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A Prolonged Outbreak of KPC-3-Producing *Enterobacter cloacae* and *Klebsiella pneumoniae* Driven by Multiple Mechanisms of Resistance Transmission at a Large Academic Burn Center

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ABSTRACT *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Enterobacter cloacae* has been recently recognized in the United States. Whole-genome sequencing (WGS) has become a useful tool for analysis of outbreaks and for determining transmission networks of multidrug-resistant organisms in health care settings, including carbapenem-resistant *Enterobacteriaceae* (CRE). We experienced a prolonged outbreak of CRE *E. cloacae* and *K. pneumoniae* over a 3-year period at a large academic burn center despite rigorous infection control measures. To understand the molecular mechanisms that sustained this outbreak, we investigated the CRE outbreak isolates by using WGS. Twenty-two clinical isolates of CRE, including *E. cloacae* ($n = 15$) and *K. pneumoniae* ($n = 7$), were sequenced and analyzed genetically. WGS revealed that this outbreak, which seemed epidemiologically unlinked, was in fact genetically linked over a prolonged period. Multiple mechanisms were found to account for the ongoing outbreak of KPC-3-producing *E. cloacae* and *K. pneumoniae*. This outbreak was primarily maintained by a clonal expansion of *E. cloacae* sequence type 114 (ST114) with distribution of multiple resistance determinants. Plasmid and transposon analyses suggested that the majority of bla_{KPC-3} was transmitted via an identical Tn*4401*b element on part of a common plasmid. WGS analysis demonstrated complex transmission dynamics within the burn center at levels of the strain and/or plasmid in association with a transposon, highlighting the versatility of KPC-producing *Enterobacteriaceae* in their ability to utilize multiple modes to resistance gene propagation.

KEYWORDS carbapenem-resistant *Enterobacteriaceae* (CRE), *Klebsiella pneumoniae* carbapenemase (KPC), outbreak, health care-associated infection, whole-genome sequencing, burn patients

Received 12 July 2016 **Returned for modification** 13 August 2016 **Accepted** 25 November 2016

Accepted manuscript posted online 5 December 2016

Citation Kanamori H, Parobek CM, Juliano JJ, van Duin D, Cairns BA, Weber DJ, Rutala WA. 2017. A prolonged outbreak of KPC-3-producing *Enterobacter cloacae* and *Klebsiella pneumoniae* driven by multiple mechanisms of resistance transmission at a large academic burn center. Antimicrob Agents Chemother 61:e01516-16. https://doi.org/10.1128/AAC.01516-16.

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Carbapenem-resistant *Enterobacteriaceae* (CRE) and more specifically carbapenemase-producing *Enterobacteriaceae* (CPE) are a major global health concern (1, 2). Infections with these pathogens have been associated with substantial mortality and are often difficult to treat (3, 4). CPE infections are frequently caused by *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* in the United States and are associated with the spread of *K. pneumoniae* sequence type 258 (ST258) (5, 6). Of particular concern, the bla_{KPC} genes responsible for this resistance are transmissible to other *Enterobacteriaceae* via plasmids in association with the mobile transposon Tn*4401* (6) and are now circulating in *Enterobacter cloacae* in the United States (e.g., Pennsylvania, North Dakota, and the upper

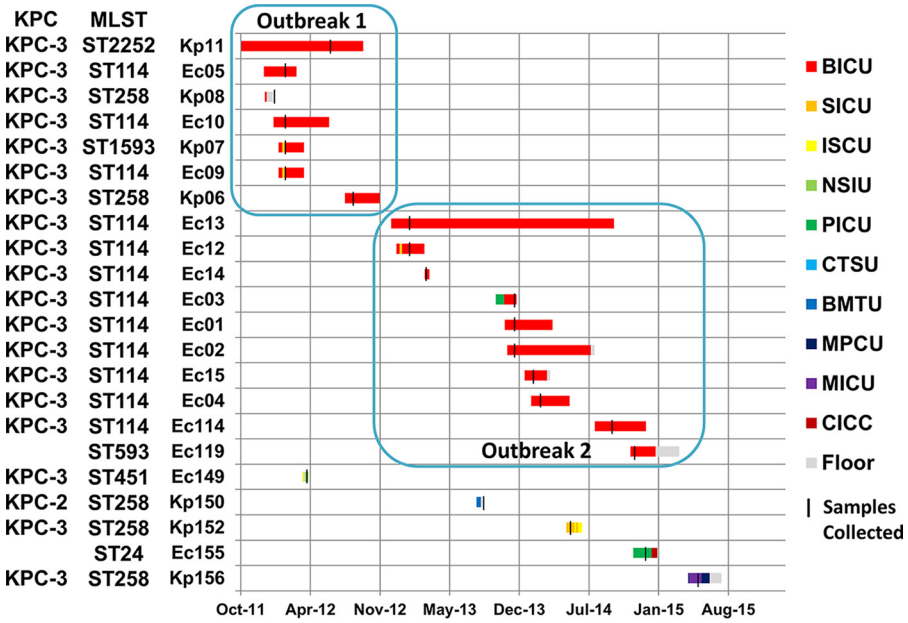

FIG 1 Timeline and location of prolonged transmission caused by carbapenem-resistant *Enterobacteriaceae*. Ec, *E. cloacae*; Kp, *K. pneumoniae*; BICU, burn intensive care unit; ISCU, intermediate surgical care unit; MICU, medicine intensive care unit; NSIU, neuroscience intensive care unit; SICU, surgery intensive care unit; PICU, pediatric intensive care unit; CTSU, cardiothoracic stepdown unit; MPCU, medicine progressive care unit; floor, general ward. Each strain ID corresponds to a case summarized in Table 1.

midwestern United States) (7–9). Despite this, KPC-producing *E. cloacae* infections have been relatively infrequent (10). A 3-year outbreak of KPC-producing *E. cloacae* and *K. pneumoniae* occurred at a large academic burn center in North Carolina. Here, we report the results of a genomic investigation into this outbreak.

Whole-genome sequencing (WGS) has become a useful tool for analysis of outbreaks and transmission networks of multidrug-resistant organisms, including CRE, in health care settings, enabling us to more comprehensively describe resistance genes and more accurately determine genotypes among epidemiologically linked strains (11–13). In contrast to multidrug-resistant *Acinetobacter baumannii* or *Pseudomonas aeruginosa* outbreaks, reports of *Enterobacter cloacae* or CRE/CPE hospital outbreaks in this particularly vulnerable population are limited (11, 14–19). In addition, there are no reports of KPC-producing *E. cloacae* outbreaks among burn patients. In this study, we used WGS to explore details of this sequential CRE outbreak, including KPC-producing *E. cloacae* and *K. pneumoniae*. We assessed the genetic relatedness of CRE strains and uncovered complex and multimodal transmission dynamics of bla_{KPC} in this prolonged hospital outbreak in the burn center.(This work was presented in part at the Society for Healthcare Epidemiology of America [SHEA] Spring 2016 Conference, Atlanta, GA [20].)

RESULTS

The timeline and location of this prolonged CRE outbreak are provided in Fig. 1. On the basis of epidemiological data, the first outbreak of CRE occurred in the burn center from January through November 2012. The index patient (defined as a patient at the earliest admission date in this CRE outbreak) had Kp11, a KPC-3-producing *K. pneumoniae* isolated from a urine culture in June 2012. After this index case, 5 additional patients with KPC-3-producing *Enterobacteriaceae* were identified: 2 patients with *K. pneumoniae*, 2 patients with *E. cloacae*, and a single patient with *K. pneumoniae* and *E. cloacae* (strain identification [ID] numbers Kp07 and Ec09). On the basis of hospital infection control surveillance, this outbreak seemed to end in November 2012, upon discharge of the last patient (Kp06). However, a second outbreak occurred in the burn center from January 2013 through March 2015. Ec13, a KPC-producing *E. cloacae* isolate, chronologically became the index case of the second outbreak. Nine patients

TABLE 1 Clinical features of patients with carbapenem-resistant *Enterobacteriaceae* infection or colonization during a prolonged outbreak

Strain ID	Organism	Specimen source[a]	Collection date	Accession no.	Age range (yrs)	Sex[b]	Underlying disease[c]	HAI[d]	Type of HAI[e]	Outcome
Ec01	*E. cloacae*	BAL fluid	2013 Nov	DRX055644	70–79	M	SJS	Yes	VAP	Died
Ec02	*E. cloacae*	Tracheal aspirate	2013 Nov	DRX055645	30–39	M	55% TBSA burn	Yes	LRI	Survived
Ec03	*E. cloacae*	Blood	2013 Nov	DRX055646	20–29	F	Desquamating rash complicated by multisystem organ failure	Yes	CLABSI	Survived
Ec04	*E. cloacae*	BAL fluid	2014 Feb	DRX055647	60–69	F	20–29% TBSA burn	Yes	LRI	Survived
Ec05	E. cloacae	BAL fluid	2012 Feb	DRX055648	70–79	F	Inhalational injury and 20% TBSA burn	Yes	LRI	Died
Kp06	*K. pneumoniae*	BAL fluid	2012 Aug	DRX055649	40–49	M	SJS/TENS	Yes	VAP	Survived
Kp07	*K. pneumoniae*	BAL fluid	2012 Feb	DRX055650	20–29	M	Inhalational burn injury	Yes	VAP	Survived
Kp08	*K. pneumoniae*	Urine	2012 Jan	DRX055651	60–69	M	Grease burn	No		Survived
Ec09	*E. cloacae*	Blood	2012 Feb	DRX055652	20–29	M	Inhalational burn injury	Yes	VAP	Survived
Ec10	*E. cloacae*	Blood	2012 Feb	DRX055653	30–39	F	30% flame burn	Yes	VAP	Survived
Kp11	*K. pneumoniae*	Rectal	2012 Jun	DRX055654	40–49	F	30% flame burn	No		Survived
Ec12	*E. cloacae*	Rectal	2013 Jan	DRX055655	60–69	M	>50% TBSA burn	No		Died
Ec13	*E. cloacae*	Wound surface	2013 Jan	DRX055656	30–39	M	Severe multitrauma	Yes	CAUTI	Survived
Ec14	*E. cloacae*	Rectal	2013 Mar	DRX055657	80–89	F	7% TBSA chemical burn	No		Survived
Ec15	*E. cloacae*	BAL fluid	2014 Jan	DRX055658	40–49	M	9% TBSA flame burn	Yes	LRI	Survived
Ec114	*E. cloacae*	Urine	2014 Sept	DRX055660	20–29	M	64%TBSA flame burn	Yes	UTI	Died
Ec119	*E. cloacae*	Lower respiratory tract	2014 Nov	DRX055661	0–9	M	50% TBSA flame burn	No		Survived
Ec149	*E. cloacae*	Blood	2012 Apr	DRX055666	50–59	F	Metastatic colon cancer, septic shock	Yes	BSI	Died
Kp150	*K. pneumoniae*	Tracheal aspirate	2013 Sept	DRX055667	40–49	F	Relapsed AML, septic shock	Yes	Pneumonia	Died
Kp152	*K. pneumoniae*	Urine	2014 May	DRX055669	60–69	M	Multiple injury by motor vehicle accident	Yes	UTI	Survived
Ec155	*E. cloacae*	Nasopharynx	2014 Dec	DRX055672	0–9	M	Congenital heart disease	Yes	LRI	Survived
Kp156	*K. pneumoniae*	Tracheal aspirate	2015 May	DRX055673	20–29	F	MRSA bacteremia from i.v. drug use, endocarditis, necrotizing pneumonia	Yes	Pneumonia	Survived

[a]BAL, bronchoalveolar lavage.
[b]M, male; F, female.
[c]AML, acute myeloid leukemia; SJS, Stevens-Johnson syndrome; TBSA, total body surface area; TENS, toxic epidermal necrolysis; MRSA, methicillin-resistant *Staphylococcus aureus* infection; i.v., intravenous.
[d]HAI, health care-associated infection.
[e]BSI, bloodstream infection; CAUTI, catheter-associated urinary tract infection; CLABSI, central line-associated bloodstream infection; LRI, lower respiratory tract infection; SSI, surgical site infection; UTI, urinary tract infection; VAE, ventilator-associated event; VAP, ventilator-associated pneumonia.

with *E. cloacae* were subsequently identified, but there were no patients with *K. pneumoniae*. Our infection control plan included strict adherence to hospital policy, meticulous hand hygiene by health care personnel, contact precautions for patients infected or colonized with CRE, enhanced environmental cleaning, decontamination of equipment, and enhanced precautions in an outbreak setting (e.g., educating and cohorting staff and patients, implementing surveillance cultures, monitoring and reinforcing interventions frequently). In this outbreak, multiple *K. pneumoniae* and *E. cloacae* isolates were PCR positive for bla_{KPC}. Three (Ec05, Ec09, and Ec10) and four (Ec1, Ec2, Ec3, and Ec13) *E. cloacae* isolates were identical based on pulsed-field gel electrophoresis.

Clinical features of patients with CRE infection or colonization are summarized in Table 1. Briefly, 17 patients (81%) were diagnosed with a health care-associated infection (HAI), including respiratory tract infections ($n = 12$), urinary tract infections ($n = 3$), and bloodstream infections ($n = 2$). Underlying diseases of the CRE cases included burns ($n = 12$, 57%), Stevens-Johnson syndrome or toxic epidermal necrolysis ($n = 2$), and other diseases ($n = 7$). Six patients (29%), including 3 burn cases (25%), died during hospitalization despite appropriate treatment.

WGS of 22 CRE isolates produced an average of 1,873,130 read pairs per isolate for *K. pneumoniae* and 2,073,037 read pairs per isolate for *E. cloacae*. Results of metagenomic sequence classification matched precisely with the results of conventional species identification techniques. After reference-guided mapping of *K. pneumoniae* isolates, all samples had at least 5× coverage at 90% of the genome or greater, with the majority of the genome covered to at least 25× coverage (see Fig. S1 in the supplemental material). For *E. cloacae* isolates, all samples had 5× coverage at nearly

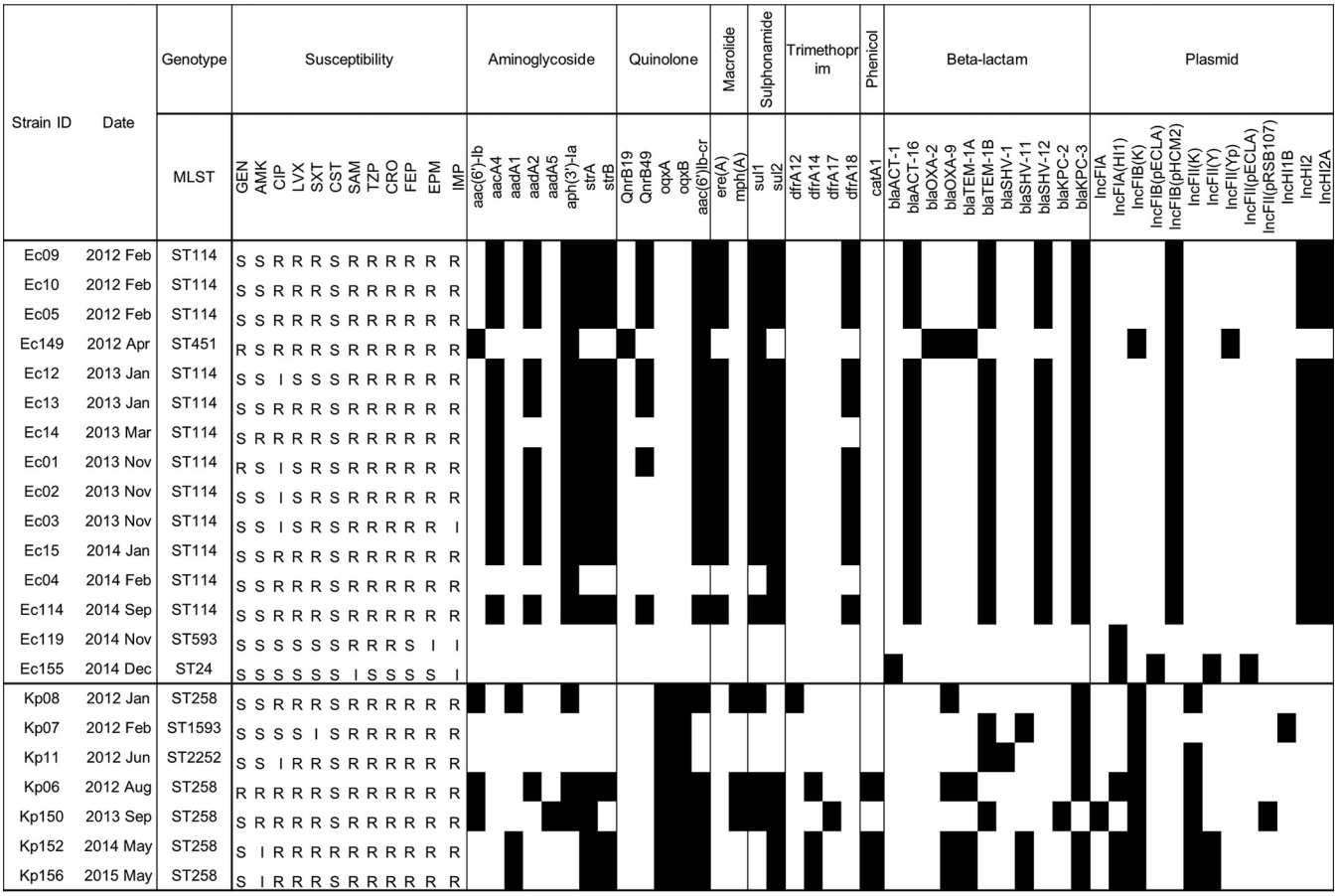

FIG 2 Characterization of acquired resistance genes and genotypes among carbapenem-resistant *Enterobacteriaceae* strains. Antimicrobial susceptibility profiles: S, susceptible; I, intermediate; R, resistant. Presence (black) or absence (white) of resistance genes and plasmids for each isolate is also shown. SAM, ampicillin-sulbactam; TZP, piperacillin-tazobactam; CRO, ceftriaxone; FEP, cefepime; IMP, imipenem; EPM, ertapenem; GEN, gentamicin; AMK, amikacin; CIP, ciprofloxacin; LVX, levofloxacin; SXT, trimethoprim-sulfamethoxazole; CST, colistin.

80% of the genome, and most had 50× coverage at more than 50% of the genome (Fig. S1). In many genomic locations, and particularly in plasmid contig assemblies, we achieved coverage depths well over 200. Thus, the vast majority of both the *K. pneumoniae* and *E. cloacae* genome assemblies were suitable for variant calling.

Antimicrobial susceptibility profiles and acquired resistance genes are shown in Fig. 2. All isolates were nonsusceptible to imipenem, but one was susceptible to ertapenem (Ec155, a non-KPC producer). Most were nonsusceptible to fluoroquinolones but were susceptible to aminoglycosides. All except one (Kp152) were susceptible to colistin, and none of them possessed *mcr-1* and *mcr-2*. We detected the following carbapenemase genes in our CRE isolates: 13 (86.7%) *E. cloacae* strains were positive for bla_{KPC-3} and 6 (85.7%) *K. pneumoniae* strains were positive for bla_{KPC-3}, as well as 1 (14.3%) *K. pneumoniae* strain positive for bla_{KPC-2}. WGS analysis suggested distribution and intra- and interspecies dissemination of multiple resistance genes and plasmids in this prolonged outbreak, as well as potential association of bla_{KPC} transmission with other β-lactamase genes and different classes of antimicrobial resistance (Fig. 2).

It is known that β-lactamase genes, including bla_{KPC}, can spread through multiple mechanisms, including clonal strain expansion, plasmid transfer, and/or mobile genetic elements, such as transposons (6). First, we explored the strain-level similarity of isolates from this CRE outbreak. Overall, CRE isolates collected within the burn unit were genetically quite distinct from samples collected outside the burn unit on the basis of *in silico* multilocus sequence typing (MLST) and chromosomal single nucleotide variant (SNV) differences (Fig. 3). Ec119 and Kp11 represented strains novel to this study and

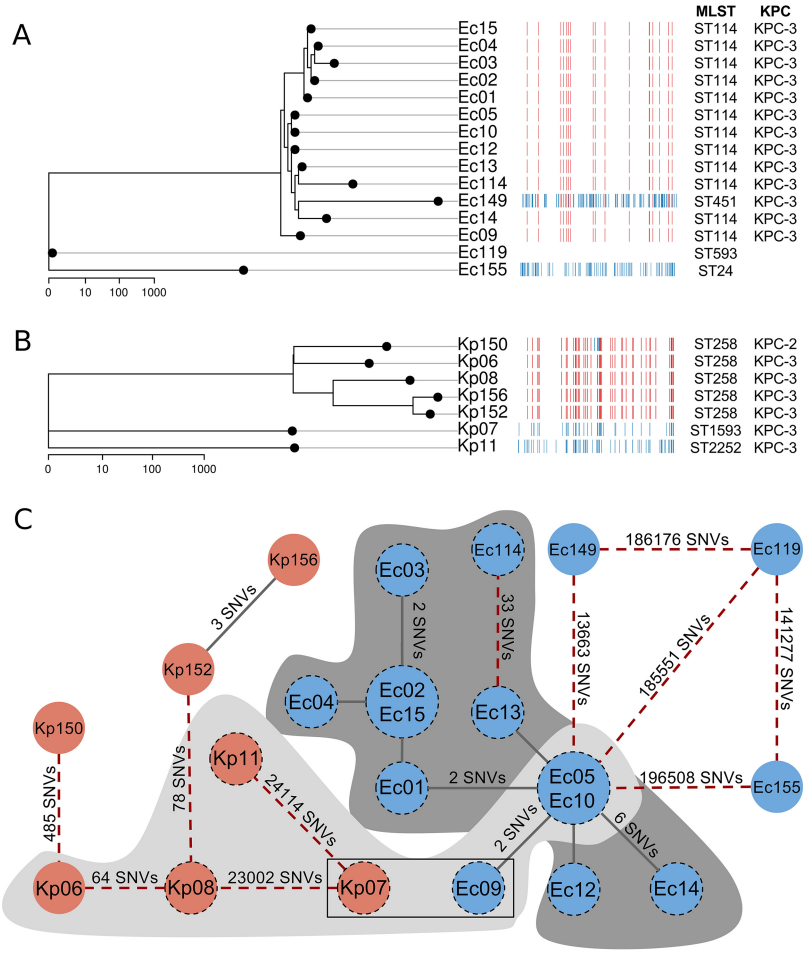

FIG 3 (A and B) Maximum-likelihood (ML) phylogram and recombination map showing the relatedness between carbapenem-resistant *Enterobacteriaceae* isolates. The ML phylogeny was constructed based on genetic variation in nonrecombinant genomic regions. In the horizontal tracks, each column represents a single nucleotide in the reference genome and each row represents an individual clinical isolate. Red blocks mark recombination events occurring on an internal branch of the phylogeny, while blue blocks mark potential recombination events or extensive mutation occurring on a terminal node. The *E. cloacae* phylogram was rooted to the most genetically distinct isolate in our sample set (Ec155). The *K. pneumoniae* phylogram was similarly rooted (using Kp11). Phylogenies are drawn on a log scale, and the scale bar below each image represents the phylogenetic distance (in point mutations). (C) Variant difference network, demonstrating Tn*4401*b-bla_{KPC-3} transmission between species and between chromosomally divergent strains. Nodes represent CRE clinical isolates of *E. cloacae* (blue) and *K. pneumoniae* (red) from the burn unit outbreak. Small nodes represent single isolates, while large nodes represent two identical isolates. The two nodes within a rectangular outline represent two samples, Kp07 and Ec09, which were isolated from a single patient. All nodes with a dashed black perimeter represent samples bearing the common Tn*4401*b genetic element. Isolates without a dashed perimeter either had no Tn*4401* element or had a non-b subtype (i.e., a or d). Lines (edges) indicate the extent of chromosomal difference between isolates. Solid lines bridging nodes indicate membership in a clonal group (defined as consecutive isolates with ≤12 variant differences; Ec_UNC for *E. cloacae* and Kp_UNC for *K. pneumoniae*), while red dashed lines connect less-related isolates. Lines are labeled with the number of SNVs that separate the clinical isolates if there are more than 2 SNVs. Line lengths are not proportional to genetic distance. Isolates depicted above a light gray matte and a dark gray matte were collected in outbreak 1 and outbreak 2 within the BICU, respectively, while isolates outside the mattes were collected outside the unit and are putatively unrelated to the outbreak.

were assigned the identifiers ST593 and ST2252, respectively. To resolve whether SNV differences within these outbreak strain types were due to recombination events or to point mutations, we constructed recombination-aware maximum-likelihood phylograms for *E. cloacae* and *K. pneumoniae* (Fig. 3A and B). Upon analysis of core genome variants, the 0-33 SNVs observed in pairwise comparisons among *E. cloacae* ST114 isolates from the burn unit were due to point mutations, while the 3-485 SNVs

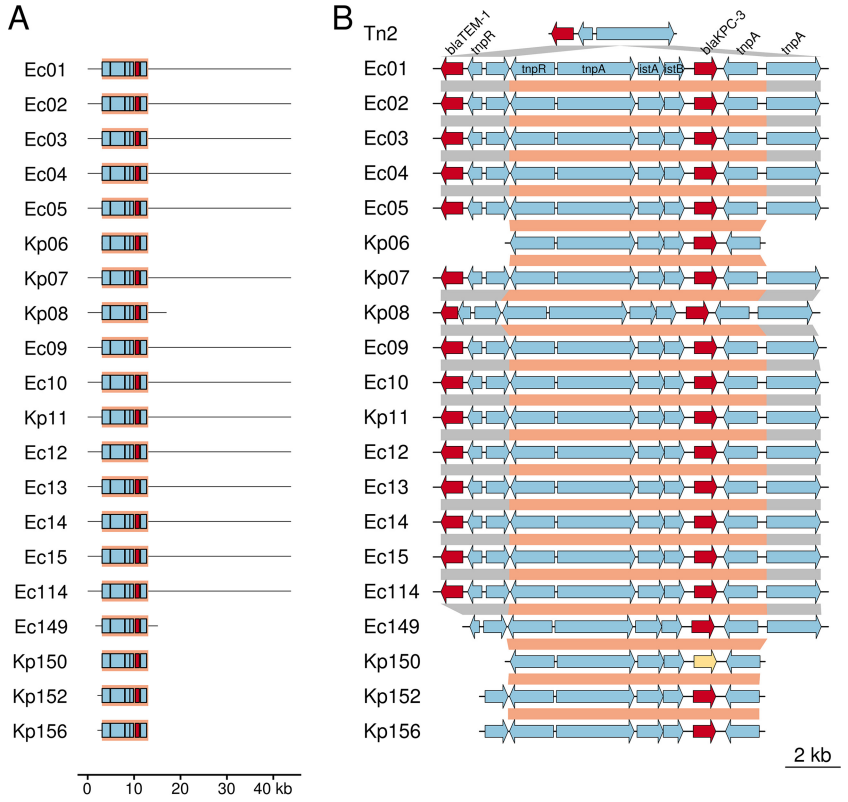

FIG 4 Plasmid and transposon maps revealing significant gene-sharing among carbapenem-resistant *Enterobacteriaceae* outbreak isolates within the burn unit. (A) The bla_{KPC-3}-containing contig from *de novo* read assemblies are depicted. In every case, bla_{KPC-3} was identified nested within a Tn*4401* element, the boundaries of which are shown with a salmon background. Tn*4401* accessory genes are shown in blue, and bla_{KPC-3} is highlighted in red. For each isolate, only the plasmid region homologous to the primary outbreak plasmid identified in most Ec and two Kp isolates are shown. (B) Structure of the Tn*4401*-bla_{KPC-3} composite genetic element as identified in each outbreak isolate. Tn*4401* boundaries are marked in salmon, and Tn*2*-like element boundaries are marked in gray. Accessory genes are colored blue, while the β-lactamase genes bla_{TEM-1} and bla_{KPC-3} are highlighted in red. The single isolate bearing bla_{KPC-2} (Kp150) is marked in yellow. A generic Tn*2*-like element is shown on the top track, and isolate-specific tracks show insertion points of Tn*4401* elements into the Tn*2*-like element.

identified among *K. pneumoniae* ST258 isolates were due to both mutation and recombination events. SNV strain typing revealed that these outbreaks, which appeared distinct based on epidemiologic data, were in fact one prolonged outbreak. The first epidemiological CRE outbreak was caused by a clone (Ec_UNC) of *E. cloacae* (Ec05, Ec09, and Ec10, all within two SNV differences) and by four distinct strains of *K. pneumoniae* (Kp06, Kp07, Kp08, and Kp11). The second epidemiological CRE outbreak was driven by a clone (Ec_UNC, identical to the first outbreak clone) of *E. cloacae* (Ec01, Ec02, Ec03, Ec04, Ec12, Ec13, Ec14, and Ec15, all within 11 SNV differences) as well as by two distinct strains of *E. cloacae* (Ec114 and Ec119). Ec114 differed from Ec13 (Ec_UNC) by 33 SNVs only. All CRE isolates outside the burn center were genetically distinct from strains inside the burn center (Fig. 3C). Of 11 patients who had the Ec_UNC clone isolates, 9 (81.8%) had an HAI and 3 (27.3%) died. Two patients who had isolates of the clone Kp_UNC had an HAI and survived.

We next evaluated the genetic similarity of these isolates at the plasmid level. We observed high sequence homology between 3 of the 35 publicly available KPC-3-containing RefSeq plasmids (NZ_CP011575.1, NZ_CP010387.1, and NZ_CP008826.1) and the KPC-3-containing contigs from *de novo* assemblies of the 12 *E. cloacae* isolates from the burn unit as well as two *K. pneumoniae* strains (Kp07 and Kp11), suggesting that a highly similar bla_{KPC-3}-containing entire or partial plasmid (pKPC-3_UNC, ~45,000 bp) was shared (Fig. 4A; Fig. S2A). Both Kp07 and Ec09 from a single patient also shared

TABLE 2 Summary of the genetic context for bla_{KPC} among KPC-producing *Enterobacter cloacae* and *Klebsiella pneumoniae* isolates during a prolonged outbreak

Strain ID	Organism	KPC	MLST	Strain relatedness	Plasmid variant[a]	Tn*4401* variant[b]	Flanking sequences[c]	Composite Tn*4401*/Tn2-like structure
Ec01	*E. cloacae*	bla_{KPC-3}	ST114	Ec_UNC	pKPC-3_UNC	Tn*4401*b	GTTCT/GTTCT	Complete, identical
Ec02	*E. cloacae*	bla_{KPC-3}	ST114	Ec_UNC	pKPC-3_UNC	Tn*4401*b	GTTCT/GTTCT	Complete, identical
Ec03	*E. cloacae*	bla_{KPC-3}	ST114	Ec_UNC	pKPC-3_UNC	Tn*4401*b	GTTCT/GTTCT	Complete, identical
Ec04	*E. cloacae*	bla_{KPC-3}	ST114	Ec_UNC	pKPC-3_UNC	Tn*4401*b	GTTCT/GTTCT	Complete, identical
Ec05	*E. cloacae*	bla_{KPC-3}	ST114	Ec_UNC	pKPC-3_UNC	Tn*4401*b	GTTCT/GTTCT	Complete, identical
Kp06	*K. pneumoniae*	bla_{KPC-3}	ST258			Tn*4401*d	GTTCT/TCTCT	
Kp07	*K. pneumoniae*	bla_{KPC-3}	ST1593		pKPC-3_UNC	Tn*4401*b	GTTCT/GTTCT	Complete, identical
Kp08	*K. pneumoniae*	bla_{KPC-3}	ST258			Tn*4401*b**	GTTCT/GTTCT	Partial, identical
Ec09	*E. cloacae*	bla_{KPC-3}	ST114	Ec_UNC	pKPC-3_UNC	Tn*4401*b	GTTCT/GTTCT	Complete, identical
Ec10	*E. cloacae*	bla_{KPC-3}	ST114	Ec_UNC	pKPC-3_UNC	Tn*4401*b	GTTCT/GTTCT	Complete, identical
Kp11	*K. pneumoniae*	bla_{KPC-3}	ST2252		pKPC-3_UNC	Tn*4401*b	GTTCT/GTTCT	Complete, identical
Ec12	*E. cloacae*	bla_{KPC-3}	ST114	Ec_UNC	pKPC-3_UNC	Tn*4401*b	GTTCT/GTTCT	Complete, identical
Ec13	*E. cloacae*	bla_{KPC-3}	ST114	Ec_UNC	pKPC-3_UNC	Tn*4401*b	GTTCT/GTTCT	Complete, identical
Ec14	*E. cloacae*	bla_{KPC-3}	ST114	Ec_UNC	pKPC-3_UNC	Tn*4401*b	GTTCT/GTTCT	Complete, identical
Ec15	*E. cloacae*	bla_{KPC-3}	ST114	Ec_UNC	pKPC-3_UNC	Tn*4401*b	GTTCT/GTTCT	Complete, identical
Ec114	*E. cloacae*	bla_{KPC-3}	ST114		pKPC-3_UNC*	Tn*4401*b	GTTCT/GTTCT	Complete, identical
Ec149	*E. cloacae*	bla_{KPC-3}	ST451			Tn*4401* novel***	GTTCT/GTTCT	
Kp150	*K. pneumoniae*	bla_{KPC-2}	ST258			Tn*4401*a	ATTGA/ATTGA	
Kp152	*K. pneumoniae*	bla_{KPC-3}	ST258	Kp_UNC		Tn*4401*d	GTTCT/TCTCT	
Kp156	*K. pneumoniae*	bla_{KPC-3}	ST258	Kp_UNC		Tn*4401*d	GTTCT/TCTCT	

[a]Isolates with shared plasmid variants indicate possible plasmid-mediated bla_{KPC-3} transmission among isolates (pKPC-3_UNC). *, Ec114 differed from others by 1 SNV.
[b]All isolates carried Tn*4401*b (except for one, marked with two asterisks, isolate Kp08 with 1 SNV) and are identical, and all isolates carrying Tn*4401*d are identical. "Tn*4401* novel" (indicated with three asterisks, isolate Ec149) is an isoform of Tn*4401* with a 91-bp deletion downstream of bla_{KPC}.
[c]Flanking sequences of Tn*4401* are shown as 5-bp target site duplications in transposition. There was no evidence of target site duplication for Tn*4401*d.

this plasmid, which we termed pKPC-3_UNC, suggesting interspecies plasmid transmission. The quality of these *de novo*-assembled KPC-bearing plasmids was further validated by assembly against plasmid reference sequence NZ_CP008826 as well as reference-guided mapping of each isolate's raw reads to its own *de novo*-assembled KPC-containing contigs (Fig. S2B) (see Materials and Methods).

Finally, we considered the immediate genetic context for bla_{KPC} among KPC-producing *E. cloacae* and *K. pneumoniae* isolates during the prolonged outbreak. Findings are summarized in Table 2. On the basis of *de novo* sequence assembly, bla_{KPC-3} was, in all isolates, nested within a Tn*4401* transposable element (Fig. 4B). All KPC-3-producing isolates within the burn unit except for one (Kp06, with Tn*4401*d), contained bla_{KPC-3} nested within the Tn*4401*b element, while isolates outside the burn unit contained a variety of Tn*4401* isoforms (Table 2). All Tn*4401*b sequences harboring bla_{KPC-3} (882 bp) from pKPC-3_UNC were 100% concordant, except for Tn*4401*b within Kp08 (1 SNV) (Fig. S3). Thus, plasmid and Tn*4401* transposon analyses suggested that the majority of bla_{KPC-3} genes were transmitted via an identical Tn*4401*b element on part of a common plasmid (Fig. 4; Fig. S2 and S3). On the other hand, Kp08, which was isolated within the burn unit, and Ec149, which was isolated outside the burn unit, both contained the Tn*4401*b-bla_{KPC-3} nested genetic element but did not share the pKPC-3_UNC plasmid. The KPC-3-bearing contigs from Kp06 and Kp08 likely represented different KPC-3-containing plasmids (IncFIA, IncI2) and different Tn*4401* isoforms (d, b), in addition to being different strains of *K. pneumoniae* (64 SNVs). Two identical strains of *K. pneumoniae* (Kp152 and Kp156; 3 SNVs) outside the burn unit had the same Tn*4401* isoform (d), which occurred on a plasmid other than pKPC-3_UNC. We found that the bla_{KPC-3}-Tn*4401*b structure was nested within 5-bp flanking sequences on the Tn*2*-like element in every case where an isolate contained the structure (Fig. 4; Table 2).

DISCUSSION

In this study, we describe the genomic analysis of two sequential CRE outbreaks that occurred at a large academic burn center over a 3-year period. Although these outbreaks seemed epidemiologically unlinked, genome-wide sequence analysis pro-

vided strong evidence that they were in fact genetically linked. We found that both *E. cloacae* and *K. pneumoniae* strains causing this outbreak utilized multiple modes of bla_{KPC-3} gene transfer. A recent genetic surveillance study involving hundreds of KPC-producing *Enterobacteriaceae* isolates at a large academic medical center clearly demonstrated these mechanisms (21). In our study, we were surprised to find multiple transmission mechanisms for the bla_{KPC} gene among strains and plasmids in association with transposons, even in the few CRE which comprised this outbreak (19 KPC-3-producing *Enterobacteriaceae* isolates), suggesting challenges for infection prevention and control measures against CRE in immunocompromised populations.

Although *E. cloacae* outbreaks were previously reported to be largely clonal (22, 23), recent genomic studies (21, 24) have identified a variety of molecular mechanisms of transmission in KPC- or New Delhi metallo-β-lactamase (NDM)-producing *E. cloacae*. We found that most cases in this outbreak were caused by a clonal strain of KPC-3-producing *E. cloacae* (Ec_UNC). Despite the importance of clonal strain expansion in the outbreak at our hospital, propagation of the outbreak was likely facilitated by plasmids in association with transposons. Specifically, we found that an *E. cloacae* strain circulating in the hospital likely acquired the bla_{KPC-3} gene via plasmid transfer from a *K. pneumoniae* isolate or vice versa. This potentially occurred among different species of isolates Kp07 and Ec09, which were isolated from a single patient.

Given the epidemiological timeline and location as well as the genetic relatedness, a majority of transmission routes in two outbreaks could represent direct transmissions. The index cases for each outbreak (Kp11 and Ec13) shared time and space with other in patients in the burn center during almost the whole outbreak period and could be the source of KPC-3. Remarkably, WGS analysis demonstrated possible transmission of KPC-3 producers in the burn center during ~2-year periods at the strain (Ec_UNC) level (e.g., Ec05 isolated in February 2012 and Ec04 isolated in February 2014) and ~2.5-year periods at the plasmid level (pKPC-3_UNC; Kp07 isolated in February 2012 and Ec114 isolated in September 2014). Although KPC-3-producing CRE isolates outside the burn center during this outbreak period initially seemed to be related to CRE isolates inside the burn center, WGS analysis clearly revealed differentiation of these strains; therefore, CRE isolates outside the burn center may have been sporadic cases in our hospital or independent introductions of bla_{KPC} into our hospital.

There were no direct contacts between the first and second outbreaks (1 month between Kp06 from the last case of the first outbreak and Ec13 from the initial case of the second outbreak) in spite of persuasive genetic relatedness, likely due to transmission through health care personnel, colonization of another patient in the burn unit, or a contaminated environmental reservoir which was not identified in this investigation. Patient-to-patient transmission is an important mode for acquisition of carbapenem-resistant *E. cloacae* (23, 25), but transmission from the hospital environment may occur since room surfaces in surroundings of CRE carriers have been frequently identified as contaminated (26). On the other hand, our previous study showed that CRE (*Enterobacter* and *Klebsiella* species) survived poorly in a hospital environment for 24 h and was infrequently isolated from environmental surfaces in rooms housing CRE-colonized/infected patients (27); thus, the role of hospital environmental surfaces for CRE transmission is yet to be clarified. Some authors have described environmental reservoirs of CPE related to transmission in health care settings (28), including sinks (29, 30), soap dispensers (24), and hospital wastewater systems (31, 32).

The index patient with *K. pneumoniae* ST2252 producing KPC-3 (Kp11) was first admitted to the burn center in this outbreak and stayed there for ~1 year, and then likely shared *E. cloacae* ST114 and other STs of *K. pneumoniae*, spreading KPC-3 among them. This transmission of KPC-3 producers was primarily maintained by *E. cloacae* ST114 with other β-lactamases, including ACT-16, TEM-1B, and SHV-12, as well as distribution of multiple classes of resistance determinants. Two molecular epidemiologic studies of *E. cloacae* isolates from Europe and other countries described that *E. cloacae* ST114 was one of the most common genotypes and was associated with producing CTX-M-15, although there were no strong correlations between a majority of

E. cloacae STs and other β-lactamase profiles (33, 34). To our knowledge, this is the first outbreak due to KPC-3-producing *E. cloacae* ST114 among a burn patient population, and this strain was identified in all of three deceased burn patients.

WGS-based genotyping had greater sensitivity for distinguishing strains than did MLST. In particular, Ec114 belonged to ST114 but was different from Ec_UNC (33 SNVs). Of CRE isolates (Kp06, Kp08, Kp152, Kp156) belonging to ST258, Kp152 and Kp156 were a clone (Kp_UNC) based on SNV typing, but others were distinct (64 to 78 SNVs). When the relatedness in hospital outbreak strains of CRE has been investigated, WGS-based SNV typing has demonstrated differentiation of strains belonging to the same ST.

There are several limitations in this study. First, we were not able to identify specific reservoirs in this outbreak. Our transmission analysis also could be affected by isolates not detected nor analyzed during this outbreak period. Nucleic acid detection and WGS analysis through broad sampling of the hospital environment and items as well as multiple specimens from a single patient could be useful for obtaining a better understanding of transmission networks in health care outbreaks (24, 35). Second, using sequencing data, we found evidence of diverse plasmids and transposons. However, even with our high-coverage short reads, we are likely unable to describe the full extent of diversity among plasmids and mobile elements that harbor resistance genes. While our careful process of *de novo* assemblies and reference-guided mapping lent confidence to our current findings, the true picture of resistance gene propagation in this outbreak may be more complex than we found. Emerging long-read sequencing technologies will prove crucial in accurately describing the full extent of resistance gene transmission in health care settings (13, 21, 36).

In conclusion, our WGS analysis provided insight into complex genetic transmissions of KPC-3-producing *E. cloacae* and *K. pneumoniae* in this prolonged burn outbreak as well as the difficulty of infection prevention and control at the burn center. WGS analysis allowed us to uncover transmission dynamics regarding resistance genes, mobile elements, and strains in health care settings. Current phenotypical detection of CRE and implementation of strict standard and contact precaution for patients with CRE infection or colonization may help prevent transmission of CRE strains but would be difficult for gene-based transmission. Further outbreak investigations using WGS will be required to elucidate the full complexity of genetic mechanisms of resistance transmission and establish infection prevention and control strategies that are responsive to specific gene-based transmission.

MATERIALS AND METHODS

Bacterial strains and clinical data. A total of 22 clinical isolates of CRE from 21 patients, including *E. cloacae* ($n = 15$) and *K. pneumoniae* ($n = 7$), between January 2012 and May 2015 at the University of North Carolina Hospitals (Chapel Hill, NC, USA), an 853-bed tertiary care academic facility, were analyzed (Table 1). A single isolate from the primary site of infection or colonization was used for each patient with CRE (*K. pneumoniae* and *E. cloacae*) at the North Carolina Jaycee Burn Center during this period. In addition, one patient was infected with both *K. pneumoniae* and *E. cloacae*, so an isolate from each species (Kp07 from bronchoalveolar lavage fluid [BAL] and Ec09 from blood) was sequenced. In addition to the isolates from the burn unit, 5 CRE isolates (two *E. cloacae*, Ec149 and Ec155, and three *K. pneumoniae*, Kp150, Kp152, and Kp156) from HAI collected during this period were included for comparison. All CRE strains were isolated from clinical specimens collected in UNC's McLendon Clinical Laboratories and were (i) nonsusceptible to one of the following carbapenems, doripenem, meropenem, or imipenem, and (ii) resistant to all of the following cephalosporins that were tested, ceftriaxone, cefotaxime, and ceftazidime, according to the 2012 Centers for Disease Control and Prevention (CDC) criteria (37).

Clinical and epidemiological data for CRE cases were obtained through chart review, an administrative database, and a comprehensive hospital-wide surveillance database, using a laboratory-based approach. HAI were prospectively ascertained using the CDC definitions (38). This study was approved by the Institutional Review Board of UNC at Chapel Hill (IRB number 06-0437).

Antimicrobial susceptibility testing. Kirby-Bauer disk-diffusion susceptibility testing was performed, and the breakpoints for each drug were interpreted according to the 2015 Clinical and Laboratory Standards Institute guideline (colistin breakpoint for *Pseudomonas aeruginosa* and the others for *Enterobacteriaceae*) (39). Antibiotics tested included ampicillin-sulbactam, piperacillin-tazobactam, ceftriaxone, cefepime, imipenem, ertapenem, gentamicin, amikacin, ciprofloxacin, levofloxacin, trimethoprim-sulfamethoxazole, and colistin.

Whole-genome sequencing. Bacterial strains were grown overnight in LB broth at 37°C. DNA from each isolate was extracted and purified using an UltraClean microbial DNA isolation kit (Mo Bio, Carlsbad, CA). Sequencing libraries were prepared using the NEBNext Ultra DNA library prep kit for Illumina (New England BioLabs, Ipswich, MA) per the manufacturer's instructions with NEBNext multiplex oligonucleotides (New England BioLabs). All libraries were pooled and sequenced on a single Illumina HiSeq2500 run using 125-base paired-end V4 chemistry at the UNC High-Throughput Sequencing Facility.

***De novo* sequence assembly.** Predicting accurate *de novo* haplotypes is crucial in an outbreak investigation where the genetic drivers are unknown. However, because predicting accurate *de novo* haplotypes from short read data can be a precarious endeavor (21), we adopted a very conservative approach to contig assembly and interpretation. We subjected our short-read WGS data to numerous *de novo* assemblers (those available at http://nucleotid.es/assemblers/), and we determined that the A5-miseq pipeline with default parameter settings produced the highest-quality assemblies, with the longest contig lengths and the highest N_{50} values (40). To further increase our certainty in the accuracy and completeness of our assembled bla_{KPC-3}-containing contigs and to check for occult duplication events that can arise during *de novo* assembly (41), we constructed multistring Burrows-Wheeler transformations of raw sequencing data (https://github.com/andrewparkermorgan/snoop) and queried these structures for the presence/absence of relevant sequence strings (42, 43).

As a single contig bearing the bla_{KPC} gene was identified in 20 of 22 UNC isolates, reads from these bla_{KPC}-containing isolates ($n = 20$) were mapped pairwise against the bla_{KPC}-containing contigs identified above ($n = 20$), for a total of $n = 400$ unique alignments. Salient comparisons and, in particular, self-alignments were examined closely for coverage gaps, misalignments, and evidence of plasmid closure in order to produce a hand-curated set of bla_{KPC}-containing plasmids, one plasmid for each bla_{KPC}-containing isolate ($n = 20$).

Reference-guided core genome mapping. To better characterize the extent of genetic diversity within the bacterial core genomes, we mapped short reads to reference genomes: NCTC9394 (RefSeq accession number NC_021046.1) in the case of *E. cloacae* and MGH78578 (GenBank accession number CP000647.1) in the case of *K. pneumoniae*. Mapping was conducted using *bwa mem*, and coverage is reported in Fig. S1 in the supplemental material (44). Mapped reads were deduplicated and subjected to local realignment through high-entropic regions using Picard (http://broadinstitute.github.io/picard/) and the Genome Analysis toolkit (GATK v3.3) (45).

SNV discovery was performed by applying the GATK unified genotyper across all reference-aligned CRE isolates simultaneously. Variants were filtered stringently using cutoffs responsive to the underlying distribution of quality scores. The following parameters were used for filtering: quality-by-depth, $\geq$25; mapping quality, $\geq$59; Fisher score, $\leq$4; map quality rank sum, greater than or equal to -4.0, and read position rank sum, greater than or equal to -4.0. In addition, only biallelic variants with at least 5× coverage in 100% of isolates were used for further comparative analysis.

Genetic analysis. First, species identification of individual isolates from short-read sequencing data was confirmed using Kraken (46). Acquired resistance genes were identified using ResFinder v2.1 (47) with a threshold of 98% identity and a minimum length of 60% on *de novo* assemblies. *In silico* MLST was performed and STs were determined from *de novo* assemblies using MLST v1.7 (48) and the PubMLST database (http://pubmlst.org/ecloacae/) for *E. cloacae* (49) and the Institut Pasteur MLST database (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html) for *K. pneumoniae* (50). Plasmid types (e.g., incompatibility groups) were identified using PlasmidFinder 1.3 (51), with a threshold of 95% identity on *de novo* assemblies. We also compared bla_{KPC}-containing contigs extracted from our *de novo* assemblies with all 35 publicly available RefSeq plasmids carrying bla_{KPC-3}, which were searched on the NCBI GenBank nucleotide database using the keyword KPC-3 as well as the categories RefSeq and plasmid (http://www.ncbi.nlm.nih.gov/nuccore). The presence/absence of the RefSeq plasmids was determined with 95% identity at the nucleotide level and 90% coverage level by BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The presence of Tn*4401* was confirmed via BLASTn, and its isoform was determined as described previously (52). Tn*4401*b-containing KPC-3 isolates from the UNC outbreak were compared to publicly available Tn*4401*b reference sequences. These were identified within the NCBI GenBank nucleotide database using the search term Tn*4401*b. After nonaligning database sequences and duplicate entries were removed, a maximum-likelihood phylogeny was constructed using the remaining database-derived Tn*4401*b sequences and our *de novo*-assembled sequences.

An analysis of recombination was undertaken using the method of Gubbins (53). Potential blocks of horizontal gene transfer were identified by at least three base substitutions, and recombinogenic regions were masked during construction of a maximum-likelihood phylogenetic tree using RAxML (54). Pairwise SNV differences between isolates were calculated using bedtools (https://bedtools.readthedocs.io/en/latest/). We used criteria for relatedness based on SNVs among strains which were proposed by Salipante et al.: genomically indistinguishable, 3 or fewer SNVs; closely related (up to 12 SNVs); and unrelated (13 or more SNVs) (12). In this study, a clone (Ec_UNC or Kp_UNC) was defined as closely related strains with up to 12 SNVs.

Accession number(s). All sequence data reported in this study have been deposited at the DDBJ/EMBL/GenBank Sequence Read Archive (SRA) under accession numbers DRX055644 to DRX055673 (see Table 1).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01516-16.

TEXT S1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

We are very grateful to Rebecca Brooks, Lauren DiBiase, and Swathi Kodavanti for their assistance in data collection. We acknowledge Maria Gergen for her experimental support. We thank Tohru Miyoshi-Akiyama for curating *Enterobacter cloacae* MLST Databases and the team of curators of the Institut Pasteur MLST for curating the data and making them publicly available.

This study was supported by internal funding from UNC Health Care and UNC School of Medicine. H.K. received financial support from the Japan Society for the Promotion of Science (JSPS) Postdoctoral Fellowship for Research Abroad. C.M.P. was supported by F30 AI109979 and T32 GM008719.

We report no conflicts of interest relevant to this article.

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