

1 **Nested Russian Doll-like Genetic Mobility Drives Rapid Dissemination of the**
2 **Carbapenem Resistance Gene *bla*_{KPC}**

3 Running title: Multiple genetic levels of *bla*_{KPC} mobility

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28 **Abstract**

29 The recent widespread emergence of carbapenem resistance in *Enterobacteriaceae* is a major
30 public health concern, as carbapenems are a therapy of last resort in this family of common
31 bacterial pathogens. Resistance genes can mobilize via various mechanisms including
32 conjugation and transposition, however the importance of this mobility in short-term evolution,
33 such as within nosocomial outbreaks, is currently unknown. Using a combination of short- and
34 long-read whole genome sequencing of 281 *bla*_{KPC}-positive *Enterobacteriaceae* isolated from a
35 single hospital over five years, we demonstrate rapid dissemination of this carbapenem
36 resistance gene to multiple species, strains, and plasmids. Mobility of *bla*_{KPC} occurs at multiple
37 nested genetic levels, with transmission of *bla*_{KPC} strains between individuals, frequent transfer
38 of *bla*_{KPC} plasmids between strains/species, and frequent transposition of the *bla*_{KPC} transposon
39 Tn4401 between plasmids. We also identify a common insertion site for Tn4401 within various
40 Tn2-like elements, suggesting that homologous recombination between Tn2-like elements has
41 enhanced the spread of Tn4401 between different plasmid vectors. Furthermore, while short-
42 read sequencing has known limitations for plasmid assembly, various studies have attempted to
43 overcome this with the use of reference-based methods. We also demonstrate that as a
44 consequence of the genetic mobility observed herein, plasmid structures can be extremely
45 dynamic, and therefore these reference-based methods, as well as traditional partial typing
46 methods, can produce very misleading conclusions. Overall, our findings demonstrate that non-
47 clonal resistance gene dissemination can be extremely rapid, presenting significant challenges
48 for public health surveillance and achieving effective control of antibiotic resistance.

49

50 **Importance**

51 Increasing antibiotic resistance is a major threat to human health, as highlighted by the recent
52 emergence of multi-drug resistant “superbugs”. Here, we tracked how one important multi-
53 drug resistance gene spread in a single hospital over five years. This revealed high levels of
54 resistance gene mobility to multiple bacterial species, which was facilitated by various different
55 genetic mechanisms. The mobility occurred at multiple nested genetic levels, analogous to a
56 Russian doll set where smaller dolls may be carried along inside larger dolls. Our results
57 challenge traditional views that drug-resistance outbreaks are due to transmission of a single
58 pathogenic strain. Instead, outbreaks can be “gene-based”, and we must therefore focus on
59 tracking specific resistance genes and their context rather than only specific bacteria.

60

61 **Introduction**

62 Although antibiotic resistance genes have been identified in ancient bacterial DNA (1), much of
63 the recent, alarming increase in pathogen antimicrobial resistance is attributable to the
64 dissemination of resistance genes via horizontal gene transfer (HGT), in response to selection
65 imposed by widespread antibiotic use in medicine and agriculture (2, 3). Many resistance genes
66 are located on plasmids, which can be transferred between different bacterial strains or
67 species, thus facilitating HGT (4). Furthermore, resistance gene mobility can be enhanced by
68 integration into transposable elements, which are short stretches of DNA (several kilobases)
69 that can autonomously mobilize between different genomic locations (5). However, the
70 importance of HGT in short-term evolution is unclear, as capturing the processes in real-time is
71 challenging, and outbreaks in health care settings are often thought to be dominated by clonal
72 transmission (6-9).

73 Carbapenem resistance in *Enterobacteriaceae* has been recognized as a key threat to modern
74 medicine (10, 11), as carbapenems often represent the therapy of last resort for serious
75 infections (12, 13). One of the most prevalent carbapenem resistance genes is the *Klebsiella*
76 *pneumoniae* carbapenemase (KPC) gene, *bla*_{KPC}, first identified in 1996 and now endemic in
77 many regions of the world (14). KPC is a beta-lactamase capable of hydrolyzing all beta-lactams,
78 including penicillins, monobactams, cephalosporins and carbapenems (15), leaving few
79 treatment options for infected vulnerable hospitalized patients and resulting in worse
80 treatment outcomes (16).

81 Most reports of *bla*_{KPC} involve *K. pneumoniae* multi-locus sequence type (ST)258 (9, 17), which
82 has been found globally, indicating that clonal dissemination of this resistant lineage has been
83 an important factor in the spread of *bla*_{KPC} (9, 17-20). Nevertheless, *bla*_{KPC} has also been
84 observed in other *K. pneumoniae* lineages, as well as other species of *Enterobacteriaceae*,
85 suggesting that *bla*_{KPC} HGT has also played a role in resistance dissemination (21-25). As *bla*_{KPC} is
86 often found on conjugative plasmids, some of which have been identified in multiple strains or
87 species, this provides a likely mechanism for HGT (21, 26, 27). In addition, *bla*_{KPC} is usually
88 present as part of the 10 kb composite Tn3-based mobile transposon Tn4401, which has been
89 identified in various different plasmids, implicating Tn4401 transposition as another mechanism
90 contributing to *bla*_{KPC} spread (28, 29).

91 While Tn4401 transposition and plasmid conjugation have been measured in the laboratory
92 (28, 30, 31), the frequencies with which these processes occur within real-world ecosystems is
93 not fully understood. In clinical contexts, it is often assumed that short-term evolution is
94 dominated by clonal propagation, such that transmission chains generally involve a single
95 pathogenic strain. However, if HGT is frequent relative to transmission (e.g. a "plasmid
96 outbreak"), then linked patients may show variation in strain composition. If transposition is
97 also frequent, then both host strain and resistance plasmid may show high variability within a
98 single outbreak. As current surveillance strategies tend to focus on the host strain, it is
99 important to establish the relevance of *bla*_{KPC} mobility within outbreak settings.

100 Traditional approaches for plasmid investigation, such as PCR-based replicon typing, are limited
101 in resolution. Next-generation sequencing has been successfully applied to molecular

102 epidemiological investigation of a number of pathogens at the host strain level, however the
103 application and limitations of this technology for transmission chains involving HGT are
104 relatively unexplored. Whole genome sequencing using short-read technologies (e.g. Illumina)
105 has become cheap and accessible, but is not ideal for plasmid analysis due to *de novo* assembly
106 limitations, as it is often not possible to accurately reconstruct the genomic context
107 surrounding repeated sequences (21, 32). Long-read sequencing (e.g. PacBio) can largely
108 overcome this, often providing single-contig plasmid assemblies, but it is currently prohibitively
109 expensive for many applications. Several studies have utilised reference-based methods for
110 plasmid assembly or inference of plasmid structures using short-read data (33, 34), however
111 these approaches make the implicit assumption that plasmid structures are relatively stable. It
112 will be important to understand the potential shortcomings of these assumptions in relation to
113 mobile genetic elements, which may frequently be involved in plasmid rearrangements.
114 Understanding when and how to successfully apply short and/or long-read sequencing
115 technologies to molecular epidemiology tracking will be important to the field as the incidence
116 of HGT is increasingly recognized (35).

117 In our institution, *bla*_{KPC} was first identified in 2007 in a patient simultaneously colonized with
118 *bla*_{KPC}-positive *K. pneumoniae* and *Klebsiella oxytoca*, harbouring the *bla*_{KPC} plasmids
119 pKPC_UVA01 and pKPC_UVA02 respectively (36, 37). Since then, we have been prospectively
120 screening all *Enterobacteriaceae* species for *bla*_{KPC} carriage despite national guidelines which
121 recommend screening of *Klebsiella* species and *Escherichia coli* only (38-41). Here we describe
122 the genetic basis of non-clonal *bla*_{KPC} emergence in a single hospital setting, using a
123 combination of short- and long-read whole genome sequencing to provide genomic

124 characterization of 281 *Enterobacteriaceae* isolates from the first five years of this multi-species
125 *bla*_{KPC} outbreak.

126

127 **Results**

128 There were 204 patients infected/colonized with *bla*_{KPC}-positive *Enterobacteriaceae* during the
129 prospective sampling period, based on clinical and surveillance sampling. We performed short-
130 read Illumina sequencing on all 294 available isolates; 13 of these were excluded due to quality
131 issues (see Methods), leaving 281 isolates, from 182/204 (89%) patients, for analysis (Table S1).
132 In all 281 isolates, *bla*_{KPC} was carried within a complete or partial Tn4401 structure.

133 ***bla*_{KPC} is found in many different host strains, indicating frequent HGT**

134 There were 13 different species carrying *bla*_{KPC} (Figure 1). The four most prevalent species were
135 *Enterobacter cloacae* (96 isolates from 80 patients), *K. pneumoniae* (94 isolates from 55
136 patients), *Klebsiella oxytoca* (35 isolates from 20 patients), and *Citrobacter freundii* (30 isolates
137 from 25 patients), each of which showed substantial genetic diversity. Across all species, there
138 were a total of 62 distinct strains (>500 chromosomal SNVs; see Methods). Of these, 18 strains
139 were identified in multiple patients, and 44 were only seen in a single patient (Figure 1), with
140 new strains continuing to appear throughout the study period. The very recent emergence of
141 *bla*_{KPC} on an evolutionary timescale (15) implies that each strain likely acquired *bla*_{KPC}
142 independently, demonstrating frequent HGT between different strains and species.

143 **The *bla*_{KPC} plasmids pKPC_UVA01 and pKPC_UVA02 are widely dispersed**

144 We hypothesised that the spread of *bla*_{KPC} could be due to conjugative transfer of the index
145 *bla*_{KPC} plasmids, pKPC_UVA01 and pKPC_UVA02. Defining plasmid presence as ≥99% sequence
146 identity over ≥80% of the plasmid length, 121 (66%) and 32 (18%) patients had isolates carrying
147 pKPC_UVA01 and pKPC_UVA02, respectively, corresponding to 39 and 5 distinct strains from 10
148 and 4 species, respectively (Figure 1). Although the wide dispersal of these plasmids supports
149 the plasmid-mediated outbreak hypothesis, short-read data is limited in the structural
150 inferences it can provide when repetitive sequences are present, and for many isolates it was
151 not possible to confirm that *bla*_{KPC} was actually co-located within pKPC_UVA01 or pKPC_UVA02
152 (Figure 1).

153 ***bla*_{KPC} is found in many different plasmids, indicating frequent Tn4401 transposition**

154 To further investigate *bla*_{KPC} plasmid structures, we performed long-read PacBio sequencing on
155 17 isolates that were chosen at random from the 281 available, yielding closed *bla*_{KPC} structures
156 in all cases. Fifteen isolates had a single *bla*_{KPC} plasmid and two isolates had two *bla*_{KPC} plasmids,
157 giving a total of 19 *bla*_{KPC} plasmids from the 17 isolates (Table 1). One isolate additionally had a
158 chromosomal insertion of Tn4401.

159 From the analysis of Illumina data described above, 11/17 of these isolates contained
160 pKPC_UVA01. As expected, the PacBio assemblies revealed a pKPC_UVA01-like plasmid in each
161 of these isolates. However, only five of these pKPC_UVA01-like plasmids actually contained
162 *bla*_{KPC} (Figure 2). The other six pKPC_UVA01-like plasmids lacked the entire Tn4401 element,

163 which was present on a different plasmid in these isolates. Importantly, this demonstrates that
164 plasmid presence (as defined by Illumina sequencing) is an unreliable indicator of the mobile
165 unit carrying *bla*_{KPC}, as the "presence" of pKPC_UVA01 was misleading in 55% (6/11) of the
166 randomly selected PacBio isolates.

167 After accounting for multiple variants of the same plasmid backbone (e.g. the pKPC_UVA01-like
168 plasmids described above), the 19 *bla*_{KPC} plasmids identified through long-read sequencing
169 represented 11 distinct plasmid structures (Table 1, Figure S1). These consisted of five
170 pKPC_UVA01-like plasmids, two pKPC_UVA02-like plasmids, four pKPC_CAV1176-like plasmids,
171 and eight *bla*_{KPC} plasmids that were each present in only a single PacBio-sequenced isolate.
172 Using Illumina data to assess the presence of each of these 11 distinct *bla*_{KPC} plasmids across
173 the entire set of isolates as described above revealed varied patterns of plasmid presence
174 (Figure 1). However, in the majority of cases it was not possible to determine from Illumina
175 data whether these plasmids contained *bla*_{KPC}, so the precise details regarding distribution of
176 *bla*_{KPC}-containing plasmids across the 281 isolates remains elusive.

177 Taken together, these results demonstrate a great deal of *bla*_{KPC} plasmid diversity, as 11 distinct
178 *bla*_{KPC} plasmids were identified through long-read sequencing of 17 isolates. Given that these
179 isolates were randomly chosen, the total number of distinct *bla*_{KPC} plasmids across the entire
180 set of 281 isolates is likely to be much greater than this. Additional Tn4401 insertion sites were
181 identified from the subset of isolates where flanking sequences could be adequately assembled
182 using short-read data, further supporting this hypothesis (Table S2). Therefore, HGT of the

183 index *bla*_{KPC} plasmids (pKPC_UVA01 and pKPC_UVA02) only partially explains *bla*_{KPC} spread, and
184 the large number of distinct *bla*_{KPC} plasmids indicates high levels of Tn4401 mobility.

185 **Tn4401 is present within a Tn2-like element in many different plasmids**

186 In 7 of the 11 distinct, fully characterised, *bla*_{KPC} plasmids, Tn4401 was surrounded by a
187 sequence element related to the *bla*_{TEM-1}-containing transposon Tn2 (Figure 3). In all cases, the
188 insertion site of Tn4401 within the *tnpA* gene of Tn2 was identical, with approximately 1 kb of
189 flanking sequence on either side of Tn4401 showing 100% identity, but the remainder of these
190 Tn2-like elements showed substantial variation. For example, while the sequence surrounding
191 Tn4401 in pKPC_CAV1176 was identical to the reference Tn2* sequence, the Tn2-like element
192 in pKPC_CAV1043 was truncated. Additionally, pKPC_CAV1344 and pKPC_CAV1596-78
193 contained a Tn2 derivative, Tn1331, which contains the additional resistance genes *bla*_{OXA-9},
194 *aadA1*, and *aac(6')-Ib* and has been seen as a prior site of insertion for Tn4401 (42).

195 **Tn4401 variation**

196 There were five different structural variants of Tn4401 (Table 2). The majority of isolates,
197 230/281 (82%), had the Tn4401b isoform, with the remaining isolates containing Tn4401a
198 (n=8), a novel Tn4401 isoform with a 188 bp deletion upstream of *bla*_{KPC} (n=39), or one of two
199 truncated Tn4401 structures (n=4). At the SNV level, there were seven sites that were variable
200 within Tn4401b. Three of these were located within *bla*_{KPC}, giving rise to three different *bla*_{KPC}
201 alleles, *bla*_{KPC-2} (n=179), *bla*_{KPC-3} (n=44), and *bla*_{KPC-4} (n=5). All non-Tn4401b isolates contained
202 *bla*_{KPC-2}. Taking all structural and SNV variation into account, there were a total of 12 different

203 Tn4401 variants. However, most of these were very rare, with seven only found in a single
204 patient.

205 ***bla*_{KPC} mobility has occurred within the hospital**

206 Based on prior healthcare exposure, *bla*_{KPC} acquisition source was classified as “imported”
207 (likely acquisition prior to admission at our institution) for 15/182 (8%) patients and “local”
208 (likely acquisition within our institution) for 167/182 (92%) patients (Figure 1; see Methods).
209 Imports were more likely to be infected/colonised with *K. pneumoniae*, particularly ST258
210 (Table S3), consistent with previous reports of this strain being the dominant *bla*_{KPC} carrier in
211 the US (9, 43). Thus, most host strain variation likely originated within the hospital via *bla*_{KPC}
212 HGT. In support of this, 15/16 (94%) patients infected/colonised with multiple strains/species
213 had shared Tn4401 variants within the patient (Table S4), suggesting recent *bla*_{KPC} HGT.
214 Notably, this included one patient with two different species carrying Tn4401-6, which is not
215 found in any other patient.

216 There was also some evidence for recent within-strain Tn4401 transposition. From the isolates
217 that were randomly chosen for long-read sequencing, 4/17 (24%) had multiple Tn4401 copies
218 (Table 1). If we assume that this randomly chosen subset is representative, this extrapolates to
219 approximately 66/281 isolates across the whole dataset. However, only 2/281 isolates had
220 multiple Tn4401 variants (Tn4401-11; Table 2), indicating that many isolates likely had multiple
221 copies of the same Tn4401 variant, consistent with recent Tn4401 transposition.

222 Taken together, these results indicate that much of the genetic diversity observed is due to
223 recent *bla*_{KPC} mobility, likely within the hospital ecosystem over the described five year
224 outbreak.

225 **Direct patient-to-patient transmission does not explain *bla*_{KPC} acquisition**

226 To further investigate *bla*_{KPC} acquisition source, we combined epidemiological and genetic data
227 to trace possible transmission chains, at two different genetic levels. We considered possible
228 transmission events where the donor and recipient were on the same ward at the same time,
229 and carried the same host strain or Tn4401 variant. Considering only “local” acquisitions (see
230 above), 48/167 (29%) patients had ward contact with another patient carrying the same *bla*_{KPC}-
231 positive strain (Figure 4, top panel). A greater proportion, 106/167 (63%), of patients had ward
232 contact with another patient carrying the same Tn4401 variant. However, as Tn4401-1 is very
233 common (66% of patients), these inferred transmissions may be spurious. With patients
234 carrying this common variant excluded, only 15/50 (30%) had ward contact with another
235 patient carrying the same Tn4401 variant (Figure 4, bottom panel). Therefore, both genetic
236 levels (strain or Tn4401 variant) demonstrated plausible transmissions for only a minority of
237 patients, indicating that direct patient-to-patient transmission is not the dominant mode of
238 *bla*_{KPC} acquisition.

239

240

241 Discussion

242 Here we have demonstrated high levels of genetic diversity in KPC-producing
243 *Enterobacteriaceae* within a single institution over five years. This diversity occurs at multiple
244 genetic levels, revealing a complex evolutionary history of the *bla*_{KPC} gene involving many
245 different host strains and plasmids.

246 In 7/11 distinct *bla*_{KPC} plasmids identified through long-read sequencing, Tn4401 was located
247 within a Tn2-like element. As these Tn2-like elements differed substantially from each other
248 (Figure 3), it is unlikely that this arose via transposition of a composite Tn4401-Tn2-like
249 structure. Instead, it suggests that Tn4401 has been repeatedly incorporated into pre-existing
250 Tn2-like elements, which are known to be widespread, and genetically divergent, in
251 *Enterobacteriaceae* (44, 45). However, the insertion site was identical in all cases, yet Tn4401
252 has been reported to have no insertion site specificity (28), suggesting that this was not
253 facilitated by a standard transposition mechanism. Therefore, we suggest that this is most likely
254 mediated by homologous recombination with other Tn2-like elements following an initial
255 integration event, as recently suggested for another multi-drug resistance gene, *bla*_{CTX-M-15} (46).
256 This implies that Tn4401 mobility may have been enhanced via integration into a second,
257 already widely dispersed, transposon. As the Tn4401-Tn2-like structure was present in the
258 index case isolate (CAV1016, Aug 2007) we presume that the initial transposition of Tn4401 into
259 a Tn2-like element occurred prior to entry into our hospital system. In support of this, one
260 particular Tn2-like element, Tn1331, has been previously described to contain Tn4401 (in
261 exactly the same position within the *tnpA* gene as described here) (21, 42, 47, 48), including

262 one report describing a *K. pneumoniae* isolated in 2005, which predates *bla*_{KPC} in our institution
263 (42). We are not aware of any previous reports describing Tn4401 within a non-Tn1331 Tn2-like
264 element.

265 The prevalence of Tn4401 insertions within Tn2-like elements also has important implications
266 with regard to plasmid tracking. We previously published a method for arbitrary PCR to track
267 the flanking regions around the Tn4401 element, as well as a PCR method to assay presence of
268 what we had wrongly assumed was a single plasmid, pKPC_UVA01. This PCR assay targeted the
269 immediate Tn4401 insertion site within a Tn2-like element (49), which we have here
270 demonstrated is present in many different plasmids, highlighting that PCR assays, and indeed
271 any partial typing methods, need to be interpreted with a great deal of caution. We were
272 further misled by the analysis of short-read whole genome sequencing data, which indicated
273 presence of pKPC_UVA01 in the majority of isolates. Taken together it was tempting to
274 conclude that horizontal transfer of pKPC_UVA01 was responsible for the vast majority of *bla*_{KPC}
275 carriage in our institution. However, long-read sequencing refuted this, revealing a far more
276 complex picture.

277 More generally, this highlights certain limitations for plasmid reconstruction from short-read
278 data. To illustrate by way of example, there were five isolates where long-read sequencing
279 revealed pKPC_UVA01-like plasmids that were identical to the reference pKPC_UVA01
280 sequence apart from the absence of Tn4401 and associated 5 bp target site duplication (Figure
281 2). We presume that in these lineages, *bla*_{KPC} may have been initially acquired via HGT of
282 pKPC_UVA01, with subsequent homologous recombination transferring Tn4401 from

283 pKPC_UVA01 to a different plasmid containing a Tn2-like element. In each of these five isolates,
284 multiple Tn2-like elements are present, which have 100% sequence identity over approximately
285 1 kb on either side of the Tn4401 insertion site. As this is longer than the fragment length used
286 for paired-end sequencing, it is not possible to resolve the plasmid context of *bla*_{KPC} using short-
287 read data. Importantly, any reference-based method for plasmid reconstruction (e.g. in this
288 case using the pKPC_UVA01 reference sequence to infer presence of the plasmid in each
289 isolate) is liable to produce misleading results. More generally, it is exactly the repetitive
290 regions that cannot be resolved using short-read data that could be expected to be involved in
291 plasmid rearrangements, either through homologous recombination as suggested here, or by
292 virtue of the fact that transposable elements are often present in multiple copies. Therefore,
293 having short-read data that is consistent with a known plasmid structure, even within the same
294 outbreak, should not be sufficient to conclude that that structure is present, if the data is also
295 consistent with an alternative structure. As several recent studies have utilised reference-based
296 approaches for plasmid assembly / inference (33, 34), our results indicate that any such
297 methods should be interpreted with extreme caution.

298 Across the *bla*_{KPC}-positive patients, there was large variation in both host strains and *bla*_{KPC}
299 plasmids, with Tn4401 being the largest genetic unit that was consistently present. Therefore,
300 surveillance strategies aimed at tracking individual strains or plasmids could be misleading, and
301 it may be more appropriate to focus on Tn4401. However, we found limited variation within the
302 transposon, as Tn4401 sequences from 121/182 (66%) patients were identical to the index case
303 (Table 2). This lack of variation implies that even the highest resolution genetic methods may be
304 insufficient for determining specific transmission routes. Even so, we have demonstrated that

305 only a minority of *bla*_{KPC} acquisition events can be explained by direct patient-to-patient
306 transmission. Future studies should therefore contemporaneously investigate the possible
307 involvement of unsampled reservoirs (e.g. environmental or silent colonization by additional
308 carriers).

309 There several limitations to this study. Because of the cost and effort involved in long-read
310 sequencing, we were only able to resolve a minority of *bla*_{KPC}-plasmids. This means that
311 although we have a compelling indicator of the diversity created by mobile genetic elements
312 within a single hospital over a five year period, we are limited in the ability to genetically
313 resolve pathways of *bla*_{KPC} mobility between host strains and plasmid vectors, even within a
314 single patient. We also speculate about the effect of Tn4401 insertion into Tn2-like elements,
315 but future *in vitro* studies could be used to illuminate the effect of this composite structure on
316 Tn4401 mobility.

317 In conclusion, our detailed genetic analysis of the evolutionary events occurring in the early
318 stages of antimicrobial resistance gene emergence in a single institution identifies several
319 distinct processes occurring at high frequency (Figure 5). First, the presence of shared *bla*_{KPC}-
320 containing strains in different patients reflects traditional (clonal) outbreak models. Second,
321 *bla*_{KPC} mobility between strains/species is facilitated by promiscuous *bla*_{KPC} plasmids such as
322 pKPC_UVA01. Third, *bla*_{KPC} transfer between plasmids is likely enhanced by homologous
323 recombination between Tn2-like elements, facilitating the movement of Tn4401 from one
324 plasmid to another. Finally, *bla*_{KPC} mobility is also enabled by standard Tn4401 transposition.
325 Rather than a single process dominating, resistance dissemination is driven by a combination of

326 these factors, resulting in a high level of diversity in KPC-producing *Enterobacteriaceae*, at
327 multiple genetic levels. As *bla*_{KPC} prevalence continues to increase, so will this genetic diversity,
328 inevitably resulting in a wider variety of more pathogenic strains carrying *bla*_{KPC}.

329 Our results indicate that the current standard practice of only screening specific species for
330 *bla*_{KPC} carriage is likely to hamper surveillance efforts by grossly underestimating true
331 prevalence. Instead of the traditional view of an outbreak involving a single pathogenic strain,
332 we propose that for KPC-producing *Enterobacteriaceae*, and possibly more generally, we should
333 instead adopt the view of a “gene-based outbreak”, with surveillance strategies tracking the
334 resistance gene itself rather than a specific host strain.

335 **Methods**

336 **Isolate collection and Illumina sequencing**

337 A subset of *K. pneumoniae* isolates, with corresponding sequence data, have been previously
338 described (37). Isolate collection, *de novo* assembly, mapping and variant calling were
339 performed as previously described (37), however here we used species-specific references for
340 mapping (Table S5). Illumina sequencing was also performed as previously described (37), with
341 some exceptions (see Supplementary Methods). In total, 281 isolates from 182 patients were
342 available for analysis; exclusion criteria for additional isolates is described in Supplementary
343 Methods.

344 **Species classification**

345 Species classification was performed using microbiological and sequenced-based methods (see
346 Supplementary Methods for details).

347 **Phylogenetic analysis and strain classification**

348 There were 52 patients with multiple isolates of the same species. One of these (patient FK)
349 involved two strains of *K. pneumoniae* that were highly divergent from each other (>20,000
350 chromosomal SNVs), clearly representing a separate *bla*_{KPC} acquisition by each strain. Excluding
351 this divergent strain pair, the remaining cases had SNV differences ranging from 0 to 60
352 (median 2 SNVs). As these could plausibly represent clonal evolution within the patient, we
353 conservatively chose to include only a single representative (the earliest isolate) for
354 phylogenetic reconstruction, in order to avoid artificially inflating genetic clusters due to

355 repeated patient sampling. Phylogenetic analysis was then performed separately for each
356 species using PhyML (50) (see Supplementary Methods). Chromosomally distinct strains were
357 defined by partitioning each phylogeny with a cutoff of ~500 SNVs (see Supplementary
358 Methods). Based on the molecular clock of *Enterobacteriaceae* (1-20 SNVs/chromosome/year)
359 (6, 37, 51), we can be relatively confident that isolates belonging to distinct strains will not have
360 a shared ancestor within the timeframe of *bla*_{KPC} dispersal, and the number of distinct strains
361 thus provides a conservative estimate of the number of distinct *bla*_{KPC} acquisition events.

362 **Long-read PacBio sequencing**

363 For long-read sequencing, 17 isolates were randomly chosen from the entire set of sequenced
364 isolates (i.e. including patient duplicates). Long-read sequencing and initial *de novo* assembly
365 were performed as previously described (37). Refinement of assemblies and closure of
366 plasmid/chromosomal sequences was performed as described in Supplementary Methods.
367 Since the isolates for PacBio sequencing were randomly chosen from the set of all Illumina
368 sequenced isolates, some of them represented within-patient strain duplicates (see previous
369 section on phylogenetic analysis), and were therefore not included in phylogenetic
370 reconstruction. For display purposes (in Figure 1), the *bla*_{KPC} structure(s) determined from long-
371 read PacBio sequencing for each of these isolates is shown alongside the representative isolate
372 of the same strain from the same patient. In all cases, the representative isolate has the same
373 short-read plasmid profile and Tn4401 variant as the PacBio sequenced isolate.

374 **Plasmid presence / absence classification**

375 The index *bla*_{KPC} plasmids pKPC_UVA01 and pKPC_UVA02, together with the additional nine
376 distinct *bla*_{KPC} plasmids identified through long-read PacBio sequencing, were used as references
377 to determine plasmid presence profiles for each isolate based on the Illumina data. Plasmid
378 presence was defined as ≥99% sequence identity over ≥80% of the length of the reference
379 sequence, as determined by BLASTn comparisons between each isolate's *de novo* assembly and
380 the reference plasmid. The high identity cutoff was chosen to reduce false positives from
381 sequences that are only distantly related (and therefore unlikely to have a common ancestor
382 within the timeframe of the outbreak), while the more permissive length cutoff allows for some
383 rearrangement. It should be noted that the method does not take any account of structural
384 continuity.

385 **Analysis of Tn4401 flanking sequences**

386 Where a plasmid was classified as being present in a particular isolate, it was not always certain
387 to contain Tn4401. The plasmid presence classification was further refined as: “containing
388 Tn4401” if the isolate's *de novo* assembly supported Tn4401 being present within the expected
389 sequence context of that plasmid, “not containing Tn4401” if the plasmid was assembled
390 without Tn4401, or “uncertain” if structure could not be determined from the *de novo*
391 assembly. The identification of novel Tn4401 insertion sites was also based on the *de novo*
392 assemblies. These methods are described in detail in Supplementary Methods.

393 **Variation in Tn4401**

394 Tn4401 isoform classification was performed by comparing each isolate's *de novo* assembly
395 with the previously described isoform b reference sequence from EU176013.1 (29) using

396 BLASTn, to identify structural variation. SNV variation was determined by mapping to a
397 reference consisting of pKPC_UVA01 plus a species-specific chromosome as described above,
398 followed by extraction of the Tn4401 region. Variation is reported for all sites where at least
399 one isolate had a non-reference call, including any ambiguity at that site in other isolates.
400 Ambiguity at non-variable sites is not reported, which may result in an underestimate of true
401 variation. However, any resulting underestimation is likely to be very minor, as the proportion
402 of called sites, excluding deleted regions described above, was >96% for all isolates.

403 **Epidemiological classification**

404 For epidemiologic analysis, patients were assigned a one or two letter code for de-
405 identification. Routine peri-rectal surveillance cultures for silent colonization began in April
406 2009 (38, 40). Patients were classified as “imported” if they did not have any prior admission to
407 University of Virginia Medical Center/Long-term Acute Care Hospital (UVaMC) and either had a
408 *bla*_{KPC}-positive *Enterobacteriaceae* isolated within 48 hours of admission, or had a carbapenem-
409 resistant *Enterobacteriaceae* culture before transfer to UVaMC with a subsequent isolate at
410 UVaMC confirmed as *bla*_{KPC} PCR positive. The index case was also classified as imported. For the
411 remaining patients, the source of *bla*_{KPC} acquisition was classified as “local”. The 48h cutoff is
412 arbitrary and may result in some misclassification if patients either acquire *bla*_{KPC} within the
413 first 48h of admission, or if *bla*_{KPC} carriage/infection remains undetected for >48h, however this
414 is expected to be minimal (see Supplementary Methods). Charts and patient contacts were
415 reviewed using bed tracing data and the electronic medical record. The study was approved by
416 the University of Virginia Institutional Review Board (protocol # 13558).

417 **Transmission analysis**

418 Possible patient-to-patient transmission events were determined on the basis of having
419 overlapping stays on the same ward, as well as genetically-related *bla*_{KPC} isolates. The analysis
420 was performed separately for two different levels of genetic relatedness (strain or Tn4401
421 variant). This is described in detail in Supplementary Methods.

422

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441 **Additional Information**

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444

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- 604
- 605

606 **Figure Legends**

607

608 **Figure 1. Diversity in bacterial species, strains, plasmids and Tn4401 variants.** For each
609 species, a phylogeny was generated from mapping to a species-specific chromosomal
610 reference, after deduplication of closely related isolates from the same patient (see Methods).
611 Distinct strains are defined by a cutoff of ~500 SNVs (see Methods); strains found in more than
612 one patient are indicated by grey background shading. Circles show plasmid “presence” as
613 determined from Illumina data, with fill colour indicating uncertainty in whether the plasmid
614 contains *bla*_{KPC}. Boxes show plasmid structure determined from long-read PacBio sequencing
615 for 17 randomly chosen isolates, as well as the previously sequenced isolates from index
616 patient B (37). Where the PacBio-sequenced isolate was excluded from the phylogeny as a
617 patient duplicate, plasmid structure is shown for the corresponding closely related isolate from
618 the same patient. Tn4401 and *bla*_{KPC} variants (Table 2) are indicated by large and small squares
619 respectively. The likely source of *bla*_{KPC} acquisition as determined from epidemiological data is
620 indicated by text colour.

621

622 **Figure 2. pKPC_UVA01-like plasmids identified through long-read PacBio sequencing.** The
623 reference pKPC_UVA01 sequence is shown, together with all 11 pKPC_UVA01-like plasmids
624 identified through long-read PacBio sequencing, including the six that do not contain *bla*_{KPC}.
625 Arrows indicate predicted open reading frames; Tn4401 is shown in purple. Pink shading
626 indicates regions of identity between adjacent sequences, with SNVs indicated by red lines.

627

628 **Figure 3. Tn4401 is commonly integrated into a Tn2-like element.** The Tn4401 and surrounding
629 region (i.e. partial plasmid sequence, except for pKPC_CAV1320) is shown for each distinct
630 *bla*_{KPC} plasmid. Variants of the same plasmid backbone (see Table 1) are not shown. Arrows
631 indicate predicted open reading frames; Tn4401 is shown in purple. Pink shading indicates
632 regions of identity between adjacent sequences, with SNVs indicated by red lines and short
633 indels (1-2 bp) by blue lines. The top panel shows the Tn2* reference sequence from AY123253
634 (44).

635

636 **Figure 4. Ward contacts between patients with genetically related isolates.** Each horizontal
637 line represents a different strain (top) or Tn4401 variant (bottom). Filled circles indicate
638 patients that had previous ward contact with another patient on the same horizontal line (i.e.
639 possible patient-to-patient transmission). As Tn4401-1 is present in two-thirds of patients,
640 many coincidental ward contacts may be expected to occur, resulting in a substantial
641 overestimate of transmission number. Therefore, for Tn4401, the total number of acquisitions
642 explainable by direct ward contact is indicated, as well as with Tn4401-1 patients excluded. The
643 vertical line indicates onset of routine patient screening.

644

645 **Figure 5. *bla*_{KPC} spreads at multiple genetic levels, resulting in a high level of diversity in**
646 ***bla*_{KPC}-positive *Enterobacteriaceae*.**

647 **Table 1. *bla*_{KPC}-containing structures ascertained from long-read PacBio sequencing of 17 randomly chosen isolates**

Isolate	Species	Patient	Date	<i>bla</i> _{KPC} plasmid	Size (bp)	Group ^a	Within-group genetic changes ^b	Tn4401 variant	Flanking sequence ^c	Tn2-like element ^d
CAV1344	<i>K. pneumoniae</i>	EP	Dec-2010	pKPC_CAV1344	176,497	Singleton	NA	Tn4401-1	GTTCT...GTTCT	Yes
CAV1392	<i>K. pneumoniae</i>	EU	Mar-2011	pKPC_CAV1392	43,621	pKPC_UVA01	1 SNV	Tn4401-5	GTTCT...GTTCT	Yes
				NA (chromosomal)	NA	NA	NA	Tn4401-5	AGATA...AGATA	No
CAV1596	<i>K. pneumoniae</i>	FK	Apr-2012	pKPC_CAV1596-78	77,801	Singleton	NA	Tn4401-5	GTTCT...GTTCT	Yes
				pKPC_CAV1596-97	96,702	Singleton	NA	Tn4401-5	TATCG...TATCG	No
CAV1099	<i>K. oxytoca</i>	AU	Apr-2009	pKPC_CAV1099	113,105	pKPC_UVA02	0 SNVs	Tn4401-1	ATGCA...GGCCA ^e	No
CAV1335	<i>K. oxytoca</i>	EQ	Dec-2010	pKPC_CAV1335	113,105	pKPC_UVA02	0 SNVs	Tn4401-1	ATGCA...GGCCA ^e	No
CAV1374	<i>K. oxytoca</i>	ED	Aug-2010	pKPC_CAV1374	332,956	Singleton	NA	Tn4401-1	GTTCT...GTTCT	Yes
CAV1043	<i>E. asburiae</i>	L	Mar-2008	pKPC_CAV1043	59,138	Singleton	NA	Tn4401-5	GTTCT...GTTCT	Yes
CAV1176	<i>E. cloacae</i>	DN	May-2010	pKPC_CAV1176	90,452	pKPC_CAV1176	0 SNVs	Tn4401-3	GTTCT...GTTCT	Yes
CAV1311	<i>E. cloacae</i>	EO	Jan-2011	pKPC_CAV1311	90,452	pKPC_CAV1176	0 SNVs	Tn4401-3	GTTCT...GTTCT	Yes
CAV1411	<i>E. cloacae</i>	FC	Jun-2011	pKPC_CAV1411	90,452	pKPC_CAV1176	1 SNV, 40 kb inversion	Tn4401-3	GTTCT...GTTCT	Yes
CAV1669	<i>E. cloacae</i>	HV	Aug-2012	pKPC_CAV1669	90,452	pKPC_CAV1176	40kb inversion	Tn4401-3	GTTCT...GTTCT	Yes
CAV1668	<i>E. cloacae</i>	HQ	Aug-2012	pKPC_CAV1668	43,433	pKPC_UVA01	1 SNV, 188 bp deletion	Tn4401-3	GTTCT...GTTCT	Yes
CAV1321	<i>C. freundii</i>	EG	Nov-2010	pKPC_CAV1321-45	44,846	pKPC_UVA01	1,225 bp insertion	Tn4401-1	GTTCT...GTTCT	Yes
				pKPC_CAV1321-244	243,709	Singleton	NA	Tn4401-1	GTTCT...GTTCT	Yes
CAV1741	<i>C. freundii</i>	ER	Oct-2012	pKPC_CAV1741	129,196	pKPC_UVA01	14,960 bp duplication, 70,615 bp insertion	Tn4401-1 ^f	GTTCT...GTTCT	Yes
CAV1151	<i>K. intermedia</i>	CD	Sep-2009	pKPC_CAV1151	43,621	pKPC_UVA01	0 SNVs ^g	Tn4401-1	GTTCT...GTTCT	Yes
CAV1320	<i>E. aerogenes</i>	EL	Nov-2010	pKPC_CAV1320	13,981	Singleton	NA	Tn4401-1	TTGTT...TTGTT	No
CAV1492	<i>S. marcescens</i>	GL	Dec-2011	pKPC_CAV1492	69,158	Singleton	NA	Tn4401-8	TTTTT...TTTTT	No

648 ^a Plasmids are defined as belonging to the same group if the sequences are largely identical, allowing for a small number of substitutions and/or rearrangements that may be
 649 expected to occur within the outbreak timeframe. Different groups have very limited homology outside the Tn4401 region, indicative of independent integrations into distinct
 650 plasmid structures. 'Singleton' indicates a plasmid backbone that is distinct from all others shown

651 ^b Differences relative to the reference sequence for that plasmid group, as specified in the previous column

652 ^c Sequences immediately flanking Tn4401; generally expected to be identical due to 5 bp target site duplication during transposition (28)

653 ^d Tn4401 integrated into the *tnpA* gene of a Tn2-like element

654 ^e No evidence of target site duplication

655 ^f 2 copies

656 ^g It is noteworthy that this plasmid from CAV1151 (*K. intermedia*) is exactly identical to pKPC_UVA01 from CAV1016 (*K. pneumoniae*), with isolation dates 2 years apart

657 **Table 2. Tn4401 variation**

Tn4401 variant	Structural isoform(29)	SNVs ^a	<i>bla</i> _{KPC} variant	Patients	Isolates	Strains
Tn4401-1 ^b	b	-	<i>bla</i> _{KPC-2}	121	176	42
Tn4401-2	a (del 7020-7118)	-	<i>bla</i> _{KPC-2}	5	8	1
Tn4401-3	novel (del 6919-7106)	-	<i>bla</i> _{KPC-2}	28	39	2
Tn4401-4	truncated (del 1-6654)	-	<i>bla</i> _{KPC-2}	2	3	1
Tn4401-5	b	8015 C→T ^c	<i>bla</i> _{KPC-3}	22	40	19
Tn4401-6	b	8015 C→T, 9621 T→C	<i>bla</i> _{KPC-3}	1	3	2
Tn4401-7	b	7199 T→A, 8015 C→T, 9621 T→C	<i>bla</i> _{KPC-3}	1	1	1
Tn4401-8	b	9663 T→C	<i>bla</i> _{KPC-2}	1	3	1
Tn4401-9	b	7509 C→G, 7917 T→G ^d	<i>bla</i> _{KPC-4}	1	1	1
Tn4401-10	b	6800 T→C, 7509 C→G, 7917 T→G	<i>bla</i> _{KPC-4}	1	4	1
Tn4401-11	b	8015 N ^e	<i>bla</i> _{KPC-2} / <i>bla</i> _{KPC-3}	1	2	1
Tn4401-12	truncated (del 1-6727)	6800 N ^f	<i>bla</i> _{KPC-2}	1	1	1

658 ^a With respect to Tn4401-1, which is considered as the reference Tn4401 sequence for this study

659 ^b Tn4401-1 differs from the reference isoform b sequence in EU176013.1 by 14 SNVs, as follows: 4939 C→G, 4989 C→T, 5099
 660 A→T, 5131 A→G, 5154 T→G, 5185 G→C, 5255 C→A, 5361 G→C, 5375 C→G, 5390 A→C, 5996 G→A, 5998 G→C, 8112 C→A,
 661 8113 A→C

662 ^c This substitution converts *bla*_{KPC-2} to *bla*_{KPC-3}

663 ^d These two substitutions convert *bla*_{KPC-2} to *bla*_{KPC-4}

664 ^e Quality filters failed at this position due to a mixture of reads supporting C and T (i.e. Tn4401-11 actually represents a mixture
 665 of Tn4401-1 and Tn4401-5)

666 ^f Quality filters failed at this position due to a lack of reads mapped in the reverse direction. All reads mapped in the forward
 667 direction supported a reference (T) call

668

669 **Supplementary Legends**

670

671 **Figure S1. Distinct *bla*_{KPC} plasmids identified through long-read PacBio sequencing.** Variants of
672 the same plasmid backbone (see Table 1) are not shown. Arrows indicate predicted open
673 reading frames; Tn4401 is shown in purple.

674

675 **Table S1. Details of sequenced isolates.**

676

677 **Table S2. Additional Tn4401 insertion sites ascertained from short-read Illumina data**

678

679 **Table S3. Association of importation status with *K. pneumoniae* and the epidemic *bla*_{KPC} *K.*
680 *pneumoniae* strain ST258**

681

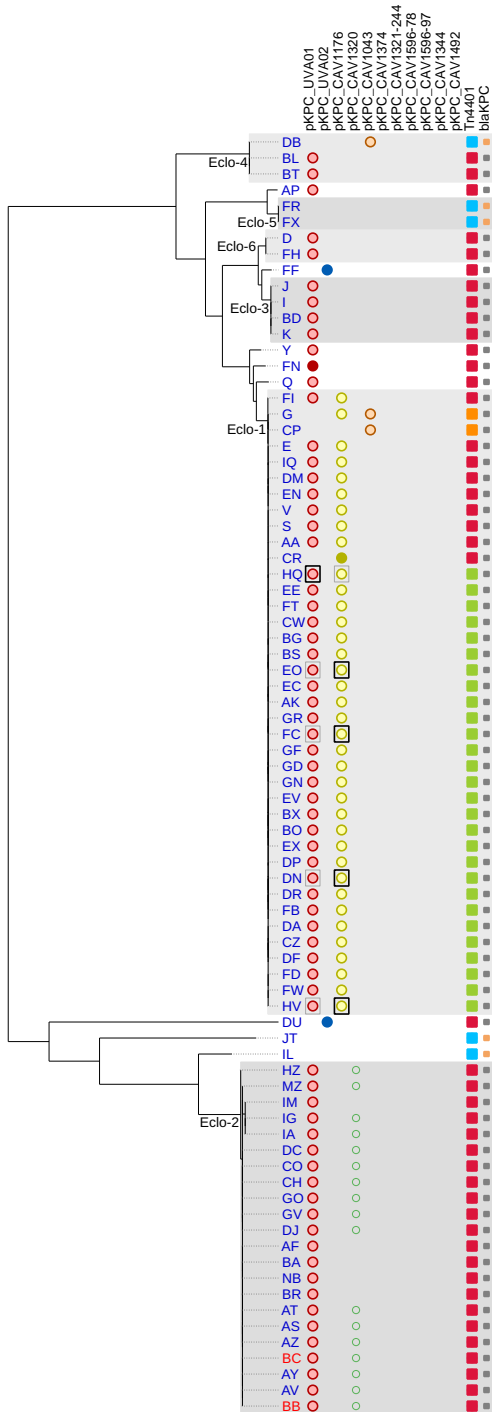
682 **Table S4. Patients with multiple *bla*_{KPC}-positive strains or species**

683

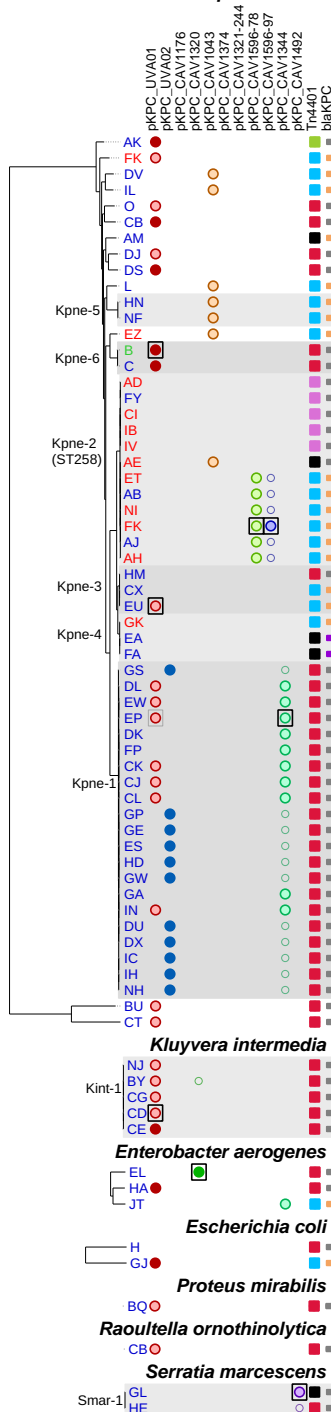
684 **Table S5. Chromosomal references used for mapping**

685

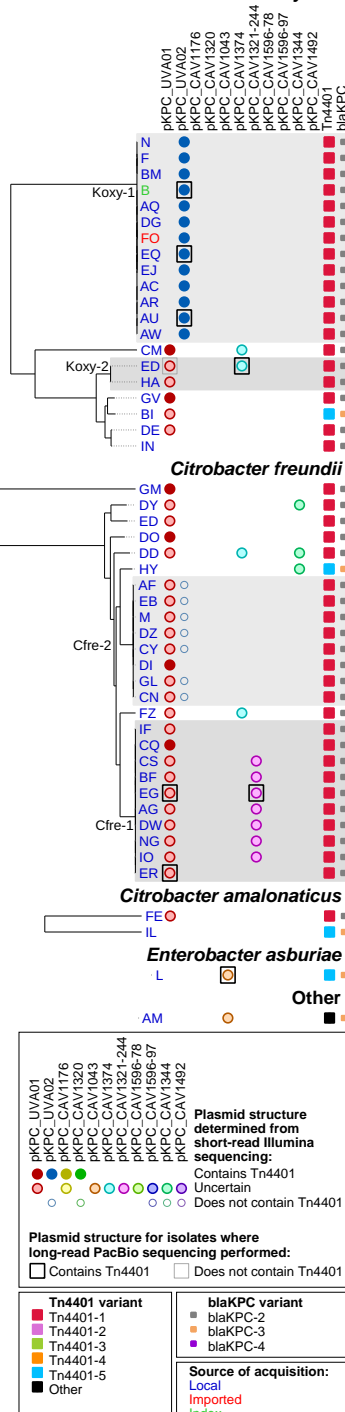
Enterobacter cloacae



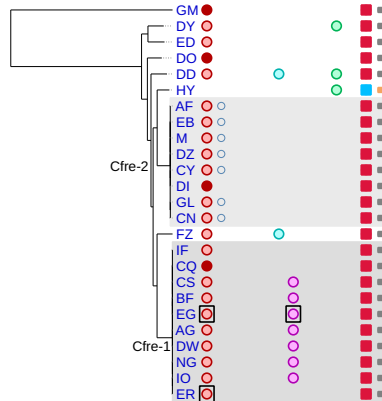
Klebsiella pneumoniae



Klebsiella oxytoca



Citrobacter freundii



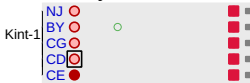
Citrobacter amalonaticus



Enterobacter asburiae



Kluyvera intermedia



Enterobacter aerogenes



Escherichia coli



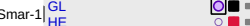
Proteus mirabilis



Raoultella ornithinolytica



Serratia marcescens



Plasmid structure determined from short-read Illumina sequencing:

- Contains Tn4401
- Uncertain
- Does not contain Tn4401

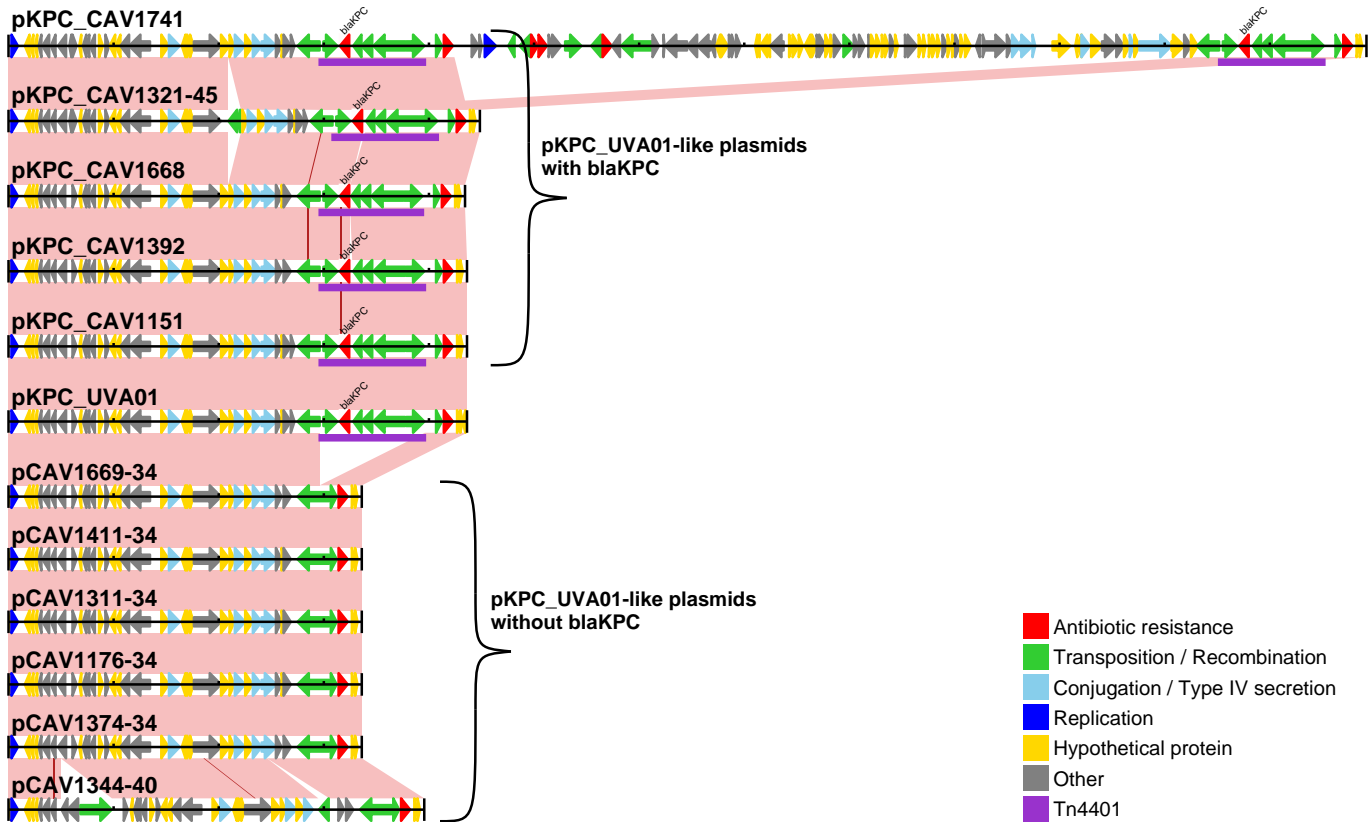
Plasmid structure for isolates where long-read PacBio sequencing performed:

- Contains Tn4401
- Does not contain Tn4401

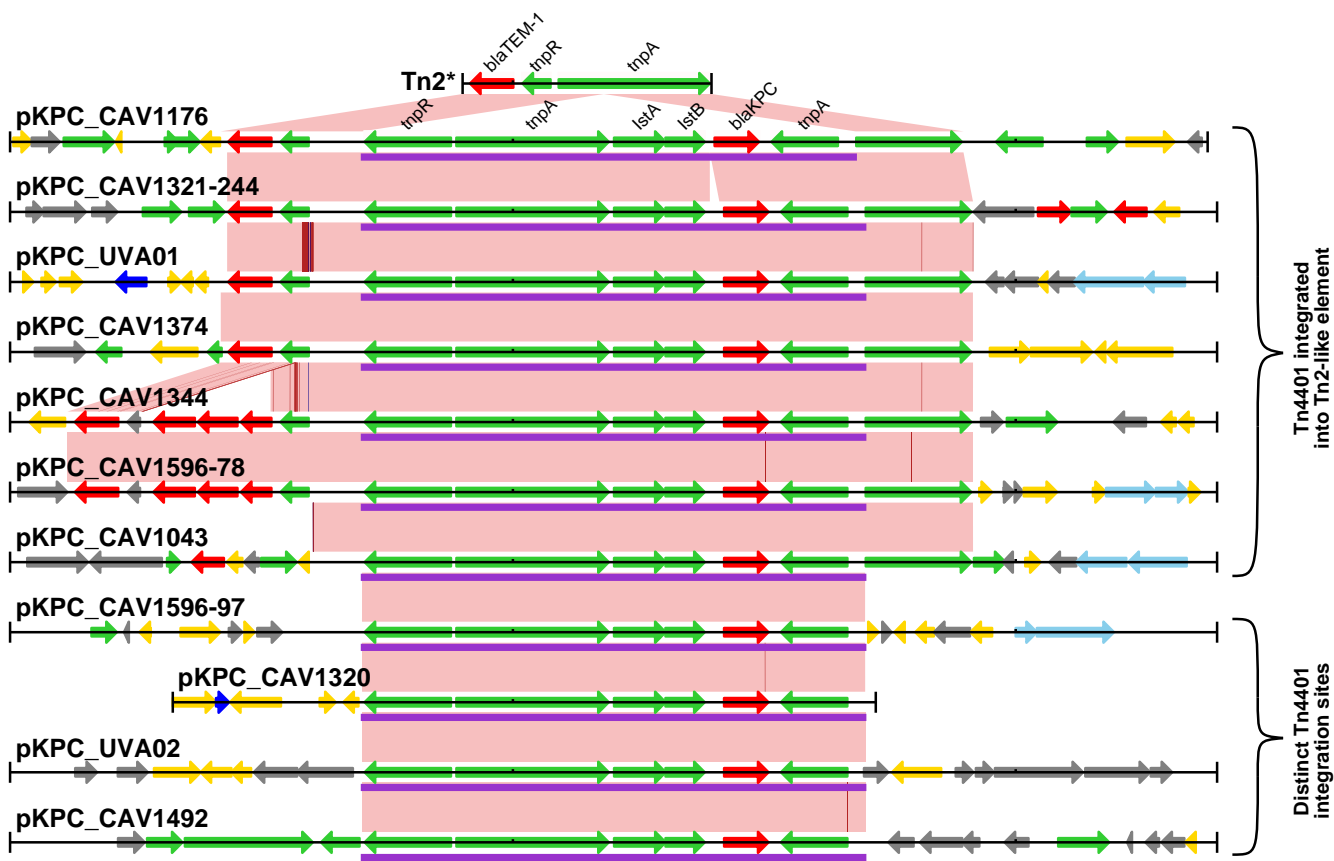
<p>Tn4401 variant</p> <ul style="list-style-type: none"> ■ Tn4401-1 ■ Tn4401-2 ■ Tn4401-3 ■ Tn4401-4 ■ Tn4401-5 ■ Other 	<p>blaKPC variant</p> <ul style="list-style-type: none"> ■ blaKPC-2 ■ blaKPC-3 ■ blaKPC-4
--	---

Source of acquisition:

- Local
- Imported
- Index

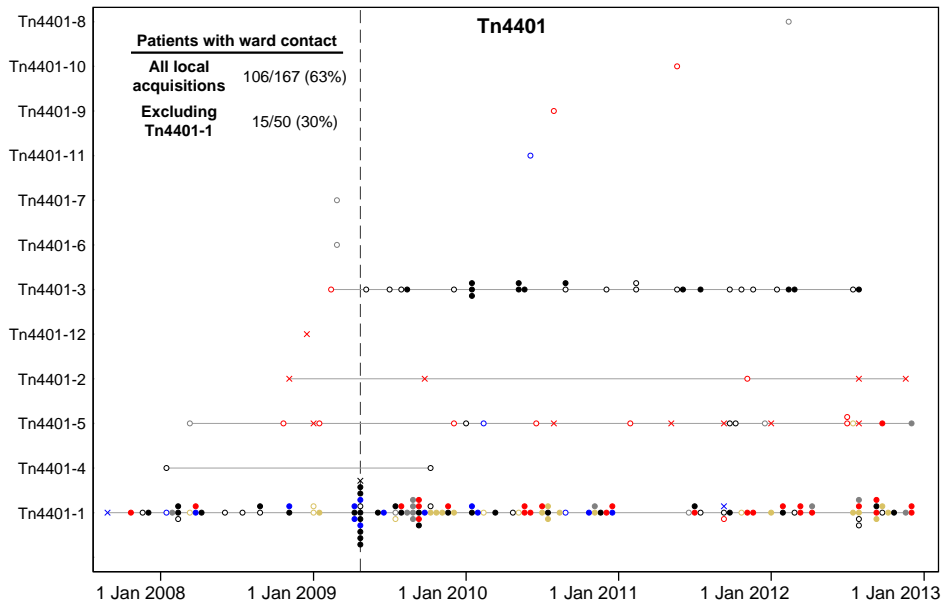
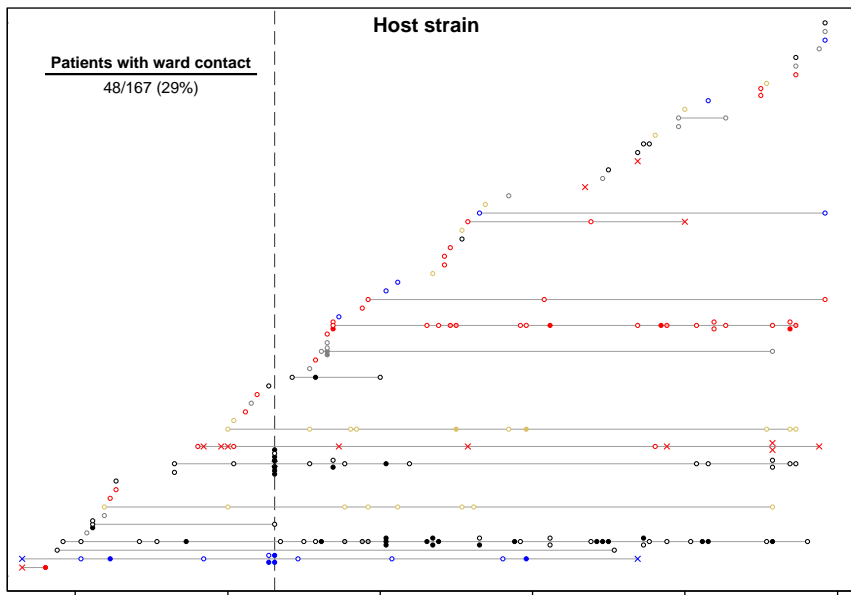


10 kb



1 kb

- █ Antibiotic resistance
- █ Transposition / Recombination
- █ Conjugation / Type IV secretion
- █ Replication
- █ Hypothetical protein
- █ Other
- █ Tn4401



○ *Klebsiella pneumoniae*

○ *Klebsiella oxytoca*

○ *Enterobacter cloacae*

○ *Citrobacter freundii*

○ Other

× Imported

● Ward contact, same Tn4401 variant

● Ward contact, different Tn4401 variant

○ No ward contact

Klebsiella pneumoniae
carbapenemase (KPC)
resistance gene



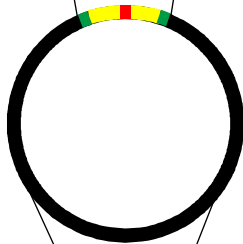
Mobile transposon
Tn4401



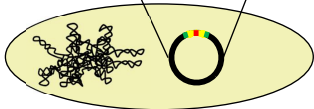
Tn4401 nested within
mobile Tn2-like element



Transposon located
within mobile
extra-chromosomal
plasmid DNA



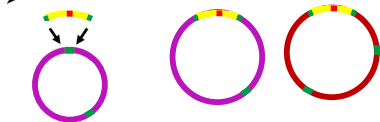
Bacterial cell with
mobile plasmid
and immobile host
chromosome
(not to scale)



Transposition of Tn4401 into different
plasmids



Proposed mobilization of Tn4401 between
Tn2-like elements in different plasmids,
via homologous recombination



Plasmid movement via conjugation into
different bacterial strains and species

