Next-generation sequencing based hospital outbreak investigation yields insight into *Klebsiella aerogenes* population structure and determinants of carbapenem resistance and virulence

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Running title: Carbapenem-resistant *Klebsiella aerogenes* outbreak

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KEY WORDS

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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 clonal cluster (<7 SNPs apart), suggestive of a protracted intra-ward 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. Genes encoding carbapenemases or 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*Kp*10), which has been associated with pathogenicity in hypervirulent *Klebsiella pneumoniae* lineages. Comparative genomics with global *K. aerogenes* genomes showed our outbreak strains to group closely with global ST4 strains, which along with ST93 likely represent dominant *K. aerogenes* lineages associated with human infections. WGS is a powerful tool that goes beyond high-resolution tracking of transmission events into identifying the genetic basis of drug-resistance and virulence, which are not part of conventional diagnostic workflows. With an increasing availability of sequenced genomes from across the globe, population structure analysis offers opportunities to identify emerging trends and dominant clones associated with specific syndromes and geographical locations for poorly characterized pathogens.

73 **INTRODUCTION**

74 *Klebsiella aerogenes* (formerly described as *Enterobacter aerogenes*) is a
75 ubiquitous member of the *Enterobacteriaceae* family and a significant nosocomial
76 pathogen associated with drug resistance and a wide variety of infections including
77 pneumonia, bacteremia, urinary tract and surgical site infections (1, 2). In vulnerable
78 patients, *K. aerogenes* infections can arise endogenously (gastrointestinal flora) or be
79 acquired from surroundings in the facility where the patient is admitted (horizontal
80 transmission through colonized healthcare workers, contaminated devices/shared
81 equipment, other patients etc.), with the most critical risk factor for acquiring infection
82 being prolonged broad-spectrum antibiotic administration. Additional risk factors for *K.*
83 *aerogenes* infections include prolonged stay at healthcare facilities especially ICUs and
84 neonatal wards, complex underlying medical conditions, immunosuppression and
85 mechanical ventilation or the presence of foreign devices. Numerous hospital ward
86 outbreaks in both pediatric and adult populations due to *K. aerogenes* have been
87 described due to a common source or spread via patient-to-patient transmission (1, 2).
88 A particularly high frequency of hospital ICU outbreaks was continually reported from
89 Western Europe in a period between the 1990s and early 2000s, that were largely
90 attributed to the spread and endemic establishment of a clonal *K. aerogenes* strain
91 harboring the extended-spectrum B-lactamase TEM-24 ($bla_{\text{TEM-24}}$) (2).

92 Within the US and other regions across the globe, *K. aerogenes* has also been
93 reported along with *Klebsiella pneumoniae, Enterobacter cloacae* and *Escherichia coli*
94 to be among the frequently isolated carbapenem resistant *Enterobacteriaceae* (3-5).
95 Clinical CR-KA strains harboring plasmid-borne serine carbapenemases have been

96 described in the US and worldwide, while metallo-β-lactamases and OXA- 48 have
97 been reported in Europe, Asia and Brazil (2). However, the primary mechanisms
98 underlying carbapenem non-susceptibility in *K. aerogenes* are thought to be
99 carbapenemase independent and mediated by mutations affecting regulation of
100 chromosomal AmpC β-lactamase expression and membrane permeability (2). The
101 latter has been well documented in *K. aerogenes* with reports describing mutations
102 impacting porin function/expression that can arise *in vivo* during antibiotherapy (6), be
103 reversible (2), and present complex diagnostic and therapeutic management challenges
104 (7).

105 Despite the role of *K. aerogenes* as an important opportunistic pathogen and its
106 epidemic potential, the clinical relevance of intra-species genetic diversity and
107 significance of specific sequence types (STs) remains unknown. In comparison, in
108 genomically closely related *K. pneumoniae* and to some extent in *E. cloacae*, clonal
109 complexes and sequence types (STs) associated with geographical distribution, multi-
110 drug resistance, hospital outbreaks and disease syndromes have been defined (8, 9).
111 Recently, a multi-locus sequence-typing (MLST) scheme has been developed for *K.*
112 *aerogenes* (10) that can help explore the above-mentioned issues, however, its
113 performance in evaluating and discriminating clinical/environmental isolates has not yet
114 been reported.

115 We pursued this study to investigate an outbreak of CR-KA in a cardiothoracic
116 intensive care unit (CICU) at our hospital, which persisted for 5 months despite
117 aggressive infection control measures. The primary goals of our study included whole-

118 genome sequencing (WGS) based investigation of the clonal relationships among the
119 CR-KA strains isolated from patients in our hospital and defining putative loci associated
120 with carbapenem resistance and virulence. In addition, the recently developed publicly
121 available *K. aerogenes* MLST scheme afforded us the opportunity to delineate the
122 population structure of CR-KA stains isolated from patients at our hospital. Our initial
123 findings led us to broadly investigate the origin and significance of specific *K. aerogenes*
124 sequence types identified in our hospital CR-KA strains by performing comparative
125 genomics using publicly available global *K. aerogenes* genomes.

126

127 **RESULTS**

128 **Epidemiological and genomic characterization of the CICU CR-KA cluster**

129 In July 2017, five CICU patients had CR-KA isolated from respiratory tract
130 specimens (room occupancy and patient demographics detailed in Fig. 1 and Table S1).
131 The first identified case (Patient A) had a past medical history significant for intravenous
132 drug use and recurrent methicillin-resistant *Staphylococcus aureus* infections. Patient
133 A's CICU course is described in Fig. S1. The temporal association of subsequent
134 positive cultures in the CICU prompted an outbreak investigation. Between August to
135 November 2017, despite extensive infection prevention interventions, 10 additional
136 CICU patients had positive CR-KA cultures (Table S1). Of the 15 positive cultures from
137 unique patients, 6 were clinical specimens (5 respiratory, 1 blood) and 9 were rectal
138 surveillance cultures. Interventions included contact precautions for all patients on the
139 unit, cohorting of staff, and weekly surveillance cultures of rectal swabs and tracheal
140 aspirates. Environmental surveillance cultures during this period were negative. An

141 epidemiological investigation into the possible risk factors among the CICU patients
142 developing CR-KA infections was non-contributory.
143 Antibiotic susceptibility testing (AST) of the CICU CR-KA strains revealed some
144 differences in phenotype (Table 1) among the carbapenems and cephalosporins. Sixty
145 percent of the CICU cluster strains were resistant to three carbapenems (ertapenem,
146 imipenem and meropenem), while 93% showed phenotypic resistance to ertapenem.
147 Susceptibility to cefepime also varied, with a subset of outbreak strains displaying
148 phenotypic resistance (Table 1).
149 To address the possibility of an outbreak event, particularly in light of variable
150 antibiotic susceptibilities, a WGS investigation was undertaken to identify phylogenetic
151 relationships and transmission links. A total of 26 URMC *K. aerogenes* isolates were
152 sequenced using Illumina WGS. The primary strains investigated in this study were 15
153 CR-KA isolates from patients admitted in the CICU, between June and Nov 2017 (Table
154 S1). For context and comparison, an additional set of 9 CR-KA strains
155 epidemiologically unlinked to the CICU-cluster strains (patient isolates between years
156 2015-17), were included in the study (Table S2). Two clinical isolates were also
157 included as controls, URMC 201 (intermediate susceptible to imipenem) and URMC 223
158 (susceptible to all carbapenems tested). The AST profiles of the non-CICU CR-KA study
159 strains are described in Table 1.
160 All of the 26 sequenced URMC *K. aerogenes* genomes showed high coverage
161 (>88%) relative to the *K. aerogenes* reference KCTC 2190 strain (ATCC 13048^{T}),
162 Dataset 1. Single nucleotide polymorphisms (SNPs) were identified across the study
163 genomes relative to the reference sequence (pair-wise SNP differences ranged from 1-

164 28,170, see Dataset 2). MLST assignment indicated that all of the CICU clinical and
165 surveillance isolates belonged to ST4, and SNP-based analyses grouped them in a tight
166 cluster separately and distantly from non-outbreak isolates (Fig. 2, Dataset 2). Within
167 the CICU cluster, strains differed from URMC 205 (first case, patient A isolate) by less
168 than 7 SNPs. In addition, these isolates bore an identical plasmid profile (based on
169 replicon and plasmid typing) (Table S3). In contrast, all of the 2017 non-CICU isolates
170 were significantly distant from the CICU outbreak isolates (>20,000 SNPs). The most
171 closely related non-CICU CR-KA strain was URMC 201 (isolated in 2015). This strain
172 was also ST4, with 433 SNPs in a pairwise comparison to URMC 205. (Fig. 2, Dataset
173 2). These relationships, coupled with the epidemiological data, indicated that the 2017
174 CICU CR-KA strains were of clonal origin.
175
176 **Carbapenem resistance in the URMC CR-KA strains was driven by adaptive**
177 **chromosomal gene alterations**
178 **A. WGS based identification of acquired antibiotic resistance genes**
179 Despite phenotypic carbapenem resistance, the CICU CR-KA did not harbor
180 genes for carbapenemases, extended-spectrum β-lactamases, or plasmid-borne AmpC
181 cephalosporinases. A single non-outbreak CR-KA isolate (URMC 203) harbored a
182 carbapenemase gene (bla_{nmcA}). No horizontally acquired genes conferring resistance to
183 non-β-lactam antibiotics were identified among the study strains, consistent with their
184 susceptibility profiles (Table 1).
185 **B. Non-synonymous sequence alterations in key chromosomal loci**
186 **implicated in carbapenem resistance**

187 In the absence of genes encoding carbapenemases and ESBLs in the CR-KA
188 outbreak strains, variations in other genetic loci associated with carbapenemase-
189 independent resistance mechanisms were investigated (11-13). Focusing on the AmpC
190 cephalosporinase and outer membrane porins, sequence variations in *ampD, ampG,*
191 *ampR, omp35*, *omp36* and *ompR* genes in the study strains were assessed relative to
192 the 'wild-type' allele in the carbapenem-susceptible reference type strain KCTC 2190
193 (14). The sequences were also compared to alleles in URMC 223 (carbapenem
194 susceptible) and URMC 201 (intermediate susceptibility). For the *omp* genes, the
195 upstream DNA sequences were also assessed. The *ampG* and *ompR* genes were wild-
196 type in all study strains. Variants were identified in all other loci and are described
197 below:

198 ***ampD.*** Mutations were identified in the *ampD* gene for each of the 24 CR-KA
199 isolates in this study (Table 2), while the control strains URMC 223 and URMC 201 bore
200 the 'wild-type' *ampD* allele. The outbreak strains harbored single missense SNPs
201 (either 284G>T or 482G>A) in *ampD* resulting in Trp95Leu or Arg161His substitutions.
202 Six non-outbreak CR-KA strains harbored independent non-synonymous single
203 substitutions while 2 missense substitutions were identified in URMC 221. A single non-
204 outbreak strain, URMC 202, harbored a nonsense mutation resulting in a truncated
205 AmpD protein (Table 2). DNA sequence corresponding to *ampD* allele was absent in
206 isolate URMC 203, (the only study strain to possess a carbapenemase-encoding gene
207 $bla_{\text{NMC-A}}$) due to a large deletion in the genomic region harboring *ampD* and the
208 neighboring *ampE* gene.

209 The potential impact of Trp95Leu or Arg161His substitutions on AmpD activity in
210 the outbreak strains was investigated by homology-based structural modeling of *K.*
211 *aerogenes* AmpD using a high-resolution crystal structure of *Citrobacter freundii* AmpD
212 (15), a close homolog (83.33% amino acid sequence identity). AmpD contains a
213 hydrophobic surface to accommodate its GlcNAc-anh-MurNAc ligand, which is made up
214 of tripeptide and glycan moieties. The tripeptide portion of GlcNAc-anh-MurNAc is
215 coordinated through three salt bridges between carboxyl groups on the tripeptide and
216 residues Arg71, Arg161 and Arg107, while the peptide backbone is oriented across the
217 hydrophobic surface. Trp95 forms a planar surface at the end of the ligand-binding
218 channel to position the diaminopimelate moiety at the distal end of the tripeptide.
219 Based on the *in silico K. aerogenes* AmpD model, the positively charged
220 guanidinium group of Arg161 forms two strong electrostatic interactions with the
221 carboxyl group of D-glutamine on the tripeptide portion of the ligand (Fig. 3). Mutation
222 of this residue to histidine was predicted to weaken ligand binding, likely affecting the
223 positioning of the ligand in the active site. In the Trp95Leu mutation, the hydrophobicity
224 in the region is preserved, but the shorter length of the leucine side chain leaves a gap
225 at the end of the binding channel likely affecting the positioning of the entire ligand (Fig.
226 3). These observations lend support that the missense mutations observed in the CICU
227 outbreak strains would alter ligand docking on AmpD likely reducing/inhibiting its
228 activity. Four out of the seven CR-KA non-outbreak strains had substitutions within
229 glycan and peptide interacting regions of AmpD (Table 2), suggesting that these
230 alterations might also impact activity.

231 ***ampR***. All of the outbreak strains harbored the reference *ampR* allele. Several
232 non-outbreak CR-KA strains harbored 2-5 substitutions that likely represent variant
233 alleles as they were also observed in the control carbapenem-susceptible strain URMC
234 223. A single non-outbreak CR-KA strain, URMC 210, bore a nonsense mutation in the
235 *ampR* gene resulting in a premature stop codon (Trp117X), likely resulting in a non-
236 functional truncated AmpR protein.

237 ***omp35* and *omp36.*** Among the 15 outbreak CR-KA strains, three different
238 *omp36* variants were identified relative to the wild-type allele (Table 3). An identical
239 profile of missense SNPs in these strains and the control strain, URMC 201
240 (intermediate carbapenem susceptibility) were observed relative to the reference
241 genome allele. Seven outbreak strains had additional mutations that resulted in
242 severely truncated proteins. Several non-outbreak CR-KA strains also harbored
243 missense SNPS of unclear significance. Two strains URMC 202 and URMC 204
244 harbored distinct frame-shift mutations yielding truncated Omp36 protein variants. A 42
245 base-pair region of high variation (nucleotides 680-724), corresponding to 15/16 amino
246 acid substitutions in loop L5 was observed in all the clinical isolates relative to the
247 reference sequence (Table 3, Fig. S2). The hypervariable region results in a different
248 charge profile in the region and has been previously reported in a *K. aerogenes* study
249 describing imipenem resistant clinical isolates (harboring ESBL TEM-24) from patients
250 in France (16). The predicted Omp36 protein in clinical strain URMC 221 and the
251 carbapenem susceptible control strain (URMC 223) bore 87% identity relative to the
252 reference genome Omp36 and were considered significantly distant variants (not
253 included in the comparative analyses).

254 All but one of our 24 CR-KA strains had the wild-type allele of *omp35*. A single
255 strain (URMC 202) bore a deletion in the N-terminal encoding region. The DNA
256 sequence upstream of the *omp35* gene was investigated to identify mutations in the
257 promoter sites of the strains, of which one (URMC 204) had a nucleotide difference of
258 unclear significance at the -22 position.

259 **Virulome analysis identifies a large pathogenicity-associated integrative and**
260 **conjugative element (ICE), ICE*Kp*10 in the CICU outbreak CR-KA strains**

261 The prolonged nature of the clonal CR-KA outbreak in our CICU led us to search
262 for putative virulence genes or pathogenicity loci, which could have promoted
263 persistence and transmission. A cluster of chromosomally encoded genes encoding
264 yersiniabactin (*ybt*) metallophore and colibactin (*clb*) genotoxin systems was identified
265 in all of the outbreak strains (Fig. 4). These loci have been implicated in invasive
266 infections of *K. pneumoniae* (17).

267 The *ybt* locus harbored putative genes involved in regulation as well as synthesis
268 of the siderophore, corresponding transport associated proteins, and a receptor protein
269 for the uptake of metal-bound siderophore. The *clb* locus included putative homologs
270 encoding enzymes, transferases and transport proteins involved in production and
271 secretion of the polyketide colibactin (Fig. 4, Dataset 3). Investigation of the genomic
272 loci associated with the virulence factor gene cluster identified them to be present on a
273 mobilizable integrative conjugative element (ICE) inserted in a tRNA-Asn site adjacent
274 to a gene encoding the glycine cleavage system (Fig. 4). The ICE element bore a
275 modular arrangement of gene clusters encoding mobile elements, P4 like integrase,
276 type IV secretion system conjugation machinery and mobilization genes (Fig. 4, Dataset

277 3). The element was identified to be ICE*Kp*10, using a recently described typing and
278 interpretation scheme of *ybt* and *clb* sequences (17).
279 Among the URMC non-outbreak *K. aerogenes* strains, these putative virulence
280 genes were identified in only 4/10 isolates, which were either ST4 or ST93. The control
281 strain URMC 223 harbored the yersiniabactin locus exclusively. Detailed descriptions of
282 these elements in the URMC *K. aerogenes* strains are described in Table S4.
283 **Comparative analyses of URMC CR-KA and publicly available *K. aerogenes***
284 **genomes**
285 To gain insights into the emergence and epidemiology of the CICU outbreak
286 clones and to place our hospital CR-KA strains in the broader context of global *K.*
287 *aerogenes* strains, comparative phylogenomic analyses were performed using Harvest
288 genomics suite (18). Publicly available *K. aerogenes* genome assemblies (n=110) were
289 included in the analyses. These included 70 clinical and surveillance strains isolated
290 from human specimens, 3 'environmental' strains, and 36 strains of unknown origin
291 (Dataset 4). Based on the newly described MLST scheme, ST4 and ST93 strains were
292 found to be markedly overrepresented in the available genomes (51.8%, 57/110).
293 Excellent correlation was observed between Harvest generated tree topologies
294 (Fig. 5) as well as pairwise SNP differences (Dataset 5) as compared to those
295 generated by the CFSAN SNP pipeline, for the URMC CR-KA study strains. Based on
296 Harvest analyses, the CICU outbreak strains clustered closely with each other and to
297 other global ST4 genomes, as compared to the other URMC CR-KA strains (outbreak-
298 unrelated), which were distantly dispersed throughout the phylogenomic distribution
299 (Fig. 5). The MLST based sequence types of URMC CR-KA and the global *K.*

300 *aerogenes* genomes also correlated tightly with HARVEST generated core-genome
301 based topologies (Fig. 5).
302 Six publicly available assembled genomes grouped closely with the CICU
303 outbreak strain genomes (< 200 SNPs apart). These included ST4 strains, UCI 27, UCI
304 28, UCI 45, which have been described in a carbapenem resistance surveillance study
305 by Cerqueria et al. (19), and GN04794, GN05662 and GN02525; strains derived from
306 varied US patients' clinical specimens (blood, sputum, wound drainage) Fig. 5, Dataset
307 4). The above-mentioned strains had fewer SNP differences in relation to the CICU
308 outbreak strains as compared to URMC 201, the closest and only non-outbreak ST4
309 strain isolated in our hospital (Fig. 5, Dataset 5).
310 Carbapenemase encoding genes were identified in a total 13/111 global *K.*
311 *aerogenes* genome assemblies (11.8%) (Fig. 5). These included genes encoding KPC-
312 2 (n=7), KPC-3 (n=1), OXA-48 (n=4) and NDM-6 (n=1). The chromosomal serine
313 carbapenemase, $bla_{\text{NMC-A,}}$ was solely present in URMC 203, a CR-KA strain isolated
314 from a patient in our hospital in 2015. Among the thirty ST4 genomes, 4 strains (13%)
315 harbored genes encoding carbapenemases (4/4, KPC-2), and these were clinical
316 strains isolated from non-USA patients. The relative contribution of carbapenemase
317 mediated versus non-carbapenemase mediated mechanisms of resistance to
318 carbapenems in the global *K. aerogenes* could not be assessed due to the absence of
319 antibiotic susceptibility metadata for most strains.
320 A characteristic genomic feature of the URMC CICU outbreak strains and a
321 subset of non-outbreak associated strains was the presence of the yersiniabactin
322 siderophore and colibactin systems. Using Kleborate (17), we investigated the

323 distribution and organization of these systems in the global *K. aerogenes* genomes to
324 identify associations, if any, with specific ST types and geographical regions (Fig. 5,
325 Datasets 6). The prevalence of yersiniabactin and colibactin encoding systems in the
326 global *K. aerogenes* genomes was found to be 53.64% (59/110) and 52.72% (58/111)
327 respectively. A 100 % association was found between the presence of colibactin in the
328 genomes and concurrent presence of yersiniabactin. Higher prevalence of the virulence
329 cluster was observed in ST4 and ST93 types; 85% (12/14) and 95% (42/44)
330 respectively, although these two ST types were also the most abundantly represented in
331 the available set of genomes. The other ST types were less well represented in the
332 study set (n<3), so the prevalence of these systems in them could not be accurately
333 established. Interestingly, the strains exclusively designated 'environmental' isolates
334 (B3, FGI35, and B) did not harbor genes encoding the above-mentioned virulence
335 systems (Fig. 5, Dataset 6). These trends correlated with analyses of our 26 hospital
336 strains, where 75% of the ST 93 strains (3/4) were positive for yersiniabactin and
337 colibactin systems, while none of the strains with novel/unassigned ST types harbored
338 genes encoding the same (0%, 5/5).
339

340 **DISCUSSION**

341 Whole genome sequencing presents a powerful resource that can be deployed
342 for prospective and comprehensive outbreak investigations offering tremendous
343 resolution in tracking transmission events and delineating genomic determinants
344 associated with drug resistance and virulence (20). This WGS study was initiated in
345 order to establish the molecular epidemiology of CR-KA strains isolated from patients in

346 our hospital. Beyond the outbreak investigation, whole genome data enabled us to
347 address additional fundamental questions associated with carbapenem resistance,
348 virulence attributes and population structure of *K. aerogenes* that are poorly understood.
349 Fifteen patients were associated with the CICU outbreak event in the period
350 between July-November 2017. Based on the differences in AST patterns in a subset of
351 study strains, it was initially questioned whether the strains were clonal. WGS
352 investigations demonstrated that the 15 CICU CR-KA isolates differed from each other
353 by less than 7 SNPs and grouped distantly from the other hospital CR-KA isolates,
354 which were used a baseline for context and comparison (Fig. 2). Based on these
355 findings, the CICU CR-KA strains were concluded to be part of a single clonal cluster
356 indicating protracted intra-ward transmission. Environmental sampling within the CICU
357 remained negative and a common source of the outbreak or contributory risk factors
358 could not be identified. After the last positive patient surveillance culture (Nov 2017), no
359 CR-KA strains were isolated in a 2-month period of continued surveillance and the
360 outbreak was deemed to have subsided. Phylogenomic analysis (albeit limited) of the
361 non-CICU CR-KA isolates did not identify specific dominant clones circulating in the
362 hospital (Fig. 2, Dataset 2).
363 Infections due to carbapenem-resistant organisms present complex diagnostic
364 and therapeutic management challenges and a better understanding of how adaptive or
365 acquired resistance emerges in the healthcare environment is needed (21). In clinical
366 CR-KA strains, carbapenem resistance has been associated with carbapenemase
367 production or adaptive mutations following antibiotic exposure (2). Among our 24 study
368 CR-KA isolates, a single non-CICU strain harbored a carbapenemase-encoding gene,

369 *bla*$_{NMC-A}$. NmcA has been reported in *E. cloacae* (22), but to our knowledge this is the
370 first report of this carbapenemase being identified in *K. aerogenes*. All but one of the 24
371 CR-KA strains were found to harbor mutations in genes involved in the synthesis or
372 regulation of the inducible AmpC cephalosporinase (*ampD, ampR*) and outer membrane
373 porins (*omp 35, omp36*). A majority of mutations were found to be within the open-
374 reading frames of *ampD* and *omp36* (catalogued in Tables 2 and 3).
375 There are limited reports regarding the role of AmpD in adaptive carbapenem
376 resistance in *K. aerogenes* strains (23, 24), although the role of AmpD in AmpC
377 expression in *E. cloacae* has been well established (25). *In silico* modeling of *K.*
378 *aerogenes* AmpD using the *C. freundii* AmpD crystal structure (15) predicted that
379 Arg161His and Trp95Leu substitutions due to SNPs in the outbreak strains would likely
380 impact enzymatic activity (Fig. 3). Other *ampD* mutations in several non-CICU CR-KA
381 isolates were found to result in substitutions that could affect substrate-enzyme
382 interactions (Table 2).
383 Mutations in *omp36* have been described in clinical CR-KA strains and functional
384 studies investigating their impact have been reported (6, 26). A diverse array of
385 mutations was identified in the *omp36* gene among the CR-KA study strains, including
386 non-synonymous mutations resulting in frame-shifts or premature stop codons resulting
387 in truncated and likely non-functional Omp36 variants (Table 3). Additional SNPs
388 relative to the carbapenem-susceptible reference genome that resulted in substitutions
389 of unknown significance in predicted β-sheets and extracellular loop regions (Table 3,
390 Fig. S2).

391 Even within the CICU CR-KA clonal cluster, heterogeneity was identified in *ampD*
392 and *omp36* genes (Table 2, Table 3). These genetic loci likely represent mutational
393 hotspots associated with adaptive carbapenem resistance in *K. aerogenes.* It is likely
394 that the CICU CR-KA cluster represents a population of clonal origin, albeit with micro-
395 heterogeneity in the above regions. The testing and archiving of single isolated
396 carbapenem resistant colonies instead of multiples during individual patient specimen
397 workup likely represents a limitation that did not allow us to capture the full complement
398 of CR-KA strain microdiversity associated with individual patients during the outbreak
399 event.
400 The detailed significance of the alleles and mutations described above in
401 development of carbapenem resistance in *K. aerogenes* needs to be verified by
402 additional genetic (allelic exchange and complementation) and biochemical approaches.
403 Moreover, complex regulatory networks including transcriptional activators, sensor
404 kinases and two-component systems have been implicated in adaptive drug resistance
405 in *Enterobacteriaceae spp*. (13), and their roles in contributing towards carbapenem
406 resistance in our study strains cannot be ruled out.
407 Despite their lack of horizontally acquired drug resistance elements, the outbreak
408 strains in this study managed to persist for several months in the face of an active
409 infection prevention effort prompting us to assess their virulence determinants. The
410 outbreak strains and a subset of non-outbreak CR-KA strains harbored an ICE
411 encoding the metallophore yersiniabactin (Ybt) and genotoxin colibactin (Clb)
412 systems (Fig. 4, Table S4). A Ybt encoding pathogenicity island has been described in
413 association with a prolonged nation-wide outbreak in the Netherlands involving multiple

414 hospitals and >100 patients due to a multi-drug resistant *Enterobacter hormaechei*
415 clone (27). A recent study by Lam *et al.* described the prevalence of *ybt* to be
416 particularly high (>80%) in certain hypervirulent *K. pneumoniae* clonal-groups (CG23)
417 (17). The study also reported a significant association of Ybt with an increased risk of
418 invasive infections (bacteremia, liver abscesses etc.). Ybt was first described in
419 pathogenic *Yersinia spp.*, encoded by a chromosomal gene cluster termed the High
420 Pathogenicity Island (HPI), with a critical role in iron scavenging during infection (28).
421 Subsequent studies reported acquisition of the HPI in other clinical *Enterobacteriaceae*
422 strains (17, 29) and additional functions ascribed to Ybt include evasion of host
423 lipocalin-2 (30) and sequestration/import of heavy metals (31). The polyketide colibactin
424 is frequently associated with yersiniabactin and has been shown to induce
425 chromosomal instability and DNA damage in eukaryotic cells (32). Our findings present
426 new avenues for research investigating the role of ICE elements encoding Ybt and Clb
427 in the virulence of clinical *K. aerogenes* strains. While horizontal transmission of
428 carbapenemases via mobile elements is increasingly recognized as a major public
429 health issue (33), the transmission of mobilizable virulence factors present an
430 underappreciated threat in the healthcare environment that warrants more surveillance.
431 In order to set our hospital strains (outbreak and non-outbreak) into a broader
432 context, core-genome comparisons and MLST were used to examine the population
433 structure of our strains relative to global *K. aerogenes* strains pulled from public
434 databases (Fig. 5, Datasets 5, 6). This analysis is the first evaluation of the nascent *K.*
435 *aerogenes* MLST scheme in discriminating clinical *K. aerogenes* isolates. The scheme
436 was found to be robust with distribution of STs correlating closely with topologies based

437 on *K. aerogenes* strains core-genomes. Our preliminary analyses suggest that ST4 and
438 ST93 might be dominant global clones associated with *K. aerogenes* infections.
439 Outbreak CR-KA strains clustered closest to other clinical US ST4 strains, suggesting a
440 clonal expansion of this ST (Fig. 5). It is noteworthy that the ST4 group also included
441 carbapenemase-producing *K. aerogenes* isolates from international sites. These
442 included CR-KA strains associated with drug-resistant intra-abdominal and urinary tract
443 infections in patient samples from Brazil, Canada, Colombia and China that had been
444 sequenced as a part of large surveillance study, SMART (Study for Monitoring
445 Antimicrobial Resistance Trends) (34). ST93 was the most prevalent sequence type in
446 the global *K. aerogenes* assembled genomes (43/110, 39%), with a wide geographical
447 distribution including the US (Fig. 5). Four of the eleven non-CICU CR-KA strains from
448 our hospital belonged to this group. Two closely related ST93 isolates, *K. aerogenes*
449 1509E and G7, have been described as representatives of clonal strains associated
450 with multiple multi-drug resistant *K. aerogenes* outbreaks in France (35, 36).
451 Incidentally, global ST4 and ST93 isolates also had a higher prevalence of HPI
452 encoding *ybt* and *clb* (85% and 95% respectively). The success of these potentially
453 "high-risk" clones needs to be examined more closely by undertaking large-scale
454 studies with strains from diverse global sites and patient populations. These studies will
455 help examine niche adaptation, emergence of antibiotic resistance and evolution of
456 pathogenicity leading to a better understanding of *K. aerogenes.*
457 In summary, genomic approaches for surveillance and outbreak investigations
458 are emerging as critical functions for infection prevention and diagnostic microbiology
459 laboratories. Apart from evaluating the effectiveness of infection measures and

460 dimension of transmission events, WGS applied across sets of isolates is a powerful
461 tool for assessing virulence factors and identifying clinically relevant and emerging
462 sequence types.
463
464 **MATERIALS AND METHODS**
465 **Setting, study design, *K. aerogenes* strains and metadata.**
466 The University of Rochester Medical Center (URMC) is an 830-bed tertiary-care medical
467 center, with a 14-bed cardiothoracic intensive care unit, serving the Greater Rochester
468 Area, New York. Following approval by the University of Rochester Institutional Review
469 Board (RSRB00068143), a total of 26 *K. aerogenes* strains isolated from patients at
470 URMC in the course of regular clinical care and/or surveillance efforts were selected for
471 the study. Each isolate corresponded to a single first CR-KA strain isolated during the
472 course of hospitalization. For context and comparison, an additional set of *K.*
473 *aerogenes* strains epidemiologically unlinked to the CICU outbreak was included in the
474 study. Ward occupancy and pertinent clinical and epidemiological information were
475 obtained through review of patient medical records and the laboratory information
476 system, and are described in Tables S1 and S2.
477
478 **Antibiotic susceptibility testing (AST) of the study *K. aerogenes* strains**
479 AST of the study strains was performed as part of routine diagnostics using the
480 VITEK®2 (BioMérieux, France) system and/or Kirby Bauer disk-diffusion methods. AST
481 interpretations were based on interpretive criteria defined by the Clinical and Laboratory
482 Standards Institute (M100 document, 27th Edition).

483 **Sequencing library preparation and raw data acquisition**

484 The isolates were cultured on standard laboratory media from archived frozen stocks,
485 examined for purity, and re-identified by Vitek MALDI-TOF MS (BioMérieux, France).
486 Single colony genomic DNA extractions were performed using MagNA Pure Compact
487 instrument (Roche Diagnostics, Indianapolis, IN); the DNA quality was analyzed using
488 QuantiFluor dsDNA system (Promega). Nextera XT kit (Illumina, San Diego, CA) was
489 used for preparing dual-indexed WGS libraries. For QC dsDNA quality of individual
490 samples was analyzed using the 4200 TapeStation system (Agilent). Following library
491 cleanup, individual libraries were pooled at equimolar ratios and denatured; DNA
492 concentration was determined using Qubit ssDNA kit (Thermo fisher Scientific). The
493 libraries were combined with20ρM PhiX control and sequenced on the Illumina Miseq™
494 benchtop sequencer (Illumina, San Diego, CA) at the URMC Genomic Core Facilities,
495 using V3 kit and 2X300 bp paired-end protocol.

496

497 **Genomic analyses**

498 Analyses were performed using an in-house bioinformatics pipeline 'URMC Bacterial
499 Genomic Analysis Pipeline' (v2.0.6), run on a high-performance computer cluster at the
500 Center for Integrated Research Computing at University of Rochester. **A) Quality**
501 **control:** For each sample, the read quality scores across all bases were assessed
502 using FastQC v0.11.5 (37) and low quality reads were trimmed using Trimmomatic
503 v0.36 (38). Genomic coverage of the sequencing reads relative to the reference *K.*
504 *aerogenes* KCTC 2190 genome (ATCC 13048^{T}; Refseq accession
505 number: NC_015663.1) were determined using Bowtie 2 v2.2.9 (39). Across the study

506 isolates, alignments showed average genomic coverage of 90.84% (minimum 89%) with
507 an average depth of coverage: 100X (excluding regions below 12X). Quality metrics
508 are detailed in Dataset 1. **B) Core-genome SNP calling workflow:** A read mapping
509 approach was used to assess SNPs in the genomes of the study CR-KA strains relative
510 to the reference genome *K. aerogenes* KCTC 2190. Mapping, variant calling and
511 phylogenetic analysis were performed by locally installed CFSAN SNP Pipeline v1.0.0
512 (40), an analysis workflow developed by the U.S Food and Drug Administration (FDA).
513 CFSAN pipeline employs a 2-phase variant calling workflow and the 'optimized' version
514 of the pipeline with criteria as applied by Saltykova et al (higher stringency in allele
515 frequency thresholds and coverage) (41) was applied. In the first phase, variants were
516 called based on *mpileup function* of SAMTools and *mpileup2snp tool* from VarScan
517 (minimum average base quality=20, minimum read depth of coverage at site=12,
518 minimum allele frequency=90%). Densely clustered SNPs that could arise due to
519 recombination were excluded (>/=3 SNPs in a 50 bp window). High-confidence SNP
520 variants meeting the criteria were composed to a list. In the 2nd phase, nucleotide sites
521 at the listed positions were determined for all sites (minimum allele frequency threshold
522 for SNP filtering=90%). SNPs located in mobile elements as annotated by RAST were
523 excluded. The final nucleotide sites post filtering corresponding to the listed positions
524 were used to build a SNP matrix. A multi FASTA file with concatenated SNP matrix
525 entries were used for inferring phylogeny by FastTree (42), which were visualized using
526 ITOL (43). **C) Sequence assembly, scaffolding, annotation and analyses:** De novo
527 genome assemblies (average N_{50}: 344943 across all isolates) were generated by
528 SPAdes genome Assembler v3.11.1 (44), and the assembly quality was assessed by

529 QUAST v4.5 (45). Ordering and orientation of contigs was performed using Medusa .
530 Draft genomes were annotated with RAST (46) and Prokka (47). MLST on the *K.*
531 *aerogenes* genomes was performed using a newly developed publicly available scheme
532 (10). Alleles and genetic markers corresponding to acquired antibiotic resistance,
533 virulence and plasmid replicons were identified using PlasmidFinder (48), ResFinder
534 (49), and Virulence Factor Database (50). Typing of genetic loci associated with
535 mobilizable yersiniabactin siderophore and genotoxin colibactin systems was performed
536 using Kleborate (17), and the loci were visualized with MacVector software (MacVector,
537 Cary, NC). Sequence variations in chromosomal genes associated with carbapenem
538 resistance in the study strains were assessed relative to alleles in control strains
539 (URMC 201, URMC 223) and 'wild-type' alleles in carbapenem susceptible (14)
540 reference genome KCTC 2190 [Genbank ID-*ampD*: 10792472 (EAE_11350), *ampG*:
541 10792757 (EAE_12735), *ampR*: 10792632 (EAE_12115), *omp35*: 10793271
542 (EAE_15245), *omp36*: 10795060 (EAE_24205) and *ompR*: 10791222 (EAE_05245)].
543 Multiple sequence alignments of gene alleles and corresponding proteins was
544 performed using Vector NTI software (Invitrogen, Carlsbad, CA) and the alignments
545 were manually inspected to identify substitutions that would likely impact function. **D)**
546 **Comparative genomics of publicly available global *K. aerogenes* assembled**
547 **genomes with URMC CR-KA assemblies**: Using a custom shell script, publicly
548 available *K. aerogenes* genomes available as of July 2018 in the NCBI genome
549 database were downloaded from the NCBI FTP site. Harvest genomics suite was used
550 to perform intraspecific core-genome alignments as described before (18); phylogenies
551 were visualized using ITOL (43). **E) Accession number(s):** WGS and metadata

552 corresponding to the study URMC *K. aerogenes* isolates were deposited at NCBI under
553 BioProject accession number PRJNA504784. Accession numbers for individual isolates
554 are listed in Table S1.
555
556 ***In silico* protein analyses**
557 For homology modeling, SWISS-MODEL (51) was used to thread *K. aerogenes*
558 AmpD (acc WP_015704411.1, aa1-187) through the structure of *C. freundii* AmpD (15)
559 (PDB 2y2c, aa1-187). The overall quaternary structure of *K. aerogenes* AmpD was
560 predicted with high precision (95% confidence, 99% coverage). Comparative analyses
561 and imaging of protein structures were performed with PyMOL (52). JalView was used
562 to create alignments (53).
563
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573
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FIGURE LEGENDS

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

Fig. 2. Dendogram showing pairwise SNP differences based phylogenetic relatedness of URMC *K. aerogenes* strains. Whole genome sequence of *K. aerogenes* KCTC 2190 (ATCC 13048) was used for reference mapping. A total of 28,170 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: IDs highlighted in red, clinical isolates in bold, patient A isolate (1st case) with yellow background. Year strain isolated: orange-2015, yellow-2016, blue-2017. SNP differences relative to patient A shown. Scale bar indicates nucleotide substitutions per site.

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

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 grey arrows-genes encoding mobilization proteins, pink arrows: vir-T4SS system.

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 in purple (outbreak strains in bold, index patient strain highlighted yellow). Presence of genes encoding yersiniabactin siderophore system (green squares), colibactin synthesis cluster (blue triangles), and carbapenamases (red circles) in assembled genomes shown. Scale bar indicates nucleotide substitutions per site.

Table 1. Phenotypic antibiotic susceptibility profiles of *K. aerogenes* strains in this study

Study Isolates		Amikacin	Gentamicin	Tobramycin	Ciprofloxacin	Moxifloxacin	Trimethoprim-Sulfa	Piperacillin-Tazobactam	Ceftriaxone	Cefepime	Ertapenem	Imipenem	Meropenem
2017 URMC CICU associated CR-KA strains	**URMC 205***	S	S	S	S	S	S	R	R	S	R	R	R
	URMC 206	S	S	S	S	S	S	R	R	S	R	R	R
	URMC 207	S	S	S	S	S	S	R	R	S	R	R	R
	URMC 208	S	S	S	S	S	S	R	R	S	S	S	R
	URMC 209	S	S	S	S	S	S	R	R	S	R	R	R
	URMC 211	S	S	S	S	S	S	R	R	D	R	R	R
	URMC 212	S	S	S	S	S	S	R	R	R	R	I	R
	URMC 213	S	S	S	S	S	S	R	R	R	R	I	R
	URMC 215	S	S	S	S	S	S	R	R	S	R	R	R
	URMC 216	S	S	S	S	S	S	R	R	S	R	S	S
	URMC 218	S	S	S	S	S	S	R	R	S	R	R	R
	URMC 219	S	S	S	S	S	S	R	R	S	R	R	R
	URMC 224	nd	S	S	S	S	S	R	R	R	R	nd	R
	URMC 225	S	S	S	S	S	S	R	R	R	R	R	R
	URMC 226	S	S	S	S	S	S	R	R	R	R	I	I
Other URMC CR-KA strains	URMC 200	S	S	S	S	nd	S	R	R	R	R	nd	R
	URMC 202	S	S	S	S	S	S	R	R	S	R	R	R
	URMC 203	S	S	S	S	S	S	R	R	S	R	R	R
	URMC 204	S	S	S	S	nd	S	R	R	S	R	nd	R
	URMC 210	S	S	S	S	nd	S	R	R	S	R	nd	S
	URMC 214	S	S	S	S	nd	S	I	R	S	R	I	S
	URMC 217	S	S	S	R	nd	S	R	R	S	R	I	S
	URMC 221	S	S	S	S	nd	S	S	S	S	R	nd	S
	URMC 222	S	S	S	S	S	S	R	R	R	R	I	S
Con	URMC 201	S	S	S	S	S	S	S	S	S	S	I	S
	URMC 223	S	S	S	S	nd	S	S	S	S	S	nd	S

R=Resistant, S= Sensitive, I =Intermediate, D= Dose Dependent, nd=not determined; *Patient A (1st case)-URMC 205; Con: control strains: URMC 223, carbapenem susceptible and URMC 201, intermediate carbapenem resistance

Table 2. Non-synonymous SNPs in *ampD* gene associated with carbapenem resistance in the URMC *K. aerogenes* isolates

Study Isolates		Differences in *ampD* gene relative to wild-type allele		Effect on encoded AmpD sequence	
		MS SNPs	NS SNPs	Single AA substitutions	PS
CICU outbreak associated CR-KA strains	URMC 205*, URMC 207, URMC 209, URMC 211, URMC 212, URMC 213, URMC 215, URMC 218, URMC 219, URMC 224, URMC 224, URMC 226	482G>A		Arg161His	
	URMC 206, URMC 208, URMC 216	284G>T		Trp95Leu	
Outbreak unrelated CR-KA strains	URMC 200	338T>G		Ile113Ser	
	URMC 202		412C>T		Gln138X
	URMC 204	117C>T		Pro39Ser	
	URMC 210	335C>T		Ser112Leu	
	URMC 214	280G>A		Ala94Thr	
	URMC 217	496G>C		Gly166Arg	
	URMC 221	478A>G, 501C>A		Ile160Val, Ala168Asp	
	URMC 222	492T>G		Glu164Asp	
Control strains	URMC 201, URMC 223				

No sequencing reads mapped to the *ampD* gene in URMC 203.

*Index patient isolate.

Abbr: SNP: single nucleotide polymorphism, MS: missense, NS: nonsense, AA: amino acids, PS: premature stop codon

Table 3. Non-synonymous genetic lesions in *omp36* genes and resulting alterations in Omp36 sequence in the URMC *K. aerogenes* isolates

Study Isolates		Differences in *omp36* gene sequence relative to wild-type allele				Effect on encoded Omp36 amino acid sequence relative to wild-type sequence				
		FS	HV region (Nt 680-724)	NS SNPs	MS SNPs	FS	HV region (AA 226-241)	PS	Truncated	Single AA substitions in protein synthesized
CICU outbreak associated CR-KA strains	URMC 205*, URMC 207	+	+		175A>G, 564T>C, 566A>G	pAsp91ThrfsX12		102X	+	Ile59Val
	URMC 206, URMC 208, URMC 212, URMC 213, URMC 216, URMC 224, URMC 225, URMC 226		+		175A>G, 564T>C, 566A>G		+			Ile59Val, Asp189Gly
	URMC 209, URMC 211, URMC 215, URMC 218, URMC 219		+	184A>T	175A>G, 564T>C, 566A>G			62X	+	Ile59Val
Outbreak unrelated CR-KA strains	URMC 200, URMC 203, URMC 210, URMC 214		+		564T>C, 566A>G, 615 T>G, 834T>C, 835A>G		+			Asp189Gly, Asp205Glu, Asn279Asp
	URMC 202	+	+		564T>C, 566A>G, 615 T>G, 834T>C, 835A>G	pTrp77LysfsX2		78X	+	Trp77Lys
	URMC 204	+	+		564T>C, 566A>G, 615 T>G, 834T>C, 835A>G	pSer304ProfsX21	+	324X	+	Asp189Gly, Asp205Glu, Asn279Asp
	URMC 217		+		564T>C, 566A>G, 569T>A		+			Asp189Gly, Phe190Tyr
	URMC 222		+		175A>G,564T>C, 566A>G, 615 T>G		+			Ile59Val, Asp189Gly, Asp205Glu
Control strain	URMC 201 (intermediate carbapenem R)		+		175A>G,564T>C, 566A>G		+			Ile59Val, Asp189Gly

*Index patient isolate.

Abbr: SNP: single nucleotide polymorphism, NS: nonsense, MS; missense, FS: frame-shift, PS: premature stop codon, HV: hypervariable region, Nt: nucleotides, AA: amino acids

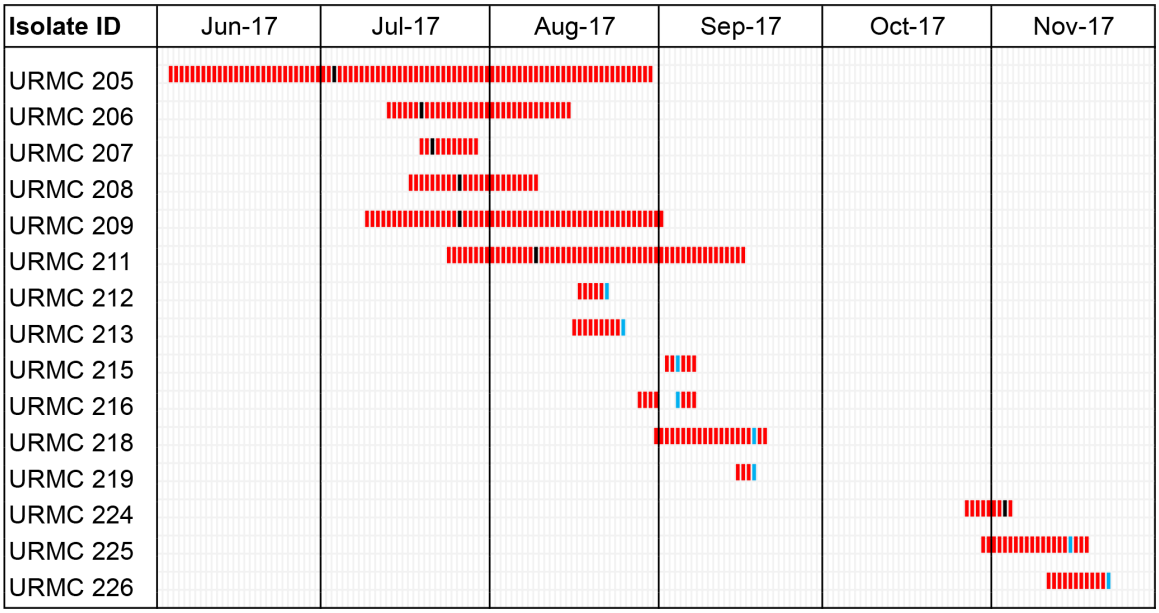

Fig. 1. Patient occupancy and overlap in CICU ward during the *K. aerogenes* outbreak. Black bars-first positive CR-KA clinical cultures, blue bars-first positive CR-KA surveillance cultures

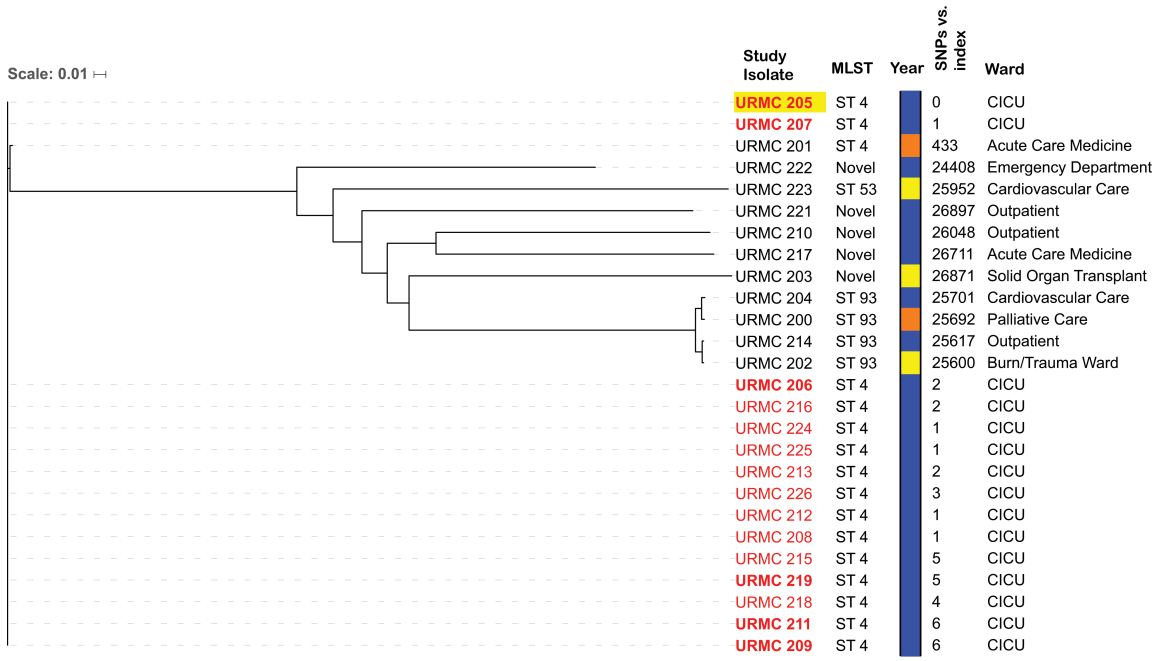

Fig. 2. Dendogram showing pairwise SNP differences based phylogenetic relatedness of URMC *K. aerogenes* strains. Whole genome sequence of *K. aerogenes* KCTC 2190 (ATCC 13048) was used for reference mapping. A total of 28,170 discriminatory high-quality SNPs in the core genomes obtained by the adapted CFSAN pipeline were used to plot the tree (excluding mobile elements and putative recombination sites). CICU outbreak strains: IDs highlighted in red, clinical isolates in bold, patient A isolate (1st case) with yellow background. Year strain isolated: orange-2015, yellow-2016, blue-2017. SNP differences relative to patient A shown. Scale bar indicates nucleotide substitutions per site.

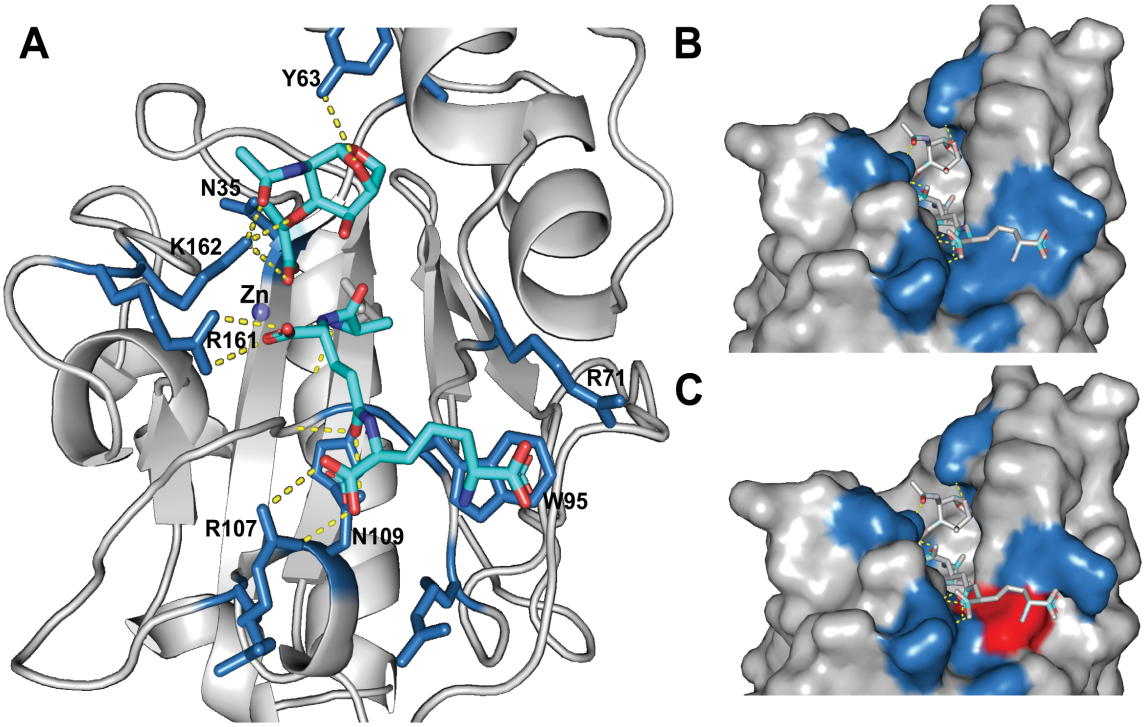

Fig. 3. Computational modeling of the impact of *ampD* mutations on AmpD in URMC outbreak CR-KA strains. A) *K. aerogenes* AmpD modeled on *C. freundii* AmpD structure. Key residues interacting with glycan and peptide portions of ligand are shown. B, C) Surface models of *K. aerogenes* AmpD depicting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

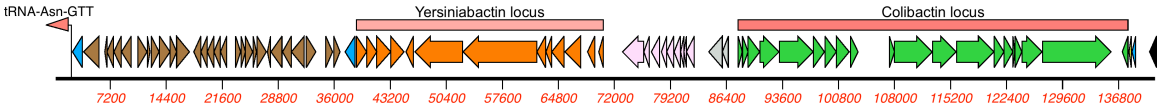

Fig. 4. Yersiniabactin and colibactin encoding gene loci on integrative conjugative element ICE*Kp*10 in the CICU outbreak CR-KA strains. Blue arrows-integrase encoding genes, brown arrows-Zn^{2+}/Mn^{2+}modules grey arrows-genes encoding mobilization proteins, pink arrows: vir-T4SS system.

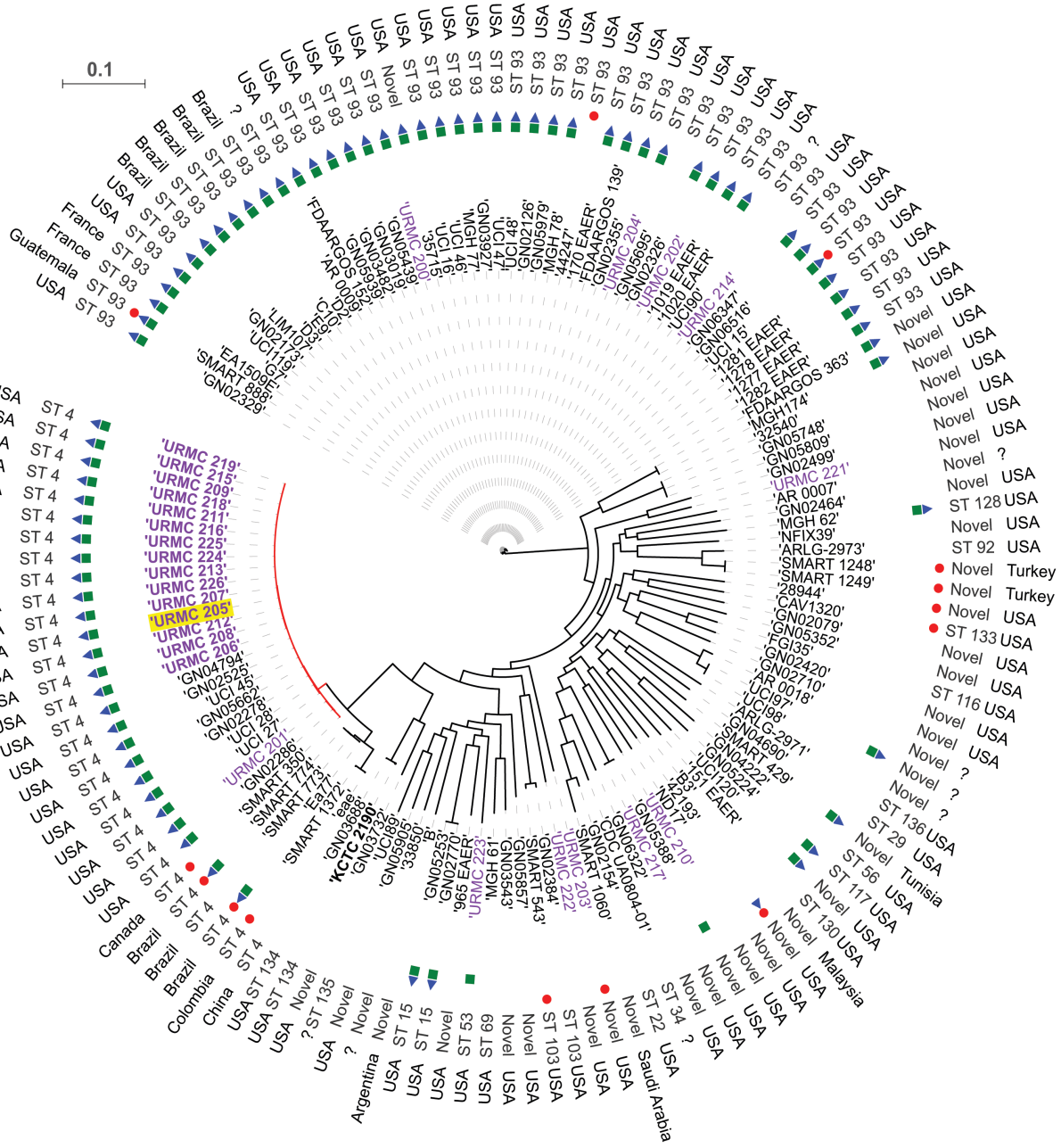

Fig. 5. Harvest based phylogenomic comparisons of URMC *K. aerogenes* genomes with global *K. aerogenes* genomes. Discriminatory SNPs based on core genome comparisons were used to plot the tree. URMC study *K. aerogenes* strains in purple (outbreak strains in bold, index patient strain highlighted yellow).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