Chromosomal Integration of the Klebsiella pneumoniae Carbapenemase Gene, bla$_{KPC}$, in Klebsiella Species Is Elusive but Not Rare

Amy J. Mathers, a,b Nicole Stoesser, c Weidong Chai, a Joanne Carroll, b Katie Barry,a Anita Cherunvanky,a Robert Sebra, d Andrew Kasarskis, d Tim E. Petoc, A. Sarah Walker,a Costi D. Sifri,a,e Derrick W. Crook,c Anna E. Sheppard,c

Division of Infectious Diseases and International Health, Department of Medicine, University of Virginia Health System, Charlottesville, Virginia, USAa; Clinical Microbiology, Department of Pathology, University of Virginia Health System, Charlottesville, Virginia, USAa; Modernizing Medical Microbiology Consortium, Nuffield Department of Clinical Medicine, University of Oxford, Oxford, United Kingdomc; Icahn Institute and Department of Genetics and Genomic Sciences, Icahn School of Medicine, Mount Sinai, New York, New York, USAad; Office of Hospital Epidemiology, University of Virginia Health System, Charlottesville, Virginia, USAe

ABSTRACT Carbapenemase genes in Enterobacteriaceae are mostly described as being plasmid associated. However, the genetic context of carbapenemase genes is not always confirmed in epidemiological surveys, and the frequency of their chromosomal integration therefore is unknown. A previously sequenced collection of bla$_{KPC}$-positive Enterobacteriaceae from a single U.S. institution (2007 to 2012; n = 281 isolates from 182 patients) was analyzed to identify chromosomal insertions of Tn4401, the transposon most frequently harboring bla$_{KPC}$. Using a combination of short- and long-read sequencing, we confirmed five independent chromosomal integration events from 6/182 (3%) patients, corresponding to 15/281 (5%) isolates. Three patients had isolates identified by perirectal screening, and three had infections which were all successfully treated. When a single copy of bla$_{KPC}$ was in the chromosome, one or both of the phenotypic carbapenemase tests were negative. All chromosomally integrated bla$_{KPC}$ genes were from Klebsiella spp., predominantly K. pneumoniae clonal group 258 (CG258), even though these represented only a small proportion of the isolates. Integration occurred via IS15-ΔI-mediated transposition of a larger, composite region encompassing Tn4401 at one locus of chromosomal integration, seen in the same strain (K. pneumoniae ST340) in two patients. In summary, we identified five independent chromosomal integrations of bla$_{KPC}$ in a large outbreak, demonstrating that this is not a rare event. bla$_{KPC}$ was more frequently integrated into the chromosome of epidemic CG258 K. pneumoniae lineages (ST11, ST258, and ST340) and was more difficult to detect by routine phenotypic methods in this context. The presence of chromosomally integrated bla$_{KPC}$ within successful, globally disseminated K. pneumoniae strains therefore is likely underestimated.

KEYWORDS carbapenemase, KPC, Klebsiella, Klebsiella pneumoniae carbapenemase, antibiotic resistance, chromosomal, plasmid analysis, plasmids, transposons, whole-genome sequencing

Carbapenem resistance in Enterobacteriaceae has become a major clinical challenge (1). Within this bacterial family, carbapenemase genes are largely located on plasmids cocirculating with various strains (2). Plasmid DNA may act as a temporary “lending library,” enabling genes of importance to survive various selective pressures (3). However, in vitro and modeling data suggest that once a gene is incorporated

Received 19 August 2016 Returned for modification 17 September 2016 Accepted 11 December 2016 Accepted manuscript posted online 28 December 2016

Citation Mathers AJ, Stoesser N, Chai W, Carroll J, Barry K, Cherunvanky A, Sebra R, Kasarskis A, Peto TE, Walker AS, Sifri CD, Crook DW, Sheppard AE. 2017. Chromosomal integration of the Klebsiella pneumoniae carbapenemase gene, bla$_{KPC}$, in Klebsiella species is elusive but not rare. Antimicrob Agents Chemother 61: e01823-16. https://doi.org/10.1128/AAC.01823-16.

Copyright © 2017 Mathers et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Address correspondence to Amy J. Mathers, ajm5b@virginia.edu.
chromosomally, it will be maintained through replication without selective pressure, and gene loss from the bacterial population becomes less likely (4–6).

The *Klebsiella pneumoniae* carbapenemase gene (bla<sub>KPC</sub>) is maintained within the self-mobilizing 10-kb transposon Tn4401 (7). The frequency of transposition in vitro is relatively high (4.4 × 10<sup>−4</sup>/recipient cell) and without site specificity, but the rate of movement outside laboratory settings is largely unknown (8). Nevertheless, Tn4401 has been described in several different genetic environments (9, 10).

Historically, most descriptions of bla<sub>KPC</sub> have been in a globally successful lineage of *K. pneumoniae*, namely, multi locus sequence type 258 (ST258) and the associated clonal group 258 (CG258), which includes ST11, ST258, and ST340 (11). CG258 isolates are widespread, albeit concentrated in geographic hotspots, with the bla<sub>KPC</sub> gene being plasmid associated in the majority of reports (9). Interestingly, the earliest observed chromosomal Tn4401 integration events have been sporadic and in non-*Klebsiella* spp.: *Pseudomonas aeruginosa* in 2006 (12), *Raoultella* spp. in 2008 (13) and *Acinetobacter baumannii* in 2009 (14). More recently, chromosomal integration among *K. pneumoniae* ST258 (n = 4) isolates has been described in the United States by three separate groups (15–17).

The rate of chromosomal integration within clinical settings for many genetic elements is largely unknown. Chromosomal integration of bla<sub>KPC</sub> may be under-reported, as its investigation often requires detailed genetic analysis, and if a plasmid copy is present then a chromosomal copy may be overlooked. Even high-resolution genetic methods, such as whole-genome sequencing (WGS) using short-read technologies, can be confounded by the presence of multiple copies of Tn4401 and/or repetitive flanking sequences, which limit the ability to accurately reconstruct the genetic context(s) of bla<sub>KPC</sub> (10, 18). Here, we use a combination of short- and long-read WGS to investigate chromosomal integration of bla<sub>KPC</sub> among 281 isolates (62 distinct strains) of bla<sub>KPC</sub>-positive Enterobacteriaceae isolated from 182 patients in a single hospital.

**RESULTS**

**Identification of chromosomal integrations of bla**<sub>KPC</sub>. There were 62 distinct strains (from 13 species) among 281 sequenced *K. pneumoniae* carbapenemase (KPC)-Enterobacteriaceae isolates derived from 182 infected/colonized patients (18). Using de novo assemblies to identify isolates where Tn4401 was adjacent to known chromosomal sequences, 44% (123/281) of isolates, from 82/182 (45%) patients, were evaluable (Table 1). In 7/123 evaluable isolates, from 3/82 patients, Tn4401 flanking sequence showed homology with chromosomal reference sequences, indicating a likely chromosomal location for bla<sub>KPC</sub>. Three different chromosomal loci were identified, one for each patient, demonstrating three distinct Tn4401 chromosomal integration events. Isolates from two of the patients were *K. pneumoniae* (ST258 and ST340, with earliest isolates CAV1453 and CAV1417, respectively), and one was *K. oxytoca* (CAV1752).

As the de novo assembly approach was only able to assess 44% of isolates for possible chromosomal integrations, we also used a mapping approach, which is unaffected by multiple Tn4401 copies. Reassuringly, all seven isolates identified by the de novo assembly approach described above were also identified by this method as having a likely chromosomal integration of Tn4401. In addition, the mapping approach identified a further 11 isolates as having putative chromosomal integrations, all of which were unevaluable by the de novo assembly approach. Six of these were *K. pneumoniae* ST11 isolates from a single patient (the earliest isolate was CAV1351). Three were *K. pneumoniae* ST340 isolates from two additional patients (earliest isolates were CAV1217 and CAV1518). One was a non-CG258 *K. pneumoniae* isolate (ST244; CAV1042) from an additional patient, and one was a *K. oxytoca* isolate (CAV1755) unrelated to CAV1752 (>40,000 single-nucleotide variant [SNV] differences), also from an additional patient.

To confirm the chromosomal locations of Tn4401, we used long-read sequencing. For 6/8 patients identified above, the earliest isolate from each was sequenced using
PacBio. In 5/6 cases, the presence of Tn4401 on the chromosome was confirmed, and in one case (CAV1042) it was refuted. For one of the two remaining patients (the earliest isolate was CAV1351), we did not perform additional PacBio sequencing, as a subsequent isolate from the same patient (CAV1392; 1 SNV difference from CAV1351) was previously PacBio sequenced as part of another study and shown to contain two copies of Tn4401, one chromosomal and one plasmid (18). CAV1518 from the final patient was not PacBio sequenced, as it was demonstrated using other methods (see below) to be a false positive from the mapping approach, since Tn4401 was not present on the chromosome.

Altogether, we found five distinct loci of chromosomal integration of blaKPC verified by long-read sequencing from six patients, with two additional patients having isolates that were falsely identified by the mapping approach (Table 2). All chromosomal integrations were in Klebsiella spp., mostly K. pneumoniae CG258, even though CG258 represented only a small proportion of the outbreak (4/18 versus 2/164 non-CG258 patients, \( P/H11005_{0.0009} \); 13/34 versus 2/247 isolates, \( P/H11021_{0.0001} \); Fisher’s exact test).

Mechanism of chromosomal integration in K. pneumoniae ST340. There were three patients with K. pneumoniae ST340 isolates where the mapping approach identified Tn4401 as having a possible chromosomal location (CAV1417, CAV1217, and CAV1518) (Table 2). To assess the molecular basis of chromosomal integration, the chromosomal region encompassing Tn4401 in the closed PacBio assembly of the ST340 CAV1417 isolate was aligned to the JUSt258.2 reference (GenBank accession number CP0006918), which belongs to the closely related ST258. Relative to the reference, CAV1417 had a 24-kb inversion and a 16-kb insertion, which included Tn4401 and several other resistance genes (Fig. 1A).

At one end of the 16-kb region was an IS15-ΔI element, highly genetically similar (3 nucleotide differences) to IS26, both of which undergo replicative transposition with 8-bp target site duplication (TSD) (19). IS26 has been shown to undergo frequent intramolecular transposition, which can result in inversion of the sequence between the original and duplicated elements. This process disrupts the pairing of TSD sequences, which can be used to trace the history of transposition events (20).

The other end of the 16-kb region terminated in a sequence with high similarity to the 14-bp IS15-ΔI inverted repeat (IR) sequence (IS15-ΔI, GGCACGTGTTGAAAA; other, GGCCTTGTGTTAATA; 10/14 nucleotide identities), suggesting that this acted as a cryptic recognition site mediating transposition of the entire 16-kb region, similar to a cryptic recognition site mediating transposition of the entire 16-kb region, similar to a...
| Chromosomal integration status | Isolate | Species | MLST | Clinical microbiology finding | Porin gene status* | No. and location of Tn4401 loci |
|--------------------------------|---------|---------|------|-------------------------------|--------------------|-------------------------------|
| No; suspected by mapping, rejected by long-read sequencing | CAV1042 | *K. pneumoniae* | ST244 | Ertapenem VITEK 2 (µg/ml) | Intact | Intact | 2 plasmid |
| Yes; suspected by mapping, confirmed by long-read sequencing of CAV1392 | CAV1351 | *K. pneumoniae* | ST11 | Meropenem VITEK 2 (µg/ml) | Intact | Intact | 1 chromosomal, 1 plasmid |
| Yes; suspected by assembly and mapping, confirmed by long-read sequencing of CAV1392 | CAV1453 | *K. pneumoniae* | ST258 | Meropenem broth dilution (µg/ml) | Intact | Intact | 1 chromosomal |
| Yes; suspected by assembly and mapping, confirmed by long-read sequencing of CAV1392 | CAV1217 | *K. pneumoniae* | ST340 | Indirect carbenapenemase test | Intact | Intact | 1 chromosomal, 1 plasmid |
| Yes; suspected by assembly and mapping, confirmed by long-read sequencing of CAV1392 | CAV1417 | *K. pneumoniae* | ST340 | Modified Hodge test | Intact | Intact | 1 chromosomal |
| No; suspected by mapping, rejected by TSD sequence examination | CAV1518 | *K. pneumoniae* | ST340 | qRT-PCR mean fold change b-1 | Intact | Intact | Unknown |
| Yes; suspected by assembly and mapping, confirmed by long-read sequencing of CAV1392 | CAV1752 | *K. oxytoca* | ST340 | Tn4401 isoform a-1 | Intact | Intact | 1 chromosomal |
| Yes; suspected by mapping, confirmed by long-read sequencing of CAV1392 | CAV1755 | *K. oxytoca* | ST340 | Tn4401 and SNV variant (18) | Intact | Intact | 1 chromosomal, 2 plasmid |

*aIntact indicates that the open reading frame is maintained with respect to the reference sequence.

bNA, not applicable.
Composite transposon. The 8-bp flanking sequences of the 16-kb region were identical to the 8-bp flanking sequences of a neighboring IS\textsubscript{15}-ΔI element located at the other end of the 24-kb inversion relative to NJST258\textsubscript{2} (Fig. 1A). This indicates that the 16-kb region initially integrated into the chromosome by intermolecular transposition, with an 8-bp TSD (GTGATGGC). Subsequently, IS\textsubscript{15}-ΔI underwent intramolecular transposition, resulting in a 24-kb inversion, with duplication of a second 8-bp sequence (TTCAGATG), with one copy flanking each of the duplicated elements.

In CAV1217, long-read sequencing demonstrated two copies of Tn\textsubscript{4401}, one chromosomal and one plasmid. For the chromosomal copy, the 8-bp sequence adjacent to the non-IS\textsubscript{15}-ΔI side of the 16-kb region (Fig. 1, right side) was identical to CAV1417 (GTGATGGC), while the plasmid copy had a different 8-bp flanking sequence (CGTAGGCA).

In CAV1518, the above-described signature of chromosomal integration (GTGATGGC sequence adjacent to the 16-kb region) is not present (Fig. 1B). Instead, the flanking sequence is identical to the plasmid copy in CAV1217 (CGTAGGCA). Therefore, the 16-kb region is most likely plasmid located in CAV1518, indicating that this isolate was a false positive from the mapping method.

Clinical microbiologic characteristics. Susceptibility results and phenotypic testing for these isolates varied substantially (Table 2). K. pneumoniae CAV1453, CAV1351,
and CAV1417 were high-level carbapenem resistant but with weak to negative carbapenemase phenotypes. All of these isolates had a sequence disruption in major *K. pneumoniae* porin channels (ompK35 and/or ompK36) (Table 2). CAV1453 had only a single Tn4401 copy in the chromosome; however, it was Tn4401a, which generally results in increased KPC expression compared to Tn4401b, as was seen here with a higher level of expression compared to the other isolates with a single copy of Tn4401b in the chromosome (21, 22). It was negative by indirect carbapenemase testing (23) but weakly positive by the modified Hodge test. CAV1417 had a single chromosomal copy of Tn4401b, and phenotypic testing for carbapenemase activity was consistently negative, with low-level blaKPC expression by quantitative PCR. Despite negative carbapenemase production by phenotypic tests, CAV1417 was highly carbapenem resistant and had disruptions in both ompK35 and ompK36. The functional consequences of these disruptions have not been confirmed, but loss of function of these genes is expected to increase carbapenem resistance above that conferred by KPC alone (11, 21, 22, 24).

*K. oxytoca* isolate CAV1755 was carbapenem resistant and phenotypically positive by carbapenemase testing. This was also true for the *K. pneumoniae* isolate CAV1042, which was ultimately rejected as having a chromosomal copy. Both of these isolates had multiple loci of Tn4401 integration (including a plasmid) with increased coverage of Tn4401b relative to the chromosome and also demonstrated increased blaKPC expression and intact porin channel sequences.

CAV1217 was not noted to be blaKPC positive until the patient was found to be colonized through perirectal screening (25) with a *K. pneumoniae* isolate with a weakly positive indirect carbapenemase test. This prompted further testing of the phenotypically carbapenem-susceptible *K. pneumoniae* urine isolate (CAV1217), which was then found to be blaKPC PCR positive. This isolate had no disruption in the ompK35 and ompK36 coding regions and had two copies of Tn4401 (one plasmid and one chromosomal) but with relatively low coverage.

*K. oxytoca* CAV1752, which had a single chromosomal copy of Tn4401b with low-level expression by quantitative PCR (qPCR), was detected on perirectal screening, again with conflicting phenotypic results (Table 2) (23). On automated susceptibility testing (VITEK 2), it was predicted to be susceptible to ceftriaxone and cefepime (MIC of $\leq 1$ μg/ml) and carbapenems, with an aztreonam MIC of 4 μg/ml. Analysis of porin genes demonstrated an insertion disrupting ompK35.

**Clinical course and epidemiology.** All six patients confirmed to have chromosomal integration of blaKPC had multiple risk factors and prolonged hospital exposure either within our health system or at outside hospitals prior to transfer (Table 3). Several patients with extensive outside health care exposure had isolates identified relatively early during hospitalization, raising the possibility of isolates circulating elsewhere in the region at outside hospitals. Interestingly, the patient with CAV1518 (the ST430 *K. pneumoniae* without chromosomal integration but with an ancestor common to CAV1217 and CAV1417) presented at an outside hospital prior to exposure to our health system, consistent with persistence of this lineage elsewhere in the region.

There were three patients who had infections (two with ventilator-associated pneumonia and one complicated urinary tract infection) with a *K. pneumoniae* isolate with chromosomal integration of blaKPC. The outcomes were complete microbiologic resolution and clinical cure at 30 days without any patients having known relapse within our health system.

The two patients with distinct strains of *K. oxytoca* (CAV1752 and CAV1755) did not demonstrate signs of infection with these isolates, thus the clinical consequences remain unclear. Interestingly, both patients had been colonized months before with other blaKPC-positive *Enterobacteriaceae*. As new acquisition at our institution is relatively rare and there were no other patients with similar isolates, this finding suggests separate within-patient horizontal transfer and chromosomal integration of Tn4401.
| Isolate | Source and date | Epidemiology (local and outside-hospital exposure) | Comorbidity(ies) | Treatment characteristic | Infection with KPC isolate with chromosomal integration | Treatment | 30-day outcome |
|---------|----------------|-----------------------------------------------------|-----------------|----------------------------|-----------------------------------------------|-----------|---------------|
| CAV1351 | Sputum, 2/2011 | Hospital day 5; extensive outside-hospital exposure | Persistent respiratory failure in setting of influenza pneumonia | Prior broad-spectrum intravenous antimicrobial in last 90 days Ventilator for >48 h (during that hospital stay) Indwelling central venous catheter Foley catheter In ICU for >48 h | Yes, VAP | Meropenem, tigecycline, amikacin | Microbiologic and clinical cure |
| CAV1453 | Perirectal, 10/2011 | Hospital day 3; extensive outside-hospital exposure | Cerebral palsy, chronic ventilator dependence | | No | NA | NA |
| CAV1217 | Urine, 8/2010 | Hospital day 3 (had similar K. pneumoniae in urine on admission which was not retained); extensive outside-hospital exposure | Diabetes mellitus, recurrent UTIs | | Yes, cUTI | Foley catheter exchange with meropenem for 72 h changed to tigecycline for lack of improvement (persistent symptoms and unchanged bacterial burden) | Microbiologic and clinical cure |
| CAV1417 | Urine, 5/2011 | Hospital day 36 | Chronic ventilator, neuromuscular weakness | | Yes, VAP | Meropenem and colistin | Microbiologic and clinical cure |
| CAV1752 | Perirectal, 12/2012 | Hospital day 100; first bla<sub>KPC</sub>-positive isolate was K. pneumoniae identified 3 mo earlier during prior hospitalization | Orthotopic liver transplant | | No | NA | NA |
| CAV1755 | Abdominal drainage, 12/2012 | Hospital day 26; first bla<sub>KPC</sub>-positive isolate was E. aerogenes, identified 10 mo earlier during prior hospitalization | End-stage renal disease, diabetes mellitus, complicated intra-abdominal infection | | No | NA | NA |

*NA, not applicable.
*cUTI, complicated urinary tract infection; VAP, ventilator-associated pneumonia.
DISCUSSION

We demonstrate, for the first time, multiple instances of chromosomal integration of Tn4401/bla\textsubscript{KPC} within a single-center outbreak. Although this is not the first description of chromosomal integration of Tn4401, the frequency of integration in a clinical setting is largely unknown and likely overlooked. With a focused effort on identifying all instances of chromosomal integration, this event does not appear to be rare. In addition, we found that phenotypic carbapenemase testing was variable in these contexts, and bla\textsubscript{KPC} could be missed by nonmolecular methods.

Illumina sequencing is increasingly used to undertake epidemiological investigation but is limited in investigating multicopy regions, which are of particular relevance to assessing resistance genes and mobile genetic elements. We utilized two bioinformatic approaches to characterize the genetic contexts of Tn4401. First, we used a de novo assembly approach, which identified three distinct chromosomal integrations. These were all verified by PacBio sequencing, demonstrating the robustness of this method. However, the method cannot identify chromosomal integration if multiple copies of Tn4401 are present or if Tn4401 is flanked by repetitive sequences, and only 44% of isolates were evaluable using this method. Second, we used a mapping approach which was able to assess all isolates but which identified several false positives. Therefore, results from the mapping approach on its own should be interpreted with caution, and a combination of methods may be most appropriate for other studies investigating sequence-based identification of resistance gene integration into the chromosome.

bla\textsubscript{KPC} chromosomal integration was overrepresented among CG258 strains in our outbreak, suggesting that it is more widespread in high-risk clones such as \textit{K. pneumoniae} ST258. This phenomenon has been seen in the high-risk \textit{Escherichia coli} ST-131 clone, with chromosomal integration of the CTX-M beta-lactamase gene followed by vertical transmission (26, 27). It remains unclear if there is a propensity for chromosomal integration among certain \textit{K. pneumoniae} strains or, more likely, whether integration is associated with the extended timespan over which these strains have been associated with Tn4401, representing greater opportunity for random integration.

Within \textit{K. pneumoniae} ST340, we identified three patients with putative chromosomal integrations of Tn4401, confirmed by PacBio sequencing in two isolates, CAV1217 and CAV1417. Comparison of the chromosomal region surrounding Tn4401 in CAV1417 to a related strain lacking Tn4401, combined with the analysis of TSD sequences, allowed us to reconstruct the history of transposition events. This revealed that chromosomal integration of Tn4401 occurred via IS\textsubscript{15}-ΔI-mediated transposition of a 16-kb region encompassing Tn4401. Further analysis of TSD sequences from Illumina data for CAV1518 revealed that the 16-kb region was not integrated into the chromosome in this isolate but rather shared flanking sequence with the plasmid copy from CAV1217. Taken together, this indicates that Tn4401 most likely was acquired once in this lineage, in a common ancestor of all three isolates, with chromosomal integration occurring after the divergence of CAV1518 (in the ancestor of CAV1217 and CAV1417) and subsequent loss of the plasmid copy in CAV1417 (Fig. 2).

Another important feature of this study was the variability in phenotypic carbapenem resistance. A prior evaluation of phenotypic carbapenemase testing in \textit{Enterobacteriaceae} from our institution (May 2010 to December 2011), using an ertapenem MIC of ≥1 μg/ml by VITEK 2 for inclusion, demonstrated that the indirect carbapenemase test had 90% sensitivity for detecting bla\textsubscript{KPC}-producing isolates (23). Isolates with chromosomally integrated bla\textsubscript{KPC} accounted for 3 of the 5 false-negative bla\textsubscript{KPC} \textit{Enterobacteriaceae} identified in that study (total of 56 isolates tested). β-Lactamase carriage on high-copy-number plasmids compared to low-copy-number plasmids is known to alter the degree of resistance seen phenotypically (22, 28). Here, we found preliminary evidence that a single chromosomal copy of Tn4401 was associated with a more subtle carbapenemase phenotype and lower expression of bla\textsubscript{KPC}. For example, CAV1752 would not have been detected with our current methods. This also could...
contribute to the misclassification of these isolates in a typical clinical microbiology laboratory, where the cost of molecular screening is often prohibitive (25). The differential impact on expression of resistance of \( \text{bla}_{\text{KPC}} \) located in the chromosome alone remains an interesting finding but will require additional in vitro studies to confirm this effect.

The impact of porin channel alterations may also affect the degree of in vitro resistance, highlighted here when comparing the related isolates CAV1417 and CAV1217. Although CAV1217 had two Tn4401 copies and CAV1417 a single copy, CAV1417 had disruptions in both porin channel genes and was more phenotypically resistant. Nevertheless, porin channel loss does not always result in high resistance (e.g., CAV1752 has a disruption in \( \text{ompK35} \) but remained carbapenem susceptible), and the prediction of phenotype from genotype in these cases is challenging.

Our small number of cases is insufficient to reliably assess the clinical impact of low MICs and subtle phenotypes associated with chromosomal integration. However, these cases are not notably distinct from other descriptions of infection with \( \text{bla}_{\text{KPC}} \)-positive \( K. \text{pneumoniae} \), with all infected patients ultimately responding to therapy (29). Interestingly, CAV1217 was phenotypically susceptible; however, despite 48 h of meropenem treatment with an initial Foley exchange, the patient had persistent symptoms and no change in bacterial burden but was ultimately treated successfully with tigecycline (Table 3).

This work also highlights that the frequency of chromosomal integration of genes of drug resistance is largely unknown and would be missed by most methods. Even with WGS, sophisticated focused analysis was required to identify chromosomal integration when a plasmid copy was also present. Currently, \( \text{bla}_{\text{KPC}} \) is carried within a replicative transposon, and although the in vitro transposition frequency of this element is relatively high, the real-world rate is unknown (8). Although the frequency of chromosomal integration cannot be fully assessed without additional study, this remains an intriguing preliminary finding from prospectively collected isolates in a clinical setting.

In summary, we present five independent examples of chromosomal integration of Tn4401-bla\(_{\text{KPC}}\) in \( K. \text{pneumoniae} \) isolates from six patients among a comprehensively characterized outbreak, indicating that chromosomal integration is not an infrequent event. We
also demonstrate that chromosomal integration often may be overlooked even with WGS, especially when multiple Tn4401 copies are present. In the clinical microbiology laboratory, phenotypic tests for carbapenem resistance/carbapenemase production were variable and could also miss these cases. Thus, chromosomal integration of \( \text{bla}_{KPC} \) was relatively common, was difficult to detect, and may facilitate stable inheritance of \( \text{bla}_{KPC} \), representing an evolutionary adaptation with potential implications for surveillance and treatment.

**MATERIALS AND METHODS**

Isolate collection, characterization, and selection for whole-genome sequencing. We previously described short-read (Illumina) sequencing of 281 \( \text{bla}_{KPC} \)-positive Enterobacteriaceae isolates, from 182 patients, collected between August 2007 and December 2012 in the University of Virginia Health System (18). These isolates comprised 62 distinct strains, defined on the basis of phylogenetic clustering, using cutoffs of 500 single-nucleotide variants (SNVs). Long-read (PacBio) sequencing was carried out on a random subset of 17 isolates (18). For the work presented here, an additional six isolates underwent long-read sequencing with PacBio technology as previously described (30).

Sequence analysis. We used three methods to identify chromosomally integrated Tn4401/\( \text{bla}_{KPC} \). First, we used a de novo assembly approach. Illumina reads from each isolate were assembled using Velvet and VelvetOptimiser (31). We then queried these with BLASTn, using 400 bp of sequence from each end of Tn4401 in order to identify contigs containing possible Tn4401 junction sequences. Isolates were evaluable if \( \geq 400 \) bp of Tn4401 sequence plus \( \geq 400 \) bp of flanking sequence were present on a single contig for at least one of the two Tn4401 ends. For evaluable isolates, Tn4401 flanking sequences were first compared to known plasmid flanking sequences from previously performed long-read sequencing of a subset of the 281 isolates (18). For isolates that did not show a match to any of these plasmid references, Tn4401 flanking sequences were used as BLASTn queries against NCBI’s nucleotide database, with homology to chromosomal sequences taken as evidence for a likely chromosomal location.

Second, we used a mapping approach. For each of the 281 isolates, Illumina reads were mapped to a reference consisting of a species-specific chromosome (Table 1) plus the \( \text{pKPC}_{\text{UVA01 Tn}} \)-contig (18). For five isolates, there was no species-specific reference available, and we used a closely related species instead (Table 1). Mapping was performed using bwa mem version 0.7.12-r1039 (32) with default parameters, and the output was filtered to remove supplementary alignments. For read pairs where one read mapped within 1 kb of either end of the Tn4401 sequence and the other read mapped to the chromosome, the corresponding position of mapping in the chromosome was extracted from the bam file. The length of the chromosome was divided into 1-kb nonoverlapping windows, and \( \geq 10 \) reads within a single window were taken as evidence for chromosomal integration of Tn4401.

Third, we used long-read PacBio sequencing (18, 30), fully resolving the genetic flanking structures around Tn4401, in order to validate the findings of putative chromosomal integration from the short-read analyses described above. In all cases where multiple isolates were identified from the same patient using the short-read approaches described above, these represented the same strain and chromosomal locus. Therefore, we performed long-read sequencing on the earliest isolate identified from each of the eight patients, with two exceptions (i.e., six isolates in total). We did not perform long-read sequencing on CAV1351, as a later isolate from the same patient (CAV1392) was previously PacBio sequenced as part of another study (18), or on CAV1518, as this isolate was demonstrated not to have a chromosomal integration of \( \text{bla}_{KPC} \) by further analysis of Illumina data (see Results).

For Tn4401 relative coverage estimation, we mapped reads to a two-contig reference (Tn4401 contig plus chromosome contig) as described above for the mapping approach. The average coverage of Tn4401 relative to the chromosome was calculated as (number of reads mapping to Tn4401/length of Tn4401 contig) \( \times \) (length of chromosome contig/number of reads mapping to chromosome).

For determination of 5-bp sequences flanking Tn4401 in ST340 isolates, reads were mapped to the Tn4401b-1 reference (18) using bwa mem as described above. For each read mapped to the start/end of Tn4401b-1 with \( \geq 5 \) bp flanking the start/end position, the 5-bp region immediately adjacent to the start/end position was extracted. Five-base-pair sequences with \( < 10 \) counts were excluded to account for sequencing errors. Determination of 8-bp sequences flanking the 16-kb composite region was performed similarly, except that the CAV1417 genome was used as a reference, with reads mapping to \( \geq 30 \) bp of the end of the 16-kb region within this extracted, and 8-bp flanking sequences were determined. Because the IS1531-\( \Delta I \) sequence exists as multiple copies in the ST340 isolates, it is not possible to determine flanking sequences using Illumina data for this end of the 16-kb region (Fig. 1, left side). Therefore, this analysis was only performed for the other (non-IS1531-\( \Delta I \)) end (Fig. 1, right side).

Analysis of porin genes was performed using tBLASTn comparisons against the de novo assembly of each patient’s earliest isolate, with translated \( \text{ompK35} \) and \( \text{ompK36} \) \( K. \text{pneumoniae} \) reference sequences (AJ011501 and JX291114, respectively) as queries. Reading frame disruptions were reported as likely loss-of-function mutations.

Expression of \( \text{bla}_{KPC} \). Total RNA was extracted using the RNeasy minikit (Qiagen, GmbH, Hilden, Germany) and underwent column DNase I digestion (Qiagen) as recommended by the manufacturer. cDNA was synthesized from RNA in accordance with the manufacturer’s instructions (qScript cDNA supermix; Quanta Biosciences, Gaithersburg, MD). Twenty nanograms of RNA equivalent DNA was used in triplicate. Quantitative reverse transcription-PCR (qRT-PCR) was performed using SsoFast EvaGreen...
supermix (Bio-Rad Laboratories, Hercules, CA), template DNA, and 500 nM each primer RT-KPC-F/RT-KPC-R, K. oxytoca rpoB-F/rpoB-R, and K. pneumoniae rpoB-F/rpoB-R. Relative quantification of gene expression was determined using the averaged cycle threshold (Ct) values for each isolate using the Pfaffl method (33). This equation uses an expression ratio to normalize the expression levels of bla<sub>KPC</sub> to the transcriptional level of the constitutively expressed rpoB gene.

**Clinical microbiology, Enterobacteriaceae** cultured from clinical (August 2007 to December 2012) and surveillance (starting in April 2009) specimens prospectively underwent carbapenemase phenotypic testing using the modified Hodge test (August 2007 to June 2008) or the indirect carbapenemase test (July 2008 to December 2012). These tests were repeated for all isolates from frozen subculture (23). The earliest available isolate with chromosomal integration from each patient underwent multiple modes of phenotypic testing, including the VITEK 2 system using a GN70 card (bioMérieux, Durham, NC), disc diffusion, Etest (bioMérieux, Durham, NC), and broth microdilution of meropenem to determine susceptibilities by following the CLSI’s and manufacturers’ guidelines (34).

**Clinical characteristics.** Electronic medical records were reviewed for treatment, clinical outcome, location and timing of transfer, and exposure to other patients with bla<sub>KPC</sub>-producing Enterobacteriaceae (approved by IRB 13558).

**Accession number(s).** Sequence data have been deposited in GenBank as follows: CAV1042, CP018671.1; CAV11392, CP011578.1; CAV1453, CP018356.1; CAV1217, CP018676.1; CAV1417, CP018352.1; CAV1752, CP018362.1; CAV1755, MRWY00000000.

**ACKNOWLEDGMENTS**

The research and AES were funded by the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Healthcare Associated Infections and Antimicrobial Resistance at Oxford University in partnership with Public Health England (PHE) (grant HPRU-2012-10041). D.W.C., T.E.A.P., and A.S.W. were supported by the National Institutes of Health Research Unit (NIHR) Oxford Biomedical Research Centre. T.E.A.P. and D.W.C. are NIHR Senior Investigators. The report presents independent research funded by the National Institute for Health Research. The views expressed in this publication are those of the authors and not necessarily those of the NHS, the National Institute for Health Research, the Department of Health, or Public Health England.

**REFERENCES**

1. Centers for Disease Control and Prevention. 2013. Antibiotic resistance threats in the United States. Centers for Disease Control and Prevention, Atlanta, GA.

2. Nordmann P, Naas T, Poirel L. 2011. Global spread of carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis 17:1791–1798. https://doi.org/10.3202/erid1710.110655.

3. Harrison E, Guymet D, Spies, AJ, Paterson S, Brockhurst MA. 2015. Parallel compensatory evolution stabilizes plasmids across the parasitism-mutualism continuum. Curr Biol 25:2034–2039. https://doi.org/10.1016/j.cub.2015.06.024.

4. Bergstrom CT, Lipsitch M, Levin BR. 2000. Natural selection, infectious transfer and the existence conditions for bacterial plasmids. Genetics 153:1505–1519.

5. Carraro N, Poulin D, Burrus V. 2015. Replication and active partition of integrative and conjugative elements (ICEs) of the SXT/R391 family: the line between ICEs and conjugative plasmids is getting thinner. PLoS Genet 11:e1005298. https://doi.org/10.1371/journal.pgen.1005298.

6. Bahl MI, Hansden LH, Sørensen SJ. 2009. Persistence mechanisms of conjugative plasmids. Methods Mol Biol 532:73–102. https://doi.org/10.1007/978-1-60327-853-9_5.

7. Naas T, Cuzon G, Villegas MV, Martígue MF, Quinn JP, Nordmann P. 2008. Genetic structures at the origin of acquisition of the beta-lactamase bla<sub>KPC</sub> gene. Antimicrob Agents Chemother 52:1257–1263. https://doi.org/10.1128/AAC.01451-07.

8. Cuzon G, Naas T, Nordmann P. 2011. Functional characterization of Tn<sub>4401</sub>, a Tn<sub>3</sub>-based transposon involved in<sub>rpoB</sub> gene mobilization. Antimicrob Agents Chemother 55:5370–5373. https://doi.org/10.1128/AAC.05202-11.

9. Mathers AJ, Peirano G, Pitout JD. 2015. The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant Enterobacteriaceae. Clin Microbiol Rev 28:565–591. https://doi.org/10.1128/CMR.00116-14.

10. Conlan S, Thomas PJ, Deming C, Park M, Lau AF, Dekker JP, Snitkin ES, Clark TA, Luong K, Song Y, Tsai YC, Boitano M, Dayal J, Brooks SY, Schmidt B, Young AC, Thomas JW, Bouffard GG, Blakesley RW, NISC Comparative Sequencing Program, Mullikin JC, Korlach J, Henderson DK, Frank KM, Palmore TN, Segre JA. 2014. Single-molecule sequencing to track plasmid diversity of hospital-associated carbapenemase-producing Enterobacteriaceae. Sci Transl Med 6:254ra126. https://doi.org/10.1126/scitranslmed.3009845.

11. Bowers JR, Kitchel B, Driebe EM, MacCannell DR, Roe C, Lemmer D, de Man T, Rasheed JK, Engelthaler DM, Keim P, Limbago BM. 2015. Genomic analysis of the emergence and rapid global dissemination of the clonal group 258 Klebsiella pneumoniae. PLoS One 10:e0133727. https://doi.org/10.1371/journal.pone.0133727.

12. Villegas MV, Lolans K, Correa A, Kattan JN, Lopez JA, Quinn JP, Colombiano Socio-Research Study Group. 2007. First identification of Pseudomonas aeruginosa isolates producing a KPC-type carbapenem-hydrolyzing beta-lactama. Antimicrob Agents Chemother 51:1553–1555. https://doi.org/10.1128/AAC.01405-06.

13. Castanheira M, Deshpande LM, DíPériso J, R., Kang J, Weinstein MP, Jones RN. 2009. First descriptions of bla<sub>KPC</sub> in Raoultella spp. (R. planticola and R. ornithinolytica): report from the SENTRY Antimicrobial Surveillance Program. J. Clin Microbiol 47:4129–4130. https://doi.org/10.1128/JCM.01502-09.

14. Martínez-T, Vázquez G, Aquino EE, Martinez J, Robledo IE. 2014. ISEcp1-mediated transposition of blakPC into the chromosome of a clinical isolate of Acinetobacter baumannii from Puerto Rico. J. Med Microbiol 63:1644–1648. https://doi.org/10.1099/jmm.0.080721-0.

15. Chen L, Chavda KD, DeLeo FR, Bryant KA, Jacobs MR, Bonomo RA, Kreiswirth BN. 2015. Genome sequence of a Klebsiella pneumoniae sequence type 258 isolate with plasmid-encoded K. pneumoniae carbapenemase. Genome Announc 3:e00569-15.

16. Conlan S, Deming C, Tsai YC, Lau AF, Dekker JP, Korlach J, Segre JA. 2014. Complete genome sequence of a Klebsiella pneumoniae isolate with chromosomally encoded carbapenem resistance and colibactin synthetase loci. Genome Announc 2:e01332-14.

17. Pecora ND, Li N, Allard M, Li C, Albano E, Delaney M, Dubois A, Onder-
donk AB, Bry L. 2015. Genomically informed surveillance for carbapenem-resistant Enterobacteriaceae in a health care system. mBio 6:e01030.

18. Sheppard AE, Stoesser N, Wilson DJ, Sebra R, Kasarskis A, Anson LW, Giess A, Pankhurst LJ, Vaughan A, Grim CJ, Cox HL, Yeh AJ, Modernising Medical Microbiology (MMM) Informatics Group, Sifri CD, Walker AS, Pankhurst LJ, Wyllie D, Crook DW, Mathers AJ. 2016. Nested Russian doll-like genetic mobility drives rapid dissemination of the carbapenem resistance gene blaKPC. Antimicrob Agents Chemother 60:3767–3778. https://doi.org/10.1128/AAC.00464-16.

19. Trieu-Cuot P, Courvalin P. 1984. Nucleotide sequence of the transposable element IS15. Gene 30:113–120. https://doi.org/10.1016/0378-1119(84)90111-2.

20. He S, Hickman AB, Varani AM, Siguiere P, Chandler M, Dekker JP, Dyda F. 2015. Insertion sequence IS26 reorganizes plasmids in clinically isolated multidrug-resistant bacteria by replicative transposition. mBio 6:e00762.

21. Naas T, Cuzon G, Truong HV, Nordmann P. 2012. Role of ISKpn7 and deletions in blaKPC gene expression. Antimicrob Agents Chemother 56:4753–4759. https://doi.org/10.1128/AAC.00334-12.

22. Kitchel B, Rasheed JK, Endimiani A, Hujer AM, Anderson KF, Bonomo RA, Patel JB. 2010. Genetic factors associated with elevated carbapenem resistance in KPC-producing Klebsiella pneumoniae. Antimicrob Agents Chemother 54:4201–4207. https://doi.org/10.1128/AAC.00008-10.

23. Mathers AJ, Carroll J, Sifri CD, Hazen KC. 2013. Modified Hodge test versus indirect carbapenemase test: prospective evaluation of a phenotypic assay for detection of Klebsiella pneumoniae carbapenemase (KPC) in Enterobacteriaceae. J Clin Microbiol 51:1291–1293. https://doi.org/10.1128/JCM.03240-12.

24. Netikul T, Kiratisin P. 2015. Genotypic characterization of carbapenem-resistant enterobacteriaceae and the spread of carbapenem-resistant Klebsiella pneumonia ST340 at a university hospital in Thailand. PLoS One 10:e0139116. https://doi.org/10.1371/journal.pone.0139116.

25. Mathers AJ, Poultet M, Dirks D, Carroll J, Sifri CD, Hazen KC. 2014. Clinical microbiology costs for methods of active surveillance for Klebsiella pneumoniae carbapenemase-producing Enterobacteriaceae. Infect Control Hosp Epidemiol 35:350–355. https://doi.org/10.1086/675603.

26. Stoesser N, Sheppard AE, Pankhurst L, De Maio N, Moore CE, Sebra R, Turner P, Anson LW, Kasarskis A, Batty EM, Kos V, Wilson DJ, Phetsouvanh R, Wylie D, Sokurenko E, Manges AR, Johnson TJ, Price LB, Peto TE, Johnson JR, Didelot X, Walker AS, Crook DW, Modernising Medical Microbiology (MMM) Informatics Group. 2016. Evolutionary history of the global emergence of the Escherichia coli epidemic clone ST131. mBio 7:e02162-15.

27. Cercueti M, Guflé M, Garcia-Fernández A, Accogli M, Fortini D, Luzzi I, Carattoli A. 2010. Ciprofloxacin-resistant, CTX-M-15-producing Escherichia coli ST131 clone in extraintestinal infections in Italy. Clin Microbiol Infect 16:1555–1558. https://doi.org/10.1111/j.1469-0691.2010.03162.x.

28. Wu PJ, Shannon K, Phillips I. 1995. Mechanisms of hyperproduction of TEM-1 beta-lactamase by clinical isolates of Escherichia coli. J Antimicrob Chemother 36:927–939. https://doi.org/10.1093/jac/36.6.927.

29. Tzouvelekis LS, Markogiannakis A, Psychogiou M, Tassios PT, Daikos GL. 2012. Carbapenemases in Klebsiella pneumoniae and other Enterobacteriaceae: an evolving crisis of global dimensions. Clin Microbiol Rev 25:682–707. https://doi.org/10.1128/CMR.05035-11.

30. Sheppard AE, Stoesser N, Sebra R, Kasarskis A, Deikus G, Anson L, Walker AS, Peto TE, Crook DW, Mathers AJ. 2016. Complete genome sequence of KPC-Producing Klebsiella pneumoniae strain CAV1193. Genome Announc 4:e01649-15.

31. Stoesser N, Batty EM, Eyre DW, Morgan M, Wylie DH, Del Ojo Elias C, Johnson JR, Walker AS, Peto TE, Crook DW, Mathers AJ. 2013. Predicting antimicrobial susceptibilities for Escherichia coli and Klebsiella pneumoniae isolates using whole genomic sequence data. J Antimicrob Chemother 68:2234–2244.

32. Li H. 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv:1303.3997v1301 [q-bio.GN].

33. Cikos S, Bukovská A, Koppel J. 2007. Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. BMC Mol Biol 8:113. https://doi.org/10.1186/1471-2199-8-113.

34. Clinical Laboratory and Standards Institute. 2015. Performance standards for antimicrobial susceptibility testing M100-S25 238. Clinical Laboratory and Standards Institute, Wayne, PA.