF078, INF151, INF237) carried novel
151 *iuc* and/or *iro* plasmids identified from short read Illumina data. We subjected these to
152 long read sequencing using a MinION R9.4 flow cell (Oxford Nanopore Technologies
153 (ONT)) device in order to resolve the complete sequences for the relevant plasmids.
154 Overnight cultures of each isolate were prepared in LB broth at 37°C, and DNA
155 extracted using Agencourt Genfind v2 (Beckman Coulter) according to a previously
156 described protocol (doi:10.17504/protocols.io.p5mdq46). Sequencing libraries
157 were prepared using a 1D Ligation library (SQK-LSK108) and Native Barcoding
158 (EXP-NBD103) as previously described (22,36). The resulting reads were combined
159 with their respective Illumina reads to generate a hybrid assembly using our Unicycler
160 software v0.4.4-beta (33,36). Annotations for the assemblies were generated as
161 described above, and the annotated sequences submitted to GenBank under accession
162 numbers TBC (**Table S1, Table S2, Table S3**).

163

164 **Multi-locus sequence typing (MLST)**

165 Chromosomal sequence types were determined for each genome assembly
166 using the BIGSdb-Kp seven-locus MLST scheme (37) screened using Kleborate
167 (<https://github.com/katholt/Kleborate>). A novel ST (ST3370) was identified and
168 added to the BIGSdb-Kp MLST database.

169 To facilitate the development of MLST schemes for the aerobactin and
170 salmochelin biosynthesis loci *iuc* and *iro*, alleles for genes belonging to each locus
171 (i.e. *iucABCD*, *iutA*; and *iroBCDN*; respectively) from strains with ‘typeable’ loci
172 (defined as those in which all genes in the locus had high quality consensus base calls
173 when mapping with SRST2) were extracted by comparison to known alleles in the
174 BIGSdb-Kp virulence database (<http://bigsdb.pasteur.fr/klebsiella/klebsiella.html>)
175 (25), using SRST2 v0.2.0 (38) to screen Illumina read sets where available and
176 BLAST+ v2.2.30 to screen assemblies. Incomplete, ‘non-typeable’ *iro* and *iuc* loci
177 were excluded from the MLST scheme (marked NT in **Table S1**). Each unique
178 combination of alleles was assigned an aerobactin sequence type (AbST) or
179 salmochelin sequence type (SmST), defined in **Table S4** and **Table S5**. The AbST

180 and SmST schemes, profiles and corresponding alleles are also available in the
181 BIGSdb-Kp database and in the Kleborate Github repository (see links above).

182

183 **Identification of other genes of interest, and genetic context of *iuc* and *iro* loci**

184 Capsule (K) loci were identified in the assembled genomes using Kaptive
185 (39). *RmpA* gene copy number was determined by BLASTn search of all genome
186 assemblies using the *rmpA* and *rmpA2* sequences from pK2044 (GenBank accession
187 AP006726.1) as queries. Similarly, BLASTn was used to screen the genomes
188 assemblies for the IncFIB_K *repA* sequence from virulence plasmids pK2044 and
189 Kp52.145 pII (GenBank accession FO834905.1), with FIB_K presence defined as
190 >90% coverage and >80% nucleotide identity to these query sequences. FII replicons
191 were identified using BLASTn search of the PlasmidFinder database (40).

192 Assemblies of all *iuc*⁺ or *iro*⁺ genomes were manually inspected to determine
193 whether the loci of interest were located on the chromosome or on previously
194 described virulence plasmids (pK2044 and Kp52.145pII). This confirmed most to be
195 located in the chromosome (*iro3* in ICEKp1, or *iuc4* in the subspecies
196 *rhinoscleromatis* lineage) or one of the known plasmids. For the remaining genomes,
197 annotated contigs containing the *iuc* and/or *iro* loci were checked for known
198 chromosomal or plasmid features, aided by BLASTn searching against the NCBI non-
199 redundant nucleotide database and inspection of the assembly graphs using Bandage
200 v0.8.0 (41).

201

202 **Phylogenetic analyses**

203 Maximum likelihood phylogenetic trees capturing the relationships between
204 AbSTs or SmSTs were constructed by aligning the allele nucleotide sequences
205 corresponding to each sequence type within each scheme using MUSCLE v3.8.31
206 (42), then using each of the two alignments (one for AbSTs, one for SmSTs) as input
207 for phylogenetic inference in RAxML v7.7.2 (43). For each alignment, RAxML was
208 run five times with the generalised time-reversible model and a Gamma distribution,
209 and the trees with the highest likelihood were selected. Lineages were defined as
210 monophyletic groups of AbSTs or SmSTs associated with the same MGE structure;
211 STs within lineages shared ≥ 2 alleles (for SmSTs) or ≥ 3 alleles (for AbST), whereas
212 no alleles were shared between lineages.

Maximum likelihood phylogenies were similarly constructed for (i) aerobactin and salmochelin locus alignments populated by sequences extracted from BLAST hits amongst representatives of the wider Enterobacteriaceae family (representatives listed in **Table S6**), and (ii) FIB_K replicon sequence alignments constructed by mapping *iuc* positive (*iuc*⁺) and *iro* positive (*iro*⁺) genomes to a reference IncFIB_K sequence (coordinates 128130 to 132007, spanning *repA* to *sopB*, of the pK2044 plasmid sequence; GenBank accession AP006726.1).

220

221 **Plasmid comparisons**

Twelve representative plasmids (10 complete and 2 partial) were chosen for comparative analysis (these are available as a set in FigShare under doi:10.6084/m9.figshare.6839981; and see **Table S2** for list of sources and GenBank accession numbers). Six of these representative plasmids were sourced from the NCTC 3000 project (<https://www.sanger.ac.uk/resources/downloads/bacteria/nctc/>). The representative plasmid sequences were compared using Mauve v2.4.0 (44), in order to identify homology blocks conserved amongst subsets of the plasmids. BLASTn comparisons of related plasmids were plotted using GenoPlotR v0.8.7 package (45) for R. All *iuc*⁺ or *iro*⁺ genomes were mapped against all 12 representative plasmids in order to calculate coverage of each plasmid in each genome. This was done using Bowtie2 v2.2.9 (46) to map Illumina reads where available, and 100 bp reads simulated from draft assemblies where raw sequence reads were not available, using the RedDog pipeline (<https://github.com/katholt/RedDog>). For every gene annotated within each reference plasmid, the proportion of strains within each group of genomes sharing the same *iuc/iro* lineage carrying the gene was calculated using the gene presence/absence table reported by RedDog (presence defined as ≥95% of the length of the gene being covered by at least five reads), and plotted as circular heatmaps using ggplot2 in R (using geom_tile to achieve a heatmap grid and polar_coord to circularize).

241

242 **RESULTS**

243

244 **Prevalence of *iuc* and *iro* in *K. pneumoniae***

Iuc and *iro* were detected only in *K. pneumoniae* genomes, and not in other members of the *K. pneumoniae* species complex. Of the 2503 *K. pneumoniae*

247 genomes screened, *iuc* was detected in 8.7% (n=217) and *iro* in 7.2% (n=181; listed
248 in **Table S1**, excluding strains with a partial *iro* locus as discussed below). The
249 presence of intact *iro* and *iuc* loci was strongly associated (odds ratio (OR) 711, 95%
250 confidence interval (CI) 386-1458, $p < 1 \times 10^{-16}$), co-occurring in 162 genomes (6.5% of
251 the genomes tested). The *iro* locus appears to be susceptible to deletion: partial *iro*
252 loci were observed in n=50 *K. pneumoniae* isolates (noted as *iro** in **Table S1**),
253 mostly those that were isolated from historical collections prior to 1960 (of 39 strains
254 isolated up to 1960 and with any *iro* genes present, 36 (92%) carried deletion variants
255 of the locus, compared to 4/163 (2.5%) amongst isolates from 1975 onwards; OR 416,
256 95% CI 88-3297, $p < 2 \times 10^{-16}$). As expected, the presence of *iuc* and *iro* were each
257 strongly associated with presence of *rmpA*, with 157 genomes carrying all three loci
258 (excluding partial *iro*). A total of 238 strains (9.5%) carried *rmpA* genes: n=110
259 (4.4%) carried one, n=127 (5.1%) carried two, and a single strain, ST23 NTUH-
260 K2044, carried three (as described previously (7,18), see **Table S1**).

261

262 **Genetic diversity of *iuc* and *iro* in *K. pneumoniae***

263 Next we explored nucleotide diversity of the genes comprising the *iro* and *iuc*
264 loci in *K. pneumoniae*. The five genes comprising the *iuc* locus (**Fig. 1a**) and four
265 genes of the *K. pneumoniae* form of the *iro* locus (**Fig. 1b**) were screened for
266 sequence variation, and each unique locus sequence variant was assigned an allele
267 number. Of the n=209 strains carrying a typeable *iuc* locus, 62 unique *iuc* allele
268 combinations were observed and assigned a unique aerobactin sequence type or AbST
269 (see **Table S4** for AbST definitions, and **Table S1** for AbSTs assigned to each
270 genome). The *iutA* alleles present in the *iuc* locus showed >28% nucleotide
271 divergence from the core chromosomal homolog of *iutA* encoding a TonB-dependent
272 siderophore receptor, which was detected in 96.4% of all genomes; the alleles of this
273 core chromosomal gene are not included in the aerobactin MLST scheme. Typeable
274 *iro* loci were identified in n=164 strains, comprising 35 unique salmochelin sequence
275 types or SmSTs (defined in **Table S5**, see **Table S1** for SmSTs assigned to each
276 genome). Maximum likelihood phylogenetic analyses of the AbST and SmST
277 sequences, and their translated amino acid sequences, revealed five highly distinct *iuc*
278 lineages and five *iro* lineages (labelled *iro1*, *iro2* etc; see **Fig. 1c**, **Fig. S1**). Nucleotide
279 divergence between lineages was 1-11% (20-1000 substitutions), and no alleles were
280 shared between lineages (**Table S7, S8**). Nucleotide divergence within lineages was

low, with mean divergence of 0.001-0.40% (*iro*) and 0.013-0.50% (*iuc*) (**Table S7, S8**), and at least two (*iro*) or three (*iuc*) shared alleles between members of the same lineage. Of note, the *iro4*, *iro5* and *iuc5* loci were quite distant from other lineages (each showing >5.5% nucleotide divergence from all other lineages, vs <4.6% divergence amongst the other lineages; **Fig. 1, Table S7, S8**). Comparison to *iuc* and *iro* genes present in other Enterobacteriaceae (see **Fig. S2, Table S9**), and the presence of the additional *iroE* gene (**Fig. 1b**), suggests that these more distant lineages derive from outside *Klebsiella*, most likely *Enterobacter* (*iro4*) and *E. coli* (*iro5*, *iuc5*). Note that genotyping of *rmpA* was not performed since most *rmpA*-positive genomes carry two copies of the gene, which complicates allele typing from short read data, however *rmpA* copy number per genome is reported in **Table S1**.

292

293 **Mobile genetic elements associated with *iuc* and *iro* loci**

294 Inspection of the genetic context surrounding the *iuc* and *iro* sequences
295 revealed that the various *iuc* and *iro* lineages were associated with distinct MGEs,
296 with the exception of *iuc4* which was restricted to the chromosome of *K. pneumoniae*
297 subspecies *rhinoscleromatis* (ST67) (**Fig. 1c, Table 1**). Most common were *iuc1* and
298 *iro1*; these were both associated with pK2044-like plasmids (hereafter called KpVP1-
299 1, see below) and the presence of two *rmpA* genes, and accounted for 74% of all
300 *iuc+iro+* genomes. These were followed by *iuc2* and *iro2*, which were associated with
301 Kp52.145 pII-like plasmids (hereafter called KpVP-2, see below), the presence of one
302 *rmpA* gene, and accounted for 14% of all *iuc+iro+* genomes. A sister clade of *iuc2*,
303 which we named *iuc2a*, was associated with diverse plasmids that shared some
304 homology with Kp52.145 pII (36-70% coverage, 99% nucleotide identity). Most
305 *iuc2a+* isolates carried a single *rmpA* gene (n=38, 88.4%) and all lacked an intact *iro*
306 locus (n=26, 60.5% had a partial *iro* locus). Lineage *iuc3* was related to the *iuc4*
307 lineage encoded on the *rhinoscleromatis* chromosome, but was present on novel
308 plasmids. *Iro3* was located within the chromosomally integrated ICEKp1, along with
309 *rmpA*. Four genomes carried *iuc5* (two of these also carried *iro5*; all lacked *rmpA*).
310 The *iuc5* sequences were distantly related to *iuc1* and *iuc2* (>8.9% nucleotide
311 divergence), but were identical to sequences found in *E. coli* and located on contigs
312 that matched closely to *E. coli* AMR plasmids (e.g. strain PCN033 plasmid
313 p3PCN033, accession CP006635.1 (47), which showed >99% nucleotide identity to
314 the best assembled of *iuc5+* *K. pneumoniae* contigs). *Iro4* was identified in a single

genome (which lacked *rmpA*), and was >6.1% divergent from *iro1* and *iro2* sequences. Its closest known relatives are *iro* sequences present in the chromosomes of *Enterobacter cloacae* and *Enterobacter hormaechei* (strains AR_0065, accession CP020053.1 and 34977, accession CP010376.2 respectively; 95% identity). Lineages *iro4* and *iro5* follow the gene configuration typical of non-*K. pneumoniae* Enterobacteriaceae *iro* loci, from which the *K. pneumoniae iro1*, *iro2* and *iro3* differ by lack of *iroE* and inversion of *iroN* (see **Fig. 1b**).

To examine the gene content and replicon differences between the various *K. pneumoniae* plasmids associated with *iuc* and/or *iro*, 12 representative plasmids associated with the various lineages were selected for comparison (**Fig. 2, Table S2**). These include six complete *K. pneumoniae* plasmid sequences identified from finished genomes: *iuc1/iro1* (n=1), *iuc2/iro2* (n=1), *iuc2a* (n=3), *iuc3* (n=1); three novel complete *K. pneumoniae* plasmid sequences that we generated for this study, carrying *iuc2a* (n=2) and *iro4* (n=1); and 2 large contigs that we identified from public *K. pneumoniae* genome data representing partial sequences for additional plasmids carrying *iuc2a* (n=1) and *iuc3* (n=1) (**Fig. 2**). The *K. pneumoniae* genomes in which *iuc5/iro5* were identified were available only as draft assemblies deposited in public databases, and the associated plasmid sequences were fragmented in these assemblies, hence we used *E. coli* strain PCN033 plasmid p3PCN033 (47) as the representative for *iuc5/iro5*. The representative plasmid sequences differed substantially in their structure and gene content (**Fig. 2**), and were differentially distributed amongst the *K. pneumoniae* population (**Fig. 3**). In order to explore structural conservation of plasmids amongst isolates with each *iro* or *iuc* lineage, we mapped the sequence data from all isolates carrying either of these loci against the 12 representative plasmid sequences (**Fig. 4**). This revealed that plasmid structures were largely conserved amongst isolates sharing the same *iuc* or *iro* lineages, although plasmids associated with *iuc2a* and *iuc3* showed more diversity than others (**Fig. 4** and see below).

All representative *iuc* or *iro* plasmids harboured an IncFIB_K (n=9) or IncFIB (n=3) replicon, including the *repA* replication gene, *oriT* origin of transfer, and *sopAB* partitioning genes (presence of these replicons in each plasmid is indicated purple in **Fig. 2c**, and listed in **Table S2**). The IncFIB_K replicon was present in n=202/208 (97%) of isolates with plasmid-encoded *iuc* or *iro*, including 100% of *iuc1/iro1*, *iuc2/iro2*, *iuc2a* and *iro4* isolates; and 82% of *iuc3* isolates. Each of these *iuc/iro*

lineages was associated with a unique sequence variant of the IncFIB_K replicon (see tree in **Fig. 5** and nucleotide identity with the IncFIB_K *rep* sequences from KpVP-1 and KpVP-2 listed in **Table S1**), supporting segregation of the *iuc* and *iro* loci with distinct FIB_K plasmid backbones. However the IncFIB_K replicon was also widely detected amongst strains that do not carry *iro* and *iuc* (77% of all *K. pneumoniae* genomes and 69% amongst other species in the complex; see **Table S1**), including MDR *K. pneumoniae* lineages such as CG258, and is known to be associated with AMR plasmids (48,49). IncFIB replicons, which are common amongst *E. coli* and display >39% nucleotide divergence from the FIB_K replicon, were found in all *K. pneumoniae* strains carrying the *E. coli* variant *iuc5* (100%) and also detected in two strains carrying *iuc3* plasmids (18%; marked in **Fig. 2**), suggesting transfer of these *iuc* variants into *K. pneumoniae* via such plasmids.

***Iuc/iro* lineages 1 and 2 are associated with two dominant *K. pneumoniae* virulence plasmids, KpVP-1 and KpVP-2**

Iuc/iro lineages 1 and 2 accounted for 64% of *K. pneumoniae* isolates carrying any aerobactin or salmochelin synthesis loci, and 88% of isolates carrying both. Whilst it was not possible to resolve the complete sequences for all plasmids associated with these lineages, read mapping to pK2044 and Kp52.145 pII reference sequences strongly supported the presence of pK2044-like plasmids in *iro1+iuc1+* genomes (mean plasmid coverage of 95.1%, range 28.8-100%; see **Fig. 4**), and Kp52.145 pII-like plasmids in *iro2+iuc2+* genomes (mean plasmid coverage of 92.4%, range 87.2-100%; see **Fig. 4**). There were limited homologous regions shared between the two plasmids (**Fig. 2**), including the *iro*, *iuc*, *rmpA* and *fec* loci, and the IncFIB_K replicon (**Table S10**). These shared regions were largely conserved across all isolates carrying *iuc/iro* lineages 1 or 2; the remaining regions unique to either pK2044 or Kp52.145 pII were largely conserved amongst the isolates that carried lineage 1 or 2 loci, respectively (**Fig. 4**). Notably, the loci encoding heavy metal resistances against copper (*pbr-pco*), silver (*sil*) and tellurite (*terXYW* and *terZABCDEF*) were highly conserved amongst lineage 1 strains but not present in any of the lineage 2 strains (**Table S10**). As noted above, *iuc/iro* lineages 1 and 2 were also each associated with a distinct variant of the IncFIB_K replicon sequence (**Fig. 5**). Hence we define pK2044-like plasmids carrying *iuc1* and *iro1* loci as *K. pneumoniae* virulence plasmid type 1 (KpVP-1), with reference plasmid pK2044; and Kp52.145

383 pII-like plasmids carrying *iuc2* and *iro2* loci as *K. pneumoniae* virulence plasmid type
384 2 (KpVP-2). Both plasmid types typically carry at least one copy of *rmpA*; neither one
385 carries genes associated with conjugation, hence we assume they are not self-
386 transmissible.

387 KpVP-1 and KpVP-2 showed distinct distributions within the *K. pneumoniae*
388 population. KpVP-1 was present in 5.0% of all isolates and accounted for 74% of
389 *iuc+**iro+* isolates. The KpVP-1 reference plasmid pK2044 originated from an ST23
390 isolate (CG23), and KpVP-1 was strongly associated with this and two other well-
391 known hypervirulent clones CG65 and CG86, in which it was present at high
392 prevalence (ranging from 79.0-96.4%, see **Fig. 3**). KpVP-1 was also detected at low
393 frequencies in other clones, including CG34, CG111, CG113 and CG25, suggesting it
394 is mobile within the *K. pneumoniae* population (**Fig. 3**). KpVP-2 was present in
395 0.96% of all isolates and accounted for 14% of *iuc+**iro+* isolates. The KpVP-2
396 reference plasmid Kp52.145 pII originated from an ST66 isolate, and KpVP-2 was
397 present in all isolates of the associated clonal group CG66 (n=11) and also all isolates
398 of CG380 (n=12) (**Fig. 3**).

400 **An *iuc* lineage 2 variant (*iuc2a*) is associated with diverse plasmids with a KpVP- 401 1-like IncFIB_K replicon**

402 *Iuc2a* was identified in 43 isolates largely belonging to three clonal groups
403 (ST3, n=4; CG90, n=19; ST82, n=19; ST382, n=1; see **Fig. 3**), with the majority
404 (n=38, 88.4%) from the historical NCTC or Murray collections and isolated between
405 1932 and 1960 (**Table S1**). Provenance information was available for only twelve of
406 the *iuc2a+* isolates (1 ST3, 9 CG90, 2 ST82); all of which originated from the human
407 respiratory tract (3 nose, 1 throat, 7 sputum, and 2 NCTC isolates recorded only as
408 respiratory tract). We used long-read sequencing to resolve plasmids in two novel
409 *iuc2a+* isolates from our own collection, INF151 and INF237, which were both CG90
410 Australian hospital sputum isolates (summarised in **Table S3**). This yielded IncFIB_K
411 plasmids in each genome, of size 138.1 kbp and 133.7 kbp, respectively (accessions:
412 pINF151_01-VP, TBC; pINF237_01-VP, TBC). Both plasmids carried *iuc2a* and one
413 *rmpA* gene, but they differed slightly from one another in structure and gene content,
414 and differed substantially from the three complete *iuc2a+* plasmid sequences
415 available from NCTC isolates (ST3 and CG90; see **Fig. 2**, **Fig. 4**). Only one of these
416 plasmids (from NCTC 12463; incomplete) carried a conjugative transfer region

(IncFII), hence we predict most are not self-transmissible. Mapping of *iuc2a*⁺ isolates to each of the five representative *iuc2a*⁺ plasmid sequences indicated a degree of conservation between plasmids in strains belonging to the same *K. pneumoniae* clone, but none particularly well conserved across all *iuc2a*⁺ strains (**Fig. 4, Fig. S3**). However all *iuc2a*⁺ isolates formed a tight monophyletic cluster in the IncFIB_K replicon tree (**Fig. 5**), consistent with recent shared plasmid ancestry followed by frequent structural and gene content changes. Notably the *iuc2a*-associated IncFIB_K replicon sequences were closely related to those of KpVP-1 and distant from those of KpVP-2, hence we hypothesise that *iuc2a* plasmids share an ancestor that was a mosaic including *iuc2*-related sequences from KpVP-2 and IncFIB_K replicon sequences from KpVP-1.

***Iuc* lineage 3 is mobilised by diverse plasmids carrying the FII_K conjugative transfer region**

Lineage *iuc3* was detected in 11 isolates from diverse sources and chromosomal STs (**Fig. 3**), and was associated with three related variants of the IncFIB_K replicon (**Fig. 5**). We identified one complete and one near-complete *iuc3* plasmid sequences: a complete 189.8 kb plasmid from NCTC 11676 (isolated 1979, ST290), and a 155.4 kb contig from NCTC 11697 (isolated 1984, ST3370) (**Fig. 2**). The plasmids share around half of their gene content (96 kbp), including the IncFII_K *tra-trb* conjugative transfer machinery, a fimbrial protein and the *fec* iron acquisition system in addition to *iuc3* (**Fig. 2, Fig. 4, Table S2**). Mapping to these sequences showed all *iuc3*⁺ isolates carried related plasmids with an FII_K transfer region (**Fig. 4, Table S10**).

Complete sequence of an *iro4* plasmid

Lineage *iro4* was identified in a single hospital UTI isolate INF078 (ST105) from Australia, whose genome sequence we completed using long reads (replicons summarised in **Table S3**). Hybrid assembly using short and long reads resolved a 399,913 kbp plasmid, pINF078-VP (accession TBC) which carried multiple copies of *iro4*, the IncFIB_K replicon (similar to the KpVP-2 variant, see **Fig. 5**) and the IncFII_K replicon and transfer region (**Fig. 2**). As noted above, the *iro4* locus is more closely related to *Enterobacter iro* than to other *K. pneumoniae iro* in terms of both structure (including the *iroE* gene; see **Fig. 1b, Fig. S4**) and sequence (**Fig. S2**), suggesting it

has been transferred from *Enterobacter* into a *K. pneumoniae* IncFIB_K/FII_K plasmid backbone (note the IncFIB_K replicon sequence of pINF078-VP was similar to those of KpVP-2; see **Fig. 5**). pINF078-VP harboured multiple tandem copies of a 17,129 bp region containing *iroBCDEN* and 12 other genes of unknown function (**Fig. S4**). Long read sequences (up to 70 kbp) spanning the non-repeat and repeat region of pINF078-VP confirmed at least n=3 copies of the 17 kbp repeated sequence, whose mean read depth in the Illumina sequence data was 13.3 times that of the rest of the plasmid sequence, suggesting approximately 13 tandem copies.

Iuc/iro* lineage 5 loci are associated with plasmids originating from *E. coli

Four *K. pneumoniae* isolates carried the *E. coli* variant *iuc5*; two of these also carried the *E. coli* variant *iro5* (see species trees in **Fig. S2**). Three *iuc5*⁺ isolates (including one with *iro5*) belonged to the globally-disseminated, carbapenemase-producing *K. pneumoniae* CG258 (ST258, KPC⁺; ST437, KPC⁺; ST833, KPC⁻) and carried several AMR genes. Unfortunately all four *iuc5*⁺ genomes were sourced from public databases and were available in draft form only, and the complete plasmid sequences could not be resolved. However the *iuc5*⁺ contig sequences from *K. pneumoniae* share close homology (99% identity, >65% coverage) with *iuc5*⁺*iro5*⁺ FII conjugative plasmids from *E. coli* that also carry AMR genes (e.g. p3PCN033, CP006635.1; D3 plasmid A, CP010141.1).

DISCUSSION

This study reveals significant genetic diversity underlying the biosynthesis of aerobactin and salmochelin in *K. pneumoniae*, but shows the distribution of *iuc* and *iro* locus variants is highly structured within the population. Our data indicate that most of the burden of these hypervirulence-associated siderophores in the *K. pneumoniae* population is associated with two dominant virulence plasmids, which we define here as KpVP-1 and KpVP-2, that differ in terms of gene content (**Fig. 2**) and are each associated with co-segregating sequences of the FIB_K replicon, *iuc* and *iro* loci (**Fig. 1, Fig. 5**). These dominant virulence plasmid types are each represented by one of the previously characterised *K. pneumoniae* virulence plasmids (18,20), pK2044 (KpVP-1, encoding *iro1* and *iuc1*) and Kp152.145pII (KpVP-2, encoding *iro2* and *iuc2*); both also carry hypermucoidy determinants, and together they account for 74% and 14% of the *iuc*⁺*iro*⁺ *K. pneumoniae* genomes analysed. Importantly, our

data indicate that each of these common virulence plasmid variants is maintained at high prevalence in a small number of known hypervirulent clones: KpVP-1 in CG23 (96%, including pK2044 (18)), CG86 (80%, including pLVPK (19)) and CG65 (79%); KpVP-2 in CG66 (100%, including Kp152.145pII) and CG380 (100%) (**Fig. 3**). This suggests that both plasmid types can persist for long periods within a host bacterial lineage as it undergoes clonal expansion; indeed our recent study of the evolutionary history of CG23 indicates that KpVP-1 has been maintained in this clonally expanding lineage for at least a century (22). Notably we also detected KpVP-1 at low prevalence in numerous other *K. pneumoniae* lineages and KpVP-2 at low prevalence in one other lineage, suggesting the possibility of wider dissemination of both plasmid types by occasional transfer to new lineages (**Fig. 3**). Given the stability of the plasmids observed in several clonal groups, we speculate that some of these transfer events will result in the emergence of novel hypervirulent strains that can stably maintain the plasmid into the future. In contrast, the non-plasmid form of *iro* (*iro3*, occasionally integrated into the chromosomes of *K. pneumoniae* via ICEKp1 was found at low prevalence (<0.5%) and included just one of the 79 ST23 isolates analysed (NTUH-K2044, in which ICEKp1 was first described), 1/1 ST5, 1/21 ST111 (13%), 1/2 ST198, 2/15 CG25, 2/2 ST493 and 5/5 ST60. Hence while ICEKp1 is somewhat dispersed in the *K. pneumoniae* population, it shows little evidence of stability within lineages, consistent with our previous observations regarding ICEKp in general (8).

We also detected several novel *iuc*⁺ or *iro*⁺ plasmid types, the most common being the group of *iuc2a* plasmids (21% of all *iuc*⁺ strains) that were detected in respiratory isolates from CG3, CG82 and CG90 and mostly originated from historical collections (50). Interestingly these combine an *iuc* sequence closely related to that of KpVP-2 (**Fig. 1**) with a FIB_K replicon sequence very close to that of KpVP-1 (**Fig. 5**), and showed substantial mosaicism and gene content variation (**Fig. 2, Fig. 4**). The *iuc3* lineage was also quite common (5.3% of all *iuc*⁺ strains) and associated with a variety of diverse plasmids, most of which carried the FII conjugative transfer region and thus are likely self-transmissible (**Fig. 2, Fig. 4**). It is notable that *iuc2a* and *iuc3* plasmids were not only quite rare in the population, but also showed less evidence of stable maintenance within *K. pneumoniae* lineages (**Fig. 3**) and lower stability of gene content (**Fig. 2**) than the dominant KpVP-1 and KpVP-2 plasmids (**Fig. 4**). The position of *iuc2a* and *iuc3* in the *iuc* trees (**Fig. 1, Fig. S2**) suggests that both derive

from other *K. pneumoniae* loci, hence we speculate it is the properties of the plasmids mobilising these loci, and not the siderophore biosynthesis loci themselves, that makes these variants less widespread in the *K. pneumoniae* population. This variation in gene content may be a consequence of self-transmissibility, exposing the plasmids to a wider gene pool of host bacteria and providing opportunities for gene content diversification, which could potentially include AMR genes. Notably the *iuc3* plasmids carry an arsenal of additional virulence loci involved in iron metabolism and resistance to heavy metals, reminiscent of KpVP-1 (**Fig. 2**).

The other novel plasmids appear to derive from outside *K. pneumoniae* (**Fig. 1, Fig. S2**). Most concerning are the four *E. coli*-derived plasmids we detected carrying *iuc5* (and occasionally *iro5*) in USA and Brazil, three of which were found in the MDR hospital outbreak-associated clone CG258. Whether these aerobactin plasmids harbour AMR genes as they do in *E. coli* is not currently resolvable; however it seems that conjugative *E. coli* plasmids such as D3 plasmid A do have the potential to deliver hypervirulence and multidrug resistance to *K. pneumoniae* strains in a single step. A recent study of *K. pneumoniae* submitted to Public Health England used PCR to screen for isolates carrying both carbapenemase genes and *rmpA*, as a marker of the virulence plasmid, and identified a plasmid harbouring *iuc*, *rmpA*, *rmpA2* and the AMR genes *sul1*, *sul2*, *armA*, *dfrA5*, *mph(A)* and *aph(3')-VIb* (28). To our knowledge this is the first report of a complete sequence of a *K. pneumoniae* plasmid harbouring both AMR and virulence genes. The isolate (ST147) was not included in our original screen, however subsequent analysis using Kleborate plus manual inspection of the plasmid sequence reveals it carries *iuc1* (AbST63, a novel single locus variant of AbST1 which is typical of hypervirulent clones CG23, CG65 and CG86) and appears to be a mosaic carrying sequences from KpVP-1 (40% coverage), an IncFII conjugative transfer region and transposons carrying AMR genes.

The presence of aerobactin synthesis loci in the *iuc5+* *K. pneumoniae* isolates we identified here was not reported in the original studies (51,52), and thus it is not known whether they actually produce aerobactin or show enhanced virulence. This highlights the need to raise awareness of the *iuc* and *iro* loci as potentially clinically relevant hypervirulence factors, and to screen for them in isolates and genome data. The latter we aim to facilitate via the genotyping schemes established here, which can be used to easily screen new genome assemblies using Kleborate

(<https://github.com/katholt/Kleborate/>) or BIGSdb-Kp (<http://bigsdb.pasteur.fr/klebsiella/klebsiella.html>), or new short read data sets using SRST2 (<https://github.com/katholt/srst2>). PCR primers suitable for screening for *iro* and *iuc* can be found in Lee *et al* (53). Notably many studies rely on the hypermucoidy phenotype to identify hypervirulent strains, however this is dependent on growth conditions (54), and recent studies indicate that aerobactin synthesis is a more important virulence determinant (16,13,14). Our data suggest that hypermucoidy screening would typically pick up most of the common aerobactin plasmids KpVP-1, KpVP-2 and *iuc2a+* plasmids; but not those carrying *iuc3*, or the *iuc5* plasmids from *E. coli*. Additionally, it is important not to conflate the presence of the core chromosomal receptor gene *iutA* with the ability to synthesise aerobactin, which is encoded in the *iuc* locus (6). False positive detection of the aerobactin locus version of *iutA* can be avoided by using an identity threshold of <20% divergence. Tellurite resistance has also been suggested as a phenotypic screen to identify hypervirulent isolates of CG23, CG65 and CG86(55); our data confirms this is a good marker for KpVP-1 (92.6% carry *ter*), but not for other aerobactin plasmid types (Table S10).

570

571 CONCLUSIONS

572 Our results illuminate that distinct virulence plasmid variants are associated
573 with the various hypervirulent *K. pneumoniae* lineages, but also highlight that these
574 alongside other plasmids and MGEs can shuttle aerobactin and salmochelin synthesis
575 loci to other lineages, threatening the emergence of novel hypervirulent strains.
576 Indeed, reports of MDR clones acquiring *iuc* plasmids appear to be increasing in
577 incidence, particularly in China (27,29,56–58), and have been associated with
578 increased morbidity and mortality. The AbST and SmST typing schemes developed in
579 this study provide an important resource to identify and monitor the movement of *iro*
580 and *iuc* loci and associated MGEs in *K. pneumoniae* genomes; which will be
581 important to detect and contain these emerging threats. Genotyping with our tools
582 reveals the *iuc* plasmid identified in the recently reported fatal hospital outbreak of
583 carbapenemase-producing ST11 in Beijing is a variant of KpVP-1 that carries *iuc1*
584 (AbST1) and a single copy of *rmpA* but lacks the *iro* locus (27). In this strain the
585 aerobactin plasmid does not carry any AMR determinants; the carbapenemase gene
586 *bla_{KPC}* and several other AMR genes were located on other plasmids. Concerningly,

the ability for the virulence plasmids to be maintained in *K. pneumoniae* lineages suggests that once established in the MDR hospital outbreak-associated clones, they may become quite stable. The initial report of *iuc*+ KPC+ ST11 in China prompted multiple other groups to report detection of the same strain in their hospitals (59–61), suggesting this strain may indeed be emerging as a persistently hypervirulent and MDR form of *K. pneumoniae*. Genomic surveillance and control of the spread of such ‘dual-risk’ strains, or indeed even plasmids combining both characteristics of MDR and hypervirulence clearly needs to be reinforced; the present work will bolster efforts to understand and limit the emergence of infections caused by *K. pneumoniae* strains carrying the high virulence determinants aerobactin and salmochelin.

List of Abbreviations:

MDR: multidrug-resistant, HA: healthcare-associated, CA: community-associated, AMR: antimicrobial resistance, MGEs: mobile genetic elements, HGT: horizontal gene transfer, ICEs: integrative and conjugative elements, ST: sequence type, CG: clonal group, MLST: multi-locus sequence typing, Ent: enterobactin, Ybt: yersiniabactin, *iuc*: aerobactin, *iro*: salmochelin, AbST: aerobactin sequence type, SmST: salmochelin sequence type, OR: odds ratio, CI: confidence interval

REFERENCES

- Podschun R, Ullmann U. *Klebsiella* spp. as Nosocomial Pathogens: Epidemiology, Taxonomy, Typing Methods, and Pathogenicity Factors. Clin Microbiol Rev. 1998;11(4):589–603.
- Martin RM, Bachman MA. Colonization, Infection, and the Accessory Genome of *Klebsiella pneumoniae*. Front Cell Infect Microbiol. 2018;8(4).
- Holt KE, Wertheim H, Zadoks RN, Baker S, Whitehouse CA, Dance D, et al. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. Proc Natl Acad Sci USA. 2015;112(27):E3574-81.
- Ramirez MS, Traglia GM, Lin DL, Tran T, Tolmasky ME. Plasmid-Mediated Antibiotic Resistance and Virulence in Gram-Negatives: the *Klebsiella pneumoniae* Paradigm. Microbiol Spectr. 2014;2(5):1–15.
- Gorrie CL, Mirceta M, Wick RR, Edwards DJ, Strugnell RA, Pratt N, et al. Gastrointestinal carriage is a major reservoir of *K. pneumoniae* infection in

- intensive care patients. Clin Infect Dis. 2017;65(2):208–15.
6. Runcharoen C, Moradigaravand D, Blane B, Paksanont S, Thammachote J, Anun S, et al. Whole genome sequencing reveals high-resolution epidemiological links between clinical and environmental *Klebsiella pneumoniae*. Genome Med; 2017;9(6).
7. Lin TL, Lee CZ, Hsieh PF, Tsai SF, Wang JT. Characterization of integrative and conjugative element ICEKp1-associated genomic heterogeneity in a *Klebsiella pneumoniae* strain isolated from a primary liver abscess. J Bacteriol. 2008;190(2):515–26.
8. Lam MMC, Wick RR, Wyres KL, Gorrie C, Judd LM, Jenney A, et al. Genetic diversity, mobilisation and spread of the yersiniabactin-encoding mobile element ICEKp in *Klebsiella pneumoniae* populations. Microb Genom. 2018; doi:10.1099/mgen.0.000196.
9. Nassif X, Sansonetti PJ. Correlation of the Virulence of *Klebsiella pneumoniae* KI and K2 with the Presence of a Plasmid Encoding Aerobactin. Infect Immun. 1986;54(3):603–8.
10. Müller S, Valdebenito M, Hantke K. Salmochelin, the long-overlooked catecholate siderophore of *Salmonella*. BioMetals. 2009;22(4):691–5.
11. Goetz DH, Holmes MA, Borregaard N, Bluhm ME, Raymond KN, Strong RK. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. Mol Cell. 2002;10(5):1033–43.
12. Fischbach MA, Lin H, Zhou L, Yu Y, Abergel RJ, Liu DR, et al. The pathogen-associated *iroA* gene cluster mediates bacterial evasion of lipocalin 2. Proc Natl Acad Sci USA. 2006;103(44):16502–7.
13. Russo TA, Olson R, Macdonald U, Beanan J, Davidson BA. Aerobactin, but not yersiniabactin, salmochelin, or enterobactin, enables the growth/survival of hypervirulent (hypermucoviscous) *Klebsiella pneumoniae* *ex vivo* and *in vivo*. Infect Immun. 2015;83(8):3325–33.
14. Russo TA, Olson R, Macdonald U, Metzger D, Maltese LM, Drake EJ. Aerobactin Mediates Virulence and Accounts for Increased Siderophore Production under Iron-Limiting Conditions by Hypervirulent (Hypermucoviscous) *Klebsiella pneumoniae*. Infect Immun. 2014;82(6):2356–67.
15. Konopka K, Bindereif A, Neilands J. Aerobactin-mediated utilization of

- transferrin iron. *Biochemistry*. 1982;21(25):6503–8.
16. Russo TA, Olson R, Fang C-T, Stoesser N, Miller M, Hutson A, et al. Identification of biomarkers for the differentiation of hypervirulent *Klebsiella pneumoniae* from classical *K. pneumoniae*. *J Clin Microbiol*. 2018; doi: 10.1128/JCM.00776-18.
17. Putze J, Hennequin C, Nougayrède JP, Zhang W, Homburg S, Karch H, et al. Genetic structure and distribution of the colibactin genomic island among members of the family *Enterobacteriaceae*. *Infect Immun*. 2009;77(11):4696–703.
18. Wu KM, Li NH, Yan JJ, Tsao N, Liao TL, Tsai HC, et al. Genome sequencing and comparative analysis of *Klebsiella pneumoniae* NTUH-K2044, a strain causing liver abscess and meningitis. *J Bacteriol*. 2009;191(14):4492–501.
19. Chen Y, Chang H, Lai Y, Pan C, Tsai S. Sequencing and analysis of the large virulence plasmid pLVPK of *Klebsiella pneumoniae* CG43. *Gene*. 2004;337:189–98.
20. Lery LM, Frangeul L, Tomas A, Passet V, Almeida AS, Bialek-Davenet S, et al. Comparative analysis of *Klebsiella pneumoniae* genomes identifies a phospholipase D family protein as a novel virulence factor. *BMC Biol*. 2014;12(1):41.
21. Nassif X, Fournier J, Arondel J, Sansonetti PJ. Muroid Phenotype of *Klebsiella pneumoniae* Is a Plasmid-Encoded Virulence Factor. *Infect Immun*. 1989;57(2):546–52.
22. Lam MMC, Wyres KL, Duchêne S, Wick RR, Judd LM, Gan Y, et al. Population genomics of hypervirulent *Klebsiella pneumoniae* clonal-group 23 reveals early emergence and rapid global dissemination. *Nat Comm*. 2018; doi: 10.1101/225359.
23. Struve C, Roe CC, Stegger M, Stahlhut SG, Hansen DS, Engelthaler DM, et al. Mapping the evolution of hypervirulent *Klebsiella pneumoniae*. *MBio*. 2015;6(4):1–12.
24. Peng H, Wang P, Wu J, Chiu C, Chang H. Molecular Epidemiology of *Klebsiella pneumoniae*. *Zhonghua Min Guo Wei Sheng Wu Ji Mian Yi Xue Za Zhi*. 1991;24:264–71.
25. Bialek-davenet S, Criscuolo A, Ailloud F, Passet V, Jones L, Garin B, et al. Genomic Definition of Hypervirulent and Multidrug-Resistant *Klebsiella*

- 689 *pneumoniae* Clonal Groups. Emerg Infect Dis. 2014;20(11):1812–20.
- 690 26. Shon AS, Russo TA. Hypervirulent *Klebsiella pneumoniae*: the next superbug?
691 Futur Microbiol. 2012;7(6):669–71.
- 692 27. Gu D, Dong N, Zheng Z, Lin D, Huang M, Wang L, et al. A fatal outbreak of
693 ST11 carbapenem-resistant hypervirulent *Klebsiella pneumoniae* in a Chinese
694 hospital: a molecular epidemiological study. Lancet Infect Dis. 2018;
695 doi:10.1016/S1473-3099(17)30489-9.
- 696 28. Turton JF, Payne Z, Coward A, Hopkins K, Turton J, Doumith M, et al.
697 Virulence genes in isolates of *Klebsiella pneumoniae* from the UK during
698 2016, including among carbapenemase gene-positive hypervirulent K1-ST23
699 and “non-hypervirulent” types ST147, ST15 and ST383. J Med Microbiol.
700 2018;67(1):118–28.
- 701 29. Chen L, Kreiswirth BN. Convergence of carbapenem-resistance and
702 hypervirulence in *Klebsiella pneumoniae*. Lancet Infect Dis. 2018;
703 doi:10.1016/S1473-3099(17)30517-0.
- 704 30. Cheong HS, Chung DR, Lee C, Kim SH, Kang C, Peck KR. Emergence of
705 serotype K1 *Klebsiella pneumoniae* ST23 strains co-producing the DHA-1 and
706 an extended-spectrum beta-lactamase in Korea. Antimicrob Resist Infect
707 Control. 2016; doi:10.1186/s13756-016-0151-2.
- 708 31. Shin J, Ko KS. Single origin of three plasmids bearing bla CTX-M-15 from
709 different *Klebsiella pneumoniae* clones. J Antimicrob Chemother.
710 2014;69:969–72.
- 711 32. Wyres KL, Wick RR, Gorrie C, Jenney A, Follador R, Thomson NR, et al.
712 Identification of *Klebsiella* capsule synthesis loci from whole genome data.
713 Microb Genomics. 2016; doi: 10.1099/mgen.0.000102.
- 714 33. Wick RR, Judd LM, Gorrie C, Holt KE. Unicycler: Resolving bacterial genome
715 assemblies from short and long sequencing reads. PLoS Comput Biol.
716 2017;13(6):e1005595.
- 717 34. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et
718 al. SPAdes: A New Genome Assembly Algorithm and Its Applications to
719 Single-Cell Sequencing. J Comp Biol. 2012;19(5):455–77.
- 720 35. Seemann T. Prokka: Rapid prokaryotic genome annotation. Bioinformatics.
721 2014;30(14):2068–9.
- 722 36. Wick RR, Judd LM, Gorrie CL, Holt KE. Completing bacterial genome

723 assemblies with multiplex MinION sequencing. *Microb Genomics*. 2017;3:1–
724 7.

725 37. Diancourt L, Passet V, Verhoef J, Grimont PAD, Brisse S. Multilocus
726 Sequence Typing of *Klebsiella pneumoniae* Nosocomial Isolates. *J Clin*
727 *Microbiol*. 2005;43(8):4178–82.

728 38. Inouye M, Dashnow H, Raven L-A, Schultz MB, Pope BJ, Tomita T, et al.
729 SRST2: Rapid genomic surveillance for public health and hospital
730 microbiology labs. *Genome Med*. 2014;6(11):90.

731 39. Wick RR, Heinz E, Holt KE, Wyres KL. Kaptive Web: user-friendly capsule
732 and lipopolysaccharide serotype prediction for *Klebsiella* genomes. *J Clin*
733 *Microbiol*. 2018; doi:10.1128/JCM.00197-18.

734 40. Carattoli A, Zankari E, García-fernández A, Larsen V, Lund O, Villa L, et al.
735 In Silico Detection and Typing of Plasmids using PlasmidFinder and Plasmid
736 Multilocus Sequence Typing. *Antimicrob Agents Chemother*.
737 2014;58(7):3895–903.

738 41. Wick RR, Schultz MB, Zobel J, Holt KE. Genome analysis Bandage:
739 interactive visualization of de novo genome assemblies. 2015;31:3350–2.

740 42. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and
741 high throughput. *Nucleic Acids Res*. 2004;32(5):1792–7.

742 43. Stamatakis A. RAxML-VI-HPC: Maximum likelihood-based phylogenetic
743 analyses with thousands of taxa and mixed models. *Bioinformatics*.
744 2006;22(21):2688–90.

745 44. Darling ACE, Mau B, Blattner FR, Perna NT. Mauve: Multiple Alignment of
746 Conserved Genomic Sequence With Rearrangements. *Methods*. 2004;14:1394–
747 403.

748 45. Guy L, Kultima J, Andersson S. genoPlotR: comparative gene and genome
749 visualization in R. *Bioinformatics*. 2010;26(18):2334–5.

750 46. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat*
751 *Methods*. 2012;9:357–9.

752 47. Liu C, Zheng H, Yang M, Xu Z, Wang X, Wei L, et al. Genome analysis and in
753 vivo virulence of porcine extraintestinal pathogenic *Escherichia coli* strain
754 PCN033. *BMC Genomics*. 2015; doi:10.1186/s12864-015-1890-9.

755 48. Carattoli A. Resistance Plasmid Families in *Enterobacteriaceae*. *Antimicrob*
756 *Agents Chemother*. 2009;53(6):2227–38.

49. Capone A, Giannella M, Fortini D, Giordano A, Meledandri M, Ballardini M, et al. High rate of colistin resistance among patients with carbapenem-resistant *Klebsiella pneumoniae* infection accounts for an excess of mortality. Clin Microbiol Infect. 2012; doi:10.1111/1469-0691.12070.
50. Wand ME, Baker KS, Benthall G, McGregor H, McCowen JWI, Deheer-Graham A, et al. Characterization of pre-antibiotic era *Klebsiella pneumoniae* isolates with respect to antibiotic/disinfectant susceptibility and virulence in *Galleria mellonella*. Antimicrob Agents Chemother. 2015;59(7):3966–72.
51. Bowers JR, Kitchel B, Driebe EM, MacCannell DR, Roe C, Lemmer D, et al. Genomic analysis of the emergence and rapid global dissemination of the clonal group 258 *Klebsiella pneumoniae* pandemic. PLoS One. 2015;10(7):1–24.
52. Davis GS, Waits K, Nordstrom L, Weaver B, Aziz M, Gauld L, et al. Intermingled *Klebsiella pneumoniae* Populations between Retail Meats and Human Urinary Tract Infections. Clin Infect Dis. 2015; 61:892–9.
53. Lee IR, Molton JS, Wyres KL, Gorrie C, Wong J, Hoh CH, et al. Differential host susceptibility and bacterial virulence factors driving *Klebsiella* liver abscess in an ethnically diverse population. Sci Rep. 2016; doi:10.1038/srep29316.
54. Catalán-Nájera JC, Garza-Ramos U, Barrios-Camacho H. Hypervirulence and hypermucoviscosity: Two different but complementary *Klebsiella spp.* phenotypes? Virulence. 2017; doi:10.1080/21505594.2017.1317412.
55. Passet V, Brisse S. Association of Tellurite Resistance with Hypervirulent Clonal Groups of *Klebsiella pneumoniae*. J Clin Microbiol. 2015;53(4):1380–2.
56. Zhan L, Wang S, Guo Y, Jin Y, Duan J, Hao Z, et al. Outbreak by Hypermucoviscous *Klebsiella pneumoniae* ST11 Isolates with Carbapenem Resistance in a Tertiary Hospital in China. Front Cell Infect Microbiol. 2017; doi: 10.3389/fcimb.2017.00182.
57. Zhang Y, Zeng J, Liu W, Zhao F, Hu Z, Zhao C, et al. Emergence of a hypervirulent carbapenem-resistant *Klebsiella pneumoniae* isolate from clinical infections in China. J Infect. 2015; doi:10.1016/j.jinf.2015.07.010
58. Araújo BF, Ferreira ML, Campos PA De, Royer S, Gonçalves IR, Fernandes MR, et al. Hypervirulence and biofilm production in KPC-2-producing

- 791 *Klebsiella pneumoniae* CG258 isolated in Brazil. J Med Microbiol.
792 2018;67:523–8.
- 793 59. Du P, Zhang Y, Chen C. Emergence of carbapenem-resistant hypervirulent
794 *Klebsiella pneumoniae*. Lancet Infect Dis. 2018; doi: 10.1016/S1473-
795 3099(17)30625-4.
- 796 60. Yao H, Qin S, Chen S, Shen J, Du X-D. Emergence of carbapenem-resistant
797 hypervirulent *Klebsiella pneumoniae*. Lancet Infect Dis. 2018; doi:
798 10.1016/S1473-3099(17)30628-X.
- 799 61. Wong MH, Shum H-P, Chen JH, Man M-Y, Wu A, Chan EW, et al.
800 Emergence of carbapenem-resistant hypervirulent *Klebsiella pneumoniae*.
801 Lancet Infect Dis. 2018; doi: 10.1016/S1473-3099(17)30629-1.

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804 **DECLARATIONS:**

805
806 **Ethics approval and consent to participate.**

807 Not applicable.

808
809 **Consent for publication.**

810 Not applicable.

811
812 **Availability of data and material.**

813 All whole-genome sequences analysed in this study are publicly available in NCBI or
814 the NCTC 3000 Project website
815 (<https://www.sanger.ac.uk/resources/downloads/bacteria/nctc/>), accession numbers
816 are listed in **Table S1**. Complete genome sequences generated for this study
817 (summarised in **Table S3**) have been deposited in NCBI GenBank under accessions
818 TBC. Accession numbers for the 12 reference plasmid sequences are listed in **Table**
819 **S2**; the set of annotated sequences and the Mauve multiple alignment of these
820 sequences are also deposited in FigShare (doi:10.6084/m9.figshare.6839981). The
821 aerobactin and salmochelin MLST schemes are available in the *K. pneumoniae*
822 BIGSdb database (<http://bigsdb.pasteur.fr/klebsiella/klebsiella.html>) and in the
823 Kleborate distribution (<https://github.com/katholt/Kleborate>).
824

825 **Competing interests.**

826 The authors declare that they have no competing interests.

827

828 **Funding.**

829 This work was funded by the National Health and Medical Research Council
830 (NHMRC) of Australia (project #1043822), a Senior Medical Research Fellowship
831 from the Viertel Foundation of Australia, and the Bill and Melinda Gates Foundation
832 of Seattle, USA.

833

834 **Authors' contributions.**

835 MMCL performed the majority of data analyses and wrote the paper together with
836 KEH. RRW, K LW, SB, and KEH contributed additional data analysis, visualisation
837 and interpretation. SB incorporated the novel MLST schemes into the BIGSdb. RRW
838 and KEH wrote code. AJ contributed clinical isolates, data and interpretations. LMJ
839 performed DNA extraction and nanopore sequencing. All authors edited and approved
840 the paper.

841

842 **Acknowledgements.**

843 We thank the team of the curators of the Institut Pasteur MLST system (Paris, France)
844 for importing novel alleles, profiles and/or isolates at <http://bigsdb.pasteur.fr>.

845 **Tables**

846 **Table 1. Summary of *iuc* and/or *iro* plasmid lineages**

Lineage(s)	N	Mobile genetic element	Reference(s)
<i>iuc1</i> (+ <i>iro1</i>)	121 (119)	<i>K. pneumoniae</i> VP-1, type I FIB _K + HI1B, <i>rmpA</i> + <i>rmpA2</i>	pK2044 (accession AP006726.1)
<i>iuc2</i> (+ <i>iro2</i>)	22 (22)	<i>K. pneumoniae</i> VP-2, type II FIB _K , <i>rmpA</i>	Kp52.145 plasmid II (accession FO384905.1)
<i>iuc2a</i>	43	Novel, diverse plasmids FIB _K + other F replicons, sometimes IncFII <i>tra</i>	Many distinct types Novel examples: pINF151 (accession TBC), pINF237 (accession TBC)
<i>iuc3</i>	11	Novel, diverse plasmids FIB _K + IncFII <i>tra</i>	NCTC11676, NCTC11697
<i>iuc4</i>	7	Chromosomal integration	<i>K. pneumoniae rhinoscleromatis</i> e.g. strain SB3432 (accession FO203501.1)
<i>iuc5</i> (+ <i>iro5</i>)	4 (2)	<i>E. coli</i> FII <i>tra</i> plasmid <i>E. coli iroBCDEN</i> +AMR	<i>E. coli</i> strain PCN033 plasmid p3PCN033 (accession CP006635.1)
<i>iro3</i>	16	Chromosomal ICEKp1	<i>K. pneumoniae</i> NTUH-K2044 ICEKp1 (accession AB298504.1)
<i>iro4</i>	1	Novel plasmid FIB _K + FII <i>tra</i> <i>E. cloacae</i> / <i>E. hormaechei iroBCDEN</i> (x13 copies)	pINF078 (accession TBC)

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856 **Supplementary Tables**

857 Table S1. Strain information for genomes included in this study.

858 Table S2. General features of reference plasmids or incomplete plasmid sequences
859 carrying *iro* and/or *iuc*.

860 Table S3. Summary of replicon sequences from strains INF151, INF237 and INF078

861 Table S4. Aerobactin sequence types (AbSTs) and corresponding alleles.

862 Table S5. Salmochelin sequence types (SmSTs) and corresponding alleles.

863 Table S6. Representative Enterobacteriaceae genome sequences included in *iro* and
864 *iuc* phylogenetic analysis

865 Table S7. Single nucleotide variants and nucleotide divergence (%) observed within
866 (shaded in grey) and between the aerobactin-encoding *iuc* lineages.

867 Table S8. Single nucleotide variants and nucleotide divergence (%) observed within
868 (shaded in grey) and between the salmochelin-encoding *iro* lineages.

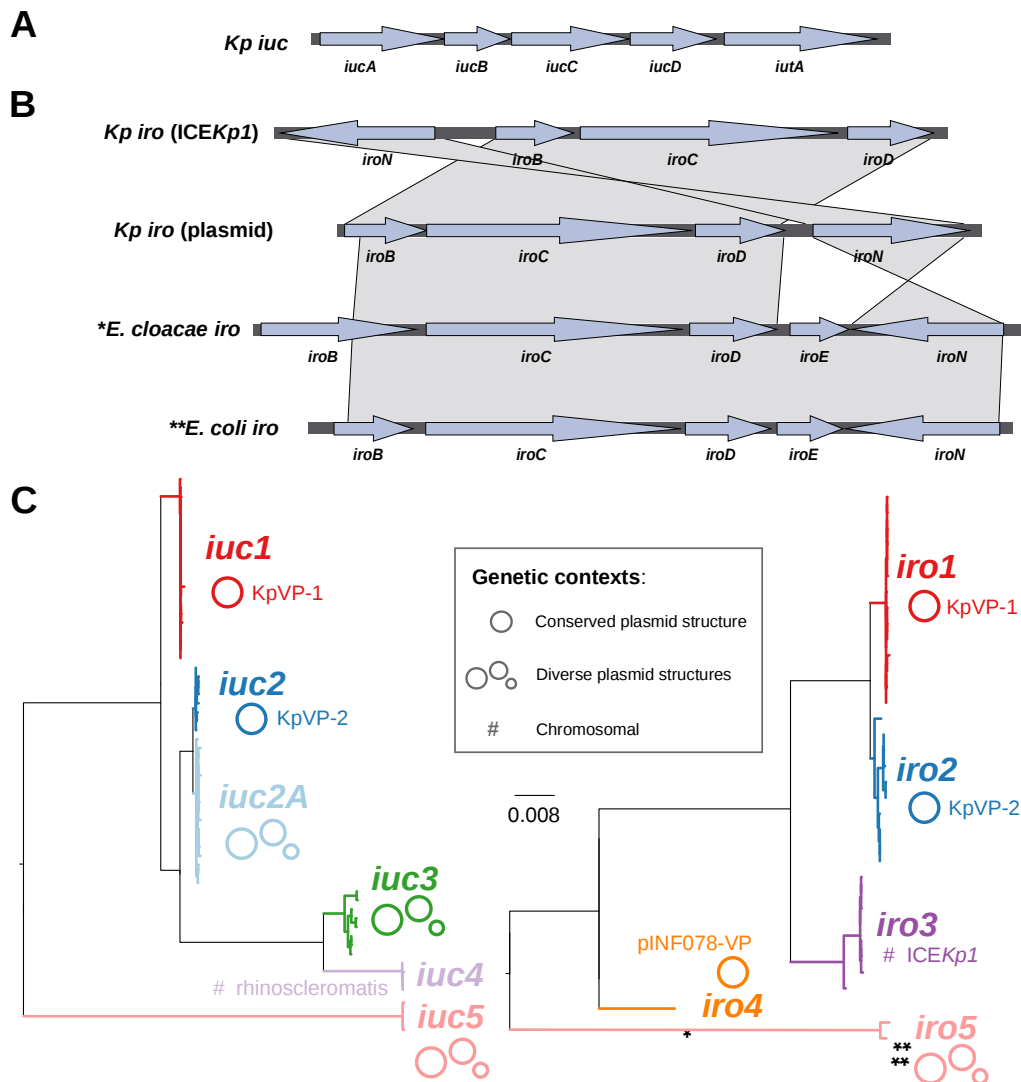
869 Table S9. Summary of aerobactin-encoding *iuc* and salmochelin-encoding *iro* loci
870 BLAST hit

871 Table S10. Prevalence of virulence loci and plasmid replication loci amongst strains
872 with virulence plasmids

873

874

875 **Figure legends:**



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877 **Figure 1. (a)** Structure of the aerobactin locus *iuc* found in *Klebsiella pneumoniae*.

878 **(b)** Structure of the salmochelin (*iro*) loci found in *K. pneumoniae* integrative

879 conjugative element ICEKp1, *K. pneumoniae* plasmids, *Enterobacter cloacae* and

880 *Escherichia coli*. *Also found in *iro4* *K. pneumoniae* plasmid; **also found in *iro5*

881 *Kp* plasmids. **(c)** Maximum likelihood phylogenetic trees inferred from *iuc* and *iro*

882 sequence types (AbSTs and SmSTs) identified in *K. pneumoniae* genomes.

883 Phylogenetic lineages discussed in the text are labelled and their mobility indicated;

884 nucleotide divergence within and between lineages is given in Tables S7 and S8.

885 * and ** indicate *iro* locus structural variants that are more typical of non-*Klebsiella*

886 species, as shown in panel **(b)**.

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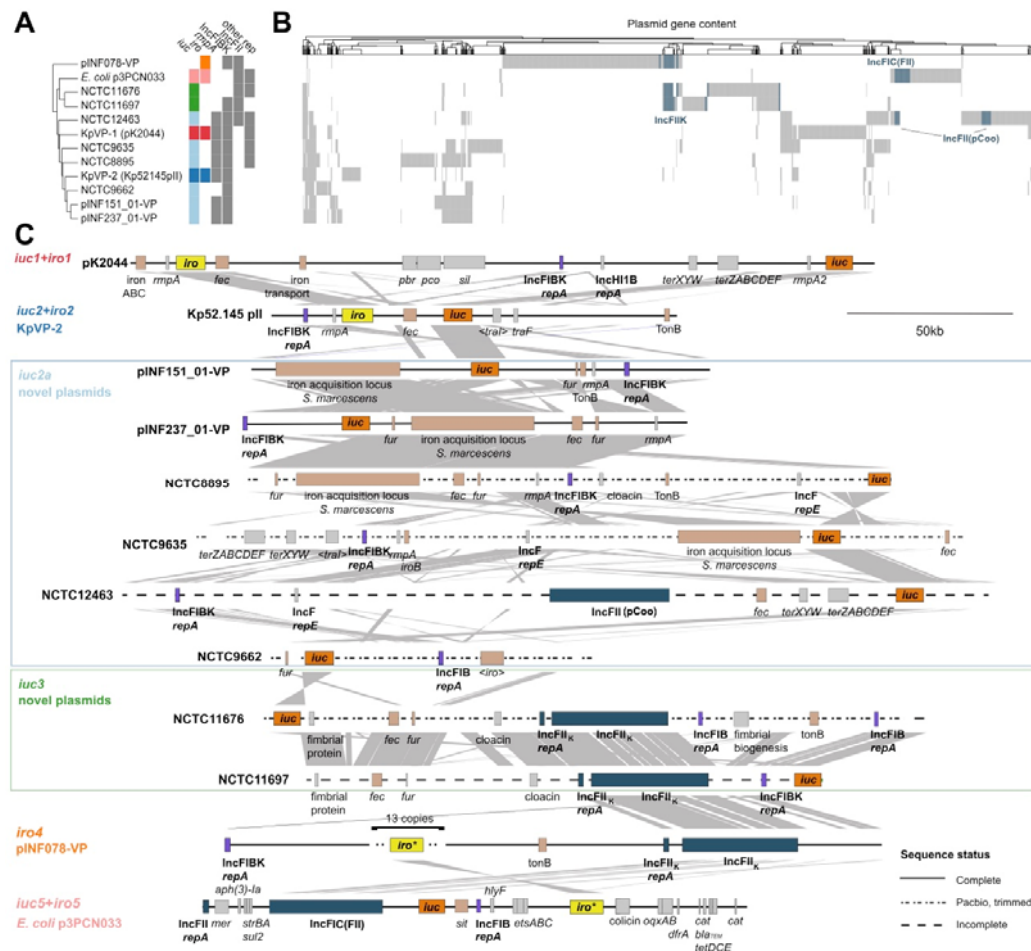


Figure 2. Plasmid variants associated with different *iro* and/or *iuc* lineages identified amongst *K. pneumoniae*. (a) Clustering of the 12 reference plasmids based on gene content, annotated with presence of *iuc* and *iro* lineages (coloured as in panel b, and Figure 1c), *rmpA*, IncFIB_K, IncFIB, IncFII and/or other plasmid replicon types. (b) Gene content matrix for reference plasmids; columns correspond to protein-coding sequences that are >10% divergent from one another. IncFII conjugal transfer region genes are coloured blue, to highlight the divergent forms of this region and labelled with the closest IncFII type as detected by PlasmidFinder. (c) Genetic maps for the reference plasmids. The positions of key loci involved in core plasmid functions (bold), virulence (*iro* highlighted in yellow, *iuc* in dark orange and other loci involved in iron acquisition/transport in light orange) and antimicrobial resistance are indicated. Grey shading indicates homology blocks sharing >60% nucleotide identity.

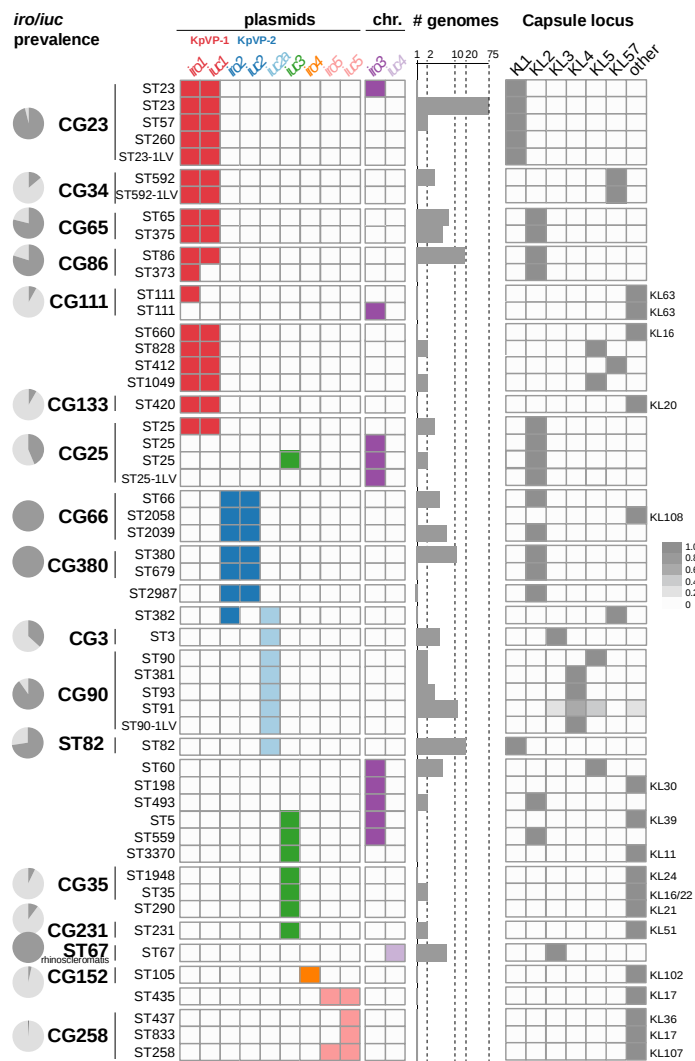


Figure 3. Distribution of plasmid and chromosomal variants of *iro* and *iuc* and capsule locus (KL) types amongst *K. pneumoniae* clones.

Rows indicate sequence types (STs, as labelled) that contain ≥ 1 genome in which *iro* and/or *iuc* was detected; vertical lines indicate STs belonging to the same clonal group (CG) as labelled. Pie charts indicate prevalence of *iro* and/or *iuc* within common *K. pneumoniae* lineages. The detection of individual *iro* and *iuc* lineages within each *K. pneumoniae* ST is indicated in the grid, coloured as per Figure 1. Bar plots indicate sample size (number of genomes per ST; note $\log_{10}$ scale). Heatmap on the right indicates prevalence of capsule (K) locus types in each *K. pneumoniae* ST, coloured as per inset legend. Individual columns are included for K types that are common amongst virulent clones; where other K types were detected these are represented in the ‘other’ column, and the relevant K type for that ST is labelled to the right.

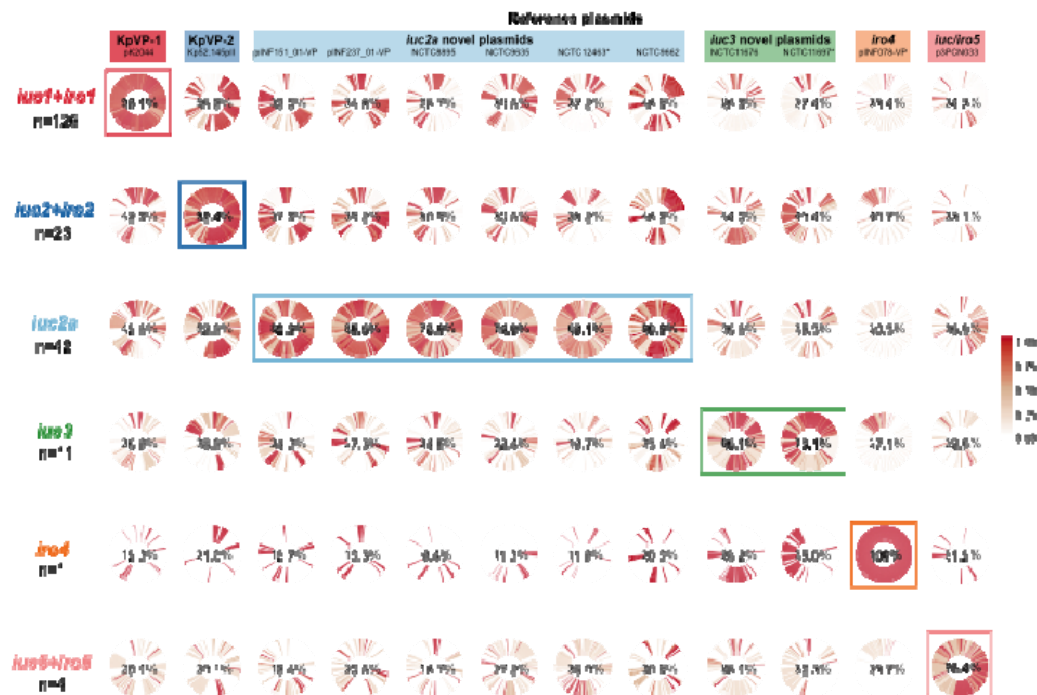


Figure 4. Conservation of reference plasmid genes amongst strains with plasmid-associated *iuc/iro* lineages. Cells show circularised heatmaps indicating the frequency of each gene in a given reference plasmid (column); amongst strains that contain a given *iro* and/or *iuc* lineage (row). Around each circle, genes are ordered by their order in the corresponding reference plasmid. Percentages in the middle of each cell indicates the mean coverage of the reference plasmid sequence (column), amongst strains belonging to each *iro/iuc* lineage (row); bold labels and boxes highlight groups of strains carrying the same *iuc/iro* lineage as the reference plasmid. * indicates the two plasmids represented by incomplete plasmid sequences.

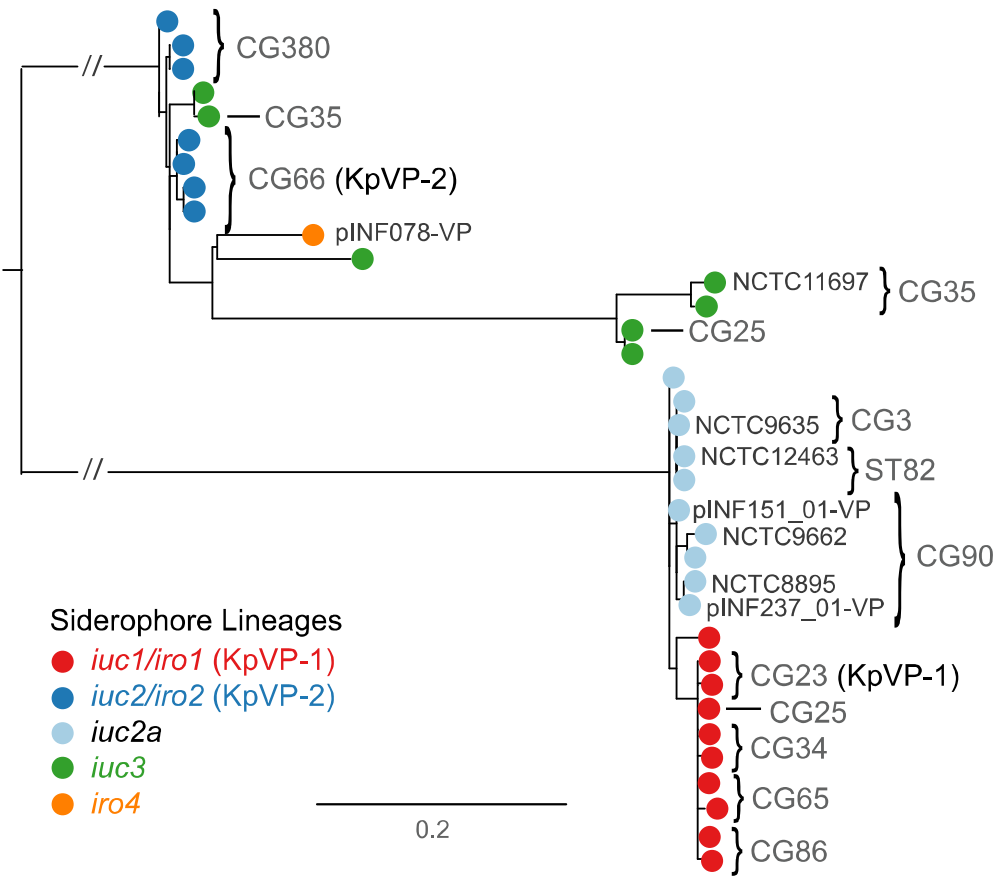


Figure 5. Maximum likelihood phylogeny of representative IncFIB_K replicon sequences from strains with *iuc/iro* plasmids.

Each tip represents a unique IncFIB_K replicon sequence (spanning *repA*, *oriT*, *sopAB*), coloured according to the *iro/iuc* lineage carried by the corresponding strains as per inset legend. FIB_K sequences found in the representative plasmid sequences (shown in **Fig. 2** and listed in **Table S2**) are labelled; tips/subclades are also annotated to indicate those found in common clonal groups (CG; see **Fig. 3**).

Supplementary figures:

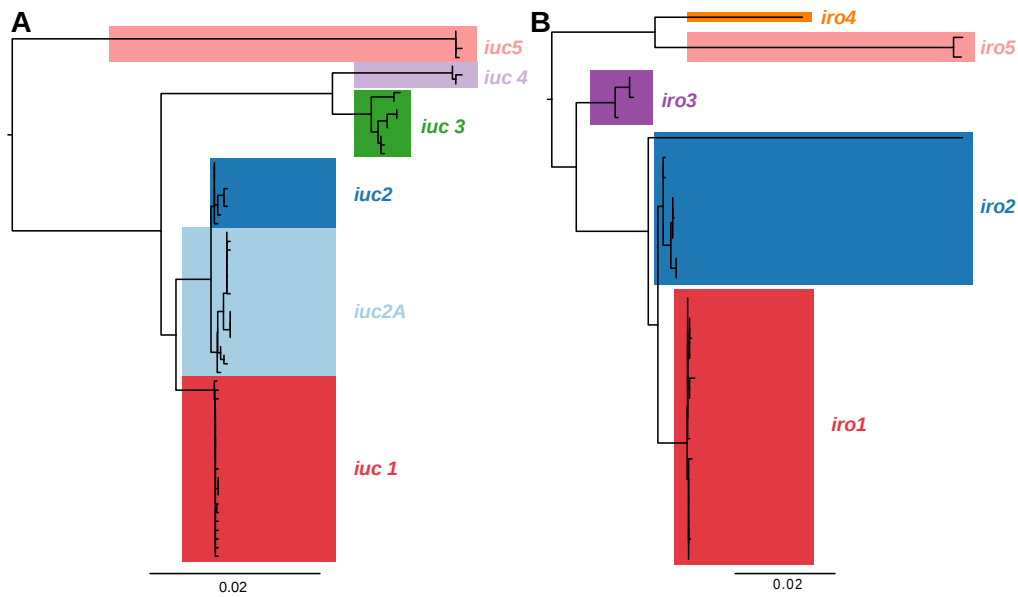


Figure S1. Phylogenetic relationships between the predicted amino acid sequences encoded by aerobactin (*iuc*) and salmochelin (*iro*) locus sequence types. Each tip represents a translated amino acid sequence for an aerobactin sequence type (AbST, in **a**) or salmochelin sequence type (SmST, in **b**). Lineages defined from nucleotide sequences (see tree in **Fig. 1**) are highlighted and labelled.

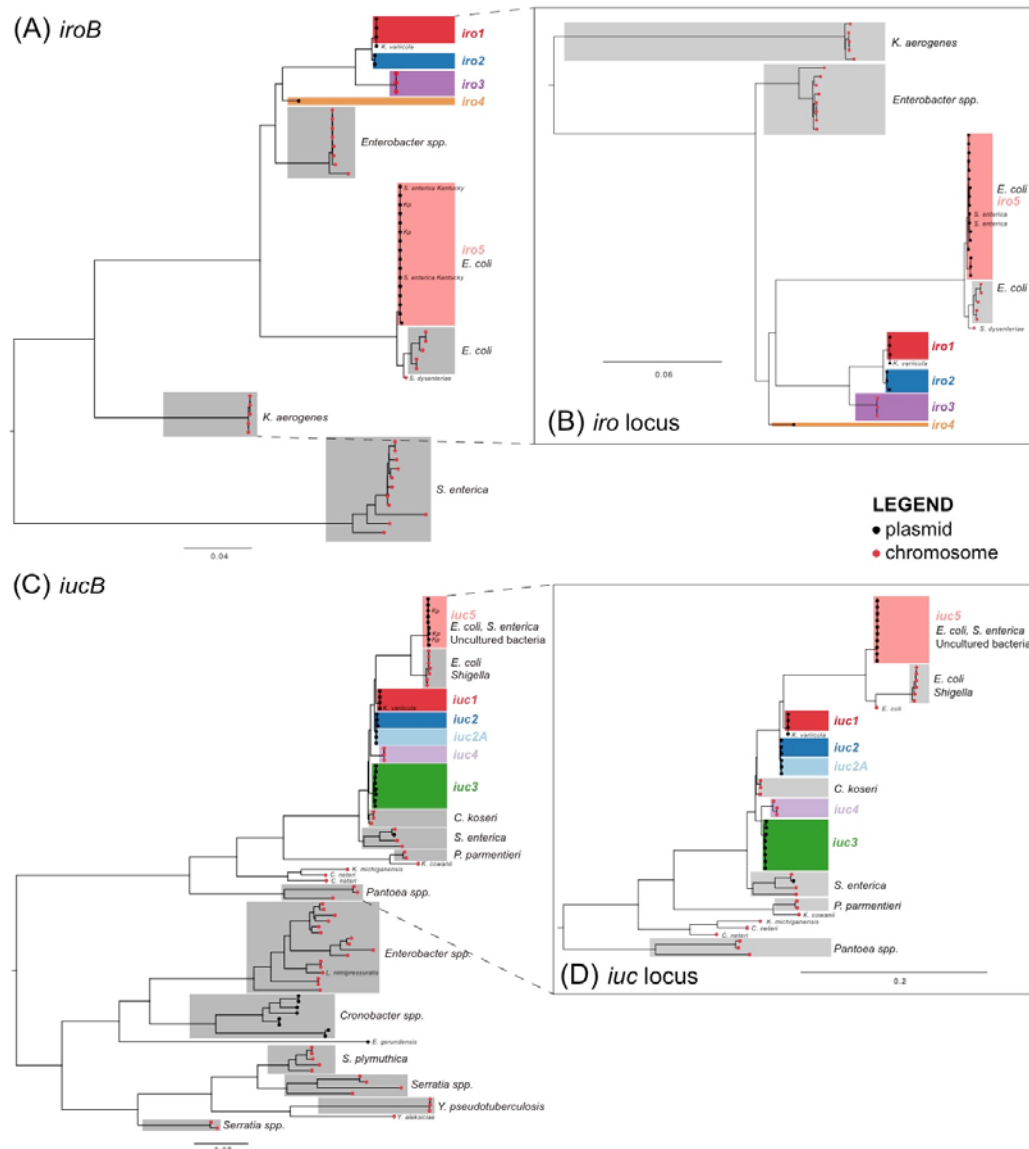
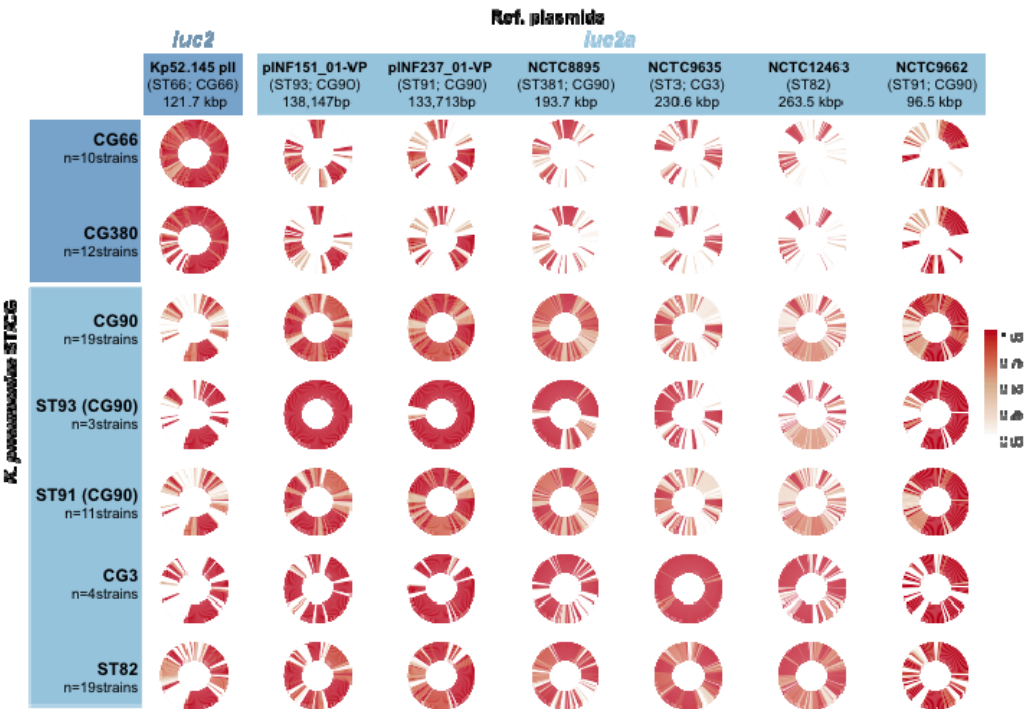


Figure S2. Phylogenetic trees for salmochelin and aerobactin encoding *iuc* locus in *K. pneumoniae* and other Enterobacteriaceae bacteria. Trees represent show a midpoint-rooted maximum likelihood phylogeny for representative sequences identified in various Enterobacteriaceae species (listed in **Table S6**). Tip colours indicate the genetic context of the locus: black=plasmid, red=chromosome. *K. pneumoniae* *iro* lineages defined in **Fig. 1** are coloured; other species-specific clades are highlighted in grey; individual labelled tips within highlighted clades indicate exceptions to the species label of the clade. Salmochelin trees were inferred using the *iroB* gene alone (panel **a**), which show a highly divergent form in *Salmonella*. Panel (b) Shows a tree inferred from all four genes of the typical *K. pneumoniae* *iro* locus (*iroBCDN*), excluding the distantly related *Salmonella* variant, to increase resolution

964 within the group containing *Klebsiella*. Similarly, aerobactin trees were inferred using
965 the *iucB* gene alone (panel c) to show the overall structure, and separately for the full
966 set of genes in the *K. pneumoniae* locus (*iucABCD*, *iutA*) to provide greater resolution
967 within the group containing *Klebsiella* (panel d).

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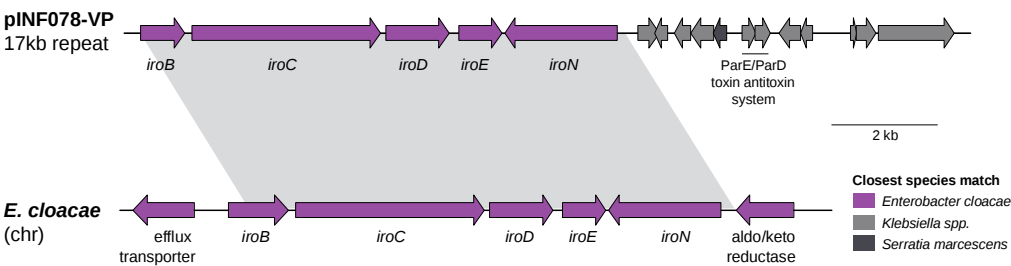


969

970 **Figure S3. Conservation of coding sequences from KpVP-2 and *iuc2a*+ reference**
971 **plasmids amongst strains carrying plasmid-encoded *iuc2* or *iuc2a* loci.** Cells
972 show circularised heatmaps indicating the frequency of each gene in a given reference
973 plasmid (column), amongst strains of a given chromosomal sequence type (ST) or
974 clonal group (CG) (rows) that carry either *iuc2* (CG66, CG380) or *iuc2a* (others).
975 Around each circle, genes are ordered by their order in the corresponding reference
976 plasmid.

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979
980 **Fig. S4 Genetic structure of 17 kbp repeat region in plasmid pINF078-VP and**
981 **the chromosomally-encoded *E. cloacae* *iro* region.** Shaded area indicates a
982 homologous region of 95% nucleotide identity shared between the two sequences.
983 Coding sequences are represented by the arrows and coloured according to the closest
984 Enterobacteriaceae species match as indicated in the legend.
985