Integrative and conjugative elements, the neglected drivers of carbapenemase-encoding genes

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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*). 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 **Introduction**

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

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

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

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

92

93 **Material and methods**

94 **Bacterial genomes and carbapenemases search**

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

102

103 **Tracing ICEs among the bacterial genomes**

104 The CEG-bearing genomes were annotated with prokka v1.13
105 (https://github.com/tseemann/prokka) [14], and the GenBank files were used as input
106 to mine ICEs on the standalone version of ICEfinder
107 (http://202.120.12.136:7913/ICEfinder/ICEfinder.html). Putative MGEs were annotated
108 as ICEs if complete conjugation and relaxase modules, plus an integrase or DDE
109 transposase superfamily were identified within the boundaries of the element, and as
110 integrative and mobilizable elements (IMEs) if the elements contained all except genes
111 involved in conjugation. The translated coding sequences of extracted MGEs were
112 analyzed on the CONJscan module of MacSyFinder (https://github.com/gem-
113 pasteur/macsyfinder) [15, 16]. A MGE was classified as an ICE when a positive prediction
114 was verified by ICEfinder and/or the CONJscan module. To predict the multi-locus
115 sequence type of each CEG-bearing MGEs, we used mlst v2.16.1
116 (https://github.com/tseemann/mlst), which scans the genomes against PubMLST typing
117 schemes (https://pubmlst.org/) [17].

118

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

120 Screening of AR genes among ICEs was performed using ResFinder v3.1
121 (https://cge.cbs.dtu.dk/services/ResFinder/) [18]. Metal and biocides tolerance genes
122 were blasted against a protein database downloaded from BacMet
123 (http://bacmet.biomedicine.gu.se) [19] using the aforementioned diamond parameters.
124 Associated MGEs were annotated using Galileo™ AMR
125 (https://galileoamr.arcbio.com/mara/) (Arc Bio, Cambridge, MA) [20]. Bagel4
126 (http://bagel4.molgenrug.nl/) was used to mine ICEs for bacteriocins and ribosomally
127 synthesized and posttranslationally modified peptides [21]. We ran our extracted ICEs
128 against REBASE (http://rebase.neb.com/rebase/rebase.html) to look for restriction-
129 modification systems [22]. We also used CRISPRCasFinder (https://crisprcas.i2bc.paris-
130 saclay.fr/CrisprCasFinder/Index) to look for CRISPR (clustered regularly interspaced
131 short palindromic repeats) arrays and their associated (Cas) proteins within ICE
132 sequences [23].

133

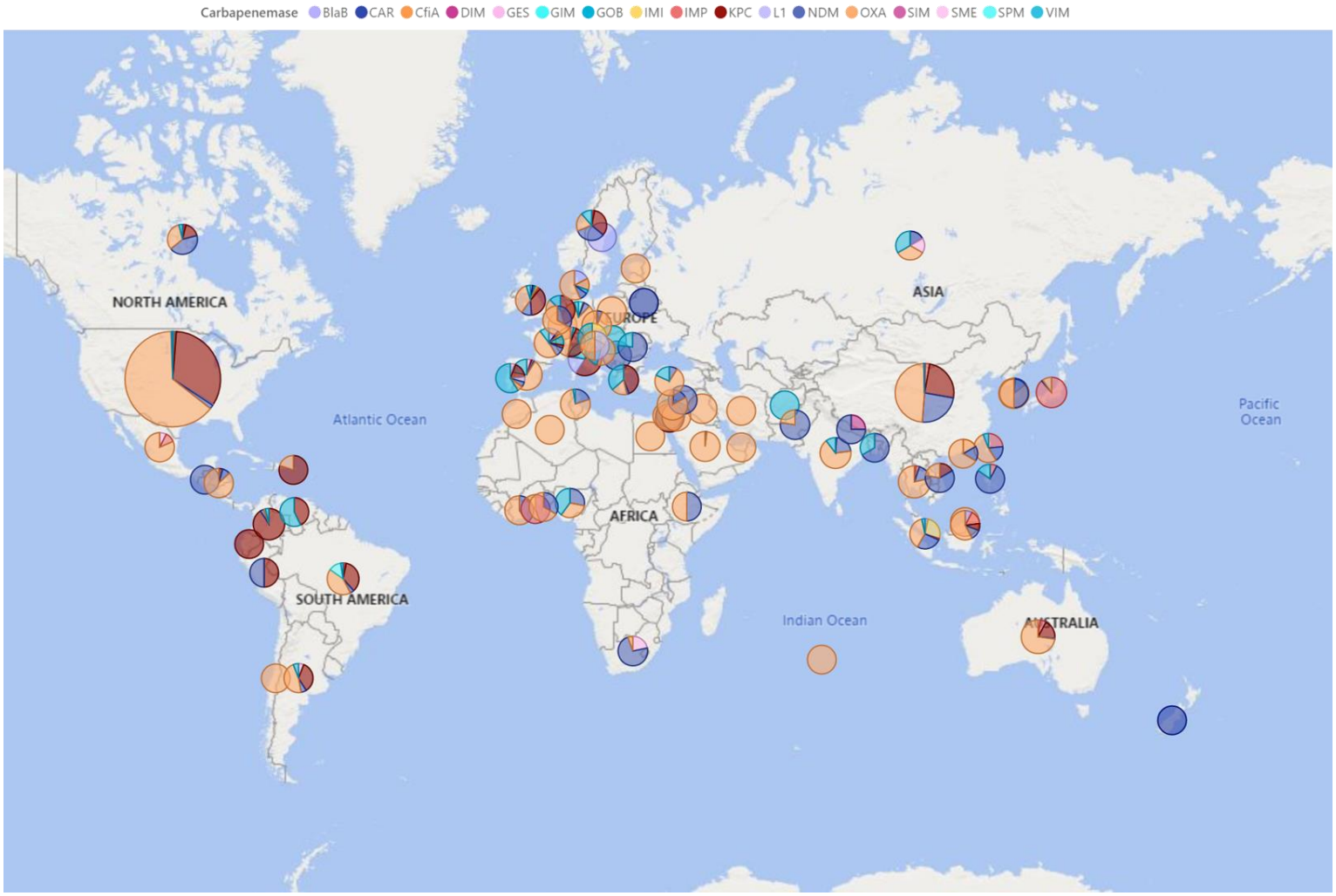
134 **Results**

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

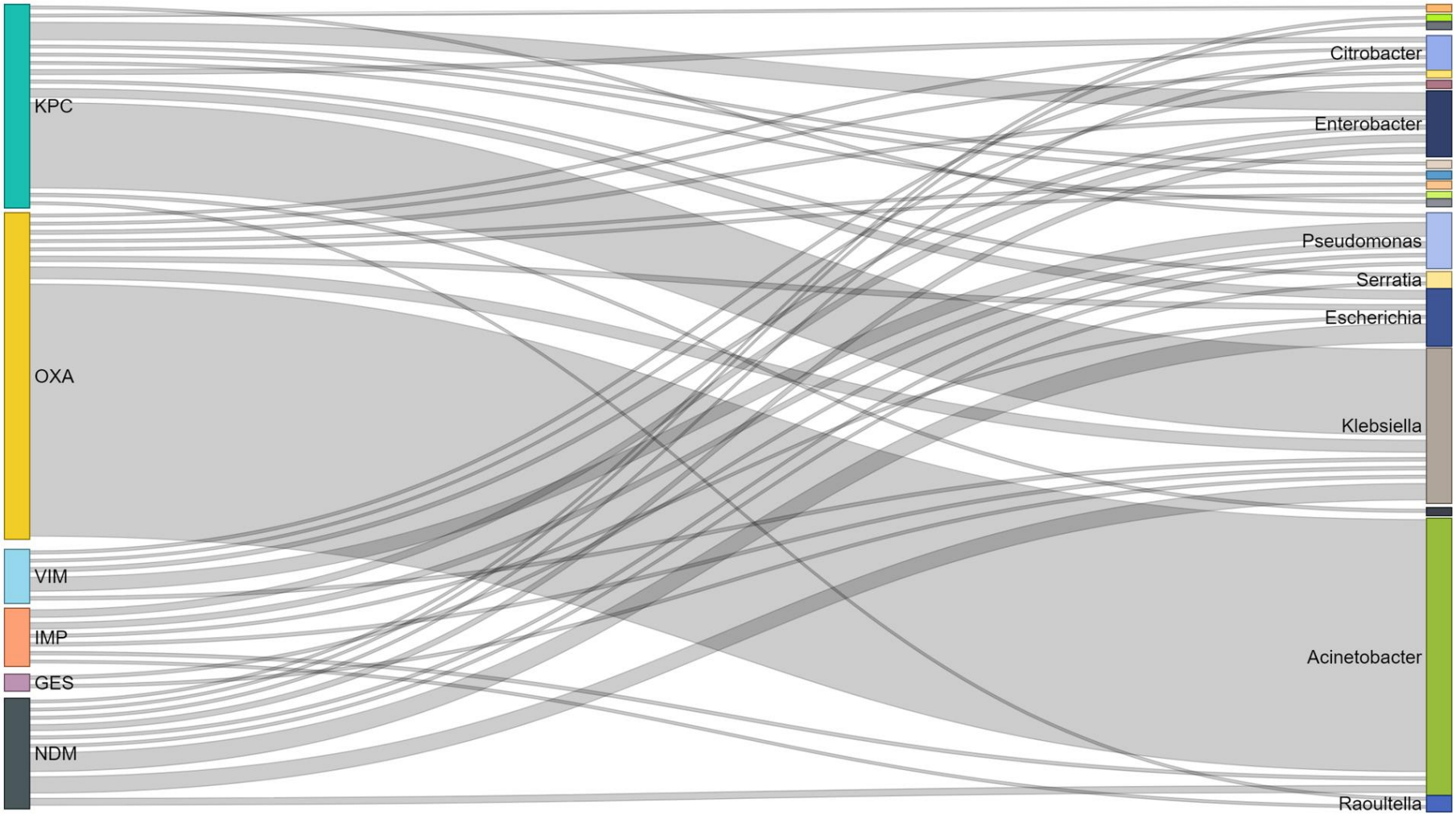
136 We retrieved a total of 107,887 bacterial genomes from NCBI and we identified 7457
137 CEGs belonging to 33 beta-lactamase families among 7343 genomes (**Table S2**). Most
138 CEG-bearing genomes were detected in the USA, China and Europe (**Figure 1A** and **Table**
139 **S2**). Also, the majority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

149 on bacterial genomes other than the *Pseudomonas* genus, a previous study has reported
150 the presence of $bla_{\text{DIM-1}}$ in *Enterobacteriaceae* [24].

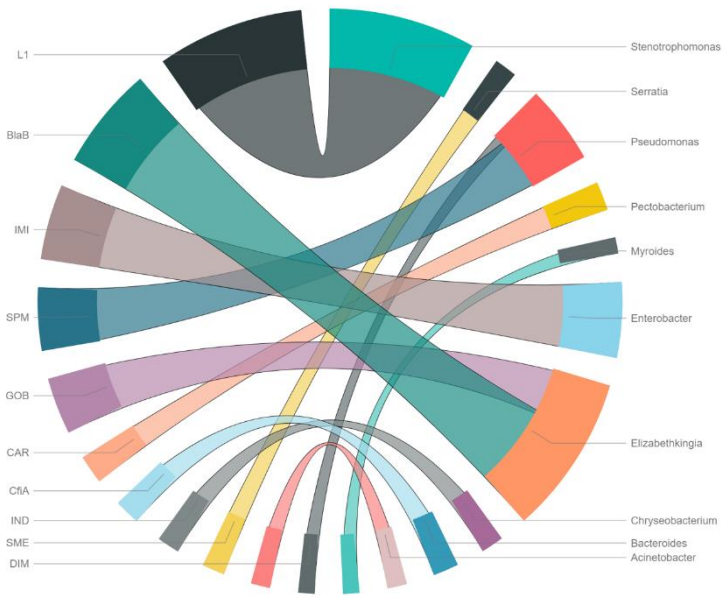
A


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

B


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

155 **C**



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

166

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

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

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

180 The bacterial hosts housing these elements belong to more than 70 sequence types
181 (STs), of which the *K. pneumoniae* ST258 is the most frequently represented (**Figure 3**
182 and **Table S3**). The worldwide spread of carbapenemase-producing *K. pneumoniae* has
183 been linked to some high-risk clones, including ST258 and close relatives that were also
184 identified in this study, such as ST11 and ST512 [25, 26]. We also reported several strains
185 belonging to ST78 from the *Enterobacter hormaechei*, recognized as a global driver of
186 CEGs [27, 28].

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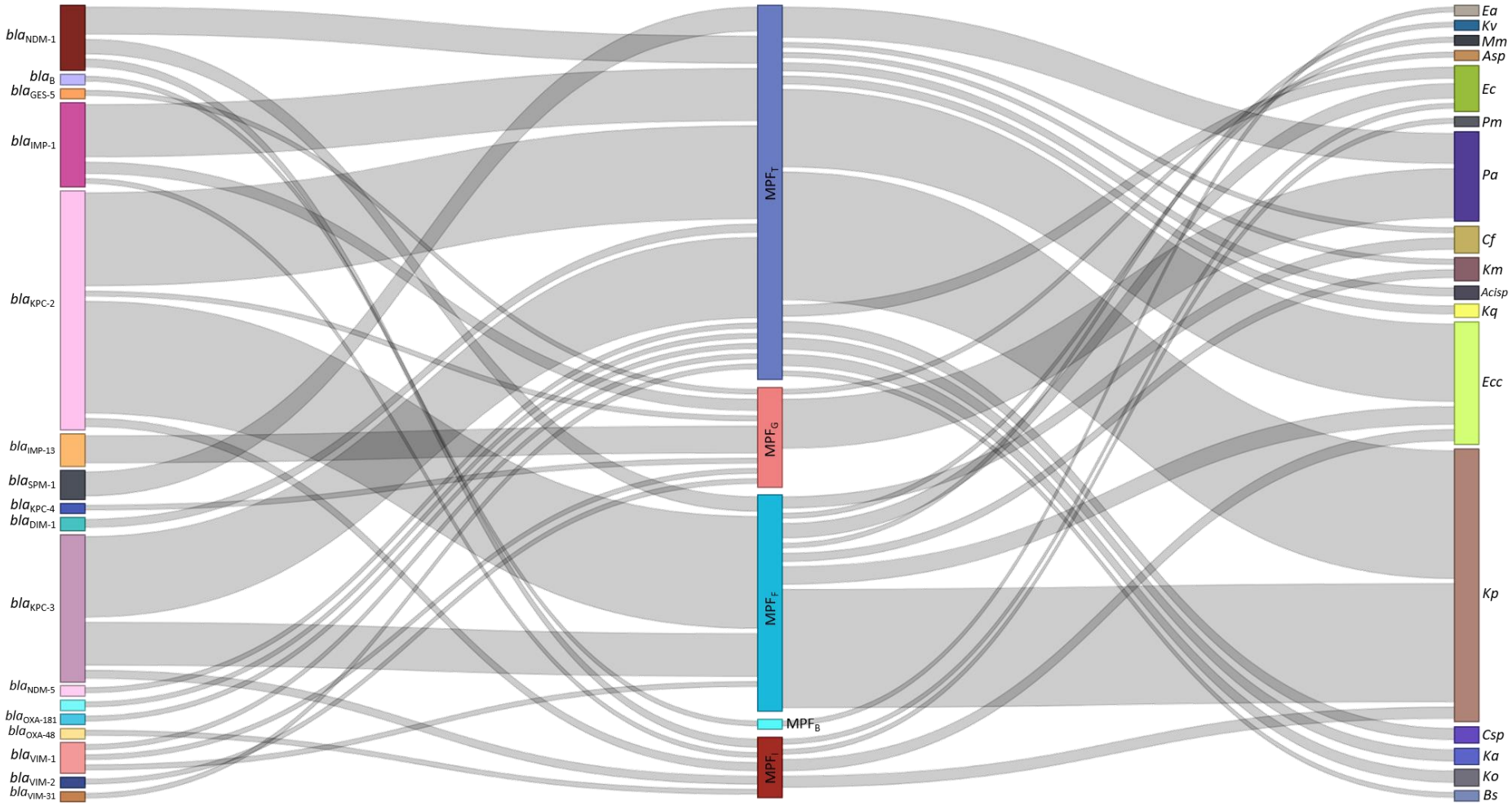
bla_{NDM-1}
bla_{B}
bla_{GES-5}
bla_{IMP-1}
bla_{KPC-2}
bla_{IMP-13}
bla_{SPM-1}
bla_{KPC-4}
bla_{DIM-1}
bla_{KPC-3}
bla_{NDM-5}
$bla_{OXA-181}$
bla_{OXA-48}
bla_{VIM-1}
bla_{VIM-2}
bla_{VIM-31}
MPF_{T}
MPF_{G}
MPF_{F}
MPF_{B}
MPF_{I}
Ea
Kv
Mm
Asp
Ec
Pm
Pa
Cf
Km
Acisp
Kq
Ecc
Kp
Csp
Ka
Ko
Bs

199

200 **Figure 2.** Sankey diagram showing the contribution of different MPF families for the spread of CEGs among several bacteria. *Aci*sp stands for
201 *Acinetobacter* sp., *A*sp for *Achromobacter* sp., *Bs* for *Bacillus subtilis*, *Cf* for *Citrobacter freundii*, *C*sp for *Citrobacter* sp., *Ea* for *Elizabethkingia*
202 *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
203 *K. pneumoniae*, *Kq* for *Klebsiella quasipneumoniae*, *Kv* for *Klebsiella variicola*, *Mm* for *Morganella morganii*, *Pa* for *P. aeruginosa* and Pm for
204 *Proteus mirabilis*.

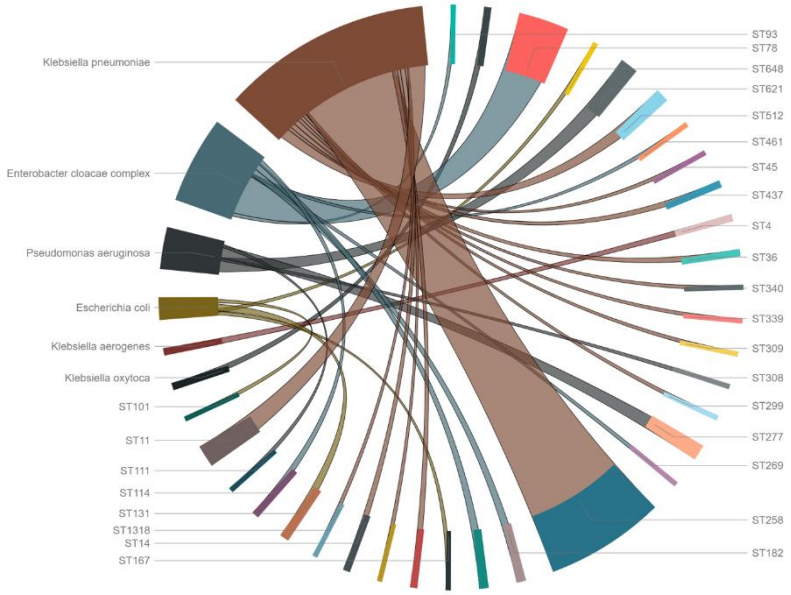

Figure 3. Sequence types (STs) of the ICEfinder hits, including ICEs, IMEs and putative conjugative regions. Only STs that were identified more than once were included on the graph. For a complete list of all STs, please refer to **Table S3**.

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

210 We identified 20 CEG variants among the 251 hits, dominated by *bla*$_{KPC-2}$, *bla*$_{KPC-3}$, *bla*$_{IMP-}$
211 $_{1}$ and *bla*$_{NDM-1}$ (**Figure 4** and **Table S3**). The *bla*$_{KPC-2/3}$ genes were spread mainly by MPF$_T$
212 and MPF$_G$ classes (**Figure 2**). We noted that composite transposons (e.g. ISCR4 and
213 ISCR24) were frequently linked to the acquisition of *bla*$_{SPM-1}$, *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$ [29,
214 30], while *bla*$_{IMP}$ and *bla*$_{VIM}$ were found on class I integrons [3]. In addition, we observed
215 that *bla*$_{KPC}$ genes were frequently flanked by the insertion sequences (ISs) IS*Kpn7* and
216 IS*Kpn6* within the Tn*4401* transposon, which is capable of conferring a high frequency
217 of transposition [31]. Interestingly, we found a *bla*$_{NDM-1}$ gene associated with a MPF$_T$
218 class ICE on a *B. subtilis* strain (**Figure 2** and **4** and **Table S3**). This is the only situation
219 where we found a CEG-bearing ICE on Gram positive bacteria. Besides CEGs, the ICEs
220 also harbor genes conferring resistance to other antibiotics, such as aminoglycosides,
221 fluoroquinolones, macrolides and trimethoprim (**Table S5**), widening the spectrum of
222 transmissible AR genes selectable by carbapenems due to linkage.

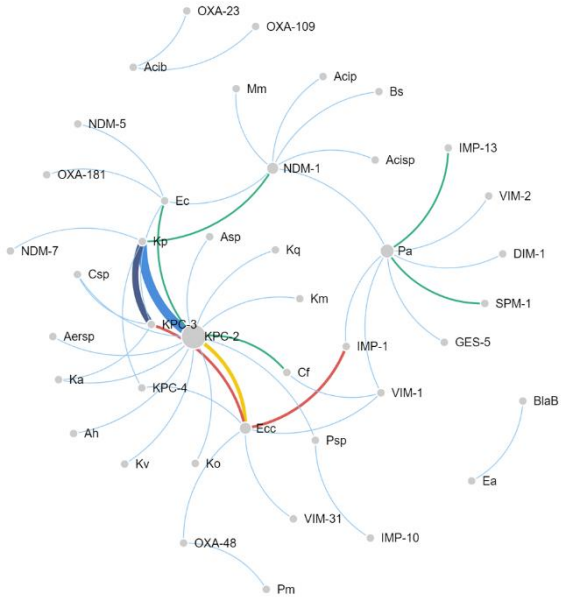

Figure 4. Distribution of carbapenemases among ICEfinder hits, including ICEs, IMEs and putative conjugative regions. Different colour links are related to the number of associations between a carbapenemase and a given bacterial species. The size of circles is proportional to the number of times each 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.

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

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

250

251 **Discussion**

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

258 Even though CEGs might rapidly spread worldwide, local selection is likely required for
259 them to reach significant fixation, as can be seen for the clonal expansion of *K.*
260 *pneumoniae* ST258 harboring $bla_{KPC-2/3}$ [25, 26]. The scenario observed for the
261 acquisition of the most important CEGs by ICEs (Tn*4401* for $bla_{KPC-2/3}$, composite
262 transposons for bla_{NDM-1} and class I integrons for bla_{IMP} and bla_{VIM}) resembles that of
263 plasmids [3] and provide additional support to the notion that the line separating these
264 elements is blurring [8, 9, 32]. We now show that besides plasmids, this promiscuous
265 repertoire of integrons and transposons frequently targets ICEs of different MPF
266 families. Surprisingly, we noted that only bla_{SPM-1} was unable to spread beyond the same
267 clonal lineage [30]. Indeed, all hits were identified in *P. aeruginosa* ST277 strains from

268 Brazil and within a MPF_T ICE family, indicating that likely these ICEs are only transferring
269 vertically. Understanding the limitations on this family of ICE's horizontal gene transfer
270 may be translatable to other, more transferable, ICEs and could underpin a control
271 strategy to prevent the spread of these elements in the future.

272 CEGs are rarely identified in Gram-positive bacteria. Local selection and enriched
273 metagenome might have also contributed to the ICE encoding NDM-1 observed in a *B.*
274 *subtilis* from a highly polluted marine environment [33]. Despite the works on ICEs in
275 Gram positive bacteria [34, 35], this first report of a CEG-bearing ICE in ubiquitous and
276 resilient bacterial groups is of remarkable epidemiological interest.

277 In addition to AR, ICEs can be involved in other traits such as heavy metal resistance,
278 carbon-source utilization, symbiosis, restriction-modification and bacteriocin synthesis
279 [9]. Since bacteria commonly inhabit highly competitive environments, the production
280 of toxins (such as bacteriocins) confers a selective advantage to the host [36]. Class III
281 bacteriocins – bacteriolysins - consists of high-molecular-weight and heat-labile toxins
282 [37]. We speculate that the presence of these toxins within the ICEs of six *K. pneumoniae*
283 ST258 strains can promote ICE stability by preferentially selecting for cells harboring the
284 ICE. Restriction-modification systems protect a bacterial host against the invasion of
285 foreign DNA through the action of a restriction enzyme that cuts DNA lacking the
286 epigenetically methylated DNA modified by the methyltransferase [38]. The presence of
287 these systems within ICEs may also allow their stable maintenance through
288 postsegregational killing. This has already been demonstrated for plasmids [39].
289 Additionally, these systems may also prevent further infection of the bacterial host by
290 another ICE or MGE lacking the methylation.

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

299 directed repeats as the tRNA-distal boundaries, meaning that if the ICE was integrated
300 into the 3' or 5' ends of protein-encoding genes and/or split into several contigs, this
301 tool may miss the element. Even though ICEfinder uses the profile Hidden Markov
302 Models (HMM) for signature proteins coded by ICEs, we identified some plasmid hits
303 among our data using PlasmidFinder (**Table S9)** [40]. Also, ICEfinder may not provide the
304 accurate boundaries of ICEs. Indeed, the relaxase and the T4SS encoded by ICEs
305 resemble those of plasmids [6, 8, 11]. Plus, it is possible that ICEs and plasmids have
306 swapped conjugation modules along their evolutionary history [6]. We believe that a
307 more thorough exploration of this issue, especially regarding the precise delimitation of
308 ICEs, will be an important further step toward an improved understanding of the
309 contribution of these elements to bacterial adaptation and evolution of AR.

310 While we have chosen to focus on CEG-bearing genomes, our computational approach
311 can be applied to trace other relevant AR genes and other cargo genes that may confer
312 a selective advantage to the ICE host. Leveraging knowledge linking the accurate
313 prediction of ICE sequences to the carriage of AR genes, will not only improve our
314 understanding of HGT, but may also uncover potential approaches to halt the spread of
315 AR.

316

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