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

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

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Abstract

The recent widespread emergence of carbapenem resistance in *Enterobacteriaceae* is a major public health concern, as carbapenems are a therapy of last resort in this family of common bacterial pathogens. Resistance genes can mobilize via various mechanisms including conjugation and transposition, however the importance of this mobility in short-term evolution, such as within nosocomial outbreaks, is currently unknown. Using a combination of short- and long-read whole genome sequencing of 281 bla_{KPC}-positive *Enterobacteriaceae* isolated from a single hospital over five years, we demonstrate rapid dissemination of this carbapenem resistance gene to multiple species, strains, and plasmids. Mobility of bla_{KPC} occurs at multiple nested genetic levels, with transmission of bla_{KPC} strains between individuals, frequent transfer of bla_{KPC} plasmids between strains/species, and frequent transposition of the bla_{KPC} transposon Tn*4401* between plasmids. We also identify a common insertion site for Tn*4401* within various Tn*2*-like elements, suggesting that homologous recombination between Tn*2*-like elements has enhanced the spread of Tn*4401* between different plasmid vectors. Furthermore, while short-read sequencing has known limitations for plasmid assembly, various studies have attempted to overcome this with the use of reference-based methods. We also demonstrate that as a consequence of the genetic mobility observed herein, plasmid structures can be extremely dynamic, and therefore these reference-based methods, as well as traditional partial typing methods, can produce very misleading conclusions. Overall, our findings demonstrate that non-clonal resistance gene dissemination can be extremely rapid, presenting significant challenges for public health surveillance and achieving effective control of antibiotic resistance.

Importance

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

Introduction

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

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

Most reports of *bla*$_{KPC}$ involve *K. pneumoniae* multi-locus sequence type (ST)258 (9, 17), which has been found globally, indicating that clonal dissemination of this resistant lineage has been an important factor in the spread of *bla*$_{KPC}$ (9, 17-20). Nevertheless, *bla*$_{KPC}$ has also been observed in other *K. pneumoniae* lineages, as well as other species of *Enterobacteriaceae*, suggesting that *bla*$_{KPC}$ HGT has also played a role in resistance dissemination (21-25). As *bla*$_{KPC}$ is often found on conjugative plasmids, some of which have been identified in multiple strains or species, this provides a likely mechanism for HGT (21, 26, 27). In addition, *bla*$_{KPC}$ is usually present as part of the 10 kb composite Tn*3*-based mobile transposon Tn*4401*, which has been identified in various different plasmids, implicating Tn*4401* transposition as another mechanism contributing to *bla*$_{KPC}$ spread (28, 29).

While Tn*4401* transposition and plasmid conjugation have been measured in the laboratory (28, 30, 31), the frequencies with which these processes occur within real-world ecosystems is not fully understood. In clinical contexts, it is often assumed that short-term evolution is dominated by clonal propagation, such that transmission chains generally involve a single pathogenic strain. However, if HGT is frequent relative to transmission (e.g. a "plasmid outbreak"), then linked patients may show variation in strain composition. If transposition is also frequent, then both host strain and resistance plasmid may show high variability within a single outbreak. As current surveillance strategies tend to focus on the host strain, it is important to establish the relevance of *bla*$_{KPC}$ mobility within outbreak settings.

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

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

In our institution, bla_{KPC} was first identified in 2007 in a patient simultaneously colonized with bla_{KPC}-positive *K. pneumoniae* and *Klebsiella oxytoca*, harbouring the bla_{KPC} plasmids pKPC_UVA01 and pKPC_UVA02 respectively (36, 37). Since then, we have been prospectively screening all *Enterobacteriaceae* species for bla_{KPC} carriage despite national guidelines which recommend screening of *Klebsiella* species and *Escherichia coli* only (38-41). Here we describe the genetic basis of non-clonal bla_{KPC} emergence in a single hospital setting, using a 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 Tn*4401* 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.

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

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

bla$_{KPC}$ is found in many different plasmids, indicating frequent Tn*4401* transposition

To further investigate *bla*$_{KPC}$ plasmid structures, we performed long-read PacBio sequencing on 17 isolates that were chosen at random from the 281 available, yielding closed *bla*$_{KPC}$ structures in all cases. Fifteen isolates had a single *bla*$_{KPC}$ plasmid and two isolates had two *bla*$_{KPC}$ plasmids, giving a total of 19 *bla*$_{KPC}$ plasmids from the 17 isolates (Table 1). One isolate additionally had a chromosomal insertion of Tn*4401*.

From the analysis of Illumina data described above, 11/17 of these isolates contained pKPC_UVA01. As expected, the PacBio assemblies revealed a pKPC_UVA01-like plasmid in each of these isolates. However, only five of these pKPC_UVA01-like plasmids actually contained *bla*$_{KPC}$ (Figure 2). The other six pKPC_UVA01-like plasmids lacked the entire Tn*4401* element,

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

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

Taken together, these results demonstrate a great deal of *bla*$_{KPC}$ plasmid diversity, as 11 distinct *bla*$_{KPC}$ plasmids were identified through long-read sequencing of 17 isolates. Given that these isolates were randomly chosen, the total number of distinct *bla*$_{KPC}$ plasmids across the entire set of 281 isolates is likely to be much greater than this. Additional Tn*4401* insertion sites were identified from the subset of isolates where flanking sequences could be adequately assembled using short-read data, further supporting this hypothesis (Table S2). Therefore, HGT of the

index *bla*$_{KPC}$ plasmids (pKPC_UVA01 and pKPC_UVA02) only partially explains *bla*$_{KPC}$ spread, and the large number of distinct *bla*$_{KPC}$ plasmids indicates high levels of Tn*4401* mobility.

Tn*4401* is present within a Tn*2*-like element in many different plasmids

In 7 of the 11 distinct, fully characterised, *bla*$_{KPC}$ plasmids, Tn*4401* was surrounded by a sequence element related to the *bla*$_{TEM-1}$-containing transposon Tn*2* (Figure 3). In all cases, the insertion site of Tn*4401* within the *tnpA* gene of Tn*2* was identical, with approximately 1 kb of flanking sequence on either side of Tn*4401* showing 100% identity, but the remainder of these Tn*2*-like elements showed substantial variation. For example, while the sequence surrounding Tn*4401* in pKPC_CAV1176 was identical to the reference Tn*2** sequence, the Tn*2*-like element in pKPC_CAV1043 was truncated. Additionally, pKPC_CAV1344 and pKPC_CAV1596-78 contained a Tn*2* derivative, Tn*1331*, which contains the additional resistance genes *bla*$_{OXA-9}$, *aadA1*, and *aac(6')-Ib* and has been seen as a prior site of insertion for Tn*4401* (42).

Tn*4401* variation

There were five different structural variants of Tn*4401* (Table 2). The majority of isolates, 230/281 (82%), had the Tn*4401*b isoform, with the remaining isolates containing Tn*4401*a (n=8), a novel Tn*4401* isoform with a 188 bp deletion upstream of *bla*$_{KPC}$ (n=39), or one of two truncated Tn*4401* structures (n=4). At the SNV level, there were seven sites that were variable within Tn*4401*b. Three of these were located within *bla*$_{KPC}$, giving rise to three different *bla*$_{KPC}$ alleles, *bla*$_{KPC-2}$ (n=179), *bla*$_{KPC-3}$ (n=44), and *bla*$_{KPC-4}$ (n=5). All non-Tn*4401*b isolates contained *bla*$_{KPC-2}$. Taking all structural and SNV variation into account, there were a total of 12 different

Tn*4401* variants. However, most of these were very rare, with seven only found in a single patient.

bla_{KPC} mobility has occurred within the hospital

Based on prior healthcare exposure, bla_{KPC} acquisition source was classified as "imported" (likely acquisition prior to admission at our institution) for 15/182 (8%) patients and "local" (likely acquisition within our institution) for 167/182 (92%) patients (Figure 1; see Methods). Imports were more likely to be infected/colonised with *K. pneumoniae*, particularly ST258 (Table S3), consistent with previous reports of this strain being the dominant bla_{KPC} carrier in the US (9, 43). Thus, most host strain variation likely originated within the hospital via bla_{KPC} HGT. In support of this, 15/16 (94%) patients infected/colonised with multiple strains/species had shared Tn*4401* variants within the patient (Table S4), suggesting recent bla_{KPC} HGT. Notably, this included one patient with two different species carrying Tn*4401*-6, which is not found in any other patient.

There was also some evidence for recent within-strain Tn*4401* transposition. From the isolates that were randomly chosen for long-read sequencing, 4/17 (24%) had multiple Tn*4401* copies (Table 1). If we assume that this randomly chosen subset is representative, this extrapolates to approximately 66/281 isolates across the whole dataset. However, only 2/281 isolates had multiple Tn*4401* variants (Tn*4401*-11; Table 2), indicating that many isolates likely had multiple copies of the same Tn*4401* variant, consistent with recent Tn*4401* transposition.

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

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

To further investigate *bla*$_{KPC}$ acquisition source, we combined epidemiological and genetic data to trace possible transmission chains, at two different genetic levels. We considered possible transmission events where the donor and recipient were on the same ward at the same time, and carried the same host strain or Tn*4401* variant. Considering only "local" acquisitions (see above), 48/167 (29%) patients had ward contact with another patient carrying the same *bla*$_{KPC}$-positive strain (Figure 4, top panel). A greater proportion, 106/167 (63%), of patients had ward contact with another patient carrying the same Tn*4401* variant. However, as Tn*4401*-1 is very common (66% of patients), these inferred transmissions may be spurious. With patients carrying this common variant excluded, only 15/50 (30%) had ward contact with another patient carrying the same Tn*4401* variant (Figure 4, bottom panel). Therefore, both genetic levels (strain or Tn*4401* variant) demonstrated plausible transmissions for only a minority of patients, indicating that direct patient-to-patient transmission is not the dominant mode of *bla*$_{KPC}$ acquisition.

Discussion

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

In 7/11 distinct bla_{KPC} plasmids identified through long-read sequencing, Tn*4401* was located within a Tn*2*-like element. As these Tn*2*-like elements differed substantially from each other (Figure 3), it is unlikely that this arose via transposition of a composite Tn*4401*-Tn*2*-like structure. Instead, it suggests that Tn*4401* has been repeatedly incorporated into pre-existing Tn*2*-like elements, which are known to be widespread, and genetically divergent, in *Enterobacteriaceae* (44, 45). However, the insertion site was identical in all cases, yet Tn*4401* has been reported to have no insertion site specificity (28), suggesting that this was not facilitated by a standard transposition mechanism. Therefore, we suggest that this is most likely mediated by homologous recombination with other Tn*2*-like elements following an initial integration event, as recently suggested for another multi-drug resistance gene, $bla_{CTX\text{-}M\text{-}15}$ (46). This implies that Tn*4401* mobility may have been enhanced via integration into a second, already widely dispersed, transposon. As the Tn*4401*-Tn*2*-like structure was present in the index case isolate (CAV1016, Aug 2007) we presume that the initial transposition of Tn*4401* into a Tn*2*-like element occurred prior to entry into our hospital system. In support of this, one particular Tn*2*-like element, Tn*1331*, has been previously described to contain Tn*4401* (in exactly the same position within the *tnpA* gene as described here) (21, 42, 47, 48), including

one report describing a *K. pneumoniae* isolated in 2005, which predates bla_{KPC} in our institution (42). We are not aware of any previous reports describing Tn*4401* within a non-Tn*1331* Tn*2*-like element.

The prevalence of Tn*4401* insertions within Tn*2*-like elements also has important implications with regard to plasmid tracking. We previously published a method for arbitrary PCR to track the flanking regions around the Tn*4401* element, as well as a PCR method to assay presence of what we had wrongly assumed was a single plasmid, pKPC_UVA01. This PCR assay targeted the immediate Tn*4401* insertion site within a Tn*2*-like element (49), which we have here demonstrated is present in many different plasmids, highlighting that PCR assays, and indeed any partial typing methods, need to be interpreted with a great deal of caution. We were further mislead by the analysis of short-read whole genome sequencing data, which indicated presence of pKPC_UVA01 in the majority of isolates. Taken together it was tempting to conclude that horizontal transfer of pKPC_UVA01 was responsible for the vast majority of bla_{KPC} carriage in our institution. However, long-read sequencing refuted this, revealing a far more complex picture.

More generally, this highlights certain limitations for plasmid reconstruction from short-read data. To illustrate by way of example, there were five isolates where long-read sequencing revealed pKPC_UVA01-like plasmids that were identical to the reference pKPC_UVA01 sequence apart from the absence of Tn*4401* and associated 5 bp target site duplication (Figure 2). We presume that in these lineages, bla_{KPC} may have been initially acquired via HGT of pKPC_UVA01, with subsequent homologous recombination transferring Tn*4401* from

pKPC_UVA01 to a different plasmid containing a Tn*2*-like element. In each of these five isolates, multiple Tn*2*-like elements are present, which have 100% sequence identity over approximately 1 kb on either side of the Tn*4401* insertion site. As this is longer than the fragment length used for paired-end sequencing, it is not possible to resolve the plasmid context of bla_{KPC} using short-read data. Importantly, any reference-based method for plasmid reconstruction (e.g. in this case using the pKPC_UVA01 reference sequence to infer presence of the plasmid in each isolate) is liable to produce misleading results. More generally, it is exactly the repetitive regions that cannot be resolved using short-read data that could be expected to be involved in plasmid rearrangements, either through homologous recombination as suggested here, or by virtue of the fact that transposable elements are often present in multiple copies. Therefore, having short-read data that is consistent with a known plasmid structure, even within the same outbreak, should not be sufficient to conclude that that structure is present, if the data is also consistent with an alternative structure. As several recent studies have utilised reference-based approaches for plasmid assembly / inference (33, 34), our results indicate that any such methods should be interpreted with extreme caution.

Across the bla_{KPC}-positive patients, there was large variation in both host strains and bla_{KPC} plasmids, with Tn*4401* being the largest genetic unit that was consistently present. Therefore, surveillance strategies aimed at tracking individual strains or plasmids could be misleading, and it may be more appropriate to focus on Tn*4401*. However, we found limited variation within the transposon, as Tn*4401* sequences from 121/182 (66%) patients were identical to the index case (Table 2). This lack of variation implies that even the highest resolution genetic methods may be insufficient for determining specific transmission routes. Even so, we have demonstrated that

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

There several limitations to this study. Because of the cost and effort involved in long-read sequencing, we were only able to resolve a minority of *bla*$_{KPC}$-plasmids. This means that although we have a compelling indicator of the diversity created by mobile genetic elements within a single hospital over a five year period, we are limited in the ability to genetically resolve pathways of *bla*$_{KPC}$ mobility between host strains and plasmid vectors, even within a single patient. We also speculate about the effect of Tn*4401* insertion into Tn*2*-like elements, but future *in vitro* studies could be used to illuminate the effect of this composite structure on Tn*4401* mobility.

In conclusion, our detailed genetic analysis of the evolutionary events occurring in the early stages of antimicrobial resistance gene emergence in a single institution identifies several distinct processes occurring at high frequency (Figure 5). First, the presence of shared *bla*$_{KPC}$-containing strains in different patients reflects traditional (clonal) outbreak models. Second, *bla*$_{KPC}$ mobility between strains/species is facilitated by promiscuous *bla*$_{KPC}$ plasmids such as pKPC_UVA01. Third, *bla*$_{KPC}$ transfer between plasmids is likely enhanced by homologous recombination between Tn*2*-like elements, facilitating the movement of Tn*4401* from one plasmid to another. Finally, *bla*$_{KPC}$ mobility is also enabled by standard Tn*4401* transposition. Rather than a single process dominating, resistance dissemination is driven by a combination of

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

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

Methods

Isolate collection and Illumina sequencing

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

Species classification

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

Phylogenetic analysis and strain classification

There were 52 patients with multiple isolates of the same species. One of these (patient FK) involved two strains of *K. pneumoniae* that were highly divergent from each other (>20,000 chromosomal SNVs), clearly representing a separate bla_{KPC} acquisition by each strain. Excluding this divergent strain pair, the remaining cases had SNV differences ranging from 0 to 60 (median 2 SNVs). As these could plausibly represent clonal evolution within the patient, we conservatively chose to include only a single representative (the earliest isolate) for 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 Tn*4401* 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 though 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 Tn*4401* flanking sequences**

386 Where a plasmid was classified as being present in a particular isolate, it was not always certain
387 to contain Tn*4401*. The plasmid presence classification was further refined as: "containing
388 Tn*4401*" if the isolate's *de novo* assembly supported Tn*4401* being present within the expected
389 sequence context of that plasmid, "not containing Tn*4401*" if the plasmid was assembled
390 without Tn*4401*, or "uncertain" if structure could not be determined from the *de novo*
391 assembly. The identification of novel Tn*4401* insertion sites was also based on the *de novo*
392 assemblies. These methods are described in detail in Supplementary Methods.

393 **Variation in Tn*4401***

394 Tn*4401* 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

BLASTn, to identify structural variation. SNV variation was determined by mapping to a reference consisting of pKPC_UVA01 plus a species-specific chromosome as described above, followed by extraction of the Tn*4401* region. Variation is reported for all sites where at least one isolate had a non-reference call, including any ambiguity at that site in other isolates. Ambiguity at non-variable sites is not reported, which may result in an underestimate of true variation. However, any resulting underestimation is likely to be very minor, as the proportion of called sites, excluding deleted regions described above, was >96% for all isolates.

Epidemiological classification

For epidemiologic analysis, patients were assigned a one or two letter code for de-identification. Routine peri-rectal surveillance cultures for silent colonization began in April 2009 (38, 40). Patients were classified as "imported" if they did not have any prior admission to University of Virginia Medical Center/Long-term Acute Care Hospital (UVaMC) and either had a *bla*$_{KPC}$-positive *Enterobacteriaceae* isolated within 48 hours of admission, or had a carbapenem-resistant *Enterobacteriaceae* culture before transfer to UVaMC with a subsequent isolate at UVaMC confirmed as *bla*$_{KPC}$ PCR positive. The index case was also classified as imported. For the remaining patients, the source of *bla*$_{KPC}$ acquisition was classified as "local". The 48h cutoff is arbitrary and may result in some misclassification if patients either acquire *bla*$_{KPC}$ within the first 48h of admission, or if *bla*$_{KPC}$ carriage/infection remains undetected for >48h, however this is expected to be minimal (see Supplementary Methods). Charts and patient contacts were reviewed using bed tracing data and the electronic medical record. The study was approved by 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 Tn*4401*
421 variant). This is described in detail in Supplementary Methods.

422

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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 Tn*4401* 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. Tn*4401* 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; Tn*4401* is shown in purple. Pink shading
626 indicates regions of identity between adjacent sequences, with SNVs indicated by red lines.

627

628 **Figure 3. Tn*4401* is commonly integrated into a Tn*2*-like element.** The Tn*4401* 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; Tn*4401* 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 Tn*2** 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 Tn*4401* 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 Tn*4401*-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 Tn*4401*, the total number of acquisitions
642 explainable by direct ward contact is indicated, as well as with Tn*4401*-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	*bla*$_{KPC}$ plasmid	Size (bp)	Group[a]	Within-group genetic changes[b]	Tn*4401* variant	Flanking sequence[c]	Tn*2*-like element[d]
CAV1344	*K. pneumoniae*	EP	Dec-2010	pKPC_CAV1344	176,497	Singleton	NA	Tn*4401*-1	GTTCT…GTTCT	Yes
CAV1392	*K. pneumoniae*	EU	Mar-2011	pKPC_CAV1392	43,621	pKPC_UVA01	1 SNV	Tn*4401*-5	GTTCT…GTTCT	Yes
				NA (chromosomal)	NA	NA	NA	Tn*4401*-5	AGATA…AGATA	No
CAV1596	*K. pneumoniae*	FK	Apr-2012	pKPC_CAV1596-78	77,801	Singleton	NA	Tn*4401*-5	GTTCT…GTTCT	Yes
				pKPC_CAV1596-97	96,702	Singleton	NA	Tn*4401*-5	TATCG…TATCG	No
CAV1099	*K. oxytoca*	AU	Apr-2009	pKPC_CAV1099	113,105	pKPC_UVA02	0 SNVs	Tn*4401*-1	ATGCA…GGCCA[e]	No
CAV1335	*K. oxytoca*	EQ	Dec-2010	pKPC_CAV1335	113,105	pKPC_UVA02	0 SNVs	Tn*4401*-1	ATGCA…GGCCA[e]	No
CAV1374	*K. oxytoca*	ED	Aug-2010	pKPC_CAV1374	332,956	Singleton	NA	Tn*4401*-1	GTTCT…GTTCT	Yes
CAV1043	*E. asburiae*	L	Mar-2008	pKPC_CAV1043	59,138	Singleton	NA	Tn*4401*-5	GTTCT…GTTCT	Yes
CAV1176	*E. cloacae*	DN	May-2010	pKPC_CAV1176	90,452	pKPC_CAV1176	0 SNVs	Tn*4401*-3	GTTCT…GTTCT	Yes
CAV1311	*E. cloacae*	EO	Jan-2011	pKPC_CAV1311	90,452	pKPC_CAV1176	0 SNVs	Tn*4401*-3	GTTCT…GTTCT	Yes
CAV1411	*E. cloacae*	FC	Jun-2011	pKPC_CAV1411	90,452	pKPC_CAV1176	1 SNV, 40 kb inversion	Tn*4401*-3	GTTCT…GTTCT	Yes
CAV1669	*E. cloacae*	HV	Aug-2012	pKPC_CAV1669	90,452	pKPC_CAV1176	40kb inversion	Tn*4401*-3	GTTCT…GTTCT	Yes
CAV1668	*E. cloacae*	HQ	Aug-2012	pKPC_CAV1668	43,433	pKPC_UVA01	1 SNV, 188 bp deletion	Tn*4401*-3	GTTCT…GTTCT	Yes
CAV1321	*C. freundii*	EG	Nov-2010	pKPC_CAV1321-45	44,846	pKPC_UVA01	1,225 bp insertion	Tn*4401*-1	GTTCT…GTTCT	Yes
				pKPC_CAV1321-244	243,709	Singleton	NA	Tn*4401*-1	GTTCT…GTTCT	Yes
CAV1741	*C. freundii*	ER	Oct-2012	pKPC_CAV1741	129,196	pKPC_UVA01	14,960 bp duplication, 70,615 bp insertion	Tn*4401*-1[f]	GTTCT…GTTCT	Yes
CAV1151	*K. intermedia*	CD	Sep-2009	pKPC_CAV1151	43,621	pKPC_UVA01	0 SNVs[g]	Tn*4401*-1	GTTCT…GTTCT	Yes
CAV1320	*E. aerogenes*	EL	Nov-2010	pKPC_CAV1320	13,981	Singleton	NA	Tn*4401*-1	TTGTT…TTGTT	No
CAV1492	*S. marcescens*	GL	Dec-2011	pKPC_CAV1492	69,158	Singleton	NA	Tn*4401*-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 Tn*4401* 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 Tn*4401*; generally expected to be identical due to 5 bp target site duplication during transposition (28)

653 [d] Tn*4401* integrated into the *tnpA* gene of a Tn*2*-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

Table 2. Tn*4401* variation

Tn*4401* variant	Structural isoform(29)	SNVs[a]	bla_{KPC} variant	Patients	Isolates	Strains
Tn*4401*-1[b]	b	-	bla_{KPC-2}	121	176	42
Tn*4401*-2	a (del 7020-7118)	-	bla_{KPC-2}	5	8	1
Tn*4401*-3	novel (del 6919-7106)	-	bla_{KPC-2}	28	39	2
Tn*4401*-4	truncated (del 1-6654)	-	bla_{KPC-2}	2	3	1
Tn*4401*-5	b	8015 C→T[c]	bla_{KPC-3}	22	40	19
Tn*4401*-6	b	8015 C→T, 9621 T→C	bla_{KPC-3}	1	3	2
Tn*4401*-7	b	7199 T→A, 8015 C→T, 9621 T→C	bla_{KPC-3}	1	1	1
Tn*4401*-8	b	9663 T→C	bla_{KPC-2}	1	3	1
Tn*4401*-9	b	7509 C→G, 7917 T→G[d]	bla_{KPC-4}	1	1	1
Tn*4401*-10	b	6800 T→C, 7509 C→G, 7917 T→G	bla_{KPC-4}	1	4	1
Tn*4401*-11	b	8015 N[e]	bla_{KPC-2} / bla_{KPC-3}	1	2	1
Tn*4401*-12	truncated (del 1-6727)	6800 N[f]	bla_{KPC-2}	1	1	1

[a] With respect to Tn*4401*-1, which is considered as the reference Tn*4401* sequence for this study

[b] Tn*4401*-1 differs from the reference isoform b sequence in EU176013.1 by 14 SNVs, as follows: 4939 C→G, 4989 C→T, 5099 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, 8113 A→C

[c] This substitution converts bla_{KPC-2} to bla_{KPC-3}

[d] These two substitutions convert bla_{KPC-2} to bla_{KPC-4}

[e] Quality filters failed at this position due to a mixture of reads supporting C and T (i.e. Tn*4401*-11 actually represents a mixture of Tn*4401*-1 and Tn*4401*-5)

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

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; Tn*4401* is shown in purple.

674

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

676

677 **Table S2. Additional Tn*4401* 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**

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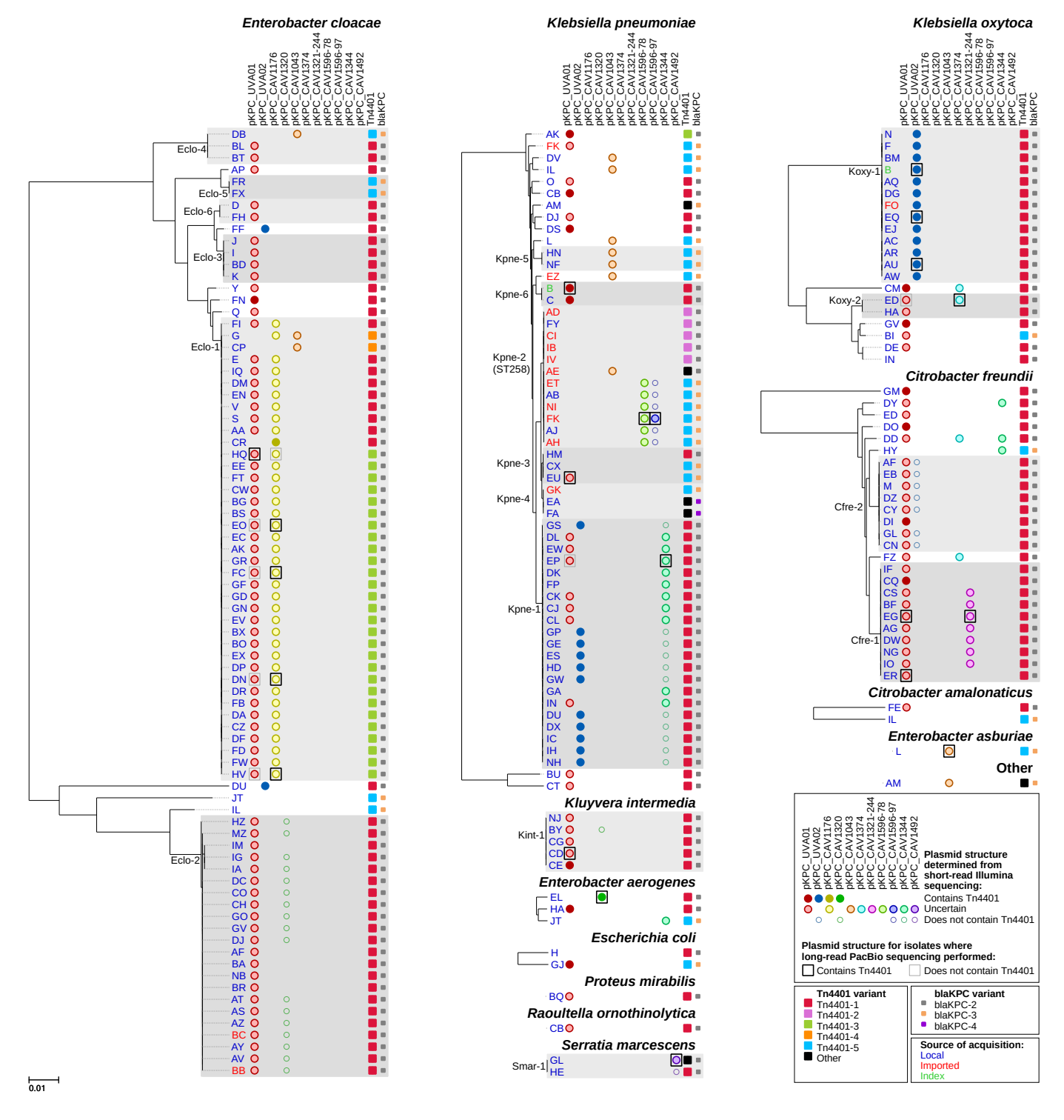
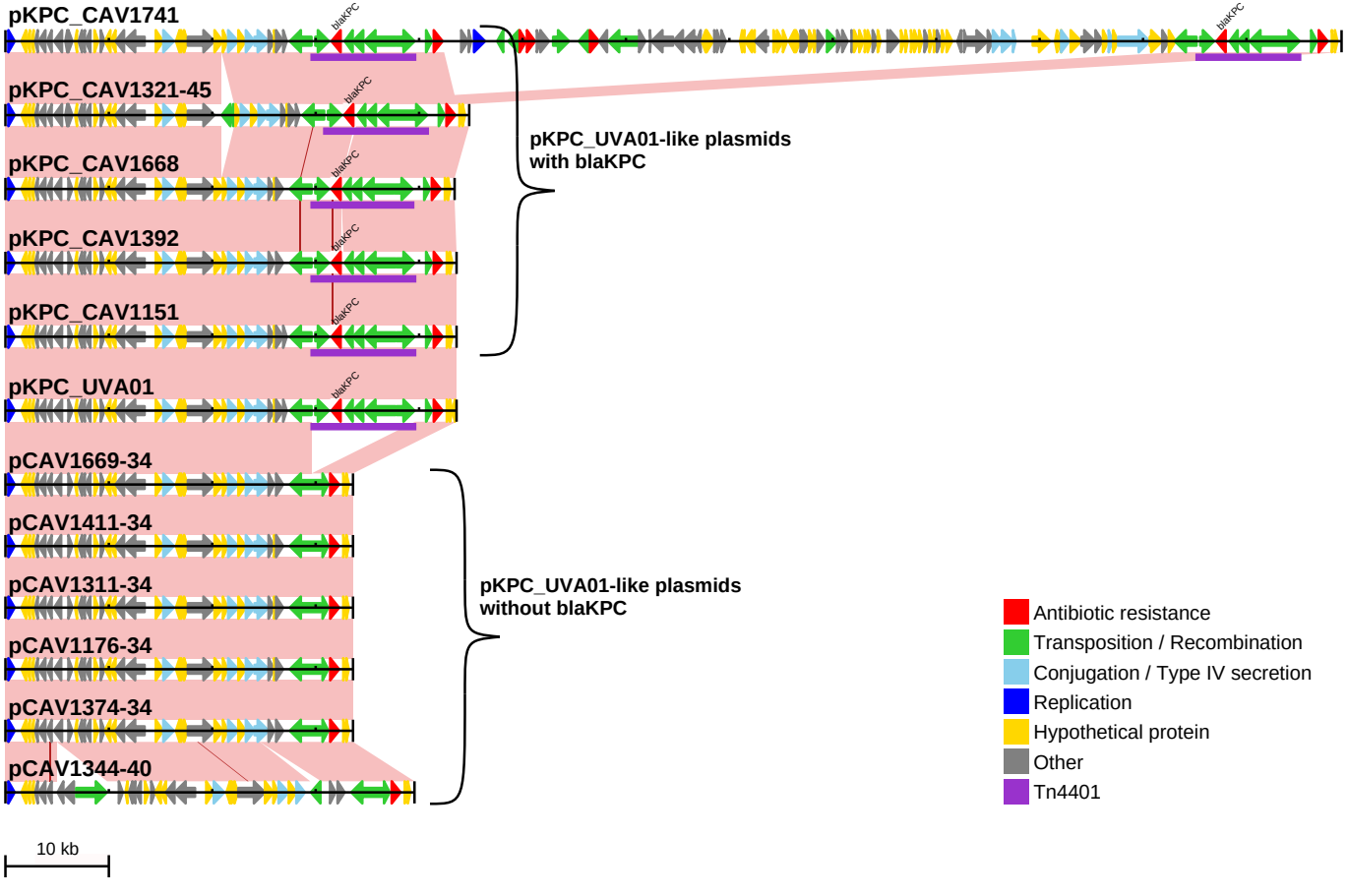
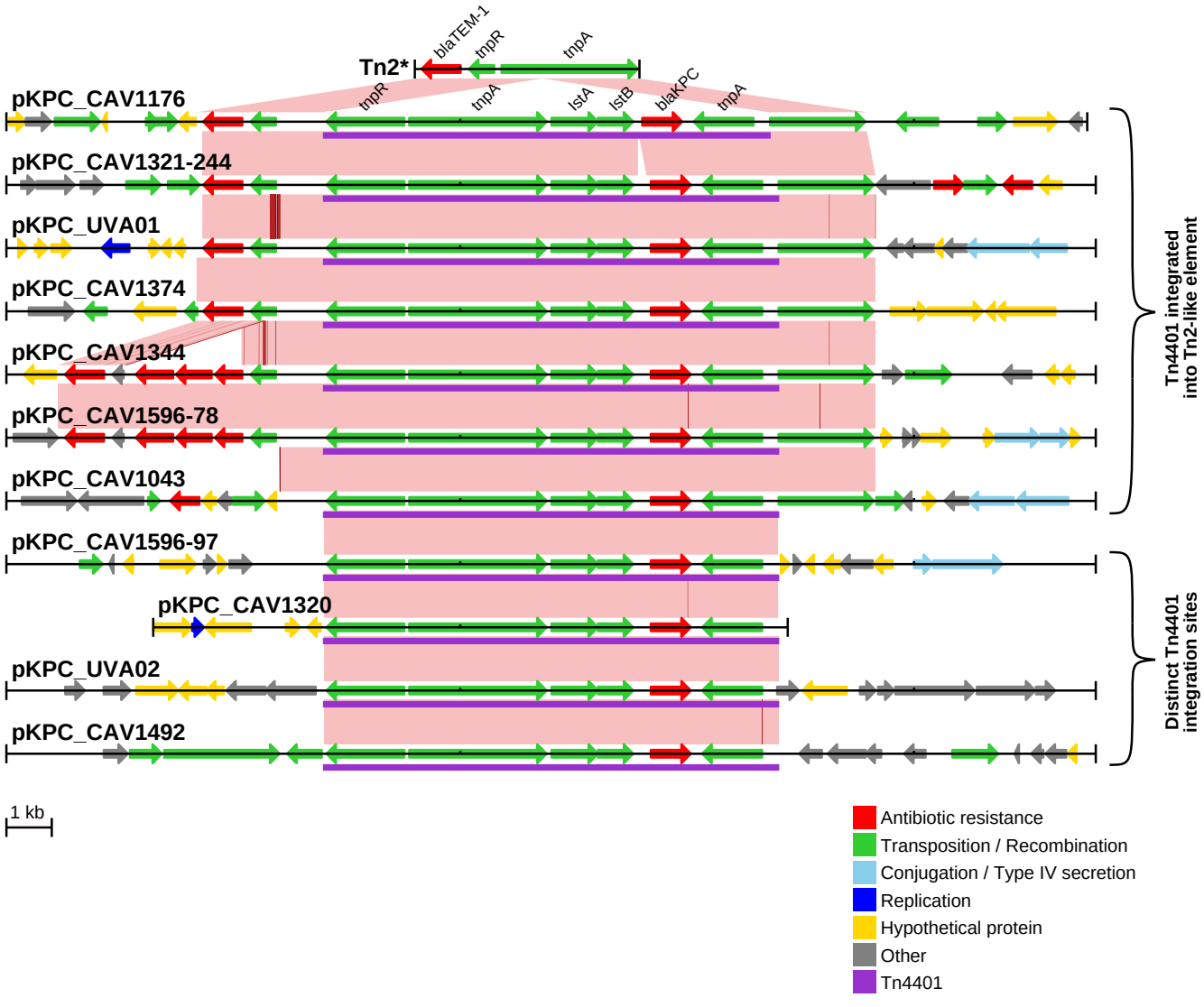
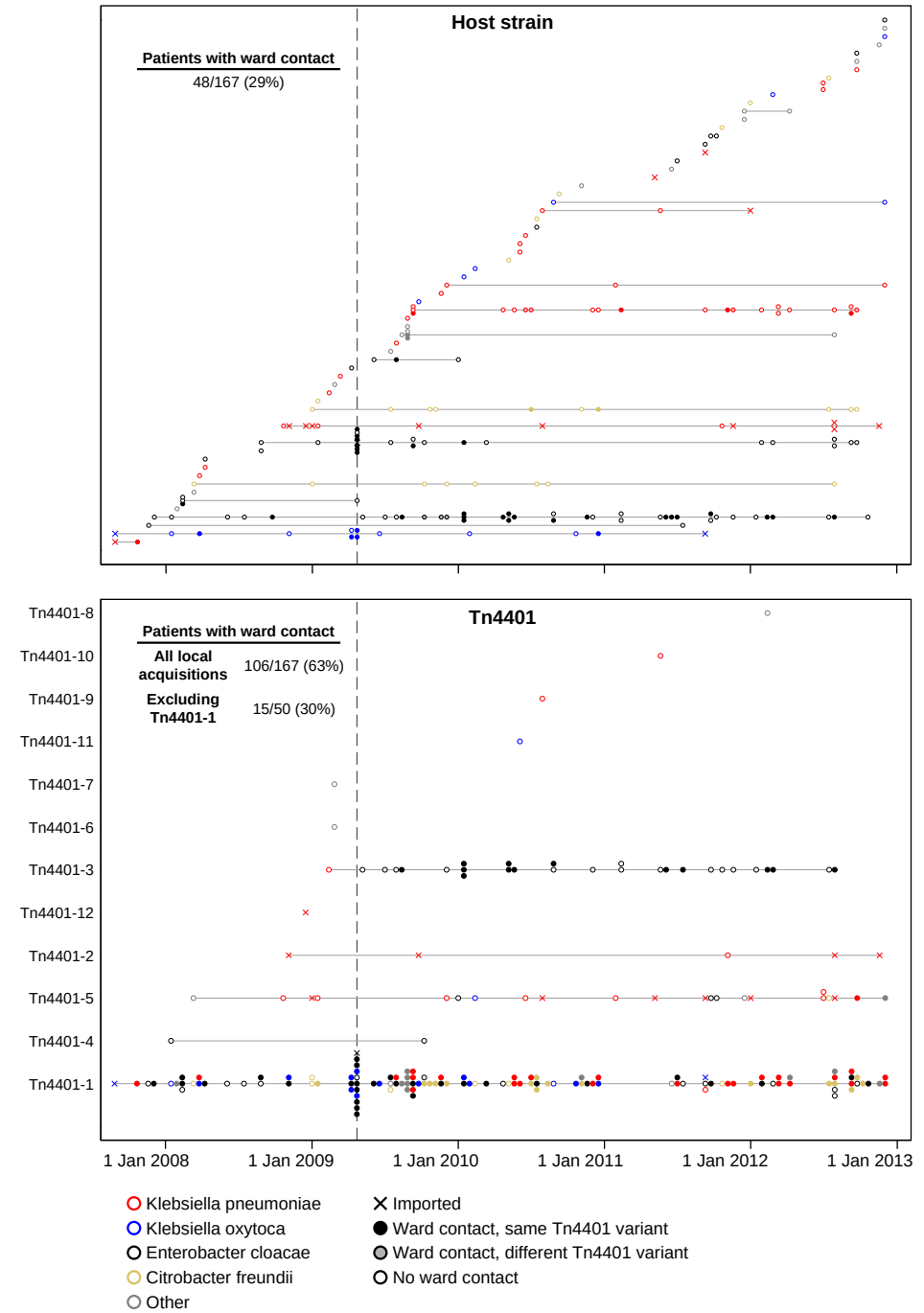
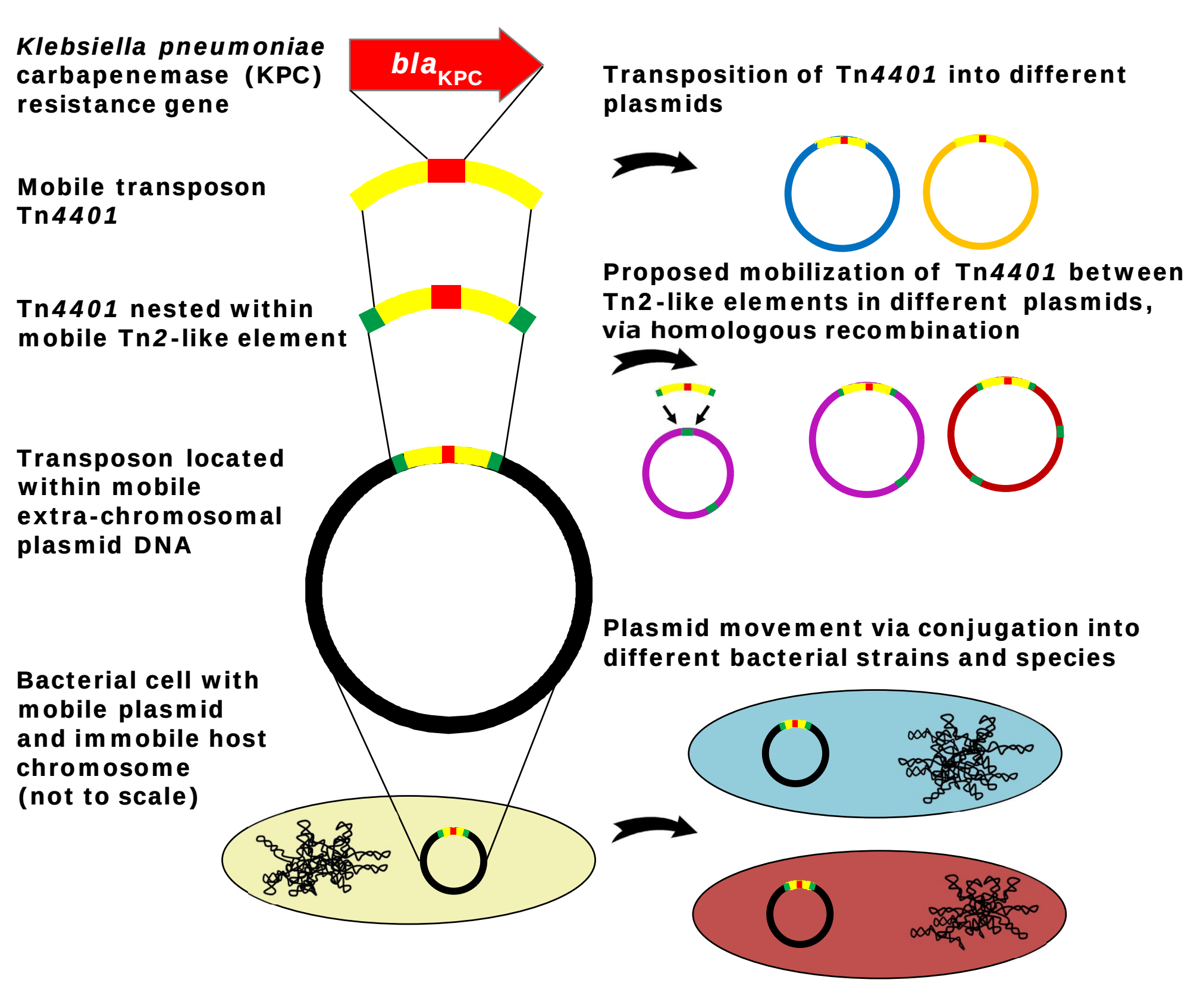

Enterobacter cloacae
Klebsiella pneumoniae
Klebsiella oxytoca
Citrobacter freundii
Citrobacter amalonaticus
Enterobacter asburiae
Other
Kluyvera intermedia
Enterobacter aerogenes
Escherichia coli
Proteus mirabilis
Raoultella ornothinolytica
Serratia marcescens
pKPC_UVA01
pKPC_UVA02
pKPC_CAV1176
pKPC_CAV1320
pKPC_CAV1043
pKPC_CAV1374
pKPC_CAV1321-244
pKPC_CAV1596-78
pKPC_CAV1596-97
pKPC_CAV1344
pKPC_CAV1492
Tn4401
blaKPC
Eclo-1
Eclo-2
Eclo-3
Eclo-4
Eclo-5
Eclo-6
Kpne-1
Kpne-2 (ST258)
Kpne-3
Kpne-4
Kpne-5
Kpne-6
Koxy-1
Koxy-2
Cfre-1
Cfre-2
Kint-1
Smar-1
0.01
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
Tn4401 variant
Tn4401-1
Tn4401-2
Tn4401-3
Tn4401-4
Tn4401-5
Other
blaKPC variant
blaKPC-2
blaKPC-3
blaKPC-4
Source of acquisition:
Local
Imported
Index


pKPC_CAV1741
blaKPC
blaKPC
pKPC_CAV1321-45
blaKPC
pKPC_CAV1668
blaKPC
pKPC_CAV1392
blaKPC
pKPC_CAV1151
blaKPC
pKPC_UVA01
pKPC_UVA01-like plasmids with blaKPC
pCAV1669-34
pCAV1411-34
pCAV1311-34
pCAV1176-34
pCAV1374-34
pCAV1344-40
pKPC_UVA01-like plasmids without blaKPC
10 kb
Antibiotic resistance
Transposition / Recombination
Conjugation / Type IV secretion
Replication
Hypothetical protein
Other
Tn4401


Tn2*
blaTEM-1
tnpR
tnpA
tnpR
tnpA
IstA
IstB
blaKPC
tnpA
pKPC_CAV1176
pKPC_CAV1321-244
pKPC_UVA01
pKPC_CAV1374
pKPC_CAV1344
pKPC_CAV1596-78
pKPC_CAV1043
pKPC_CAV1596-97
pKPC_CAV1320
pKPC_UVA02
pKPC_CAV1492
Tn4401 integrated into Tn2-like element
Distinct Tn4401 integration sites
1 kb
Antibiotic resistance
Transposition / Recombination
Conjugation / Type IV secretion
Replication
Hypothetical protein
Other
Tn4401


Host strain
Patients with ward contact
48/167 (29%)
Tn4401
Patients with ward contact
All local acquisitions
106/167 (63%)
Excluding Tn4401-1
15/50 (30%)
Tn4401-8
Tn4401-10
Tn4401-9
Tn4401-11
Tn4401-7
Tn4401-6
Tn4401-3
Tn4401-12
Tn4401-2
Tn4401-5
Tn4401-4
Tn4401-1
1 Jan 2008
1 Jan 2009
1 Jan 2010
1 Jan 2011
1 Jan 2012
1 Jan 2013
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
bla_{KPC}
Mobile transposon Tn*4401*
Tn*4401* nested within mobile Tn*2*-like element
Transposon located within mobile extra-chromosomal plasmid DNA
Bacterial cell with mobile plasmid and immobile host chromosome (not to scale)
Transposition of Tn*4401* into different plasmids
Proposed mobilization of Tn*4401* between Tn2-like elements in different plasmids, via homologous recombination
Plasmid movement via conjugation into different bacterial strains and species