Prevalence of Hypervirulence-Associated Pathogenicity Loci Among *Klebsiella pneumoniae* Bloodstream Isolates at a United States Hospital

Travis J. Kochan[1], Natalia Khalatyan[1], Bettina H. Cheung[1], Sophie H. Nozick[1], Egon A. Ozer[2], Alan R. Hauser[1,2]

[1]Department of Microbiology-Immunology, Northwestern University, Feinberg School of Medicine, Chicago, IL, USA

[2]Division of Infectious Diseases, Department of Medicine, Northwestern University, Feinberg School of Medicine, Chicago, IL, USA

Abstract

Klebsiella pneumoniae is a major threat to human health worldwide. “Classical” *K. pneumoniae* (cKp) strains commonly cause multidrug-resistant (MDR) infections in debilitated patients residing in hospitals and long-term care facilities. In contrast, hypervirulent *K. pneumoniae* (hvKp) strains may cause invasive, community-acquired infections in otherwise healthy individuals. These highly virulent strains were first identified in Taiwan in 1986 and have since disseminated across the globe. The prevalence of hvKp has been well described in Southeast Asia, but is not well understood in the United States. In this work, 141 consecutive *K. pneumoniae* blood stream isolates were collected at Northwestern Memorial Hospital from 2015-2017. To screen this collection for hvKp, isolates were tested for the hypermucoviscous phenotype and the presence of aerobactin and salmochelin biosynthesis genes, biomarkers that have been used to distinguish hvKp from cKp. These biomarkers were identified in 12 (8.5%), 6 (4.3%), and 5 (3.5%) isolates, respectively. Whole genome

sequencing was performed on the six isolates that contained aerobactin biosynthesis genes and demonstrated that these genes were contained on large plasmids. Five isolates contained plasmids nearly identical to previously described hvKp virulence plasmids while one was similar to a plasmid from a swine isolate of *K. pneumoniae*. These findings indicate that several non-clonal hvKp-like strains are present in the U.S.

Introduction

Klebsiella pneumoniae (Kp) is a leading cause of nosocomial infections worldwide including pneumonia, bloodstream, and urinary tract infections (1). Kp can cause severe, secondary pneumonia in patients with respiratory viral infections and was recently identified as a leading cause of secondary pneumonia in patients with the novel coronavirus SARS-CoV-2 (2). Kp pathogenicity is due to a variety of virulence factors, including siderophores, fimbriae, and a polysaccharide capsule of various types. The polysaccharide capsule is the major virulence factor for Kp as it allows this bacterium to evade host immune defenses, such as complement-mediated phagocytosis (3, 4). The most common type of Kp are referred to as classical strains (cKp), which infect debilitated patients residing in hospitals and long-term care facilities, (5). cKp strains are increasingly resistant to antibiotics, including carbapenems (6-11). Genes conferring antimicrobial resistance are often carried by conjugative plasmids allowing for extensive dissemination among cKp strains. Thus, it is not surprising that the IDSA, WHO, and the CDC have each deemed Kp as a serious public health priority in need of new therapeutic development (12-14). As such, multidrug-resistant (MDR) cKp strains have been the focus of research within the United States.

A second type of Kp, referred to as hypervirulent Kp (hvKp), was first identified in Taiwan in 1986 as a common cause of pyogenic liver abscesses (PLA) in young, otherwise healthy individuals living in the community (15-22). These strains are now

50 common in Asia, but little is known about their prevalence in the U.S. Case reports
51 document the presence of hvKp infections and PLA in North America, but only a few
52 surveillance studies have been performed (21, 24-26). hvKp strains frequently express a
53 highly mucoid capsule, leading to a “hypermucoviscous” colony phenotype, and are
54 considerably more virulent in mouse models than cKp strains (23). Their increased
55 virulence is usually attributed to the presence of one of a number of large, non-
56 conjugative, “hypervirulence” plasmids (27-29). The limitations imposed by the hyper-
57 mucoid capsule on genetic exchange and the non-conjugative nature of these virulence
58 plasmids may restrict the hypervirulence phenotype to few sequence types (e.g. ST23,
59 ST86, ST66) and capsule types (e.g. K1, K2). The best characterized hypervirulence
60 plasmids are pK2044 and KP52.145pII (Fig 1A, B) (29, 30). In addition, we previously
61 identified a unique plasmid with hvKp features, pTK421_2 (Fig 1C), in a bloodstream
62 isolate from Northwestern Memorial Hospital (NMH), suggesting considerable diversity
63 among the hypervirulence plasmids of hvKp (31). Each hypervirulence plasmid contains
64 two distinct pathogenicity loci (PAL-1 and PAL-2) (Fig 1D). PAL-1 usually consists of a
65 mucoid regulator gene (*rmpA2*), the aerobactin receptor gene (*iutA*), and aerobactin
66 biosynthesis genes (*iucABCD*). PAL-2 usually consists of a distinct mucoid regulator
67 operon (*rmpADC*), the salmochelin receptor gene (*iroN*), and salmochelin biosynthesis
68 genes (*iroBD*) (Fig 1D). Plasmids KP52.145pII and pTK421_2 contain an incomplete
69 PAL-1 that lacks *rmpA2* (Fig 1D). The mucoid regulators *rmpA* and *rmpA2* play a major
70 role in virulence by increasing capsule production and hypermucoviscosity (28), but the
71 roles of the different siderophores (aerobactin, salmochelin, and yersiniabactin) in hvKp
72 virulence remain controversial (30, 32). A recent report suggested that aerobactin was
73 the only siderophore required for the hypervirulent phenotype of the ST86-K2 capsule
74 type strain hvKp1 (32). In contrast, another report showed that only a triple siderophore
75 mutant (yersiniabactin, aerobactin, and salmochelin) of the ST23-K1 strain NTUH-K2044

76 was attenuated in the mouse intraperitoneal model (30). In addition, strain ATCC43816
77 (also called KPPR1) is hypervirulent in mice (4, 33) but is otherwise atypical in that it
78 lacks a hypervirulence plasmid and PAL-1 but contains a version of PAL-2 integrated
79 into a chromosomal integrative and conjugative element (ICEKp1, Fig 1D). In KPPR1,
80 yersiniabactin promoted disease in the lung and salmochelin promoted dissemination to
81 tissues (34). Thus, it is clear that the mucoid regulators and siderophores play roles in
82 conferring the hypervirulent phenotype, but significant strain-to-strain variability of
83 virulence factors exists.

84 A microbiological definition of hvKp has remained elusive. Initially,
85 hypermucoviscosity was used as a proxy for the hvKp phenotype. However, several
86 reports have described hypermucoviscous Kp strains that are not hypervirulent in mice
87 and lack pathogenicity loci usually associated with hvKp (35-37). As a result, genetic
88 biomarkers have been used to define hvKp, but these definitions have varied from study-
89 to-study. Recently, Russo and colleagues systematically examined the accuracy of using
90 *rmpA*, *rmpA2*, *peg-344*, *iucA*, and *iroB,* genes found on virulence plasmids, as diagnostic
91 biomarkers for hvKp (23, 38). In their cohort of 175 isolates, the presence of *iucA* or *iroB*
92 or a hypermucoviscosity phenotype distinguished hvKp from cKp with an accuracy of
93 96%, 97%, and 90%, respectively. Here, we use these biomarkers to describe the
94 prevalence of hvKp at NMH in Chicago, Illinois. From 2015 – 2017, we collected 141
95 consecutive Kp bloodstream isolates and tested them for these biomarkers by
96 polymerase chain reaction and the string test. We identified twelve hypermucoviscous
97 isolates and six isolates that contain hypervirulence pathogenicity loci located on large
98 hvKp-like plasmids.

99 **Results**

100 **Identification of hvKp using diagnostic biomarkers**

101 To assess the prevalence of hvKp-like strains at NMH, we collected 141
102 consecutive Kp isolates from patients with positive blood cultures from 2015–2017.
103 Given that hvKp strains are thought to disseminate through the bloodstream, we
104 hypothesized that bloodstream isolates would be enriched for hvKp. We screened all
105 141 isolates for the following previously published diagnostic biomarkers of hvKp: *iucA,*
106 *iroB,* and the hypermucoviscous phenotype. The presence of *iucA* and *iroB* was
107 evaluated by PCR. Among NMH bloodstream isolates, six (4.3%) were positive for *iucA*
108 and five (3.5%) were positive for *iroB* (Table 1). Five isolates were positive for both, and
109 a single isolate was *iucA*+ and *iroB*- (Table 1). The collection was screened for
110 hypermucoviscosity by both string test and centrifugation. Twelve (6.7%) and 24 (13.5%)
111 isolates, respectively, were positive for hypermucoviscosity by these two tests (Table 1).
112 In addition, isolates were tested for tellurite resistance, a phenotype commonly encoded
113 by pK2044 plasmids (Fig 1). Six isolates (4.3%) were able to grow on tellurite, including
114 three isolates that were positive for *iroB* and *iucA* (Table 1). This suggested that these
115 three isolates may contain pK2044-like plasmids while the other double-positive (*iroB*+,
116 *iucA*+) isolates may contain KP52.145pII-like plasmids or unique hypervirulence
117 plasmids. Collectively, these findings indicate that Kp strains with features of hvKp are
118 circulating at NMH and suggest that these strains may contain multiples types of hvKp-
119 associated pathogenicity loci.

120 **Analysis of hvKp Plasmid Content**

121 Since hvKp pathogenicity loci are commonly encoded on a large virulence
122 plasmid, we next focused on the six isolates that contained either salmochelin or
123 aerobactin biosynthesis genes. To determine whether hvKp-associated genes of these
124 six isolates were carried on large plasmids similar to those found in characterized hvKp
125 isolates, complete genome sequences were obtained using both Nanopore and Illumina
126 platforms (Table 2). Whole-genome sequencing revealed that each of these isolates

127 harbored large plasmids with some hvKp pathogenicity loci. Three isolates (KpN8,
128 KpN89, and KpN115) contained plasmids with near complete alignment with pK2044,
129 including PAL-1, PAL-2, and tellurite resistance genes (Fig 2A). KpN49 and KpN165
130 harbored plasmids with poor overall alignment with plasmid pK2044, (Fig 2B) but with
131 nearly complete alignment with KP52.145pII (Fig 3). Similar to KP52.145pII, these two
132 plasmids contained a version of PAL-1 with *iucABCD* and *iutA* but lacking *rmpA2* (Fig 1).
133 The remaining isolate, KpN23, harbored a conjugative virulence plasmid containing only
134 the aerobactin biosynthesis genes and receptor (Fig 3, 4A and 4B). pKpN23_1 was
135 highly homologous to a plasmid harbored by a swine isolate collected in Thailand in
136 2016, KpCTRSRTH01_p2, but had minimal alignment with pK2044 or KP52.145pII. We
137 had previously identified pTK421_2, a Kp plasmid with PAL-1, PAL-2, and conjugation
138 genes (Fig 1). This plasmid was also distinct from pK2044 or KP52.145pII (Fig 2B & 3).
139 We therefore aligned pKPN23_1 to pTK421_2 but found that these plasmids shared
140 relatively little alignment beyond PAL-1 (Fig 4B). Overall, among the 141 bloodstream
141 isolates, we identified six isolates that cumulatively contained three distinct plasmids with
142 pathogenicity loci associated with hypervirulent Kp.

143 Discussion

144 Case reports indicate that hvKp strains exist in the U.S., but their prevalence is
145 unclear and has only been investigated in two surveillance studies (24, 25). In this work,
146 we determined the prevalence of hvKp at NMH by identifying isolates using diagnostic
147 criteria of hvKp. The three criteria we tested were *iroB*, *iucA,* and hypermucoviscosity.
148 We identified five isolates (3.5%) that met all three of these examined diagnostic criteria
149 for hvKp; each contained a large plasmid nearly identical to previously described hvKp
150 plasmids. We identified one additional isolate that contained *iucA* on a large plasmid but
151 was not hypermucoviscous and lacked *iroB*. Currently, it is unclear if isolates positive for

152 only a single biomarker are highly virulent and cause the types of infections
153 characteristic of hvKp. Further studies are warranted to understand the level of virulence
154 in strains such as this one with incomplete pathogenicity loci. In addition, we identified
155 12 isolates that were hypermucoviscous by string test and 24 that were
156 hypermucoviscous by centrifugation. It is unclear why some isolates were
157 hypermucoviscous by centrifugation but not the string test. These data agree with
158 previous reports suggesting that the string test is less sensitive than centrifugation for
159 measuring hypermucoviscosity and that hypermucoviscosity does not accurately predict
160 hypervirulence (37, 39). However, all strains that contained *rmpA* were positive by both
161 string and centrifugation tests (Table 2).

162 Since hvKp pathogenicity loci are normally encoded on a large plasmid, we
163 performed long-read sequencing of the six isolates that contained *iucA*. Whole genome
164 sequencing confirmed that each of these isolates harbored a large plasmid. Five of
165 these isolates harbored a plasmid highly similar to one of the two previously described
166 hvKp virulence plasmids, pK2044 or KP52.145pII, and the other, KpN23, harbored a
167 unique plasmid containing only aerobactin. Taken together, these data suggest that
168 multiple clones of hvKp isolates are circulating at NMH.

169 Our study agrees with the two published U.S. surveillance studies, which
170 describe the hvKp prevalence in the U.S. as 3.5%-6.25% of Kp infections. In 2010, 64
171 Kp strains were analyzed from two hospitals in the greater Houston area (25). They
172 identified four strains (6.25%) containing hvKp pathogenicity-associated genes, based
173 on the presence of *rmpA* and K1 capsule type by PCR amplification. In 2018, a larger
174 study in New York City characterized Kp isolates from 462 patients (24). This study
175 identified 17 (3.7%) isolates by PCR amplification that contained the hvKp biomarkers
176 *rmpA* and *iucA*. This is much lower than the prevalence of hvKp isolates in China in

177 2013 (37.8%, defined as *iuc*+), where hvKp is endemic (40). A recent genomic
178 surveillance study of seven countries in South and Southeast Asia analyzed the
179 sequences of 365 bloodstream isolates collected from 2010-2017 (41). Sequencing
180 revealed that 26% (95/365) were *iuc*+, 17% (63/365) were *iuc*+ and either *rmpA*+ or
181 *rmpA2*+, and an additional 0.8% (3/365) were *iro*+ and *rmpA*+. Of the *iuc*+ isolates,
182 13.7% (13/95) contained carbapenamase genes, but only 1 was positive for *iuc*, *rmpA/2*
183 and a carbapenamase gene. Our results together with these reports indicate that while
184 hvKp-like isolates are circulating within the United States, they remain relatively rare
185 compared to Southeastern Asia.

186 The presence of hvKp in U.S. hospitals has important clinical implications. In
187 patients, hvKp infections are associated with invasive infections most commonly
188 characterized by the development of PLA. Extrahepatic complications such as
189 endogenous endophthalmitis, necrotizing fasciitis, and meningitis have also been
190 reported (42). These invasive infections are associated with significant mortality and
191 morbidity; patients with PLA take longer to recover and 9-18% have recurrent PLA (43,
192 44). In addition, one study showed that 10/14 patients with endogenous endophthalmitis
193 progressed to blindness (45). Interestingly, while hvKp isolates cause severe invasive
194 infections, several studies reported no difference in mortality following bloodstream
195 infection with hvKp or cKp (46, 47). This is likely related to the fact that community
196 acquired hvKp infections tend to occur in young healthy patients, and outcome of
197 bloodstream infection is largely dependent on the time it takes to initiate appropriate
198 antimicrobial therapy. Delay in effective antimicrobial therapy, which is more likely to
199 occur in infections cause by multidrug-resistant bacteria, leads to poor outcomes in
200 bloodstream infection (48, 49). None of the six hvKp-like isolates identified at NMH
201 contained antimicrobial resistance genes. Thus far, antimicrobial resistant hvKp

202 infections are exceedingly rare in the United States. However, antimicrobial resistant
203 hvKp infections are on the rise in Asia, likely due to the increase in hospital acquired
204 hvKp infections (46, 50, 51). While this study is limited to a single institution, the findings
205 presented here describe the prevalence and diversity of hvKp plasmids at a major U.S.
206 academic medical center and call attention to the need for further studies evaluating the
207 risk and clinical outcomes of these diverse hvKp isolates.
208

209 **Materials and Methods**

210 **Bacterial isolates and growth conditions**

211 The isolates used in this study were collected from patients with a positive blood culture
212 from 2015-2017 and are described in Table S1. These isolates were identified as Kp on
213 a VITEK-2 (52).. Kp was grown at 37°C in Lysogeny broth (LB) or on LB agar plates. For
214 screening of tellurite resistance, Kp was grown at 37°C on LB agar plates supplemented
215 with 3 μg/mL potassium tellurite (Sigma-Aldrich, USA).

216 **Screening for *iroB* and *iucA***

217 Aerobactin and salmochelin biosynthesis genes were detected by polymerase chain
218 reaction using custom designed primers:
219 iroB Fwd- ATTTCCGCCGCTACCTCTTCAG; iroB Rev-
220 TGGGCTATTGTATCCTGTGCTATCTCTG; iucA Fwd-
221 GTTGTGGTCGTTCAGATAACAGAAAAAGTT; iucA Rev-
222 AATTCTCATTATCATGACGAGTGAGATCTGT

223 **Hypermucoviscosity testing**

224 A string test was used to assess the hypermucoviscosity of each Kp isolate as described
225 previously (53). Isolates were grown overnight at 37°C on LB agar. A single colony was
226 lifted with a loop to evaluate the formation of a viscous string between the loop and the

227 colony. A positive string test was defined as a string length ≥ 5 mm. Hypermucoviscosity
228 was also assessed by centrifugation of overnight cultures (5 mL LB) at 3,220 rcf for 10
229 minutes (39). Hypermucoviscous isolates were identified by the persistence of turbidity.

230 **Whole-Genome Sequencing**

231 Genomic DNA (gDNA) was prepared from a single colony cultured overnight at 37°C in
232 LB using the Maxwell 16 system (Promega Corp., Madison, WI). Nanopore sequencing
233 was performed as described previously (54, 55). Briefly, libraries were prepared from
234 gDNA using the ligation sequencing kit (SQK-LSK109, Oxford Nanopore, UK). Libraries
235 were sequenced on the MinION using a FLO-MIN106 flow cell and base calling and
236 demultiplexing of sequence reads was performed using Guppy v3.4.5. Libraries for
237 Illumina sequencing were prepared using Nextera XT library kits (Illumina, Inc., San
238 Diego, CA) and sequenced on an Illumina NextSeq instrument to generate paired-end
239 150-bp reads. Hybrid assembly and circularization of Nanopore and Illumina reads were
240 performed using Flye v2.6. Nanopore sequencing errors were corrected by aligning
241 Illumina reads to the assembly using BWA v0.7.17 and correcting the errors with serial
242 rounds of Pilon v1.23. Annotation was performed using the NCBI Prokaryotic Genome
243 Annotation Pipeline.

244

245 **Molecular typing and identification of virulence genes**

246 Assembled whole genome sequences were analyzed with the bioinformatics tools
247 Kleborate and Kaptive to evaluate multi locus sequence type, capsule locus typing, and
248 virulence gene content (56, 57). Plasmids identified by whole genome sequencing were
249 aligned using BLAST Ring Image generator (BRIG) with the alignment threshold set at
250 85% identity (58).

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455

456

457 **Figure Legends**

458 **Figure 1. Virulence plasmids associated with hypervirulence in *Klebsiella***

459 ***pneumoniae*.** Genomic elements and plasmid architecture are depicted for

460 pK2044 **(A)**, KP52.145pII **(B)** and pTK421 **(C)**. PAL-1 and PAL-2 contents and

461 organization are depicted for pK2044, KP52.145pII, pTK421_2, and ICEKp1 **(D)**.

462 (Drawings not to scale.)

463

464

465

466

467 **Figure 2. The bloodstream isolates KpN115, KpN8, and KpN89 harbor**
468 **pK2044-like plasmids.** DNA sequences for plasmids identified by nanopore
469 sequencing were aligned with either pK2044 **(A)**, or KP52.145pII **(B)** using blast
470 ring image generator (BRIG). A sequence identity threshold of 85% was used.
471 Aerobactin biosynthesis genes are indicated with green arrows, salmochelin with
472 orange arrows, *rmpA* with blue arrows, and *rmpA2* with red arrows. Plasmid and
473 PALoc alignments are shown in the indicated colors.
474
475 **Figure 3. The bloodstream isolates KpN49_2 and KpN165_1 harbor**
476 **KP52.145pII-like plasmids.** DNA sequences for plasmids identified by Nanopore
477 sequencing were aligned with KP52.145pII using blast ring image generator
478 (BRIG). A sequence identity threshold of 85% was used. Aerobactin biosynthesis
479 genes are indicated with green arrows, salmochelin with orange arrows, and
480 *rmpA* with blue arrows. Plasmid and PALoc alignments are shown in the
481 indicated colors.
482
483 **Figure 4. A unique plasmid containing aerobactin biosynthesis genes was**
484 **identified in strain KpN23.** DNA sequences for plasmid KpCTRSRTH01_p2
485 and plasmids from NMH bloodstream isolates **(A)** or pK2044, KP52.145pII, and
486 pTK421_2 **(B)** were aligned with pKpN23_1 using blast ring image generator
487 (BRIG). A sequence identity threshold of 85% was used. Aerobactin biosynthesis
488 genes are indicated with green arrows. Plasmid and PALoc alignments are
489 shown in the indicated colors.

490

Table 1. Prevalence of hypervirulence features in blood stream isolates

	Isolates n = 141
	Positive
Hypervirulence Marker	n(%)
iroB	5 (3.5)
iucA	6 (4.3)
iroB/iucA	5 (3.5)
String Test	12 (8.5)
Centrifugation Test	24 (17.0)
Tellurite Resistance	6 (4.3)

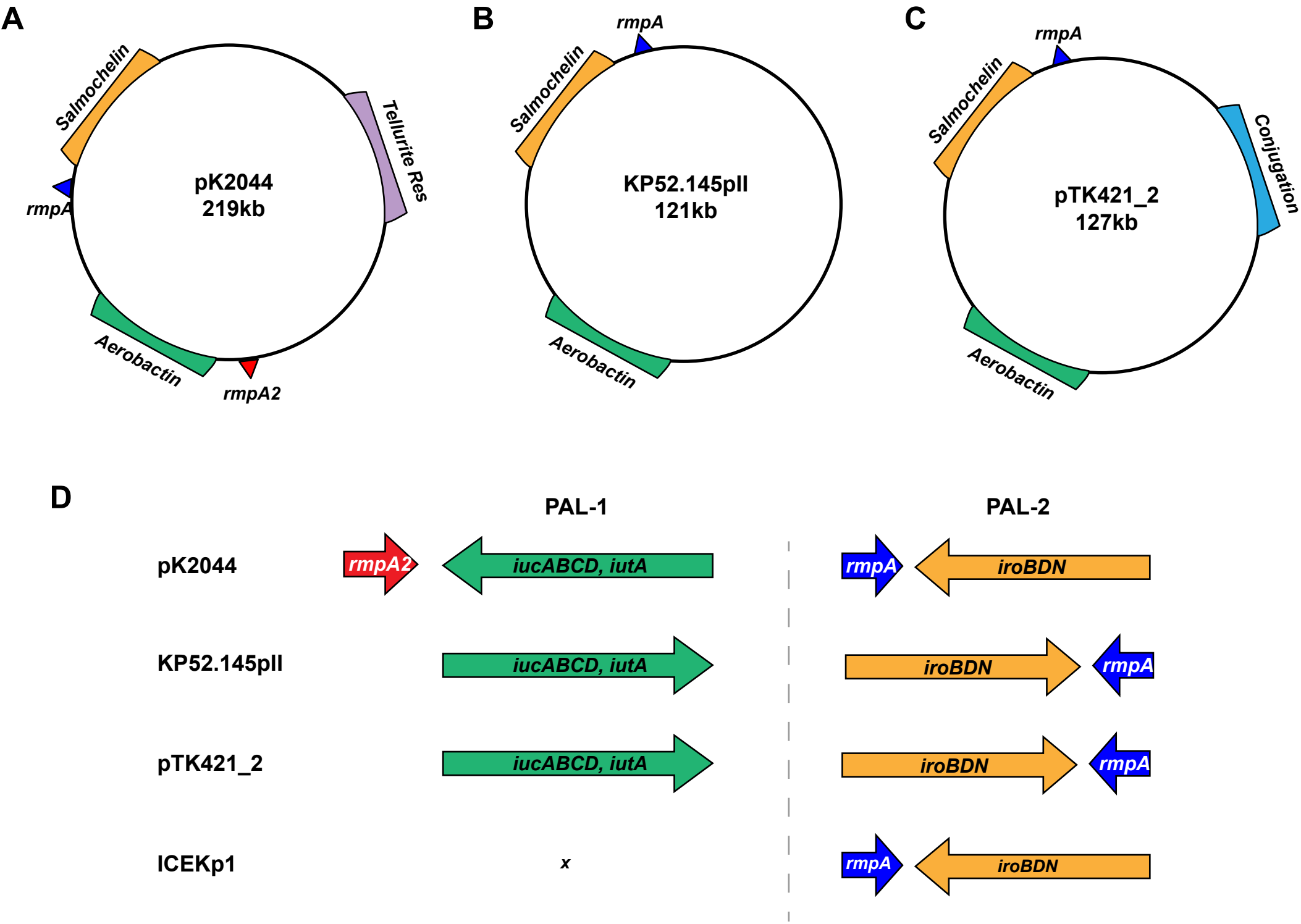
491

492

Table 2. Characteristics of bloodstream isolates containing hvKp pathogenicity loci

Strain	ST	K-Locus	*iuc*	*iro*	hmv*	*rmpA/2*	Plasmid
KPN8	ST23	KL1	*iuc 1*	*iro 1*	+	*rmpA, rmpA2*	pK2044
KPN23	ST881	KL2	*iuc 3*	-	-	-	pKPN23_1
KPN49	ST66	KL2	*iuc 2*	*iro 2*	+	*rmpA*	KP52.145pII
KPN89	ST23	KL1	*iuc 1*	*iro 1*	+	*rmpA, rmpA2*	pK2044
KPN115	ST23	KL1	*iuc 1*	*iro 1*	+	*rmpA, rmpA2*	pK2044
KPN165	ST380	KL2	*iuc 2*	*iro 2*	+	*rmpA*	KP52.145pII

493 *hmv = hupermucoviscous; ST = Sequence Type


A
Salmochelin
Tellurite Res
pK2044
219kb
rmpA
Aerobactin
rmpA2
B
rmpA
Salmochelin
KP52.145pII
121kb
Aerobactin
C
rmpA
Salmochelin
Conjugation
pTK421_2
127kb
Aerobactin
D
PAL-1
PAL-2
pK2044
rmpA2
iucABCD, iutA
rmpA
iroBDN
KP52.145pII
iucABCD, iutA
iroBDN
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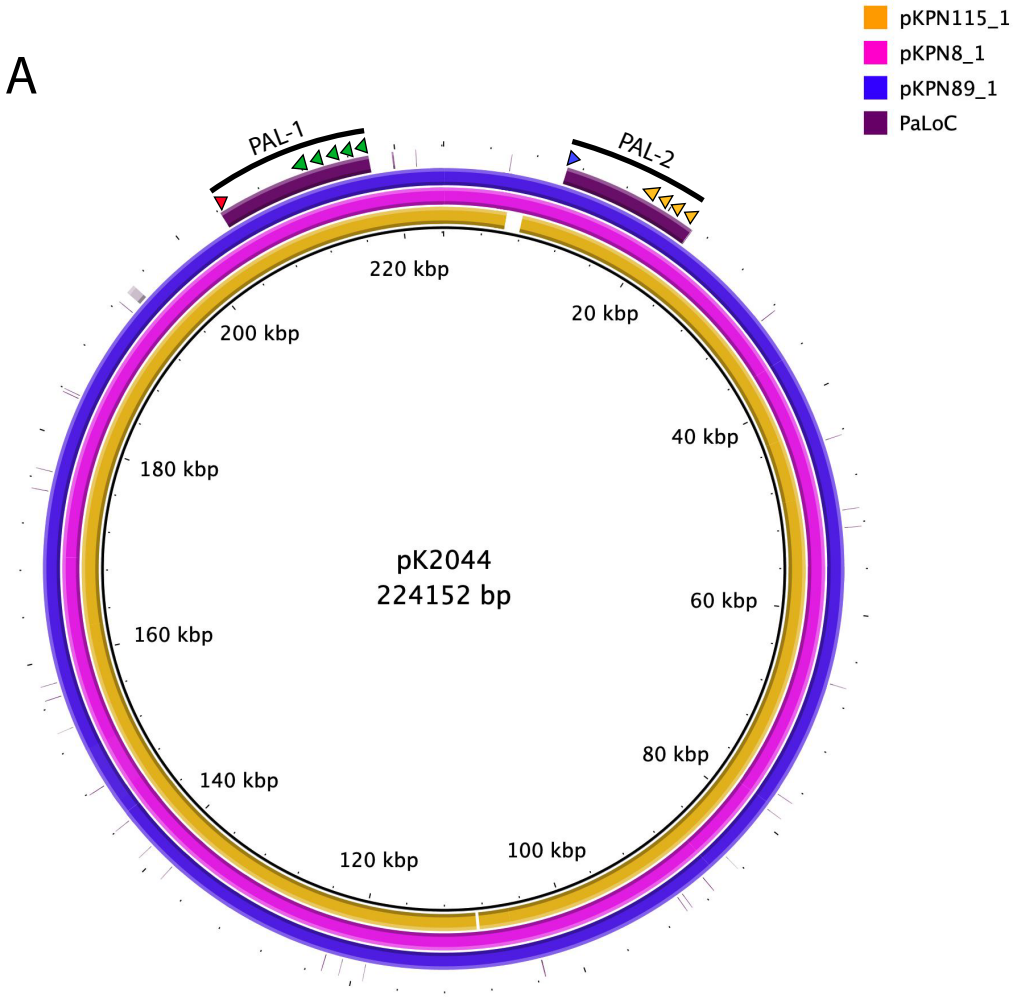
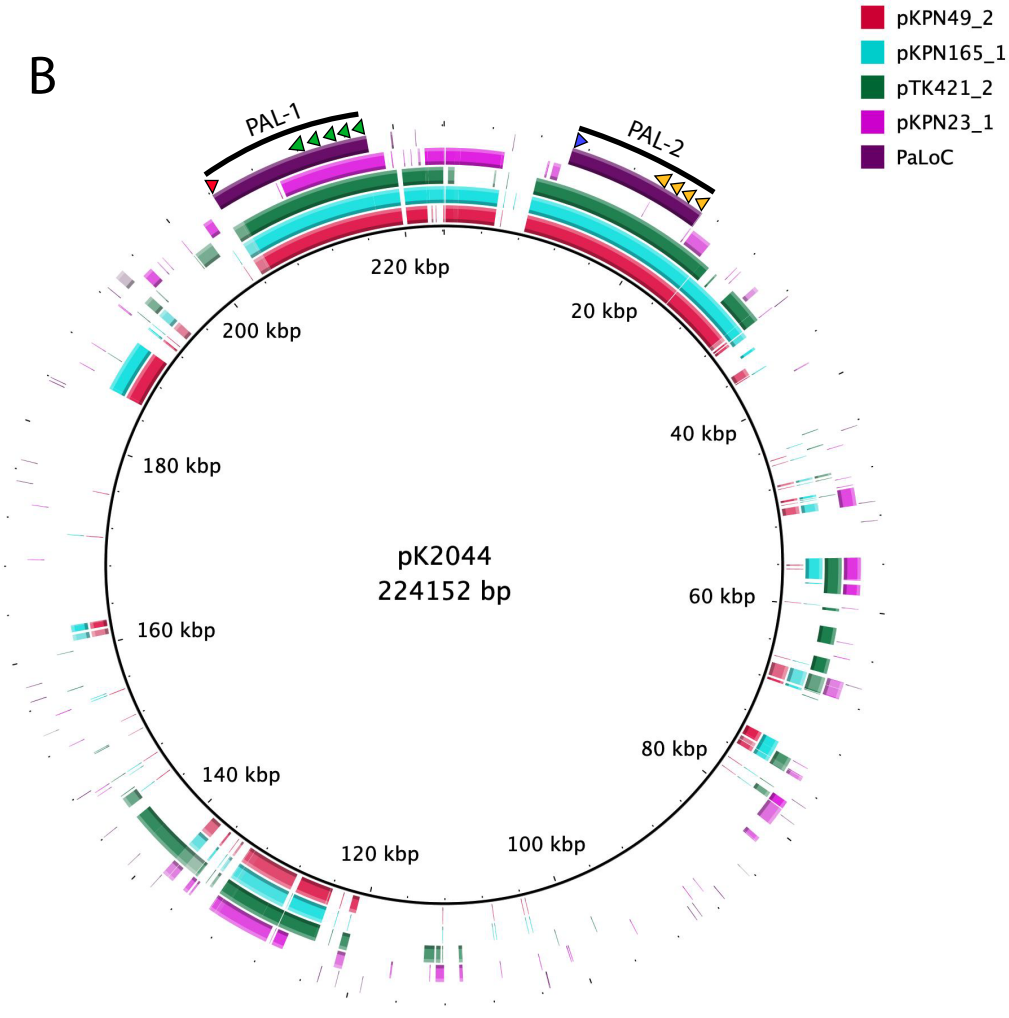
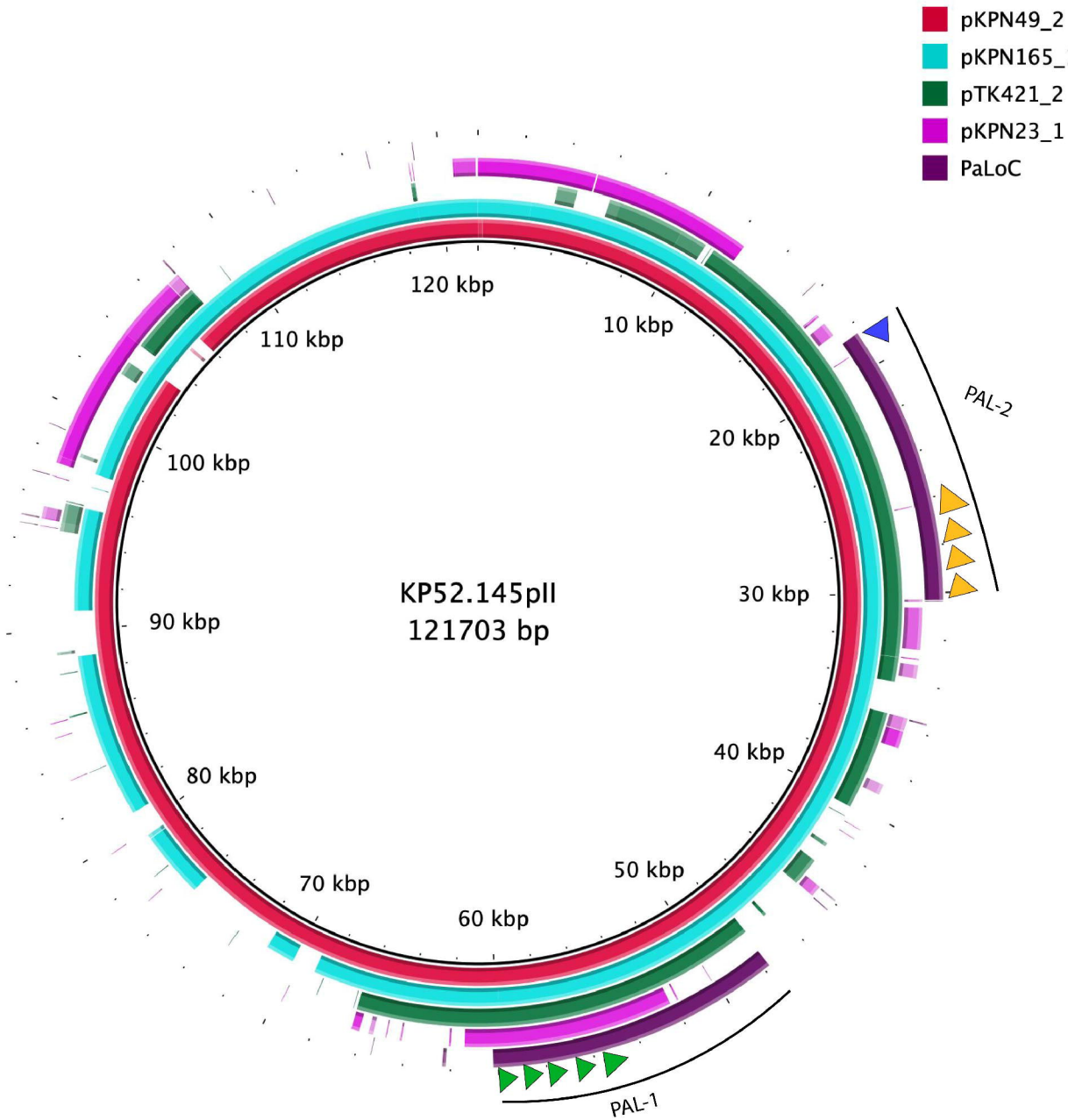
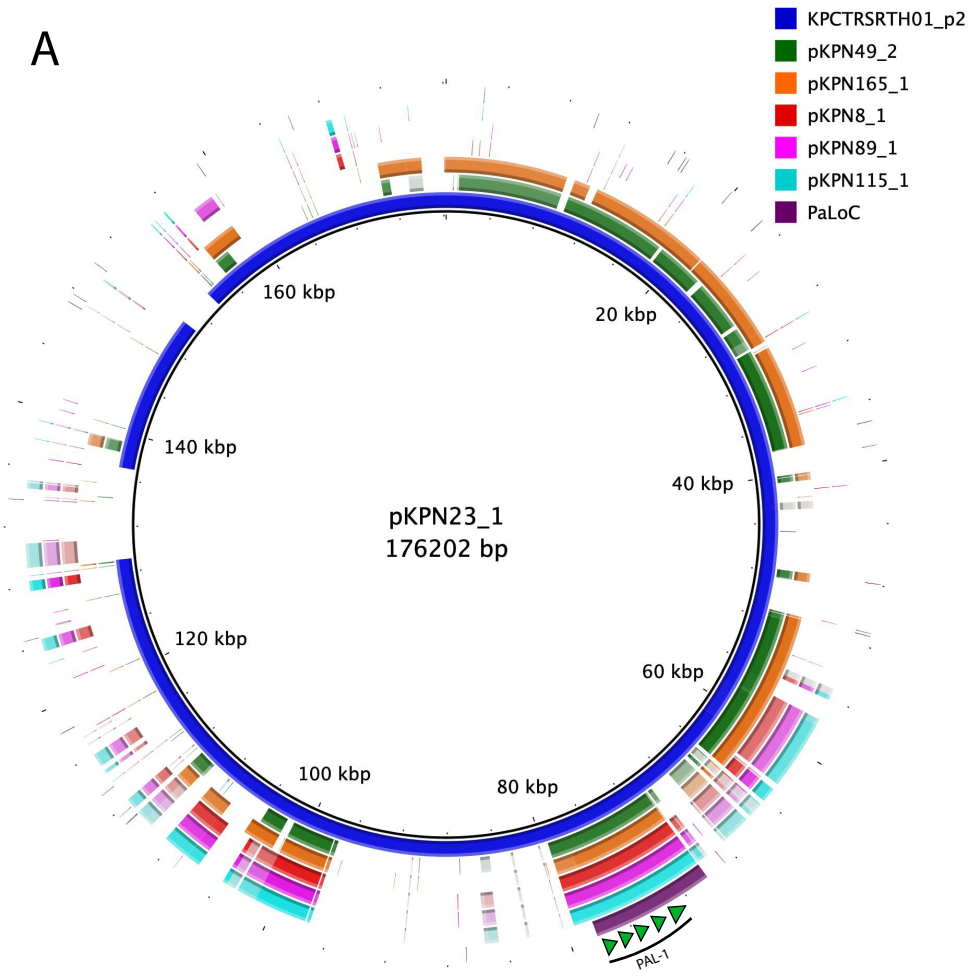
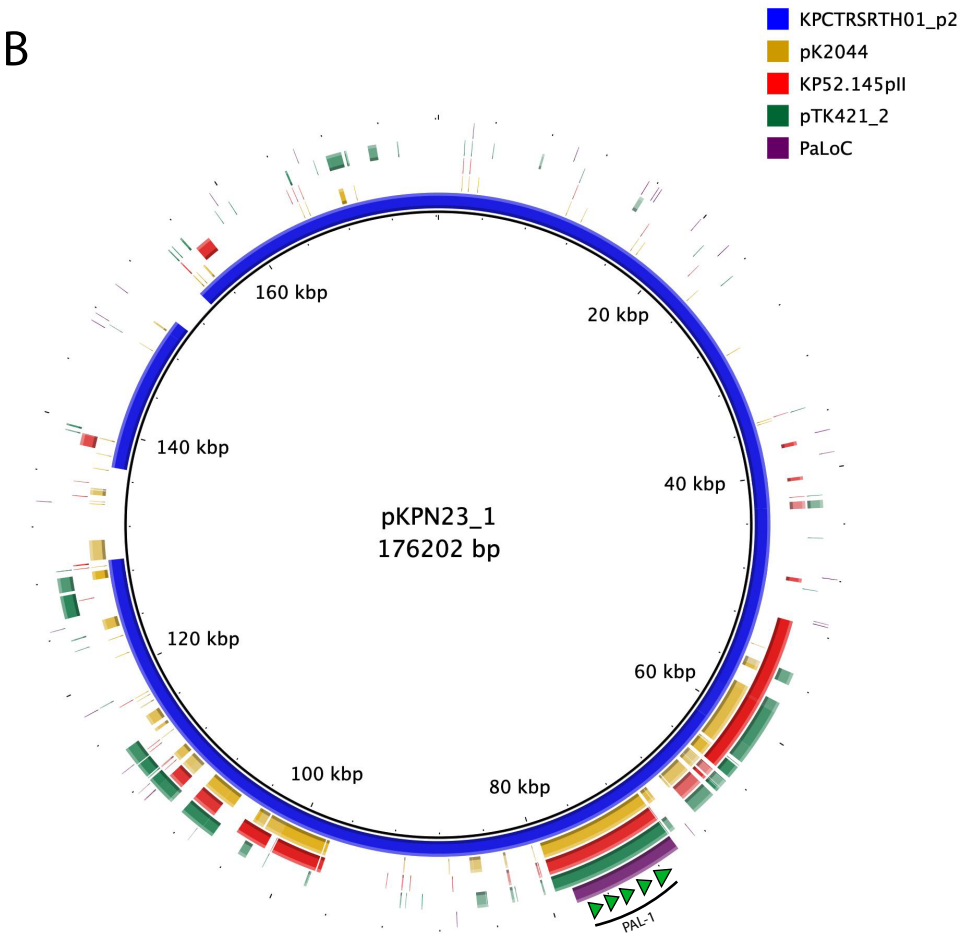
pTK421_2
iucABCD, iutA
iroBDN
rmpA
ICEKp1
x
rmpA
iroBDN


A
pKPN115_1
pKPN8_1
pKPN89_1
PaLoC
PAL-1
PAL-2
220 kbp
20 kbp
200 kbp
40 kbp
180 kbp
pK2044
224152 bp
60 kbp
160 kbp
80 kbp
140 kbp
120 kbp
100 kbp


B
pKPN49_2
pKPN165_1
pTK421_2
pKPN23_1
PaLoC
PAL-1
PAL-2
220 kbp
20 kbp
200 kbp
40 kbp
180 kbp
pK2044
224152 bp
60 kbp
160 kbp
80 kbp
140 kbp
120 kbp
100 kbp


pKPN49_2
pKPN165_1
pTK421_2
pKPN23_1
PaLoC
120 kbp
10 kbp
20 kbp
30 kbp
40 kbp
50 kbp
60 kbp
70 kbp
80 kbp
90 kbp
100 kbp
110 kbp
KP52.145pII
121703 bp
PAL-2
PAL-1


A
KPCTRSRTH01_p2
pKPN49_2
pKPN165_1
pKPN8_1
pKPN89_1
pKPN115_1
PaLoC
160 kbp
20 kbp
140 kbp
40 kbp
pKPN23_1
176202 bp
120 kbp
60 kbp
100 kbp
80 kbp
PAL-1


B
KPCTRSRTH01_p2
pK2044
KP52.145pII
pTK421_2
PaLoC
160 kbp
20 kbp
140 kbp
40 kbp
pKPN23_1
176202 bp
120 kbp
60 kbp
100 kbp
80 kbp
PAL-1