

# **KPC-3, GES-5 and VIM-1-producing *Enterobacterales* isolated from urban ponds**

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## **SUPPLEMENTARY MATERIAL**

Tables S1-S3

Figures S1-S5

**Table S1.** PCR primers used in molecular typing, 16S rRNA based affiliation and screening of ARGs and Integrons

Gene	Sequences	Fragment size(bp)
<i>blaKPC</i> <sup>[76]</sup>	FW 5' - CATTCAAGGGCTTTCTTGCTGC REV 5' - ACGACGGCATAGTCATTTGC	538
<i>blaNDM</i> <sup>[76]</sup>	FW 5' - GGTTCGGCGATCTGGTTTC REV 5' - CGGAATGGCTCATCACGATC	621
<i>blaVIM</i> <sup>[76]</sup>	FW 5' - GATGGTGTTCGGTCGCATATCG REV 5' - GCCACGTTCCCCGCAGACG	475
<i>blaGES</i> <sup>[76]</sup>	FW 5' - AGTCGGCTAGACCGGAAAG REV 5' - TTTGTCCGTGCTCAGGAT	399
<i>blaOXA-48</i> <sup>[76]</sup>	FW 5' - TTGGTGGCATCGATTATCGG REV 5' - GAGCACTTCTTTGTGATGGC	744
<i>blaCTX-M</i> <sup>[76]</sup>	FW 5' - GTGCAGTACCAGTAAAGTTATGG REV 5' - CGCAATATCATTGGTGGTGCC	538
<i>mcr-1</i> <sup>[117]</sup>	FW 5' - CGGTCAGTCCGTTTGTTTC REV 5' - CTTGGTCGGTCTGTAGGG	350
16S rRNA <sup>[118]</sup>	FW 5' - AGAGTTTGATCCTGGCTCAG REV 5' - GGTTACCTTGTTACGACTTT	1467
<i>Int1</i> <sup>[119]</sup>	FW 5' - ACATGCGTGTAATCGTC REV 5' - CTGGATTTTCGATGACGGCACG	280
<i>Int2</i> <sup>[119]</sup>	FW 5' - ACGGCTACCCTCTGTTAT REV 5' - TTATTGCTGGGATTAGGC	233
<i>Int3</i> <sup>[119]</sup>	FW 5' - AGTGGGTGGCGAATGAGTG REV 5' - TGTTCTTGATCGGCAGGTG	600
BOX element <sup>[120]</sup>	BOXA1R: CTACGGCAAGGCGACGCTGAC	variable

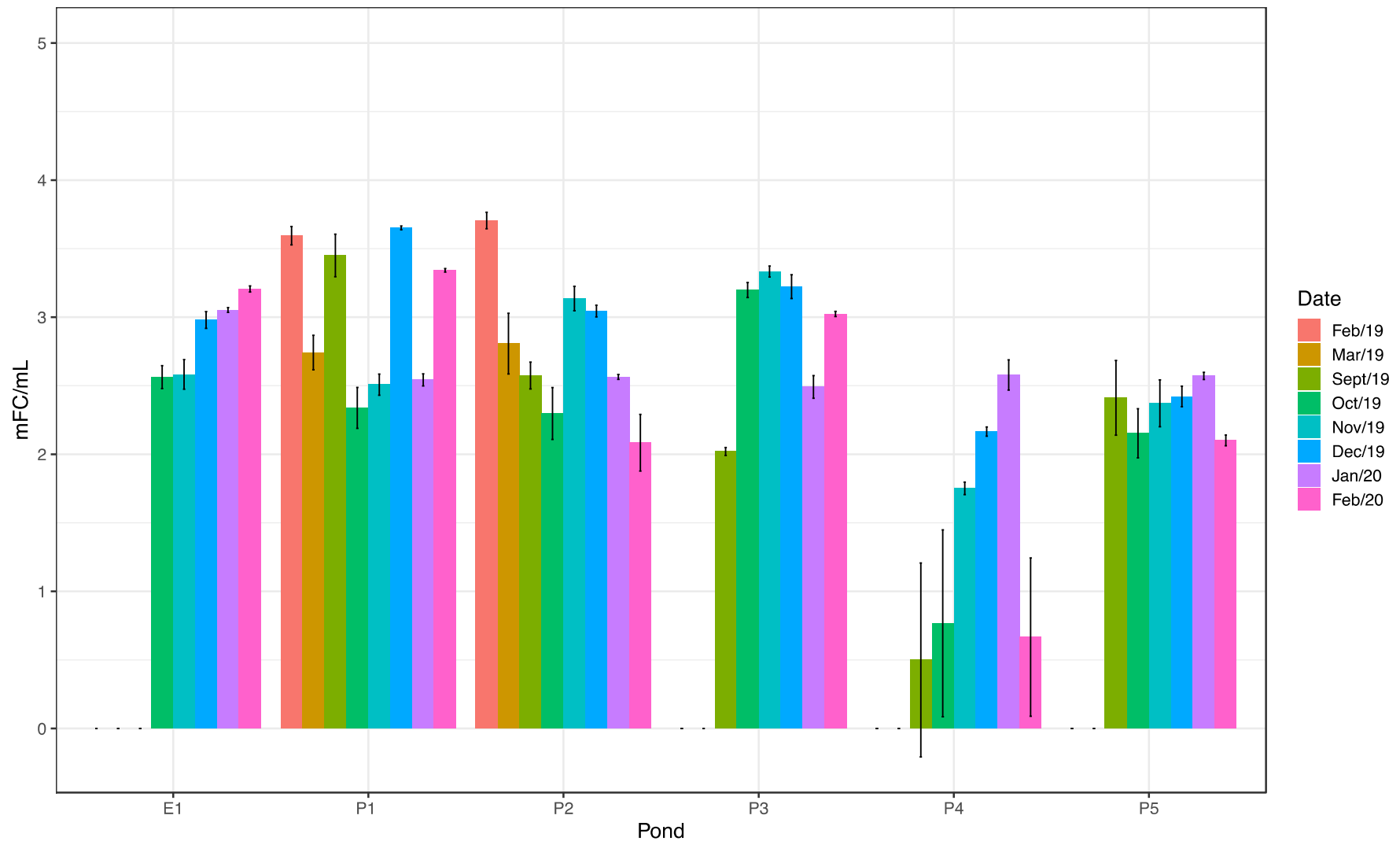
**Table S2.** Percentage of imipenem resistant CFUs in the sampled ponds and estuarine canal in February and March 2019, and from September 2019 to February 2020. No imipenem-resistant colonies were obtained from Pond P4 and P5. Hyphens are exhibited whenever no imipenem resistant CFUs were detected.

Sampling sites	feb/19	mar/19	sept/19	oct/19	nov/19	dec/19	jan/20	feb/20
P1	0.0244	0.0018	0.1609	0.0058	0.0535	0.0045	0.0019	0.0018
P2	0.0003	0.0010	-	-	-	-	-	-
P3	-	-	-	-	0.0005	-	-	-
E1	-	-	-	0.0045	0.0624	0.0032	0.0047	0.0025

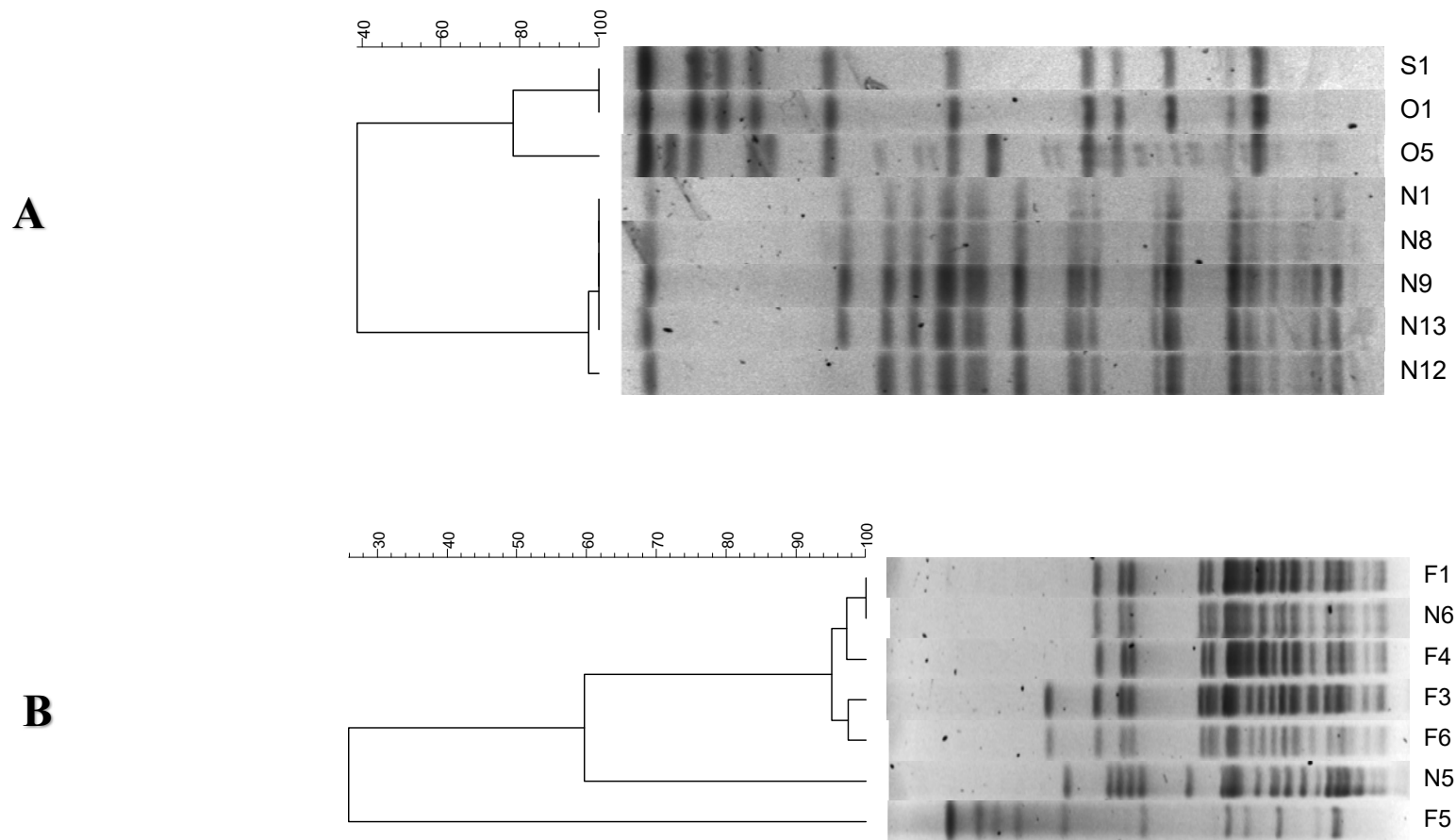
**Table S3.** General features of *C. freundii* F6, *R. ornithinolytica* N9 and *E. kobei* N10 draft genomes and WGS-based parameters used for phylogenetic affiliation.

	<i>C. freundii</i> F6	<i>R. ornithinolytica</i> N9	<i>E. kobei</i> N10
Size (bp)	5,117,230	6,252,962	5,397,864
GC content (%)	51.7	55.2	54.4
Nº Contigs	230	420	431
Coverage	180x	170x	115x
N50	88,228	109,201	65,421
Nº of CDS	4997	6014	5128
Nº of tRNAs	80	84	80
Nº of rRNAs	2	2	1
Sequence Type	ST270	-	ST1378
ANIb (%)*	98.32	99.16	99.25
ANIm (%)*	98.98	99.53	99.85
dDDH(%)*	90.50	94.90	94.30
Difference in %G+C*	0.01	0.47	0.01

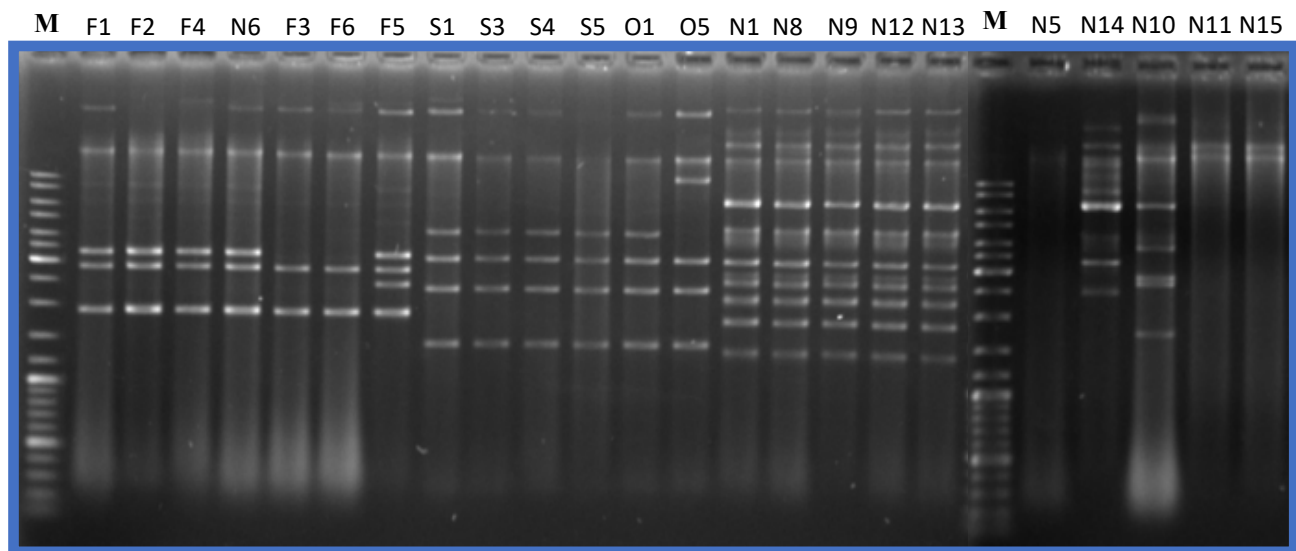
\*For species identification, *C. freundii* ATCC 8090 (JMTA01000050), *R. ornithinolytica* NBRC 105727 (BCYR0100000000) and *E. kobei* DSM13645 (CP017181) were used as reference genomes.



**Figure S1.** Average counting of typical coliforms in CFU/mL (log<sub>10</sub>) collected in February and March 2019, and from September 2019 to February 2020, from five ponds and an estuarine canal. In both February and March 2019, samples were only collected from ponds P1 and P2, while in September 2019, no samples were collected from E1.

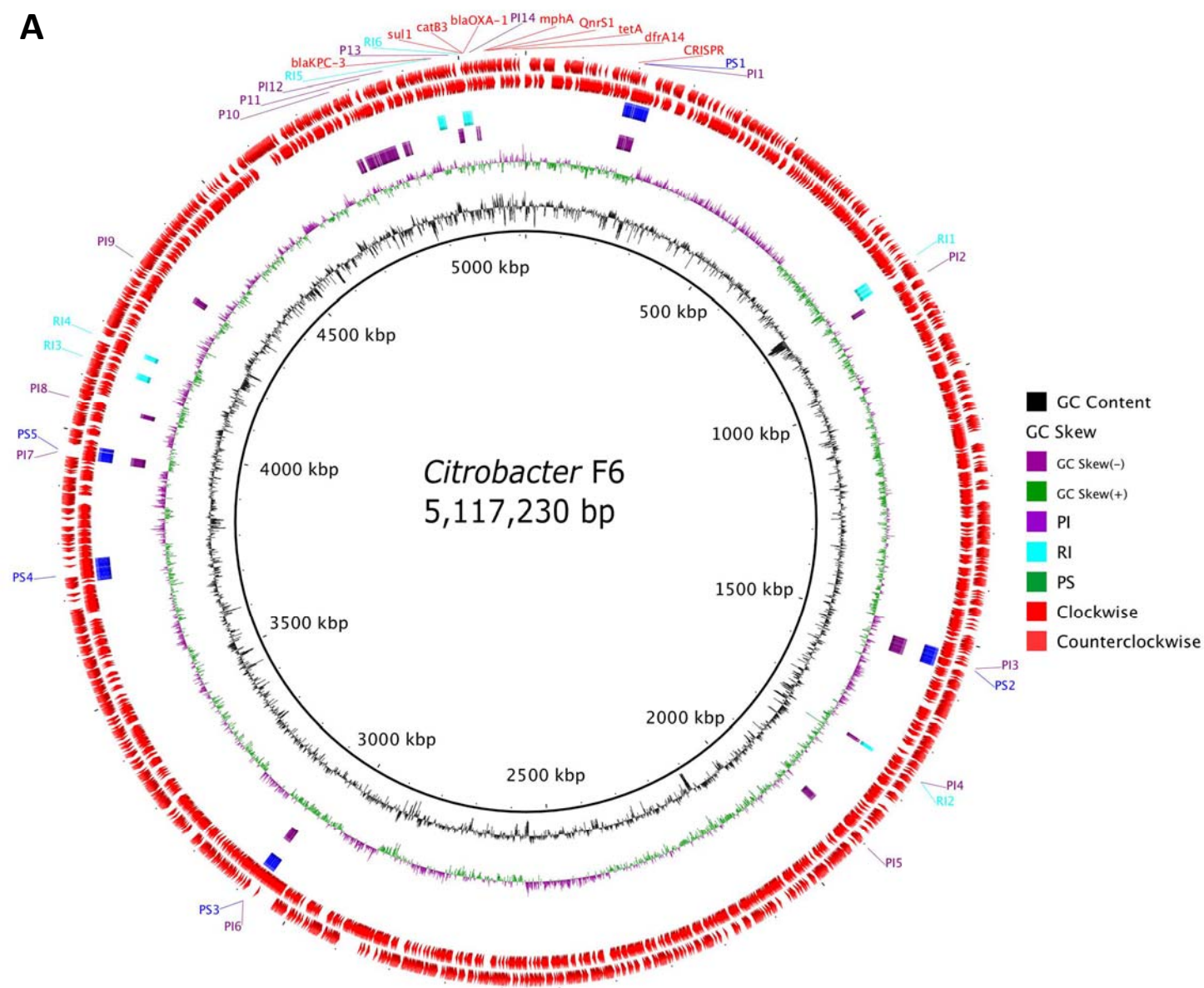


**Figure S2.** Dendrogram with the PFGE restriction patterns of genomic DNA from the 8 *Raoultella* (A) and the 7 *Citrobacter* (B) selected strains digested with XbaI. The dendrogram was constructed using GelCompar II version 6.1 (Applied Maths, Belgium) with Dice coefficient and UPGMA clustering (Opt. 2.00%; Tol 1.0%; H > 0.0%; S > 0.0%).

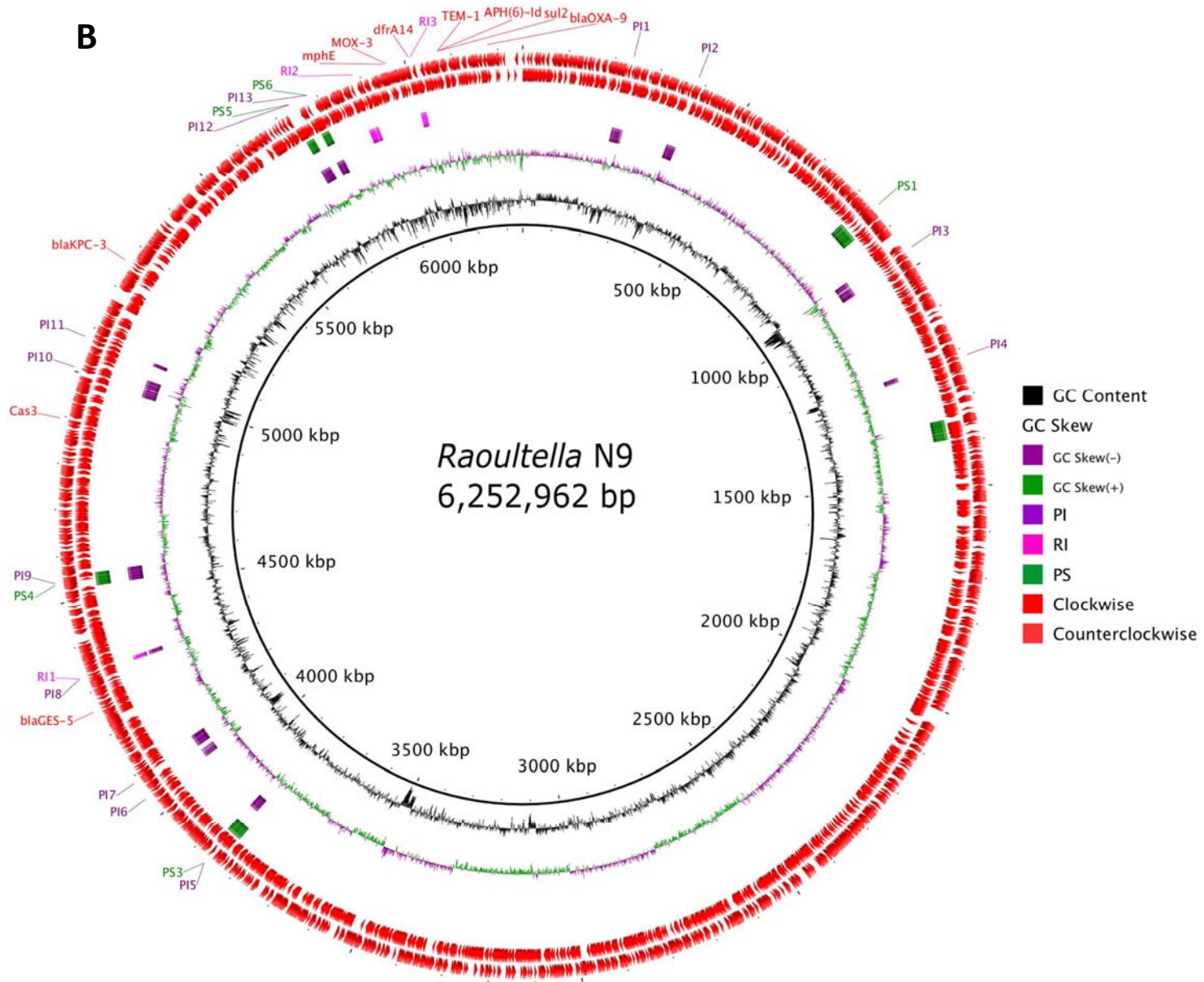


**Figure S3.** Plasmid DNA fingerprint of the entire CRE collection comprising 23 isolates. The molecular weight marker 1.5 Kb DNA NZYDNA Ladder VI (NzyTech, Portugal) is represented by the letter M.

A

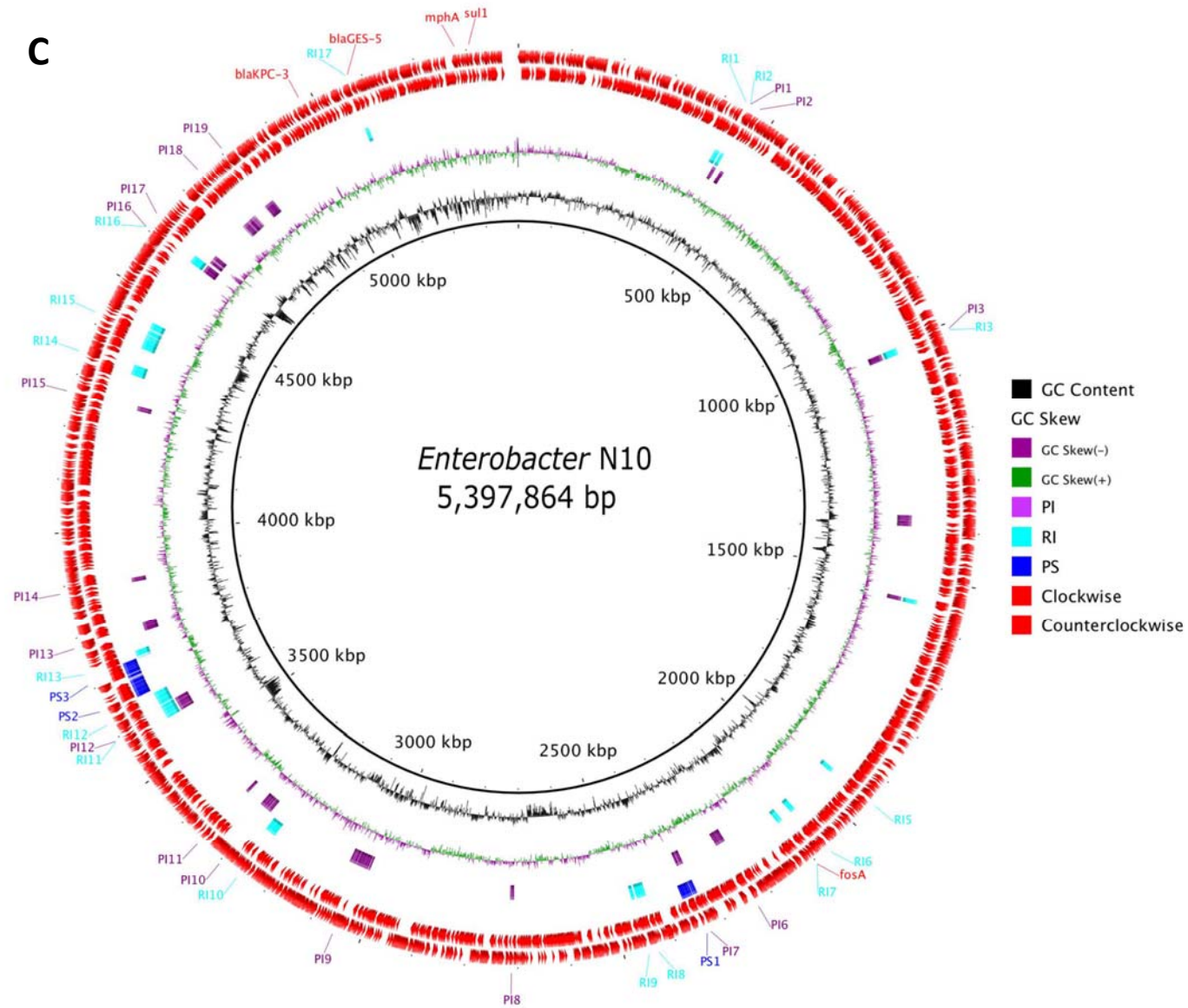


**B**

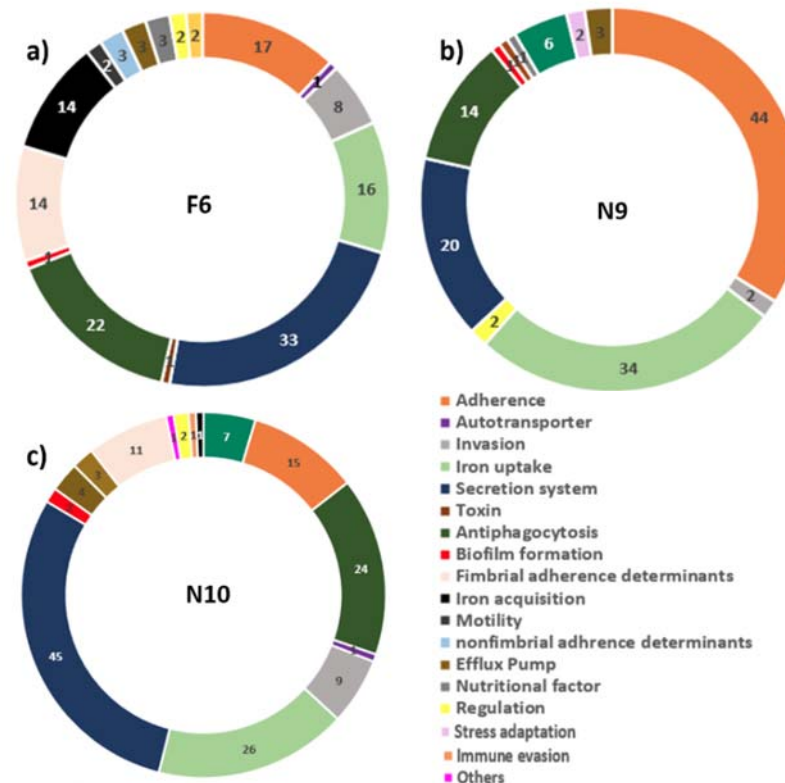




C



**Figure S4.** Circular genome representation of *C. freundii* F6 (A), *R. ornithinolytica* N9 (B) and *E. kobei* N10 (C). BRIG performed the alignment using a local BLAST+ with the standard parameters (50% lower-70% upper cut-off for identity and e-value of 10). The ring colour gradients correspond to varying degrees of identity of BLAST matches. Circular genomic maps include information on GC Skew, GC content, putative pathogenicity islands (PIs), putative resistance islands (RIs), prophage sequences (PS) and the location of ARGs and CRISPRs within the clockwise and counterclockwise CDS strands.



**Figure S5.** Category of bacterial virulence factors found in *C. freundii* F6 a), *R. ornithinolytica* N9 b) and *E. kobei* N10 c) according to the VFDB analysis.