Big data analysis of all bacterial genomes establishes a triple whammy of carbapenemases, ICEs and multiple clinically-relevant bacteria

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Abstract

Carbapenemases inactivate most β-lactam antibiotics, including carbapenems and have been frequently reported among *Enterobacteriaceae*, *Acinetobacter* spp. and *Pseudomonas* spp. Traditionally, the horizontal gene transfer of carbapenemase-encoding genes (CEGs) has been linked to plasmids. However, given that integrative and conjugative elements (ICEs) are possibly the most abundant conjugative elements among prokaryotes, we conducted an *in silico* analysis to ascertain the likely role of ICEs in the spread of CEGs among all bacterial genomes. We detected 7457 CEGs, of which 224 were located within putative ICEs among several clinically-relevant bacterial species (including *Klebsiella pneumoniae*, *Escherichia coli, Citrobacter freundii, Enterobacter hormaechei* and *Pseudomonas aeruginosa*). We also identified a *Bacillus subtilis* strain with an NDM-1-encoding ICE. Most CEGs detected within ICEs belong to the KPC, IMP and NDM families and different mechanisms were likely responsible for acquisition of these genes. The majority of CEG-bearing ICEs belong to the MPF_T, MPF_F, MPF_G and MPF_I classes and often encode additional adaptive traits such as resistance to other antibiotics (e.g. aminoglycosides and fluoroquinolones), metal and biocide tolerance and bacteriocin production. This study provides a snapshot of the different CEGs associated with ICEs among all bacterial genomes and sheds light on the underappreciated contribution of ICEs to the spread of carbapenem resistance globally.

60

61 **Introduction**

62 Due to the importance of carbapenems for the treatment of serious infections in
63 humans, the WHO has stated these antibiotics should be reserved for infections caused
64 by multidrug resistant Gram-negative bacteria in humans [1]. Recently, the same agency
65 presented a list of bacterial pathogens for which research and development of new
66 antibiotics are urgently required, and the top priority pathogens were the carbapenem-
67 resistant strains of *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and
68 *Enterobacteriaceae* [2].

69 The evolution of carbapenem-resistance in bacteria is often driven by the horizontal
70 gene transfer (HGT) of carbapenemase-encoding genes (CEGs) [3]. Carbapenemases are
71 beta-lactamases able to hydrolyze carbapenems as well as most of other beta-lactam
72 antibiotics. The CEGs are often located on integrons or transposons that themselves
73 target mobile genetic elements (MGEs) such as plasmids [3, 4], which makes the
74 dissemination of these genes unpredictably complex within bacterial communities.
75 Recently, we demonstrated that another type of MGE, the integrative and conjugative
76 elements (ICEs), play a significant role as vehicles for the dissemination of CEGs among
77 *P. aeruginosa* [5]. ICEs are self-transmissible MGEs that can integrate into and excise
78 from the genome (as transposons and phages do) and can exist as circular, sometimes
79 replicable, extrachromosomal elements and be transferred by conjugation (as plasmids
80 do) [6–9]. ICEs appear to have a bipartite lifestyle that shifts between vertical and
81 horizontal transmission [7, 10]. Eight mating pair formation (MPF) classes were
82 proposed (B, C, F, FA, FATA, G, I and T), based on the phylogeny of VirB4, the only
83 ubiquitous protein in all known type-IV secretion systems (T4SS) involved in conjugation
84 [11]. The MPF_T is widely distributed both in conjugative plasmids and ICEs, while MPF_F
85 is more prevalent in plasmids and MPF_G on ICEs [12].

86 Given that ICEs were identified in most bacterial clades and were proposed to be more
87 prevalent than conjugative plasmids [6], we conducted an *in silico* analysis to explore
88 the distribution of CEG-bearing ICEs among all sequenced bacterial genomes. Our results
89 demonstrate that CEG-bearing ICEs mainly belong to three MPF families and are
90 primarily located in several bacterial pathogens. Our analysis highlights the importance

91 of thoroughly investigating these elements as important vehicles for the spread of
92 antibiotic resistance (AR), particularly to carbapenems.

93

94 **Material and methods**

95 **Bacterial genomes and carbapenemases search**

96 All RefSeq bacterial genomes available in NCBI (accessed on 02/03/2018), including
97 complete and draft genome sequences were downloaded and blasted against a local
98 database of 688 carbapenemases (**Table S1**) adapted from AMRfinder
99 (https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/AMRFinder/) [13],
100 using diamond v0.9.21 (https://github.com/bbuchfink/diamond) [14] with the following
101 parameters: 'diamond blastx –d DB.dmnd –o hits.txt --id 100 --subject-cover 100 -f 6 --
102 sensitive'.

103

104 **Tracing ICEs among the bacterial genomes**

105 The CEG-bearing genomes were annotated with prokka v1.13
106 (https://github.com/tseemann/prokka) [15], and the GenBank files were used as input
107 to mine ICEs on the standalone version of ICEfinder
108 (http://202.120.12.136:7913/ICEfinder/ICEfinder.html). Putative MGEs were annotated
109 as ICEs if complete conjugation and relaxase modules, plus an integrase or DDE
110 transposase superfamily were identified within the boundaries of the element, and as
111 integrative and mobilizable elements (IMEs) if the elements contained all except genes
112 involved in conjugation. The translated coding sequences of extracted MGEs were
113 analyzed on the CONJscan module of MacSyFinder (https://github.com/gem-
114 pasteur/macsyfinder) [16, 17]. A MGE was classified as an ICE when a positive prediction
115 was verified by ICEfinder and/or the CONJscan module. To predict the multi-locus
116 sequence type of each CEG-bearing MGEs, we used mlst v2.16.1
117 (https://github.com/tseemann/mlst), which scans the genomes against PubMLST typing
118 schemes (https://pubmlst.org/) [18].

119

120 **Characterization of the CEG-bearing ICEs**

121 Screening of AR genes among ICEs was performed using ResFinder v3.1
122 (https://cge.cbs.dtu.dk/services/ResFinder/) [19]. Metal and biocides tolerance genes
123 were blasted against a protein database downloaded from BacMet
124 (http://bacmet.biomedicine.gu.se) [20] using the aforementioned diamond parameters.
125 Associated MGEs were annotated using Galileo™ AMR
126 (https://galileoamr.arcbio.com/mara/) (Arc Bio, Cambridge, MA) [21]. Bagel4
127 (http://bagel4.molgenrug.nl/) was used to mine ICEs for bacteriocins and ribosomally
128 synthesized and posttranslationally modified peptides [22]. We ran our extracted ICEs
129 against REBASE (http://rebase.neb.com/rebase/rebase.html) to look for restriction-
130 modification systems [23]. We also used CRISPRCasFinder (https://crisprcas.i2bc.paris-
131 saclay.fr/CrisprCasFinder/Index) to look for CRISPR (clustered regularly interspaced
132 short palindromic repeats) arrays and their associated (Cas) proteins within ICE
133 sequences [24].

134

135 **Results**

136 **Carbapenemase-encoding genes are mainly found in Gram-negative bacteria**

137 We retrieved a total of 107,887 bacterial genomes from NCBI and we identified 7457
138 CEGs belonging to 33 beta-lactamase families among 7343 genomes (**Table S2**). Most
139 CEG-bearing genomes were detected in the USA, China and Europe (**Figure 1A** and **Table**
140 **S2**). Also, the majority of the bacterial genomes were sequenced in these countries. A
141 total of 43 genera were detected, although dominated by a limited number of bacterial
142 genus. Our results show that CEGs are mostly present on Gram-negative bacteria.
143 Exceptions included the report of OXA-72 in *Staphylococcus aureus* and NDM-1 in
144 *Bacillus subtilis*. As suspected, we observed that the ESKAPEEc pathogens *K.*
145 *pneumoniae*, *A. baumannii*, *P. aeruginosa, Enterobacter spp.* and *Escherichia coli* carry a
146 wide diversity of CEG families including the most promiscuous OXA, KPC, NDM, IMP, VIM
147 and GES-types (**Figure 1B**). In addition, we observed that several CEGs (such as bla_{SPM-1})
148 have a narrow host range and were only identified within strains belonging to the same
149 genus (**Figure 1C**). Even though carbapenemases of the DIM family were not identified

150 on bacterial genomes other than the *Pseudomonas* genus, a previous study has reported
151 the presence of $bla_{\text{DIM-1}}$ in *Enterobacteriaceae* [25].

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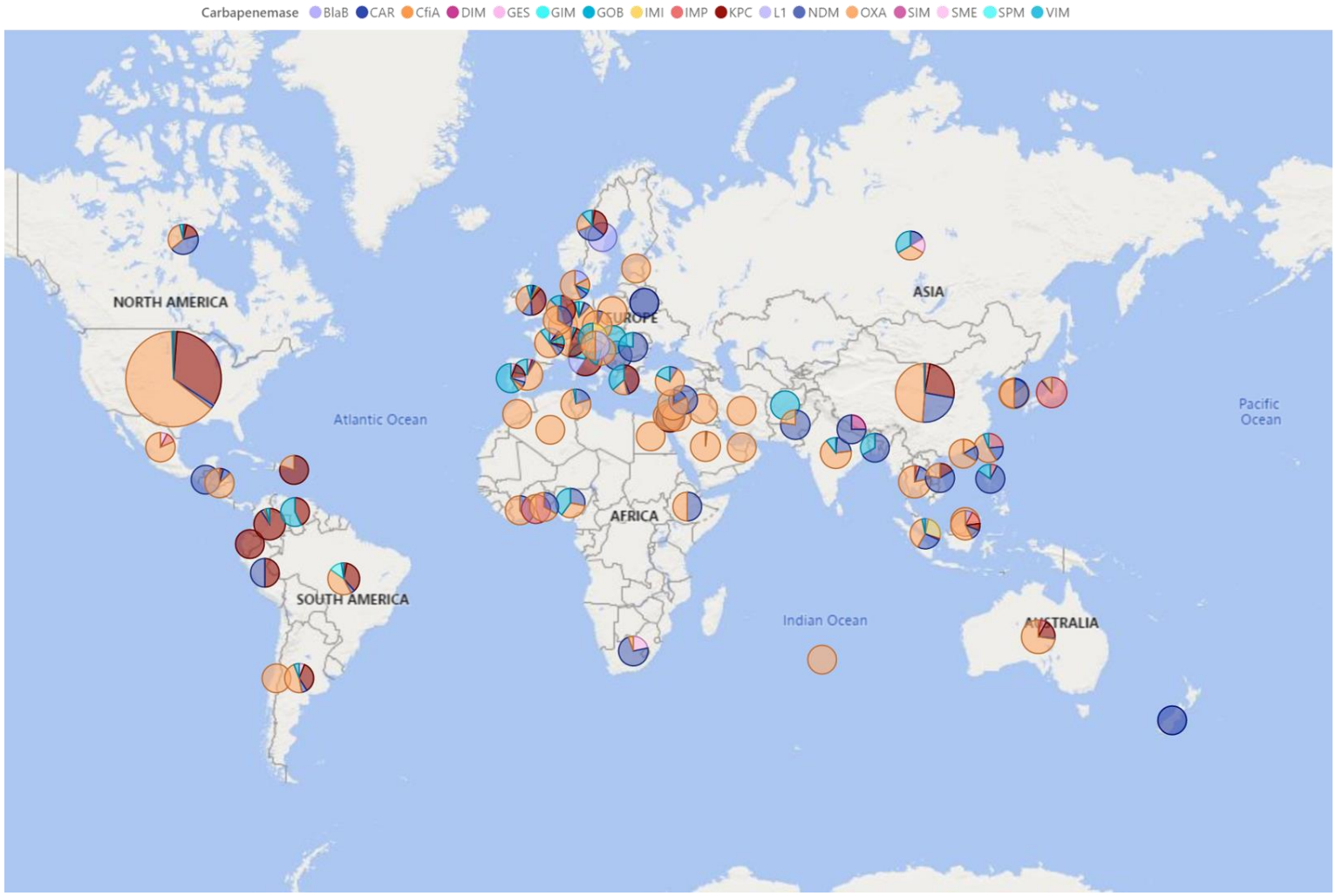
Carbapenemase
BlaB
CAR
CfiA
DIM
GES
GIM
GOB
IMI
IMP
KPC
L1
NDM
OXA
SIM
SME
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NORTH AMERICA
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Atlantic Ocean
Pacific Ocean
AFRICA
SOUTH AMERICA
Indian Ocean
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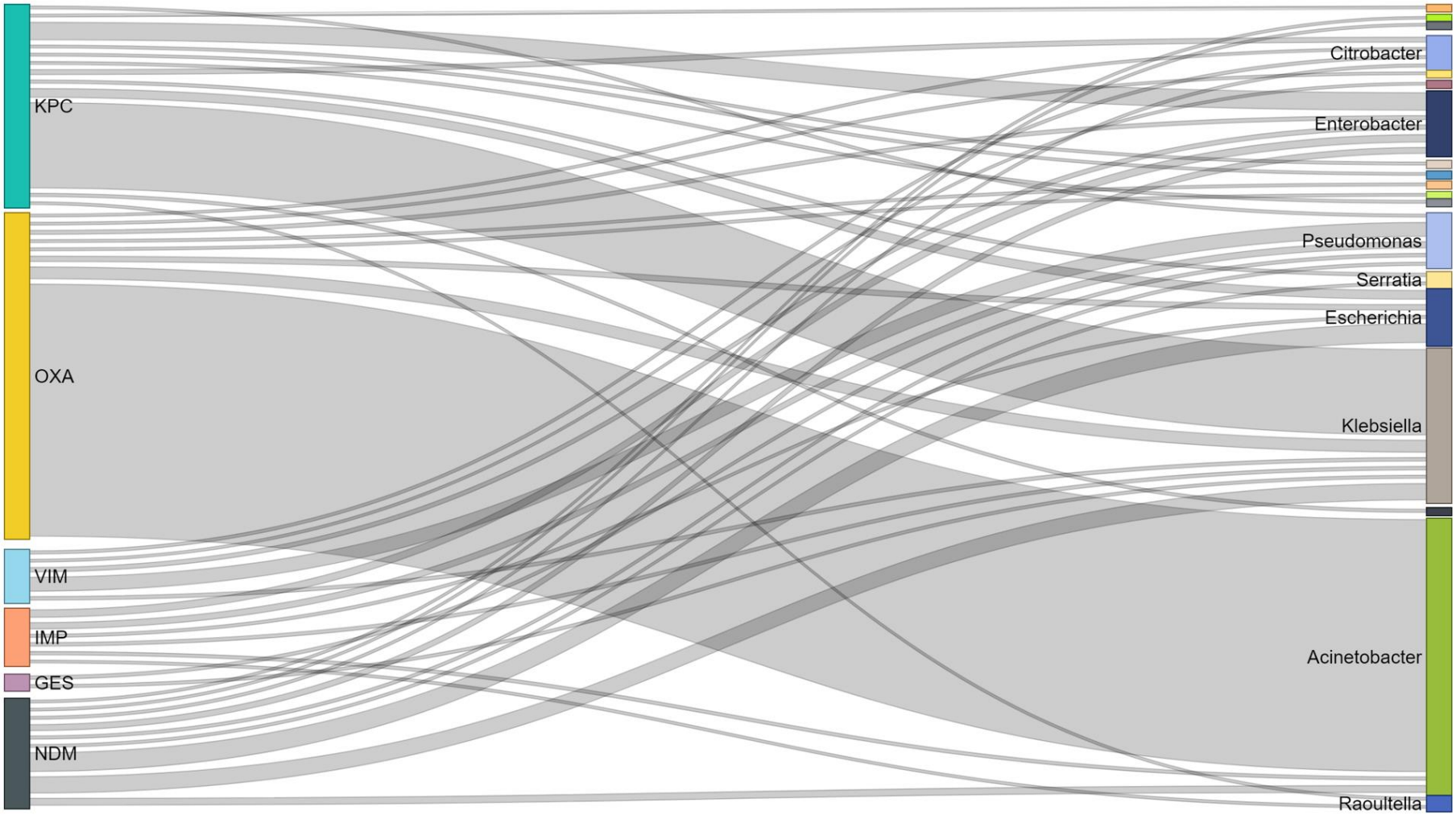
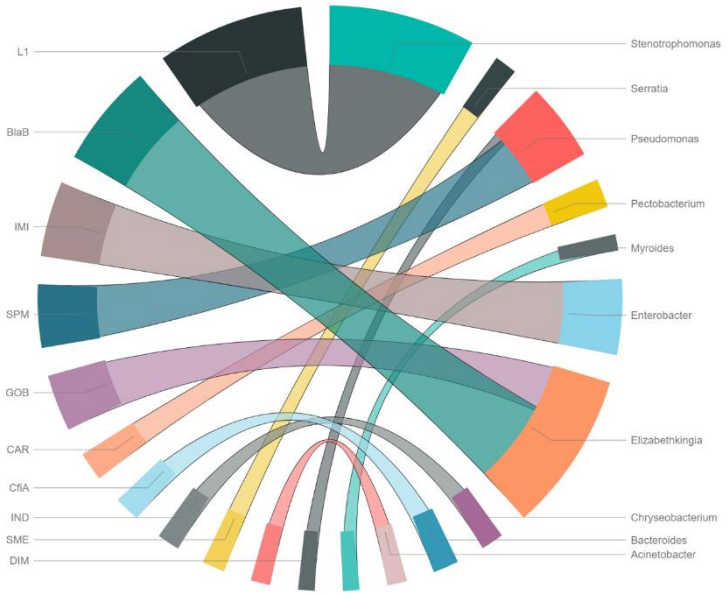

KPC
OXA
VIM
IMP
GES
NDM
Citrobacter
Enterobacter
Pseudomonas
Serratia
Escherichia
Klebsiella
Acinetobacter
Raoultella

156 **C**



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159 **Figure 1.** Dispersion of carbapenemase-encoding bacterial genomes. **A.** Worldwide
160 distribution of carbapenemase families. The size of the circles reflects the number of
161 hits. **B.** Distribution of carbapenemases observed in more than one genus. The legend
162 for the most frequently identified carbapenemases and bacterial genus is present on the
163 left and right axis, respectively. **C.** Representation of carbapenemases identified in only
164 one bacterial genus. Only carbapenemases that were identified more than 4 times were
165 included on the map and on the graphs. For a complete list of all carbapenemases,
166 please refer to **Table S2**.

167

168 **A large proportion of CEG-bearing ICEs belong to three MPF families and target**
169 **clinically-relevant gram-negative bacteria**

170 Our analysis with ICEfinder resulted in the identification of 251 CEGs within 248 putative
171 MGEs (**Table S3 and Table S4**). Of these, 224 putative ICEs were predicted. The
172 remaining 24 MGEs were considered as IMEs or putative conjugative regions. The later
173 designation is attributed to regions where an integrase and a qualified T4SS but no
174 relaxase or a type-IV coupling protein were identified. Using the CONJscan module of

175 MacSyFinder, we noted that the majority of the ICEfinder hits belong to three MPF
176 families: MPF_T, MPF_F and MPF_G (**Figure 2** and **Table S3**). ICEs of the MPF_T class were
177 particularly promiscuous, being responsible for the spread of several CEGs such as bla_{KPC-}
178 $_{2/3}$, bla_{NDM-1} and bla_{IMP-1} among clinically-relevant pathogens as *P. aeruginosa*, *E. coli* and
179 species belonging to the *Klebsiella* genus and the *Enterobacter cloaceae* complex. The
180 bla_{SPM-1} gene was exclusively identified in ICEs of the MPF_T class.

181 The bacterial hosts housing these elements belong to more than 70 sequence types
182 (STs), of which the *K. pneumoniae* ST258 is the most frequently represented (**Figure 3**
183 and **Table S3**). The worldwide spread of carbapenemase-producing *K. pneumoniae* has
184 been linked to some high-risk clones, including ST258 and close relatives that were also
185 identified in this study, such as ST11 and ST512 [26, 27]. We also reported several strains
186 belonging to ST78 from the *Enterobacter hormaechei*, recognized as a global driver of
187 CEGs [28, 29].

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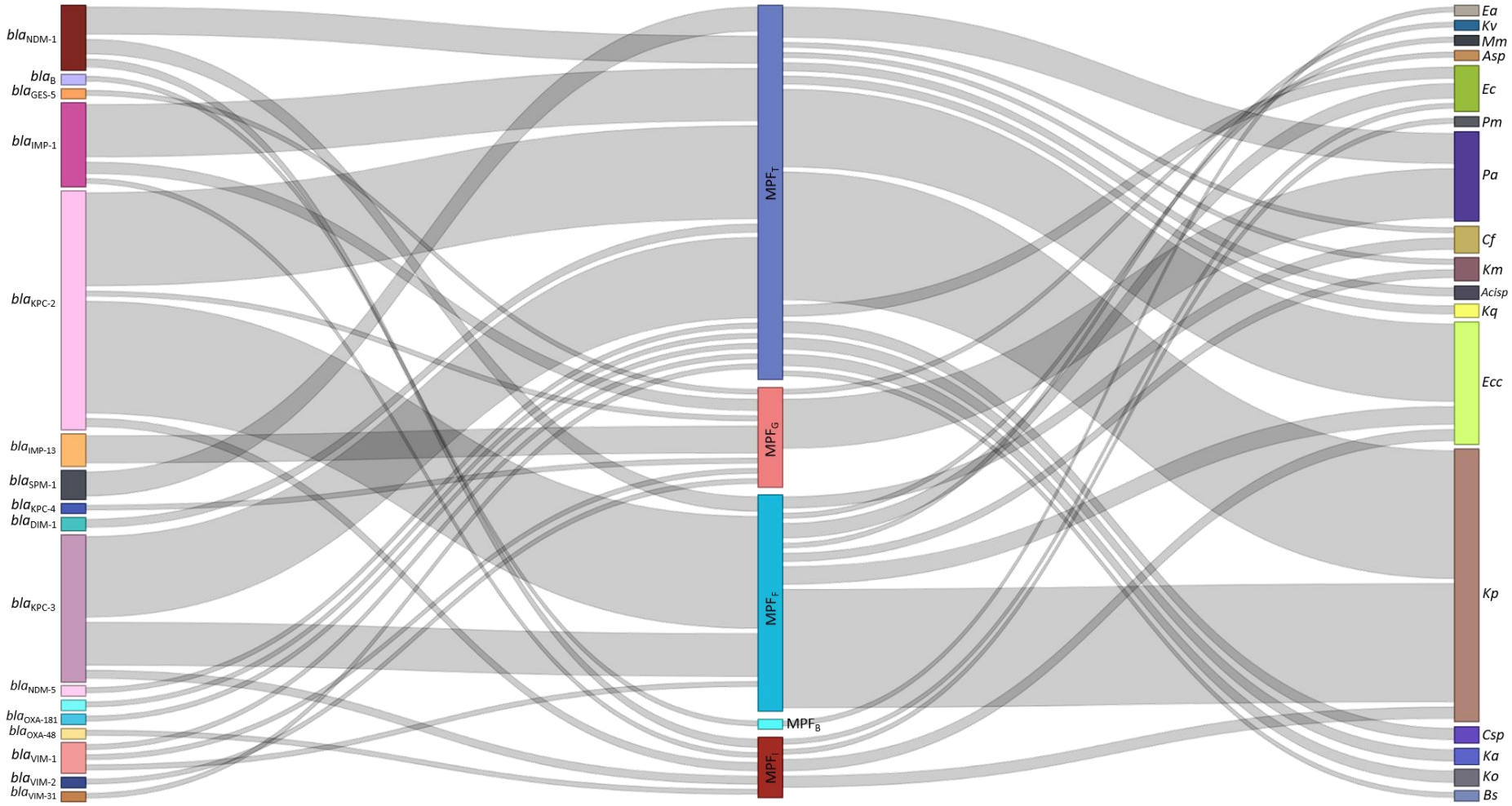
blaNDM-1
blaB
blaGES-5
blaIMP-1
blaKPC-2
blaIMP-13
blaSPM-1
blaKPC-4
blaDIM-1
blaKPC-3
blaNDM-5
blaOXA-181
blaOXA-48
blaVIM-1
blaVIM-2
blaVIM-31
MPFT
MPFG
MPFF
MPFB
MPFI
Ea
Kv
Mm
Asp
Ec
Pm
Pa
Cf
Km
Acisp
Kq
Ecc
Kp
Csp
Ka
Ko
Bs

200

201 **Figure 2.** Sankey diagram showing the contribution of different MPF families for the spread of CEGs among several bacteria. *Aci*sp stands for
202 *Acinetobacter* sp., *A*sp for *Achromobacter* sp., *Bs* for *Bacillus subtilis*, *Cf* for *Citrobacter freundii*, *C*sp for *Citrobacter* sp., *Ea* for *Elizabethkingia*
203 *anophelis*, *Ec* for *E. coli*, *Ec*c for *E. cloaceae* complex, *Ka* for *Klebsiella aerogenes*, *Km* for *Klebisella michiganensis*, *Ko* for *Klebsiella oxytoca, Kp* for
204 *K. pneumoniae*, *Kq* for *Klebsiella quasipneumoniae*, *Kv* for *Klebsiella variicola*, *Mm* for *Morganella morganii*, *Pa* for *P. aeruginosa* and Pm for
205 *Proteus mirabilis*.

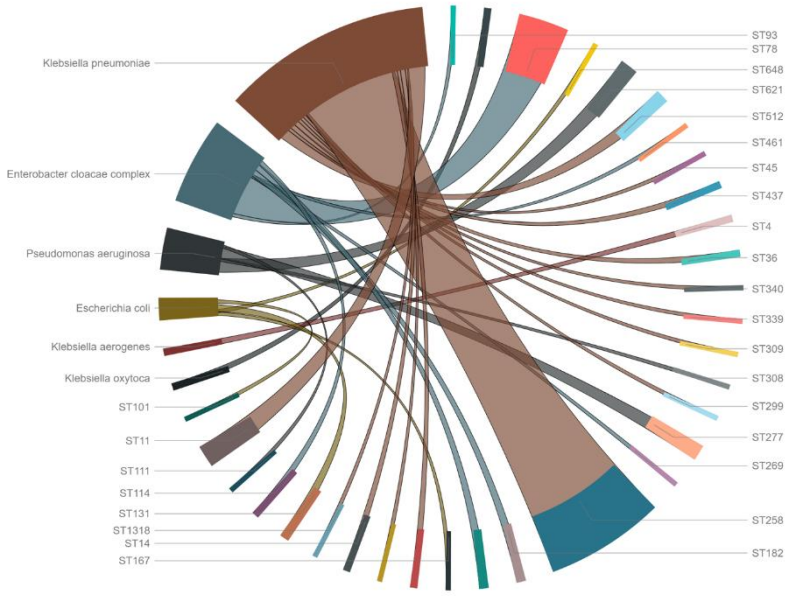

Figure 3. Sequence types (STs) of the ICEfinder hits, including ICEs, IMEs and putative conjugative regions. Only STs that were identified more than once were included on the graph. For a complete list of all STs, please refer to **Table S3**.

210 **A variable repertoire of CEG-bearing integrons and transposons target ICEs**

211 We identified 20 CEG variants among the 251 hits, dominated by *bla*$_{KPC-2}$, *bla*$_{KPC-3}$, *bla*$_{IMP-}$
212 $_{1}$ and *bla*$_{NDM-1,}$ and rare descriptions of *bla*$_{KPC-4}$, *bla*$_{IMP-10/13}$, *bla*$_{VIM-1/34}$, *bla*$_{DIM-1}$, *bla*$_{SPM-1}$
213 and *bla*$_{OXA-23/48/181}$ (**Figure 4** and **Table S3**). KPC-encoding ICEs although mostly observed
214 in *Klebsiella* spp. were also identified in *E. coli*, *Enterobacter* spp., *Citrobacter* spp. and
215 *Aeromonas* spp. IMP-encoding ICEs were restricted to *Enterobacter* spp. and
216 *Pseudomonas* spp.

217 The *bla*$_{KPC-2/3}$ genes were spread mainly by MPF$_T$ and MPF$_G$ classes (**Figure 2**). We noted
218 that composite transposons (e.g. ISCR4 and ISCR24) were frequently linked to the
219 acquisition of *bla*$_{SPM-1}$, *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$ [30, 31], while *bla*$_{IMP}$ and *bla*$_{VIM}$ were found
220 on class I integrons [3]. In addition, we observed that *bla*$_{KPC}$ genes were frequently
221 flanked by the insertion sequences (ISs) IS*Kpn7* and IS*Kpn6* within the Tn*4401*
222 transposon, which is capable of conferring a high frequency of transposition [32].
223 Interestingly, we found a *bla*$_{NDM-1}$ gene associated with a MPF$_T$ class ICE on a *B. subtilis*
224 strain (**Figure 2** and **4** and **Table S3**). This is the only situation where we found a CEG-
225 bearing ICE on Gram positive bacteria. Besides CEGs, the ICEs also harbor genes
226 conferring resistance to other antibiotics, such as aminoglycosides, fluoroquinolones,
227 macrolides, sulfonamides and trimethoprim (**Table S5**), widening the spectrum of
228 transmissible AR genes selectable by carbapenems due to linkage.

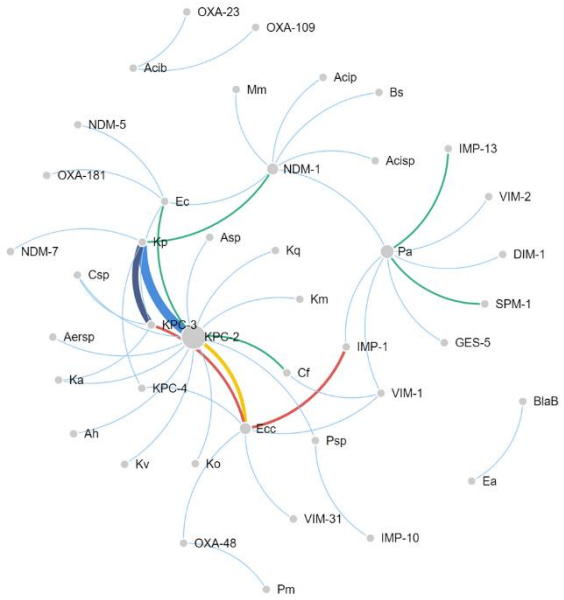

Figure 4. Distribution of carbapenemases among ICEfinder hits, including ICEs, IMEs and putative conjugative regions. Different colour links are related to the number of associations between a carbapenemase and a given bacterial species. The size of circles is proportional to the number of times each carbapenemase or bacterial strain was identified. *Acib* stands for *A. baumannii*, *Acip* for *Acinetobacter pittii*, *Aci*sp for *Acinetobacter* sp., *Aer*sp for *Aeromonas* sp., *Ah* for *Aeromonas hydrophila*, *A*sp for *Achromobacter* sp., *Bs* for *Bacillus subtilis*, *Cf* for *Citrobacter freundii*, *C*sp for *Citrobacter* sp., *Ea* for *Elizabethkingia anophelis*, *Ec* for *E. coli*, *Ec*c for *E. cloaceae* complex, *Ka* for *Klebsiella aerogenes*, *Km* for *Klebisella michiganensis*, *Ko* for *Klebsiella oxytoca, Kp* for *K. pneumoniae*, *Kq* for *Klebsiella quasipneumoniae*, *Kv* for *Klebsiella variicola*, *Mm* for *Morganella morganii*, *Pa* for *P. aeruginosa*, Pm for *Proteus mirabilis* and *Ps*p for *Pseudomonas* sp.

244 **Acquisition of additional traits by ICEs include competitive weapons as bacteriocins**

245 Besides genes conferring antibiotic resistance, some of the CEG-bearing ICEs harbor
246 other cargo genes that may confer a selective advantage to the ICE host, as bacteriocins
247 and restriction-modification systems. We identified class III bacteriocins among 6 *K.*
248 *pneumoniae* ST258 strains and associated with MPF_F and MPF_T classes (**Figure S1** and
249 **Table S6**). Additionally, our results show that some representatives of this ST and other
250 *Enterobacteriaceae* (e.g. *E. coli*, *Enterobacter* sp.) strains carry type I and II restriction-
251 modification systems or "orphan" methyltransferases (**Table S7**). Ammonium
252 quaternary compounds and metal tolerance genes, such as the *qac* (ammonium
253 quaternary), *mer* (mercury), *ars* (arsenic) and *cus/czc* (copper) operons were also found
254 in several genera, including *Enterobacter*, *Klebsiella* and *Pseudomonas* (**Table S8**). No
255 CRISPR-Cas systems were identified within these elements.

256

257 **Discussion**

258 We have set out to comprehensively identify the CEGs among all bacterial genomes
259 deposited in NCBI and the CEG-bearing ICE sequences. Our study considerably expands
260 the knowledge of the repertoire of CEGs-bearing ICEs. We uncovered more than 200
261 putative MGEs that may be involved in HGT of CEGs among all bacterial genomes. To
262 expand our predictions, we also used the CONJscan module of Macsyfinder to trace the
263 MPF families likely involved in spreading these genes.

264 Even though CEGs might rapidly spread worldwide, local selection is likely required for
265 them to reach fixation, as can be seen for the clonal expansion of *K. pneumoniae* ST258
266 harboring $bla_{KPC-2/3}$ [26, 27]. The scenario observed for the acquisition of the most
267 important CEGs by ICEs (Tn*4401* for $bla_{KPC-2/3}$, composite transposons for bla_{NDM-1} and
268 class I integrons for bla_{IMP} and bla_{VIM}) resembles that of plasmids [3] and provide
269 additional support to the notion that the line separating these elements is blurred [8, 9,
270 33]. We now show that besides plasmids, this promiscuous repertoire of integrons and
271 transposons frequently targets ICEs of different MPF families. Surprisingly, we noted
272 that only bla_{SPM-1} was unable to spread beyond the same clonal lineage [31]. Indeed, all
273 hits were identified in *P. aeruginosa* ST277 strains from Brazil and within a MPF_T ICE

274 family, indicating that likely these ICEs are only transferring vertically. Understanding
275 the limitations on this family of ICE's horizontal gene transfer may be translatable to
276 other, more transferable, ICEs and could underpin a control strategy to prevent the
277 spread of these elements in the future.

278 CEGs are rarely identified in Gram-positive bacteria. Local selection and enriched
279 metagenome might have also contributed to the ICE encoding NDM-1 observed in a *B.*
280 *subtilis* from a highly polluted marine environment [34]. Despite the works on ICEs in
281 Gram positive bacteria [35, 36], this first report of a CEG-bearing ICE in ubiquitous and
282 resilient bacterial groups is of remarkable epidemiological interest.

283 In addition to AR, ICEs can be involved in other traits such as heavy metal resistance,
284 carbon-source utilization, symbiosis, restriction-modification and bacteriocin synthesis
285 [9]. Since bacteria commonly inhabit highly competitive environments, the production
286 of toxins (such as bacteriocins) confers a selective advantage to the host [37]. Class III
287 bacteriocins – bacteriolysins - consists of high-molecular-weight and heat-labile toxins
288 [38]. We speculate that the presence of these toxins within the ICEs of six *K. pneumoniae*
289 ST258 strains can promote ICE stability by preferentially selecting for cells harboring the
290 ICE. Restriction-modification systems protect a bacterial host against the invasion of
291 foreign DNA through the action of a restriction enzyme that cuts DNA lacking the
292 epigenetically methylated DNA modified by the methyltransferase [39]. The presence of
293 these systems within ICEs may also allow their stable maintenance through
294 postsegregational killing. This has already been demonstrated for plasmids [40].
295 Additionally, these systems may also prevent further infection of the bacterial host by
296 another ICE or MGE lacking the methylation.

297 One caveat of our studies is that these results do not yet expose the complete set of
298 CEG-bearing ICEs present in all bacteria. There is an inherent bias in the number of times
299 we detect a particular CEG in certain pathogen genomes as some are over represented
300 in the database compared to others. It is possible that certain observations will flatten
301 out as more genomes are analyzed. This analysis also underestimates the extent of host
302 range because we only used assembled genomes. Less than 10% of the bacterial
303 genomes currently present on NCBI are complete. This is a major drawback since
304 ICEfinder can only detect ICEs within complete genomes or contigs with or without

305 directed repeats as the tRNA-distal boundaries, meaning that if the ICE was integrated
306 into the 3' or 5' ends of protein-encoding genes and/or split into several contigs, this
307 tool may miss the element. Even though ICEfinder uses the profile Hidden Markov
308 Models (HMM) for signature proteins coded by ICEs, we identified some plasmid hits
309 among our data using PlasmidFinder (**Table S9)** [41]. Also, ICEfinder may not provide the
310 accurate boundaries of ICEs. Indeed, the relaxase and the T4SS encoded by ICEs
311 resemble those of plasmids [6, 8, 11]. Plus, it is possible that ICEs and plasmids have
312 swapped conjugation modules along their evolutionary history [6]. We believe that a
313 more thorough exploration of this issue, especially regarding the precise delimitation of
314 ICEs, will be an important further step toward an improved understanding of the
315 contribution of these elements to bacterial adaptation and evolution of AR.

316 While we have chosen to focus on CEG-bearing genomes, our computational approach
317 can be applied to trace other relevant AR genes and other cargo genes that may confer
318 a selective advantage to the ICE host. Leveraging knowledge linking the accurate
319 prediction of ICE sequences to the carriage of AR genes, will not only improve our
320 understanding of HGT, but may also uncover potential approaches to tackle the spread
321 of AR.

322

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326

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