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EPIDEMIOLOGY AND SURVEILLANCE

Next-Generation-Sequencing-Based Hospital Outbreak Investigation Yields Insight into *Klebsiella aerogenes* Population Structure and Determinants of Carbapenem Resistance and Pathogenicity

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ABSTRACT *Klebsiella aerogenes* is a nosocomial pathogen associated with drug resistance and outbreaks in intensive care units. In a 5-month period in 2017, we experienced an increased incidence of cultures for carbapenem-resistant *K. aerogenes* (CR-KA) from an adult cardiothoracic intensive care unit (CICU) involving 15 patients. Phylogenomic analysis following whole-genome sequencing (WGS) identified the outbreak CR-KA isolates to group together as a tight monoclonal cluster (with no more than six single nucleotide polymorphisms [SNPs]), suggestive of a protracted intraward transmission event. No clonal relationships were identified between the CICU CR-KA strains and additional hospital CR-KA patient isolates from different wards and/or previous years. Carbapenemase-encoding genes and drug-resistant plasmids were absent in the outbreak strains, and carbapenem resistance was attributed to mutations impacting AmpD activity and membrane permeability. The CICU outbreak strains harbored an integrative conjugative element (ICE) which has been associated with pathogenic *Klebsiella pneumoniae* lineages (ICE*Kp*10). Comparative genomics with global *K. aerogenes* genomes showed our outbreak strains to group closely with global sequence type 4 (ST4) strains, which, along with ST93, likely represent dominant *K. aerogenes* lineages associated with human infections. For poorly characterized pathogens, scaling analyses to include sequenced genomes from public databases offer the opportunity to identify emerging trends and dominant clones associated with specific attributes, syndromes, and geographical locations.

KEYWORDS AmpD, carbapenem-resistant *Klebsiella aerogenes*, MLST, ST4, cardiothoracic intensive care unit, genomic epidemiology, integrative conjugative element, outbreak, porins, yersiniabactin

Klebsiella aerogenes (formerly described as *Enterobacter aerogenes*) is a ubiquitous member of the *Enterobacteriaceae* family and a significant nosocomial pathogen associated with drug resistance and a wide variety of infections, including pneumonia, bacteremia, and urinary tract and surgical site infections (1–3). *K. aerogenes* infections can arise endogenously (gastrointestinal flora) or be acquired from surroundings in the facility where the patient is admitted (horizontal transmission through colonized health care workers, contaminated devices/shared equipment, other patients, etc.), with the most critical risk factor for acquiring infection being prolonged broad-spectrum antibiotic administration (4). Risk factors for *K. aerogenes* infections include prolonged stay at health care facilities, especially for patients who are immunosuppressed, on me-

Citation Malek A, McGlynn K, Taffner S, Fine L, Tesini B, Wang J, Mostafa H, Petry S, Perkins A, Graman P, Hardy D, Pecora N. 2019. Next-generation-sequencing-based hospital outbreak investigation yields insight into *Klebsiella aerogenes* population structure and determinants of carbapenem resistance and pathogenicity. Antimicrob Agents Chemother 63:e02577-18. https://doi.org/10.1128/AAC.02577-18.

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Received 14 December 2018
Returned for modification 21 January 2019
Accepted 21 March 2019
Accepted manuscript posted online 25 March 2019
Published 24 May 2019

chanical ventilation, or harbor foreign devices (4). Numerous hospital ward outbreaks in both pediatric and adult populations due to *K. aerogenes* have been described due to a common source (5, 6) or spread via patient-to-patient transmission (7–10). A particularly high frequency of hospital intensive care unit (ICU) outbreaks was continually reported from Western Europe in the period between the 1990s and early 2000s and was largely attributed to the spread and endemic establishment of a clonal *K. aerogenes* strain harboring the extended-spectrum β-lactamase (ESBL) TEM-24 ($bla_{\text{TEM-24}}$) (2, 11, 12).

Within the United States and other regions across the globe, *K. aerogenes* has also been reported, along with *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Escherichia coli*, to be among the frequently isolated carbapenem-resistant *Enterobacteriaceae (*CRE) (13–15). Clinical carbapenem-resistant *K. aerogenes* (CR-KA) strains harboring plasmid-borne serine carbapenemases have been described in the United States and worldwide, while metallo-β-lactamases and OXA-48 have been reported in Europe, Asia, and Brazil (2, 16). However, the primary mechanisms underlying resistance to carbapenems in *K. aerogenes* are considered carbapenemase independent and attributed to chromosomal AmpC β-lactamase overexpression and mutations affecting membrane permeability (2). The latter has been well documented in *K. aerogenes* with reports describing mutations impacting porin function/expression that can arise *in vivo* during antibiotherapy (17), be reversible (2), and present complex diagnostic and therapeutic management challenges (18).

Despite the role of *K. aerogenes* as an important opportunistic pathogen and its epidemic potential, the clinical relevance of intraspecies genetic diversity and significance of specific sequence types (STs) remain unknown. In comparison, in the genomically more closely related *K. pneumoniae* and, to some extent, in *E. cloacae*, clonal complexes and STs associated with geographical distribution, multidrug resistance, hospital outbreaks, and disease syndromes have been defined (19, 20). Recently, a multilocus sequence typing (MLST) scheme has been developed for *K. aerogenes* that can help explore the above-mentioned issues; however, its performance in evaluating and discriminating clinical/environmental isolates has not yet been reported.

We pursued this study to investigate an outbreak of CR-KA in a cardiothoracic intensive care unit (CICU) at our hospital, which persisted for 5 months despite aggressive infection control measures. The primary goals of our study included whole-genome sequencing (WGS)-based investigation of the clonal relationships among the CR-KA strains isolated from patients in our hospital and defining putative loci associated with carbapenem resistance and virulence. In addition, the recently developed publicly available *K. aerogenes* MLST scheme afforded us the opportunity to delineate the population structure of CR-KA strains isolated from patients at our hospital. Our initial findings led us to broadly investigate the origin and significance of specific *K. aerogenes* sequence types identified in our hospital CR-KA strains by performing comparative genomics using publicly available global *K. aerogenes* genomes.

RESULTS

Epidemiological and genomic characterization of the CICU CR-KA cluster. In July 2017, five CICU patients had CR-KA isolated from respiratory tract specimens (unit occupancy and patient demographics are detailed in Fig. 1 and in Table S1 in the supplemental material). The first identified case (patient A) had a past medical history significant for intravenous drug use and recurrent methicillin-resistant *Staphylococcus aureus* infections. Patient A's CICU course is described in Fig. S1. The temporal association of subsequent positive cultures from patients in the CICU prompted an outbreak investigation.

In response to the event, the Infection Prevention team implemented universal contact precautions, weekly surveillance cultures, distribution of educational materials pertaining to hand hygiene, equipment disinfection, and the use of UV irradiation for all patient rooms as census allowed. Infected patients were separated into a cohort on one side of the unit with dedicated nursing staff. Environment-of-care rounds were

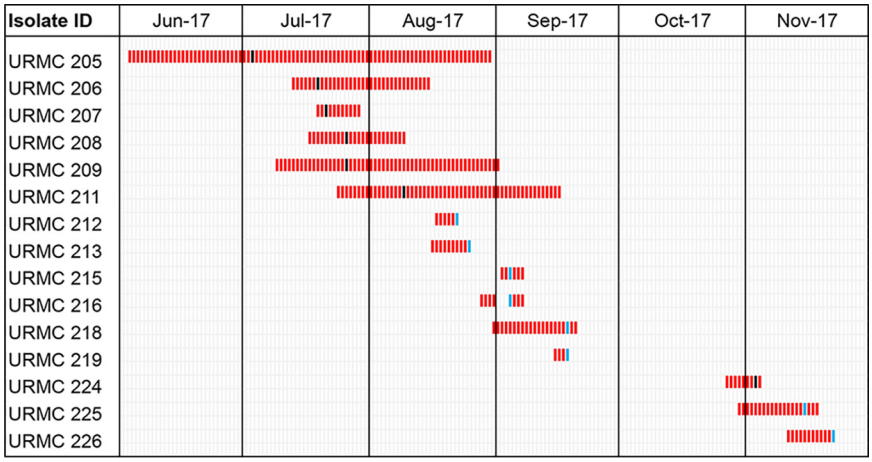

FIG 1 Patient occupancy and overlap in CICU ward during the *K. aerogenes* outbreak. Black bars, first positive CR-KA clinical cultures; blue bars, first positive CR-KA surveillance cultures.

performed in the CICU and all cardiac operating rooms. Limited environmental surveillance cultures ($n = 4$; hand-washing sinks near/inside the ward) were performed and were negative. Antibiotic stewardship efforts were also reinforced during the investigation.

Despite interventions, 10 additional CICU patients had positive CR-KA cultures between August and November 2017 (Fig. 1 and Table S1). Of the total 15 positive cultures from unique patients, 6 were clinical specimens (5 respiratory and 1 blood), and 9 were rectal surveillance cultures. Epidemiological investigation of possible risk factors among the CICU patients for developing CR-KA infection/colonization was performed. A retrospective case-control study (cases, $n = 15$; controls, $n = 30$) revealed no significant differences for gender, age, mortality, unit location, dialysis, ventilator support, transport from another hospital, or carbapenem exposure (Table S2). Infected patients were significantly more likely to have undergone a surgical procedure ($P = 0.04$) but were not found to be associated with a specific operating room (data not shown). After November 2017, no CR-KA strains were isolated in either clinical or surveillance specimens. Active surveillance in the ward was discontinued at the end of January 2018, and the outbreak was deemed to have subsided.

Antibiotic susceptibility testing (AST) of the CICU CR-KA strains revealed some differences in phenotypic susceptibilities to carbapenems and cephalosporins (Table 1 and Data Set S1). Sixty percent of the CICU cluster strains were resistant to three carbapenems (ertapenem, imipenem, and meropenem), while 93% showed phenotypic resistance to ertapenem only. Susceptibility to cefepime also varied, with a subset of outbreak strains displaying phenotypic resistance (Table 1; Data Set S1).

To address the possibility of an outbreak, particularly in light of variable antibiotic susceptibilities, WGS was undertaken to identify phylogenetic relationships and potential transmission links. Clinical strains detected between July and August were sequenced retrospectively, while isolates from clinical specimens and surveillance cultures obtained between August and November were sequenced prospectively as part of the active-outbreak investigation. A total of 26 University of Rochester Medical Center (URMC) *K. aerogenes* isolates were sequenced using Illumina WGS. These included 15 CR-KA isolates from patients admitted in the CICU between June and November 2017 and an additional set of 9 CR-KA strains epidemiologically unlinked to the CICU cluster strains (patient isolates from between 2015 and 2017) (Table S3) for context and comparison. Two clinical isolates were included as controls, URMC 201 (intermediate susceptibility to imipenem) and URMC 223 (susceptible to all carbapenems tested). The AST profiles of the non-CICU CR-KA study strains are also described in Table 1.

TABLE 1 Phenotypic antibiotic susceptibility profiles of *K. aerogenes* strains in this study[a]

URMC strain group and no.[b]	Amikacin	Gentamicin	Tobramycin	Ciprofloxacin	Moxifloxacin	Trimethoprim-sulfa	Piperacillin-tazobactam	Ceftriaxone	Cefepime	Ertapenem	Imipenem	Meropenem
2017 CICU-associated CR-KA strains												
205[c]	S	S	S	S	S	S	R	R	S	R	R	R
206	S	S	S	S	S	S	R	R	S	R	R	R
207	S	S	S	S	S	S	R	R	S	R	R	R
208	S	S	S	S	S	S	R	R	S	S	S	R
209	S	S	S	S	S	S	R	R	S	R	R	R
211	S	S	S	S	S	S	R	R	D	R	R	R
212	S	S	S	S	S	S	R	R	R	R	I	R
213	S	S	S	S	S	S	R	R	R	R	I	R
215	S	S	S	S	S	S	R	R	S	R	R	R
216	S	S	S	S	S	S	R	R	S	R	S	S
218	S	S	S	S	S	S	R	R	S	R	R	R
219	S	S	S	S	S	S	R	R	S	R	R	R
224	ND	S	S	S	S	S	R	R	R	R	ND	R
225	S	S	S	S	S	S	R	R	R	R	R	R
226	S	S	S	S	S	S	R	R	R	R	I	I
Other CR-KA strains												
200	S	S	S	S	ND	S	R	R	R	R	ND	R
202	S	S	S	S	S	S	R	R	S	R	R	R
203	S	S	S	S	S	S	R	R	S	R	R	R
204	S	S	S	S	ND	S	R	R	S	R	ND	R
210	S	S	S	S	ND	S	R	R	S	R	ND	S
214	S	S	S	S	ND	S	I	R	S	R	I	S
217	S	S	S	R	ND	S	R	R	S	R	I	S
221	S	S	S	S	ND	S	S	S	S	R	ND	S
222	S	S	S	S	S	S	R	R	R	R	I	S
Controls[d]												
201	S	S	S	S	S	S	S	S	S	S	I	S
223	S	S	S	S	ND	S	S	S	S	S	ND	S

[a]R, resistant; S, sensitive; I, intermediate; D, dose dependent; ND, not determined. See Data Set S1 in the supplemental material for MIC values and Kirby-Bauer disk zone sizes and interpretations.
[b]All strain numbers are prefixed by URMC.
[c]Patient A (first case).
[d]URMC 223, carbapenem susceptible; URMC 201, carbapenem intermediate.

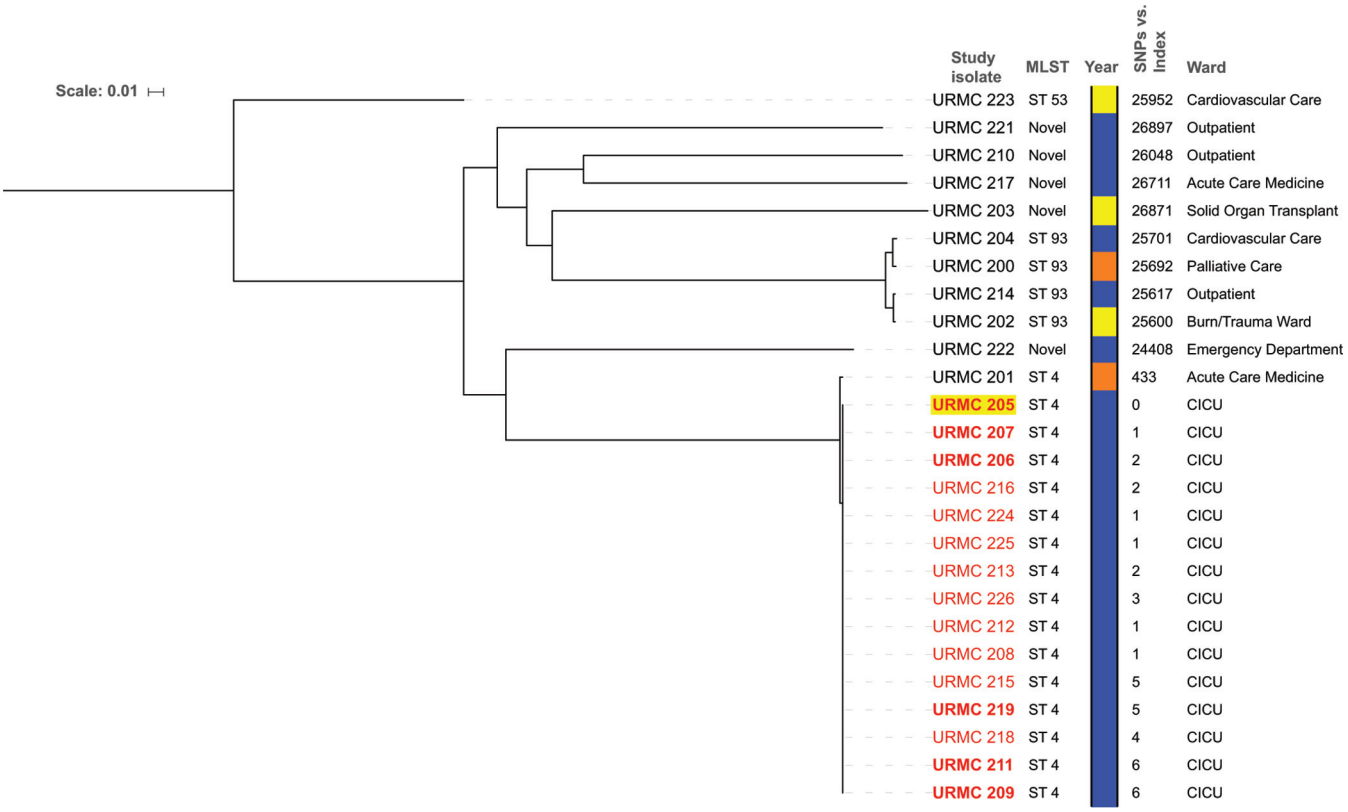

FIG 2 Dendogram showing pairwise SNP differences based on the phylogenetic relatedness of URMC *K. aerogenes* strains. Whole-genome sequence of *K. aerogenes* KCTC 2190 (ATCC 13048) was used for reference mapping. Discriminatory high-quality SNPs in the core genomes obtained by the CFSAN SNP pipeline were used to plot the tree (excluding mobile elements and putative recombination sites). CICU outbreak strains are identified by number (in red); clinical isolates are in bold, and the patient A isolate (1st case) is highlighted in yellow. The year of strain isolation is color coded as follows: orange, 2015; yellow, 2016; blue 2017. SNP differences relative to patient A are shown. The scale bar indicates the number of nucleotide substitutions per site.

The 26 sequenced URMC *K. aerogenes* genomes showed high genomic coverage (>88%) relative to that of the *K. aerogenes* KCTC 2190 reference strain (ATCC 13048^T; GenBank accession number NC_015663.1) (Data Set S2). Single nucleotide polymorphisms (SNPs) were identified across the study genomes relative to the reference sequence (pairwise SNP differences ranged from 1 to 28,170) (Data Set S3). MLST assignment indicated that all of the CICU clinical and surveillance isolates belonged to ST4, and SNP-based analyses grouped them in a tight cluster separately and distantly from nonoutbreak isolates (Fig. 2; Data Set S3). Within the CICU cluster, strains differed from URMC 205 (first case, patient A isolate) by no more than 6 SNPs. In addition, these isolates bore identical plasmid profiles (based on replicon and plasmid typing) (Table S5). In contrast, the 2017 non-CICU isolates were significantly distant from the CICU outbreak isolates (>20,000 SNPs). The most closely related non-CICU CR-KA strain was URMC 201 (isolated in 2015). This strain was also ST4, with 433 SNPs in a pairwise comparison to the sequence of URMC 205. The next most closely related non-CICU isolates, identified by pairwise SNP comparisons, belonged to ST93: the pair URMC 214 and URMC 202 (244 SNPs apart) and the pair URMC 200 and URMC 204 (517 SNPs apart) (Fig. 2; Data Set S3).

A potential limitation of mapping-based SNP identification approaches using a divergent reference and distantly related genomes is the possible underestimation of variation and decreased analyses resolution (21). To address this, intracluster comparative SNP analysis was performed for the CICU CR-KA isolates. The genome of CR-KA isolated from the earliest case (patient A, strain URMC 205) was *de novo* assembled and used as a mapping reference, and the rest of the closely related CICU CR-KA cluster strains were exclusively used as query genomes. The resulting phylogenetic analyses

TABLE 2 Nonsynonymous SNPs in the *ampD* gene associated with carbapenem resistance in the URMC *K. aerogenes* isolates

URMC strain group and no.[a]	SNP relative to *ampD* wild-type allele[c]	SNP effect on encoded AmpD sequence
CICU outbreak-associated CR-KA strains		
205,[b] 207, 209, 211, 212, 213, 215, 218, 219, 224, 225, 226	482G→A	Arg161His
206, 208, 216	284G→T	Trp95Leu
Outbreak-unrelated CR-KA strains		
200	338T→G	Ile113Ser
202	412C→T[d]	
204	117C→T	Pro39Ser
210	335C→T	Ser112Leu
214	280G→A	Ala94Thr
217	496G→C	Gly166Arg
221	478A→G	Ile160Val
	501C→A	Ala168Asp
222	492T→G	Glu164Asp

[a]All strain numbers are prefixed by URMC. Control strains were URMC 201 and URMC 203; no sequencing reads mapped to the *ampD* gene in URMC 203. Isolates additionally resistant to cefepime are underlined.
[b]Index patient isolate.
[c]Unless noted otherwise, all SNPs produced missense mutations.
[d]This nonsense mutation produced the substitution Gln138X, resulting in a premature stop codon.

showed the strains to differ from URMC 205 by not more than 7 SNPs and from each other in a pairwise comparison by less than 11 SNPs (Table S4 and Data Set 4). These relationships, coupled with the epidemiological data, indicated the 2017 CICU CR-KA cluster to be a monoclonal outbreak.

Carbapenem resistance in the URMC CR-KA strains was driven by adaptive chromosomal gene alterations. (i) WGS-based identification of acquired antibiotic resistance genes. Despite phenotypic carbapenem resistance, the CICU CR-KA strains did not harbor genes for carbapenemases, extended-spectrum β-lactamases, or plasmid-borne AmpC cephalosporinases. A single nonoutbreak CR-KA isolate (URMC 203) harbored a carbapenemase gene ($bla_{\text{NMC-A}}$). No horizontally acquired genes conferring resistance to non-β-lactam antibiotics were identified among the study strains, consistent with their susceptibility profiles (Table 1).

(ii) Nonsynonymous sequence alterations in key chromosomal loci implicated in carbapenem resistance. In the absence of genes encoding carbapenemases and ESBLs in the CR-KA outbreak strains, variations in other genetic loci associated with carbapenemase-independent resistance mechanisms were investigated (22–24). By focusing on the AmpC cephalosporinase and outer membrane porins, sequence variations in *ampD*, *ampG*, *ampR*, *omp35*, *omp36*, and *ompR* genes in the study strains were assessed relative to the wild-type alleles in the carbapenem-susceptible reference type strain KCTC 2190 (25). The sequences were also compared to alleles in URMC 223 (carbapenem susceptible) and URMC 201 (intermediate susceptibility). For the *omp* genes, the upstream DNA sequences were also assessed. The *ampG* and *ompR* genes were wild type in all study strains. Variants were identified in all other loci and are described below.

*(a) ampD***.** Mutations were identified in the *ampD* gene for each of the 24 CR-KA isolates in this study (Table 2), while the control strains URMC 223 and URMC 201 bore the wild-type *ampD* allele. The outbreak strains harbored single missense SNPs (either 284G→T or 482G→A) in *ampD*, resulting in a Trp95Leu or Arg161His substitution. Six nonoutbreak CR-KA strains harbored independent nonsynonymous single substitutions while two missense substitutions were identified in URMC 221. A single nonoutbreak strain, URMC 202, harbored a nonsense mutation resulting in a truncated AmpD protein (Table 2). DNA sequence corresponding to the *ampD* allele was absent in isolate URMC 203 (the only study strain to possess a carbapenemase-encoding gene $bla_{\text{NMC-A}}$) due to a large deletion in the genomic region harboring *ampD* and the neighboring *ampE* gene.

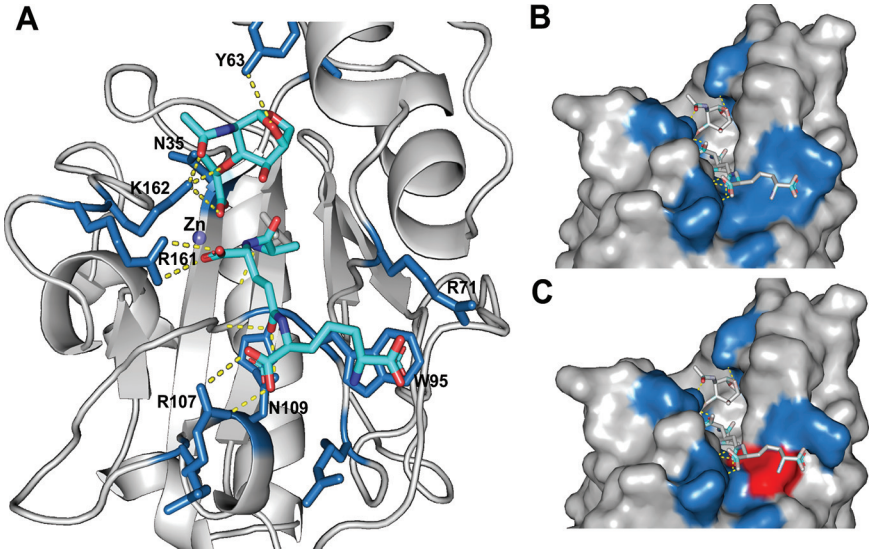

FIG 3 Computational modeling of the impact of *ampD* mutations on AmpD in URMC outbreak CR-KA strains. (A) *K. aerogenes* AmpD modeled on the *C. freundii* AmpD structure. Key residues interacting with glycan and peptide portions of ligand are shown. (B and C) Surface models of *K. aerogenes* AmpD depicting the wild-type binding surface for the diaminopimelate moiety (B, W95) and the binding surface of AmpD containing the W95L mutation (C; blue surfaces indicate amino acid positions from panel A, and the altered surface is highlighted in red).

The potential impact of a Trp95Leu or Arg161His substitution on AmpD activity in the outbreak strains was investigated by homology-based structural modeling of *K. aerogenes* AmpD using the high-resolution crystal structure of *Citrobacter freundii* AmpD (26), a close homolog (83.33% amino acid sequence identity). AmpD contains a hydrophobic surface to accommodate its GlcNAc-anh-MurNAc (*N*-acetylglucosaminyl-β-1,4-anhydro-*N*-acetylmuramic acid) ligand, which is made up of tripeptide and glycan moieties. The tripeptide portion of GlcNAc-anh-MurNAc is coordinated through three salt bridges between carboxyl groups on the tripeptide and residues Arg71, Arg161, and Arg107, while the peptide backbone is oriented across the hydrophobic surface. Trp95 forms a planar surface at the end of the ligand-binding channel to position the diaminopimelate moiety at the distal end of the tripeptide.

Based on the *in silico K. aerogenes* AmpD model, the positively charged guanidinium group of Arg161 forms two strong electrostatic interactions with the carboxyl group of D-glutamine on the tripeptide portion of the ligand (Fig. 3). Mutation of this residue to histidine was predicted to weaken ligand binding, likely affecting the positioning of the ligand in the active site. In the Trp95Leu substitution, the hydrophobicity in the region is preserved, but the shorter length of the leucine side chain leaves a gap at the end of the binding channel, likely affecting the positioning of the entire ligand (Fig. 3). These observations lend support to the idea that the missense mutations observed in the CICU outbreak strains would alter ligand docking on AmpD, likely reducing/inhibiting its activity. Four out of the seven CR-KA nonoutbreak strains had substitutions within glycan- and peptide-interacting regions of AmpD (Table 2), suggesting that these alterations might also impact activity.

(b) ampR. All of the outbreak strains harbored the reference *ampR* allele. Several nonoutbreak CR-KA strains harbored two to five substitutions that likely represented variant alleles as they were also observed in the control carbapenem-susceptible strain URMC 223. A single nonoutbreak CR-KA strain, URMC 210, bore a nonsense mutation in the *ampR* gene, resulting in a premature stop codon (Trp117X) which likely resulted in a nonfunctional truncated AmpR protein.

(c) omp35 and *omp36*. Among the 15 outbreak CR-KA strains, three different *omp36* variants were identified relative to the wild-type allele (Table 3). An identical profile of

TABLE 3 Nonsynonymous genetic mutations in *omp36* genes and resulting alterations in Omp36 sequence in the URMC *K. aerogenes* isolates

URMC strain group and no.[a]	Type of mutation in *omp36*[b]				Effect of mutation on Omp36 sequence[c]				
	FS	HV region	NS SNP	MS SNP	FS	HV region	PS	Truncation	Single substitution(s)
CICU outbreak associated CR-KA strains									
205,[d] 207	+	+		175A→G, 564T→C, 566A→G	pAsp91ThrfsX12		102X	+	Ile59Val
206, 208, 212, 213, 216, 224, 225, 226		+		175A→G, 564T→C, 566A→G		+			Ile59Val, Asp189Gly
209, 211, 215, 218, 219		+	184A→T	175A→G, 564T→C, 566A→G			62X	+	Ile59Val
Outbreak unrelated CR-KA strains									
200, 203, 210, 214		+		564T→C, 566A→G, 615 T→G, 834T→C, 835A→G		+			Asp189Gly, Asp205Glu, Asn279Asp
202	+	+		564T→C, 566A→G, 615 T→G, 834T→C, 835A→G	pTrp77LysfsX2		78X	+	Trp77Lys
204	+	+		564T→C, 566A→G, 615 T→G, 834T→C, 835A→G	pSer304ProfsX21	+	324X	+	Asp189Gly, Asp205Glu, Asn279Asp
217		+		564T→C, 566A→G, 569T→A		+			Asp189Gly, Phe190Tyr
222		+		175A→G,564T→C, 566A→G, 615 T→G		+			Ile59Val, Asp189Gly, Asp205Glu
Control strain									
201[e]		+		175A→G, 564T→C, 566A→G		+			Ile59Val, Asp189Gly

[a]All strain numbers are prefixed by URMC. Isolates additionally resistant to cefepime are underlined.
[b]Changes shown are nucleotide positions relative to the wild-type sequence. FS, frameshift; HV, hypervariable region (nucleotides 680 to 724); NS, nonsense; MS, missense; SNP, single nucleotide polymorphism. Plus signs indicate the presence of the feature.
[c]Changes shown are amino acid positions relative to the wild-type sequence. FS, frameshift; HV, hypervariable region (amino acids 226 to 241); PS, premature stop codon.
[d]Index patient isolate.
[e]Intermediate carbapenem resistance.

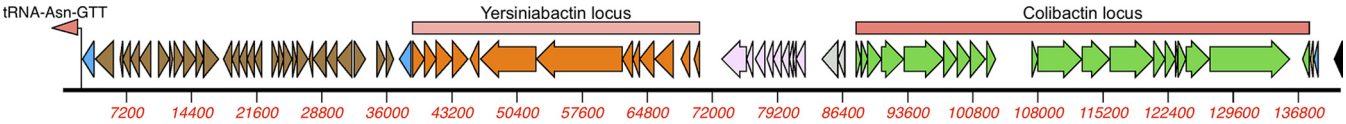

FIG 4 Yersiniabactin- and colibactin-encoding gene loci on integrative conjugative element ICE*Kp*10 in the CICU outbreak CR-KA strains. Blue arrows, integrase-encoding genes; brown arrows, Zn^{2+}/Mn^{2+} modules; gray arrows, genes encoding mobilization proteins; pink arrows, *vir*-T4SS.

missense SNPs in these strains and the control strain, URMC 201 (intermediate carbapenem susceptibility), was observed relative to the reference genome allele. Seven outbreak strains had additional mutations that resulted in severely truncated proteins. Several nonoutbreak CR-KA strains also harbored missense SNPs of unclear significance. Two strains, URMC 202 and URMC 204, harbored distinct frameshift mutations yielding truncated Omp36 protein variants. A 42-bp region of high variation (nucleotides 680 to 724), corresponding to 15/16 amino acid substitutions in loop L5, was observed in all the clinical isolates relative to the reference sequence (Table 3; Fig. S2). The hypervariable region results in a different charge profile in the region and has been previously reported in a *K. aerogenes* study describing imipenem-resistant clinical isolates (harboring ESBL TEM-24) from patients in France (27). The predicted Omp36 proteins in clinical strain URMC 221 and the carbapenem-susceptible control strain (URMC 223) bore 87% identity relative to the sequence of the reference genome Omp36 and were considered significantly distant variants (not included in the comparative analyses).

All but one of the study CR-KA strains had the wild-type allele of *omp35*. A single strain (URMC 202) bore a deletion in the 5′ end of the gene. The DNA sequence upstream of the *omp35* gene was investigated to identify mutations in the promoter sites of the strains, of which one (URMC 204) had a nucleotide difference of unclear significance at the −22 position.

Identification of a large pathogenicity-associated integrative and conjugative element, ICE*Kp*10, in the CICU outbreak CR-KA strains. The prolonged nature of the clonal CR-KA outbreak in our CICU led us to search for pathogenicity loci that could have promoted persistence and transmission. A cluster of chromosomally located genes encoding yersiniabactin (*ybt*) metallophore and colibactin (*clb*) genotoxin systems were identified in all of the outbreak strains (Fig. 4). These loci have been implicated in invasive infections due to pathogenic lineages of *K. pneumoniae* (28).

The *ybt* locus harbored putative genes involved in regulation as well as synthesis of the siderophore, corresponding transport-associated proteins, and a receptor protein for the uptake of metal-bound siderophore. The *clb* locus included putative homologs encoding enzymes, transferases, and transport proteins involved in production and secretion of the polyketide colibactin (Fig. 4; Data Set S5). Investigation of the genomic loci associated with the virulence factor gene cluster identified them to be present on a mobilizable integrative conjugative element (ICE) inserted in a tRNA-Asn site adjacent to a gene encoding the glycine cleavage system (Fig. 4). The ICE bore a modular arrangement of gene clusters encoding mobile elements, P4-like integrase, type IV secretion system (T4SS) conjugation machinery, and mobilization genes (Fig. 4; Data Set S5). The element was identified to be ICE*Kp*10 using BLAST (99% identity to the ICE*Kp*10 mobile element in *K. pneumoniae* strain 16703761; GenBank accession number KY454634) and a recently described virulence genomic typing scheme for *Klebsiella* spp. (28).

Among the URMC nonoutbreak *K. aerogenes* strains, these loci were identified in only 4/10 isolates, which were either ST4 or ST93. The control strain URMC 223 harbored the yersiniabactin locus exclusively. Detailed descriptions of these elements in the URMC *K. aerogenes* strains are given in Table S5.

Comparative analyses of URMC CR-KA and publicly available *K. aerogenes* genomes. To gain insights into the emergence and epidemiology of the CICU outbreak clones and to place our hospital CR-KA strains in the broader context of global *K. aerogenes* strains, comparative phylogenomic analyses were performed using the

Harvest genomics suite (29). Publicly available *K. aerogenes* genome assemblies ($n = 110$) were included in the analyses. These included 71 clinical and surveillance strains isolated from human specimens, 3 environmental strains, and 36 strains of unknown origin (Data Set S6). Based on the newly described MLST scheme, ST4 and ST93 strains were found to be markedly overrepresented in the available genomes (51.8%, or 57/110).

Excellent correlation was observed between Harvest-generated tree topologies (Fig. 5), as well as pairwise SNP differences (Data Set S7), and those generated by the CFSAN SNP pipeline for the URMC CR-KA study strains. Based on Harvest analyses, the CICU outbreak strains clustered closely with each other and with other global ST4 genomes compared to clustering of the other URMC CR-KA strains (outbreak unrelated), which were distantly dispersed throughout the phylogenomic distribution (Fig. 5). The MLST-based sequence types of URMC CR-KA strains and the global *K. aerogenes* genomes also correlated tightly with Harvest-generated core-genome-based topologies (Fig. 5).

Six publicly available assembled genomes grouped closely with the CICU outbreak strain genomes (<200 SNPs apart). These included ST4 strains UCI 27, UCI 28, and UCI 45, prospective isolates collected in the year 2013 from patients in Irvine, CA (described in a carbapenem resistance surveillance study by Cerqueria et al. [30]), and GN04794, GN05662, and GN02525, representing strains derived from various U.S. patient clinical specimens (blood, sputum, and wound drainage) in the years 2012, 2013, and 2007, respectively (Fig. 5; Data Set S7). The above-mentioned strains had fewer SNP differences in relation to the CICU outbreak strains than URMC 201, the closest and only nonoutbreak ST4 strain isolated in our hospital (Fig. 5; Data Set S7).

Carbapenemase-encoding genes were identified in a total of 13/110 global *K. aerogenes* genome assemblies (11.8%) (Fig. 5). These included genes encoding KPC-2 ($n = 7$), KPC-3 ($n = 1$), OXA-48 ($n = 4$), and NDM-6 ($n = 1$). The chromosomal serine carbapenemase, $bla_{\text{NMC-A,}}$ was solely present in URMC 203, a CR-KA strain isolated from a patient in our hospital in 2015. Among the 30 ST4 genomes, 4 strains (13%) harbored genes encoding carbapenemases (4/4, KPC-2), and these were clinical strains isolated from non-U.S. patients. The relative contribution of carbapenemase-mediated versus non-carbapenemase-mediated mechanisms of resistance to carbapenems in the global *K. aerogenes* strains could not be assessed due to the absence of antibiotic susceptibility metadata for most strains.

A characteristic genomic feature of the URMC CICU outbreak strains and a subset of non-outbreak-associated strains was the presence of the yersiniabactin siderophore and colibactin systems. Using the program Kleborate (28), we investigated the distribution and organization of these systems in the global *K. aerogenes* genomes to identify associations, if any, with specific STs and geographical regions (Fig. 5; Data Set S8). The prevalences of yersiniabactin- and colibactin-encoding systems in the global *K. aerogenes* genomes were found to be 53.64% (59/110) and 52.72% (58/110), respectively. A 100% association was found between the presence of colibactin in the genomes and the concurrent presence of yersiniabactin. Higher prevalence of the virulence cluster was observed in ST4 and ST93, with 85% (12/14) and 95% (42/44), respectively, although these two STs were also the most abundantly represented in the available set of genomes. The other STs were less well represented in the study set ($n < 3$), so the prevalence of these systems in them could not be accurately established. Interestingly, the strains exclusively designated environmental isolates (B3, FGI35, and B) did not harbor genes encoding the above-mentioned virulence systems (Fig. 5; Data Set S8). These trends correlated with analyses of our 26 hospital strains, where 75% of the ST93 strains (3/4) were positive for yersiniabactin and colibactin systems, while none of the strains with novel/unassigned STs harbored genes encoding the same (0%, 5/5).

DISCUSSION

This WGS study was initiated in order to establish the molecular epidemiology of CR-KA strains isolated from patients in our hospital following an outbreak event. Data

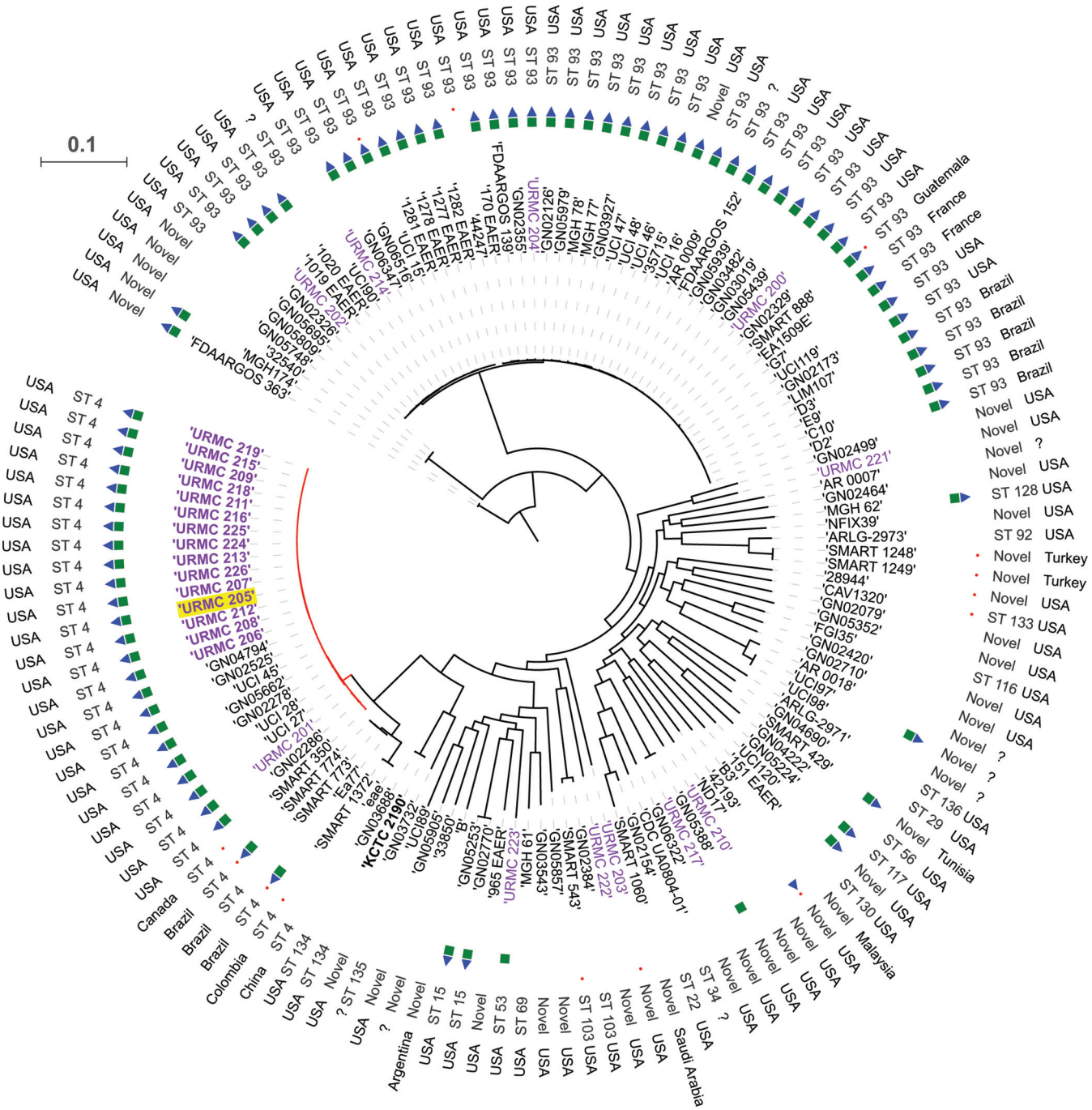

FIG 5 Harvest-based phylogenomic comparisons of URMC *K. aerogenes* genomes with global *K. aerogenes* genomes. Discriminatory SNPs based on core-genome comparisons were used to plot the tree. URMC study *K. aerogenes* strains are shown in purple (outbreak strains in bold; index patient strain highlighted in yellow). The presence of genes encoding a yersiniabactin siderophore system (green squares), colibactin synthesis cluster (blue triangles), and carbapenemases (red circles) in the assembled genomes is shown. Scale bar indicates the number of nucleotide substitutions per site.

from prospective WGS performed during the period between August and November 2017 informed the Infection Prevention team regarding the extent of the transmission event and effectiveness of infection control interventions. However, the source of the outbreak and how transmission occurred were not identified. The protracted nature of the outbreak and the significant number of cases at our institution highlight that non-carbapenemase-producing CRE also represent an important threat in health care systems. Additionally, *K. aerogenes* is a relatively poorly understood opportunistic

pathogen. The population structure and clinically important clones are undefined while genetic attributes associated with multidrug resistance and virulence have not been detailed and led us to perform additional comparative analyses.

Infections due to carbapenem-resistant organisms present complex diagnostic and therapeutic management challenges, and a better understanding of how adaptive or acquired resistance emerges in the health care environment is needed (31). In clinical CR-KA strains, carbapenem resistance has been associated with either carbapenemase production or coupling of adaptive mutations affecting membrane permeability and AmpC hyperproduction (2). However, chromosomal mutations associated with AmpC overexpression in CR-KA strains have not been reported in the literature. In our study, 23/24 CR-KA isolates were found to harbor mutations in genes involved in the synthesis or regulation of the inducible AmpC cephalosporinase (*ampD* and *ampR*) and outer membrane porins (*omp 35* and *omp36*). A majority of mutations were found to be within the open reading frames of *ampD* and *omp36* (catalogued in Tables 2 and 3).

While the role of AmpD in AmpC expression in *E. cloacae* has been well established (32), there are limited and conflicting reports regarding the role of AmpD in adaptive carbapenem resistance in *K. aerogenes* strains (33, 34). *In silico* modeling of *K. aerogenes* AmpD using the *C. freundii* AmpD crystal structure (26) predicts that substitutions resulting from SNPs in the outbreak strains likely impact enzymatic activity (Fig. 3). Separate *ampD* mutations in several non-CICU CR-KA isolates were also found to result in substitutions that could affect substrate-enzyme interactions (Table 2). This signifies that missense mutations in *ampD* likely play an important role in AmpC-mediated carbapenem resistance in *K. aerogenes*. Studies have also reported a minority of *E. cloacae* clinical isolates to have chromosomal *ampR* mutations that can confer high-level constitutive AmpC expression (35, 36). To our knowledge, this is the first report of *ampR* mutation associated with carbapenem resistance in a clinical *K. aerogenes* isolate (URMC 210). A single non-CICU strain harbored a carbapenemase-encoding gene, $bla_{\text{NMC-A}}$. NmcA has been reported in *E. cloacae* (37) but had not been described in *K. aerogenes* previously.

In clinical CR-KA strains, mutations in *omp36* have been described, and functional studies investigating their impact have been reported previously (11, 17). A diverse array of mutations was identified in the *omp36* gene among our study strains, including nonsynonymous mutations resulting in frameshifts or premature stop codons resulting in truncated and likely nonfunctional Omp36 variants (Table 3). Additional SNPs relative to the carbapenem-susceptible reference genome resulted in substitutions of unknown significance in predicted β-sheets and extracellular loop regions (Table 3; see Fig. S2 in the supplemental material). Interestingly, mutations resulting in substitutions in the "eyelet" region of internal loop 3 were not identified in our study CR-KA strains. A highly conserved region in the OmpC/Omp36 family of porins, the internal loop 3 forms a constriction pore that regulates β-lactam penetration, and mutations in this region have been reported to be associated with carbapenem resistance in *K. aerogenes* (27, 38, 39).

Within the CICU CR-KA monoclonal cluster, heterogeneity was observed in *ampD* and *omp36* genes (Tables 2 and 3). These genetic loci likely represent mutational hot spots associated with adaptive and reversible carbapenem resistance in *K. aerogenes*. The testing and archiving of single isolated carbapenem-resistant colonies instead of multiple colonies during individual patient specimen workup likely represent a limitation that did not allow us to capture the full complement of CR-KA strain microdiversity associated with individual patients during the outbreak event.

The detailed significance of alleles and mutations described above in development of carbapenem resistance in *K. aerogenes* needs to be verified by additional genetic (allelic exchange and complementation) and biochemical approaches. Complex regulatory networks, including transcriptional activators, sensor kinases, and two-component systems, have been implicated in adaptive drug resistance in *Enterobacteriaceae* spp. (24), and their roles in contributing toward carbapenem resistance in our study strains also cannot be ruled out.

Despite the lack of horizontally acquired drug resistance elements, the outbreak strains in this study managed to persist for several months in the face of an active infection prevention effort, prompting us to assess determinants that could promote transmission/persistence. The outbreak CR-KA strains harbored an ICE encoding the metallophore yersiniabactin (Ybt) and genotoxin colibactin (Clb) systems (Fig. 4; Table S6), raising the intriguing possibility that the element was instrumental in the success of this clone. A Ybt-encoding pathogenicity island has been described in association with a prolonged nationwide outbreak in the Netherlands involving multiple hospitals and >100 patients due to a multidrug-resistant *Enterobacter hormaechei* clone (40). A recent study by Lam et al. described the prevalence of *ybt* to be higher among the global carbapenemase-associated *K. pneumoniae* clonal group CG258 (40%) and hypervirulent *K. pneumoniae* clonal group CG23 (~87%) than in the wider *K. pneumoniae* population (~32%) (28). The study also reported a significant association of Ybt with an increased risk of invasive infections (bacteremia, liver abscesses, etc.). Ybt was first described in pathogenic *Yersinia* spp., encoded by a chromosomal gene cluster, termed the high-pathogenicity island (HPI), with a critical role in iron scavenging during infection (41). Subsequent studies reported acquisition of the HPI in other clinical *Enterobacteriaceae* strains (28, 42), and additional functions ascribed to Ybt include evasion of host lipocalin-2 (43) and sequestration/import of heavy metals (44). The polyketide colibactin is frequently associated with yersiniabactin and has been shown to induce chromosomal instability and DNA damage in eukaryotic cells (45). CRE surveillance studies (carbapenem-resistant isolates, years 2013 to 2018) at our institution did not identify concurrent or recent outbreaks due to ICE*Kp*10-harboring *K. pneumoniae* strains in our hospital CICU (data not shown). The outbreak clone could represent an entry of a *K. aerogenes* strain already harboring the ICE; however, recent acquisition from HPI-harboring *K. pneumoniae* strains silently colonizing patients cannot be ruled out. These findings present new avenues for research investigating the role of ICEs encoding Ybt and Clb in the pathogenicity and transmission of clinical *K. aerogenes* strains and other *Enterobacteriaceae*. While horizontal transmission of carbapenemases via mobile elements is increasingly recognized as a major public health issue (46), the transmission of mobilizable virulence factors presents an underappreciated threat in the health care environment that warrants more surveillance.

In order to set our hospital strains (outbreak and nonoutbreak) into a broader context, core-genome comparisons and MLST were used to examine the population structure of our strains relative to that of global *K. aerogenes* strains pulled from public databases (Fig. 5; Data Sets S6 and S8). This analysis is the first evaluation of the nascent *K. aerogenes* MLST scheme in discriminating clinical *K. aerogenes* isolates. The scheme was found to be robust, with distribution of STs correlating closely with topologies based on *K. aerogenes* strain core genomes. Our preliminary analyses suggest that ST4 and ST93 might be dominant global clones associated with *K. aerogenes* infections. Outbreak CR-KA strains clustered closest to other clinical U.S. ST4 strains, suggesting a clonal expansion of this ST (Fig. 5; Data Set S7). These strains were isolated from patients in the years 2007 to 2013 in Irvine, CA, and in undisclosed parts of the United States. It is noteworthy that the ST4 group also included carbapenemase-producing *K. aerogenes* isolates from international sites (Fig. 5; Data Set S8). These included CR-KA strains associated with drug-resistant intra-abdominal and urinary tract infections in patient samples from Brazil, Canada, Colombia, and China that had been sequenced as part of the SMART (Study for Monitoring Antimicrobial Resistance Trends) large surveillance study (47). ST93 was the most prevalent sequence type in the global *K. aerogenes* assembled genomes (43/110, or 39%), with wide global geographical distribution, including the United States (Fig. 5). Four of the 11 non-CICU CR-KA strains from our hospital belonged to this group. Two closely related ST93 isolates, *K. aerogenes* 1509E and G7, have been described as representatives of clonal strains associated with multiple multidrug-resistant *K. aerogenes* outbreaks in France (48, 49). Incidentally, global ST4 and ST93 isolates also had higher prevalences of HPI harboring *ybt* and *clb* (85% and 95%, respectively). Apart from the HPI, additional genetic traits or metabolic capabilities may also play a role in the success of these STs. These potentially high-risk clones need to be

examined more closely by undertaking large-scale studies with strains from diverse global sites and patient populations. This will help in the examination of niche adaptation, emergence of antibiotic resistance, and evolution of pathogenicity, leading to a better understanding of *K. aerogenes*.

In summary, genomic approaches for surveillance and outbreak investigations have emerged as critical functions for infection prevention and diagnostic microbiology laboratories. Along with evaluating the effectiveness of infection measures and the dimension of transmission events, WGS applied across sets of local and global isolates is a powerful approach for identifying emerging clinically relevant trends.

MATERIALS AND METHODS

Setting, study design, *K. aerogenes* strains and metadata. The University of Rochester Medical Center (URMC) is an 830-bed tertiary-care medical center, with a 14-bed cardiothoracic intensive care unit, serving the Greater Rochester Area, New York. Following approval by the University of Rochester Institutional Review Board (RSRB00068143), a total of 26 *K. aerogenes* strains isolated from patients at URMC in the course of regular clinical care and/or surveillance efforts were selected for the study. Each isolate corresponded to a single first CR-KA strain isolated during the course of hospitalization. Historical and contemporary patient *K. aerogenes* isolates epidemiologically unlinked to the CICU outbreak were also included in the study for context and comparison. Ward occupancy and pertinent clinical and epidemiological information were obtained through review of patient medical records and the laboratory information system and are described in Tables S1 and S3 in the supplemental material.

Clinical case-control study. For investigating patient risk factors associated with CR-KA infection/colonization, a retrospective case-control analysis was performed for patients admitted in the CICU in the outbreak period (July to November 2017; CRE cases, $n = 15$; controls, $n = 30$). Patient demographics and mortality data, unit location, carbapenem exposure, transfer record, procedures, and surgical histories were collected by chart review (Table S2). Significance was determined by a two-tailed Student's t test and z test for continuous and categorical variables, respectively. The significance level cutoff was set at a P value of ≤ 0.05.

AST of the study *K. aerogenes* strains. Antibiotic susceptibility testing (AST) of the study strains was performed as part of routine diagnostics using a Vitek2 (bioMérieux, France) system and/or Kirby-Bauer disk diffusion methods. AST interpretations were based on interpretive criteria defined by the Clinical and Laboratory Standards Institute (50).

Sequencing library preparation and raw data acquisition. The isolates were cultured on standard laboratory medium from archived frozen stocks, examined for purity, and reidentified by Vitek matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (bioMérieux, France). Dual-indexed sequencing libraries were prepared from genomic DNA extracted from single colonies and sequenced on an Illumina Miseq benchtop sequencer (Illumina, San Diego, CA) at the URMC Genomic Core Facilities (see the supplemental material for detailed methods).

Genomic analyses. Analyses were performed using an in-house bioinformatics pipeline, URMC Bacterial Genomic Analysis Pipeline (version 2.0.6), run on a high-performance computer cluster at the Center for Integrated Research Computing at the University of Rochester (see the supplemental material for detailed methods).

***In silico* protein analyses.** For homology modeling, SWISS-MODEL (51) was used to thread *K. aerogenes* AmpD (GenBank accession number WP_015704411.1; amino acids 1 to 187) through the structure of *C. freundii* AmpD (26) (PDB accession number 2Y2C; amino acids 1 to 187). The overall quaternary structure of *K. aerogenes* AmpD was predicted with high precision (95% confidence, 99% coverage). Comparative analyses and imaging of protein structures were performed with PyMOL (52). JalView was used to create alignments (53).

Data availability. WGS and metadata corresponding to the study URMC *K. aerogenes* isolates were deposited at NCBI under BioProject accession number PRJNA504784. Individual isolates were deposited under BioSample accession numbers SAMN10405413 to SAMN10405438 (Data Set S2).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02577-18.

SUPPLEMENTAL FILE 1, XLSX file, 0.01 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.05 MB.
SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 4, XLSX file, 0.04 MB.
SUPPLEMENTAL FILE 5, XLSX file, 0.03 MB.
SUPPLEMENTAL FILE 6, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 7, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 8, XLSX file, 0.01 MB.
SUPPLEMENTAL FILE 9, PDF file, 1.9 MB.

ACKNOWLEDGMENTS

We are grateful to the URMC Clinical Microbiology Laboratories and Infection Prevention staff in specimen processing, data collection, and epidemiological investigations. We acknowledge the URMC Genomics Research Center for support with WGS. We also thank Steve Gill (URMC, Genomics Research Center) for reviewing the manuscript draft.

Internal funding from the University of Rochester Department of Pathology and Laboratory Medicine supported this study.

We report no conflicts of interest relevant to this article.

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