Comprehensive genome data analysis 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 (n=107,887). We detected 7457 CEGs, of which 118 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_F, MPF_T and MPF_G classes and often encode additional adaptive traits such as resistance to other antibiotics (e.g. aminoglycosides and fluoroquinolones), restriction-modification systems and bacteriocin production. This study provides a snapshot of the different CEGs associated with ICEs among available bacterial genomes and sheds light on the underappreciated contribution of ICEs to the spread of carbapenem resistance globally.

48 ## Introduction

49 Due to the importance of carbapenems for the treatment of severe infections in
50 humans, the WHO has stated these antibiotics should be reserved for infections caused
51 by multidrug resistant Gram-negative bacteria in humans [1]. Recently, the same agency
52 presented a list of bacterial pathogens for which research and development of new
53 antibiotics are urgently required, and the top priority pathogens were the carbapenem-
54 resistant strains of *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and
55 *Enterobacteriaceae* [2].

56 The evolution of carbapenem-resistance in bacteria is often driven by the horizontal
57 gene transfer (HGT) of carbapenemase-encoding genes (CEGs) [3, 4]. Carbapenemases
58 are beta-lactamases able to hydrolyze carbapenems as well as most of other beta-
59 lactam antibiotics. The CEGs are often located on integrons or transposons that
60 themselves target mobile genetic elements (MGEs) such as plasmids [3, 4], which makes
61 the dissemination of these genes unpredictably complex within bacterial communities.
62 Recently, we demonstrated that another type of MGE, the integrative and conjugative
63 elements (ICEs), are likely to play a significant role as vehicles for the dissemination of
64 CEGs among *P. aeruginosa* [5]. ICEs are self-transmissible MGEs that can integrate into
65 and excise from the genome (as transposons and phages do) and can exist as circular,
66 sometimes replicable, extrachromosomal elements and be transferred by conjugation
67 (as some plasmids do) [4, 6–9]. ICEs appear to have a bipartite lifestyle that shifts
68 between vertical and horizontal transmission [10, 11]. HGT by conjugation requires
69 three main components: a relaxase, a mating pair formation system (MPF) and a type-
70 IV coupling protein (T4CP), with the last two forming a spanning-membrane multi-

71 protein complex named Type-IV Secretion System (T4SS). Until now, eight MPF classes
72 were proposed (B, C, F, FA, FATA, G, I and T), based on the phylogeny of VirB4, the only
73 ubiquitous protein among the T4SS [6]. The MPF_T is widely distributed both in
74 conjugative plasmids and ICEs, while MPF_F is more prevalent in plasmids and MPF_G on
75 ICEs [12].

76 Given that ICEs were identified in most bacterial clades and were proposed to be more
77 prevalent than conjugative plasmids [6], we conducted an *in silico* analysis to explore
78 the distribution of CEG-bearing ICEs among all sequenced bacterial genomes. Our results
79 demonstrate that CEG-bearing ICEs mainly belong to three MPF families and are
80 primarily located in several bacterial pathogens. Our analysis highlights the importance
81 of thoroughly investigating these elements as important vehicles for the spread of
82 antibiotic resistance (AR), particularly to carbapenems.

83

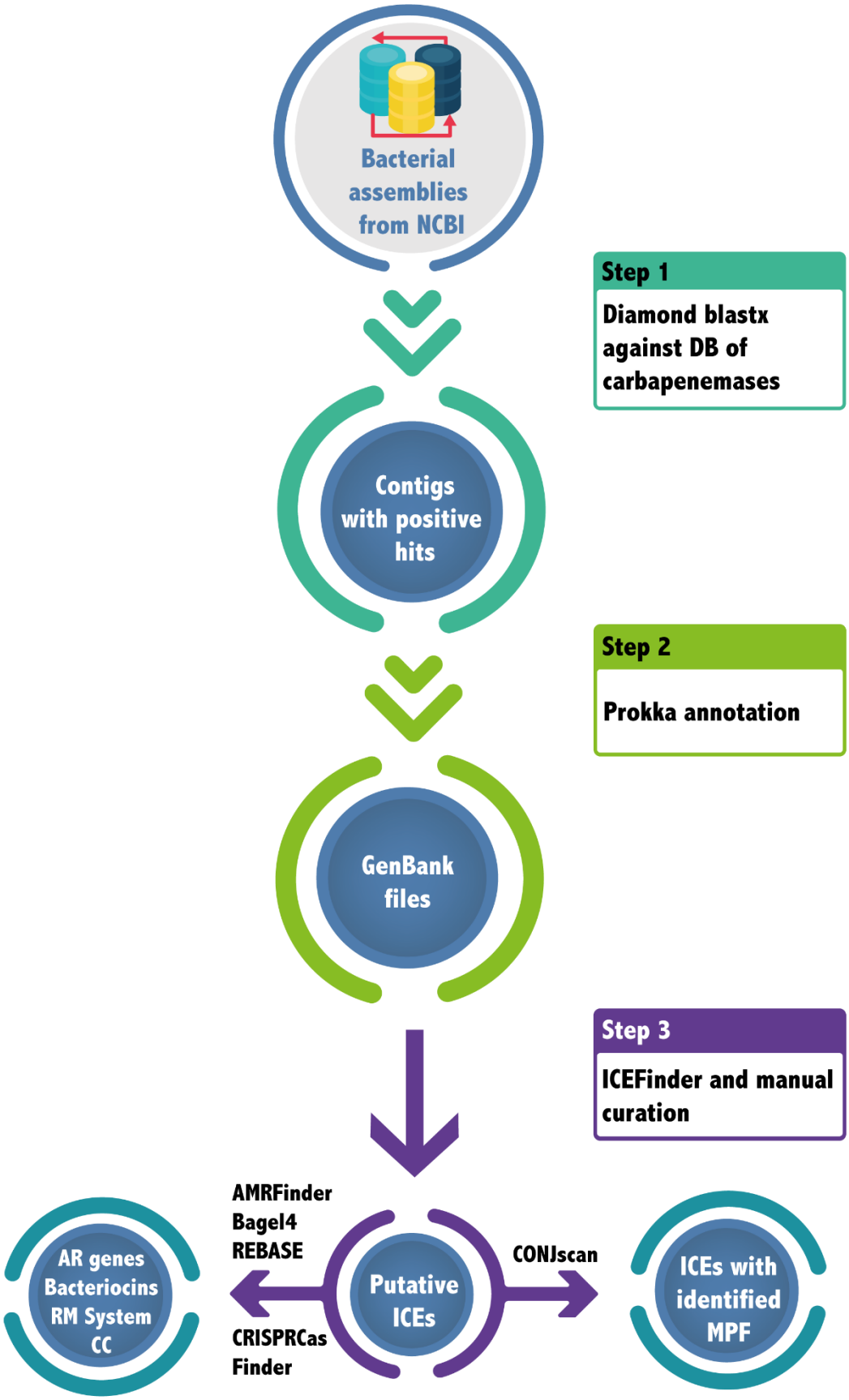
84 Material and methods

85 Bacterial genomes and carbapenemases search

86 All RefSeq bacterial genomes available in NCBI (accessed on 02/03/2018), including
87 complete and draft genome sequences, were downloaded and blasted against a local
88 database of 688 carbapenemases (**Table S1**), adapted from AMRfinder
89 (https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/AMRFinder/) [13],
90 using diamond v0.9.25 (https://github.com/bbuchfink/diamond) [14] with the following
91 parameters: 'diamond blastx –d DB.dmnd –o hits.txt --id 100 --subject-cover 100 -f 6 --

92 sensitive'. A workflow with all the analyses performed in the downloaded bacterial

93 genomes is presented in **Figure 1**.



Abbreviations: AR, Antibiotic Resistance; CC, CRISPR-Cas; DB, Database; ICEs, Integrative and Conjugative Elements; MPF, Mating-Pair Formation; RM, Restriction-Modification.

95 **Figure 1.** Overview of the workflow followed in this study. All complete and draft
96 genomes available in NCBI were downloaded and blasted against a database of
97 carbapenemases using diamond (Step 1). Hits with CEGs were annotated with prokka
98 (Step 2) and the resulting GenBank files were used as input in ICEfinder (Step 3).
99 CONJscan was used to identify the MPF family of each putative ICE. ICEs were then
100 mined for AR genes, bacteriocins, restriction-modification systems and CRISPR arrays
101 and their associated (Cas) proteins.

102

103 Tracing ICEs among the bacterial genomes

104 The CEG-bearing genomes were annotated with prokka v1.14.0
105 (https://github.com/tseemann/prokka) [15], and the GenBank files were used as input
106 to mine ICEs on the standalone version of ICEfinder [16]. Putative MGEs were annotated
107 as ICEs if complete conjugation and relaxase modules, plus an integrase or DDE
108 transposase superfamily were identified within the boundaries of the element (**Table**
109 **S2**). The translated coding sequences of extracted ICEs were analyzed on the standalone
110 CONJscan module of MacSyFinder v1.0.5 (https://github.com/gem-
111 pasteur/macsyfinder) [17, 18] to predict MPF families. Hits corresponding to putative
112 plasmids (**Table S3**) were identified by blasting the extracted nucleotide sequences
113 against NCBI and were excluded from the analyses. To identify the multi-locus sequence
114 type of each CEG-bearing ICE, we used mlst v2.16.1
115 (https://github.com/tseemann/mlst), which scans the genomes against PubMLST typing
116 schemes (https://pubmlst.org/) [19].

117

118 ## Characterization of the CEG-bearing ICEs

119 Screening of AR genes among ICEs was performed using amrfinder v3.2.3
120 (https://github.com/ncbi/amr) [13]. Associated MGEs were annotated using Galileo™
121 AMR (https://galileoamr.arcbio.com/mara/) (Arc Bio, Cambridge, MA) [20]. Bagel4
122 (http://bagel4.molgenrug.nl/) was used to mine ICEs for bacteriocins and ribosomally
123 synthesized and posttranslationally modified peptides [21]. We ran our extracted ICEs
124 against REBASE (http://rebase.neb.com/rebase/rebase.html) to look for restriction-
125 modification systems [22]. We also used CRISPRCasFinder (https://crisprcas.i2bc.paris-
126 saclay.fr/CrisprCasFinder/Index) to look for CRISPR (clustered regularly interspaced
127 short palindromic repeats) arrays and their associated (Cas) proteins within ICE
128 sequences [23].

129

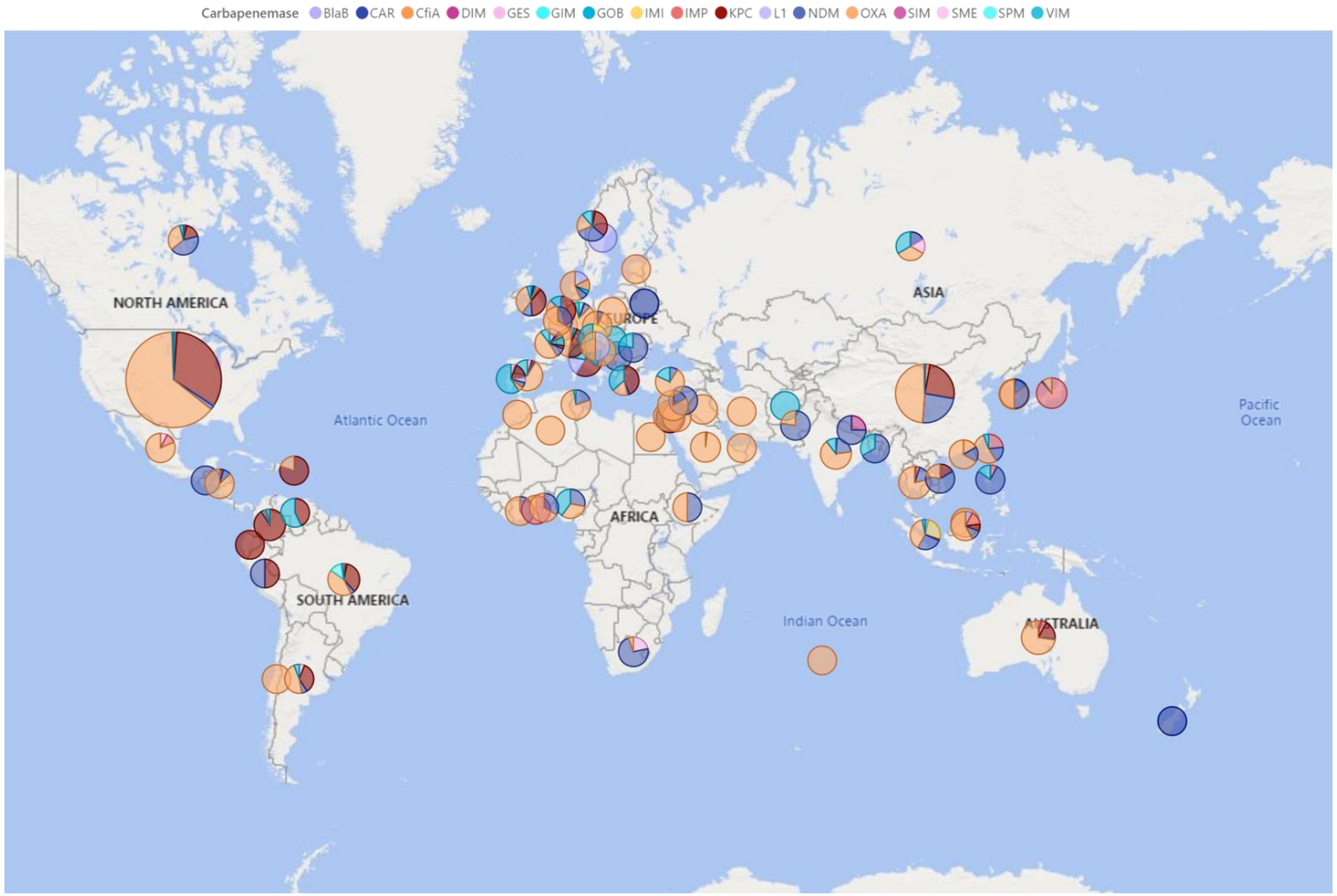
130 ## Results

131 ## Carbapenemase-encoding genes are mainly found in Proteobacteria

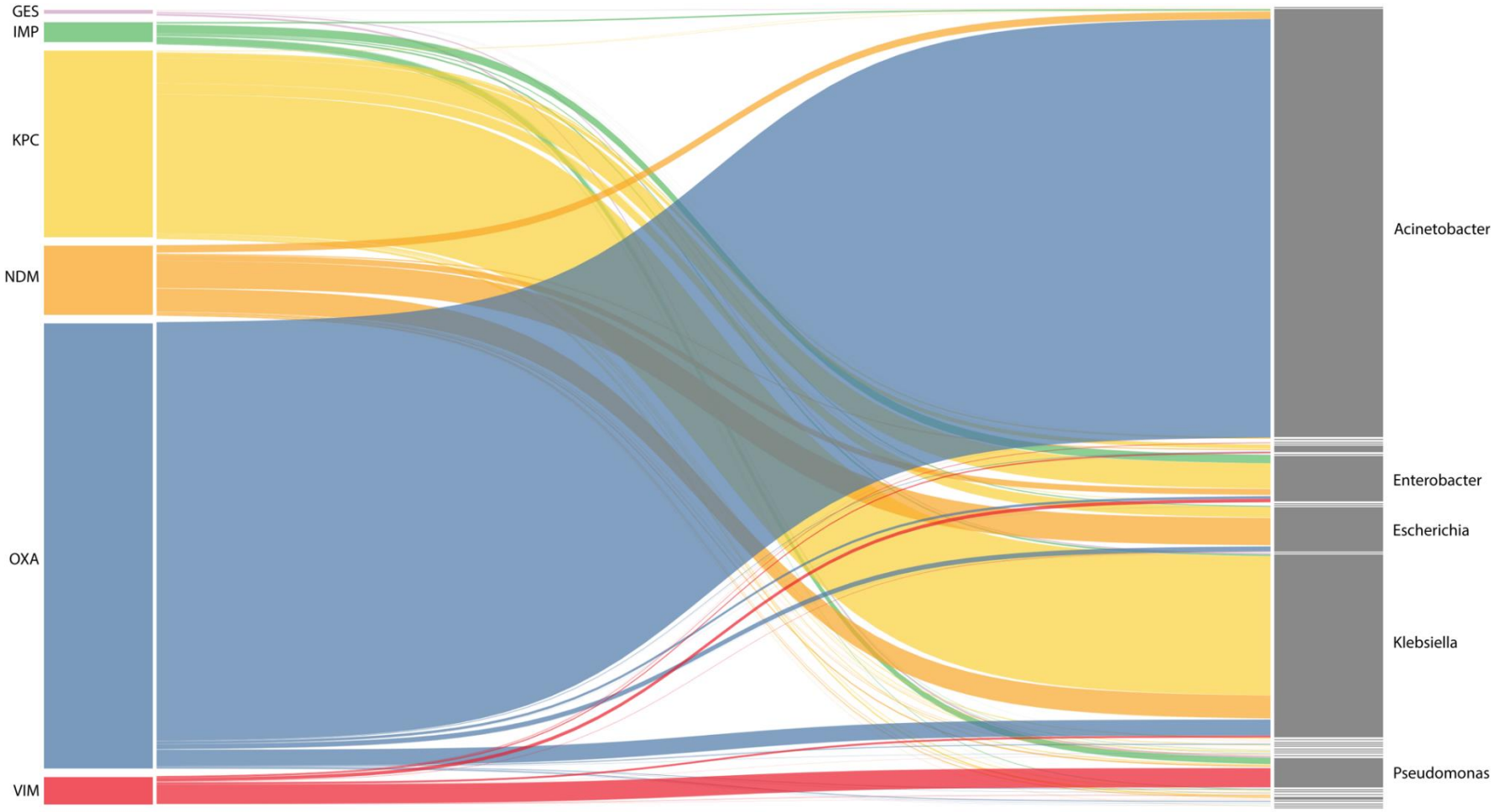
132 We retrieved a total of 107,887 bacterial genomes from NCBI and we identified 7457
133 CEGs belonging to 33 beta-lactamase families among 7343 genomes (**Table S4**). Most
134 CEG-bearing genomes were detected in the USA, China and Europe (**Figure 2A** and **Table**
135 **S4**), where the majority of the bacterial genomes were sequenced. Our results show
136 that CEGs are present on 43 genera, with most of them belonging to Proteobacteria
137 (79%, n=34/43). We observed that CEGs are mainly associated with specific bacterial
138 genera, with some of them belonging to the ESKAPEEc pathogens *Klebsiella*

139 *pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa, Enterobacter* spp.
140 and *Escherichia coli*. These genomes carry a wide diversity of CEG families, including the
141 most promiscuous OXA (58%, n=4292/7457), KPC (24%), NDM (9%), VIM (4%), IMP (3%)
142 and GES-types (0.4%) (**Figure 2B**). Exceptions included OXA-72 in *Staphylococcus aureus*
143 and NDM-1 in *Bacillus subtilis*. In addition, we observed that several CEGs (such as
144 $bla_{\text{SPM-1}}$) have a narrow host range and were only identified within strains belonging to
145 the same genus (**Figure 2C**).

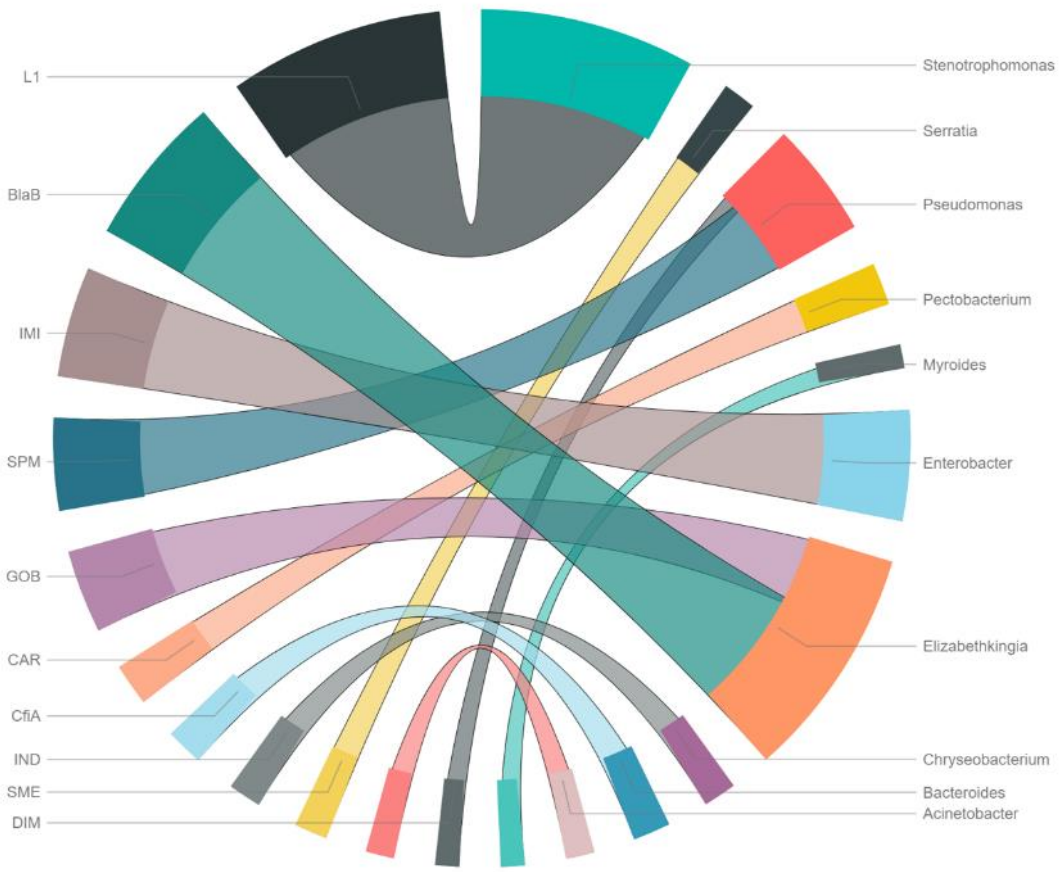
A


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


GES
IMP
KPC
NDM
OXA
VIM
Acinetobacter
Enterobacter
Escherichia
Klebsiella
Pseudomonas

150 **C**



151

152 **Figure 2.** Dispersion of CEGs in bacterial genomes. **A.** Worldwide distribution of
153 carbapenemase families. The color on each circle graph represents the
154 carbapenemases, and the size of the arc reflects the number of positive hits; **B.** Sankey
155 diagram representing the distribution of the most frequent carbapenemases. The left
156 and right axis represents the association between the identified carbapenemase and the
157 bacterial genus, respectively, while the width of each connection is proportional to the
158 number of positive hits; **C.** Chord diagram displaying relevant carbapenemases
159 identified in only one bacterial genus. In each chord, the bacterial genera are shown on
160 the right, and the carbapenemases are on the left. The width of each link represents the

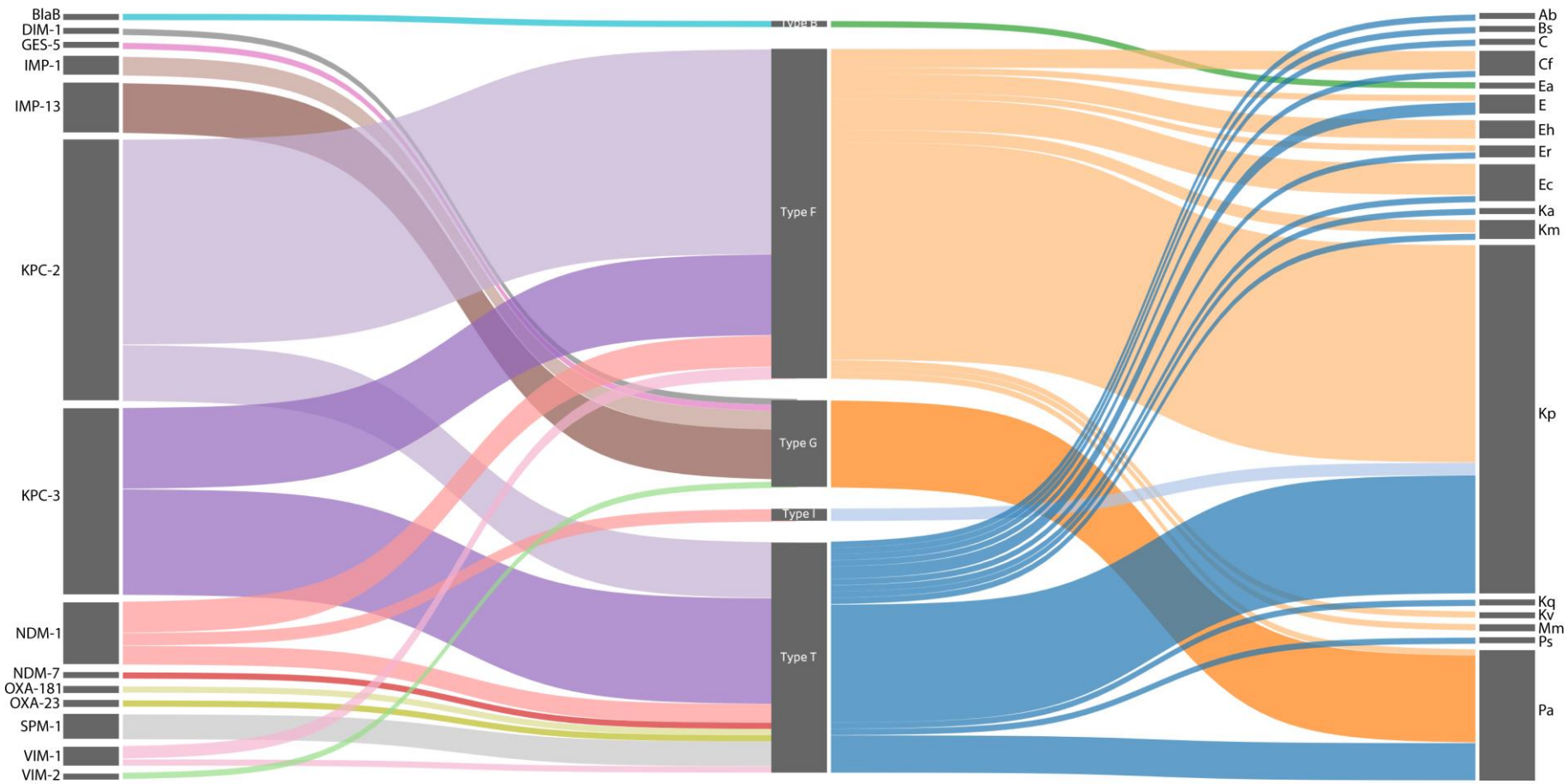
161 number of positive hits in the association. For a complete list of all carbapenemases,
162 please refer to **Table S4**.

163

164 A large proportion of CEG-bearing ICEs belong to three families and target 165 clinically-relevant gram-negative bacteria

166 Our analysis with ICEfinder resulted in the identification of 121 CEGs within 118 putative
167 ICEs (**Table S2** and **Table S5**). The *Pseudomonas* spp. hits were described in a previous
168 publication [5]. The bla_{NDM-1} and bla_{SPM-1}-harboring ICEs in *P. aeruginosa* were identified
169 in clinical isolates from Singapore and Brazil, respectively [24–26]. Using the CONJscan
170 module of MacSyFinder we identified the MPF family for 107 hits and we noted that the
171 majority of the putative ICEs belong to three families: MPF_F (49%, n=53/107), MPF_T
172 (35%) and MPF_G (13%) (**Figure 3** and **Table S5**). ICEs of the MPF_F and MPF_T classes were
173 particularly promiscuous, being responsible for the spread of several CEGs such as bla_{KPC-}
174 $_{2/3}$, bla_{NDM-1} and bla_{VIM-1} among clinically-relevant pathogens as *K. pneumoniae*, *E. coli*
175 and species belonging to the *Enterobacter cloaceae* complex. The bla_{SPM-1} gene was
176 exclusively identified in ICEs of the MPF_T class.

177 The bacterial hosts housing these elements belong to several sequence types (STs), of
178 which the *K. pneumoniae* ST258 is the most frequently represented (**Table S5**). The
179 worldwide spread of carbapenemase-producing *K. pneumoniae* has been linked to some
180 high-risk clones, including ST258 and close relatives that were also identified in this
181 study, such as ST11 and ST512 [27, 28]. We also detected several *Enterobacter*
182 *hormaechei* strains belonging to ST78, recognized as a global driver of CEGs [29, 30].



Abbreviations: Ab, Acinetobacter baumannii; Bs, Bacillus subtilis; C, Citrobacter; Cf, Citrobacter freundii; Ea, Elizabethkingia anophelis; E, Enterobacter; Eh, Enterobacter hormaechei; Er, Enterobacter roggenkampii; Ec, Escherichia coli; Ka, Klebsiella aerogenes ; Km, Klebsiella michiganensis; Kp, Klebsiella pneumoniae; Kq, Klebsiella quasipneumoniae; Kv, Klebsiella variicola; Mm, Morganella morganii; Ps, Pseudomonas; Pa, Pseudomonas aeruginosa.

183

184 **Figure 3 -** Sankey diagram showing the contribution of different MPF families for the spread of CEGs among several bacteria. The left, center and

185 right axis represents the association between the identified carbapenemases, MPF type and the bacterial genus, respectively, while the width of

186 each connection is proportional to the number of positive hits.

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

188 **ICEs**

189 We identified 16 CEG variants among the 121 hits, dominated by *bla*$_{KPC-2}$, *bla*$_{KPC-3}$, *bla*$_{IMP-}$

190 $_{13,}$ *bla*$_{NDM-1}$ and *bla*$_{SPM-1}$, and rare descriptions of *bla*$_{KPC-4}$, *bla*$_{IMP-1}$, *bla*$_{VIM-1/2}$, *bla*$_{DIM-1}$, and

191 *bla*$_{OXA-23/109/181}$. KPC-encoding ICEs although mostly observed in *Klebsiella* spp. were also

192 identified in *E. coli*, *Enterobacter* spp. and *Citrobacter* spp. IMP-encoding ICEs were

193 restricted to *P. aeruginosa* (**Figure 3** and **Table S5**).

194 The *bla*$_{KPC-2/3}$ genes were located mainly on ICEs belonging to MPF$_F$ and MPF$_T$ classes

195 (**Figure 3**). We noted that insertion sequences (IS, e.g. ISCR4 and ISCR24) were

196 frequently linked to the acquisition of *bla*$_{SPM-1}$ and *bla*$_{NDM-1}$ [24, 26], while *bla*$_{IMP}$ and

197 *bla*$_{VIM}$ were found on class I integrons [3]. In addition, we observed that *bla*$_{KPC}$ genes

198 were frequently flanked by the ISs IS*Kpn7* and IS*Kpn6* within the Tn*4401* transposon,

199 which is capable of conferring a high frequency of transposition [31]. Interestingly, we

200 found a *bla*$_{NDM-1}$ gene associated with a MPF$_T$ class ICE on a *B. subtilis* strain (**Figure 3**

201 and **Table S5**). This is the only situation where we found a CEG-bearing ICE on Firmicutes.

202 Besides CEGs, the ICEs also harbor genes conferring resistance to other antibiotics, such

203 as aminoglycosides, fluoroquinolones, macrolides, sulfonamides and trimethoprim

204 (**Table S6**), widening the spectrum of transmissible AR genes selectable by carbapenems

205 due to linkage.

206

207 **Acquisition of additional traits by ICEs include competitive weapons as**

208 **bacteriocins**

209 Besides genes conferring antibiotic resistance, some of the CEG-bearing ICEs harbor
210 other cargo genes that may confer a selective advantage to the ICE host, as bacteriocins
211 and restriction-modification systems. We identified class III bacteriocins among *bla*$_{KPC-}$
212 $_{2/3}$-harboring MPF$_F$ ICEs in five *K. pneumoniae* strains belonging to ST258 (**Table S7**).
213 Additionally, our results show that some representatives of this ST258 as well as other
214 *Enterobacteriaceae* (e.g. *E. coli*, *Enterobacter* sp.) strains carry type I and II restriction-
215 modification systems or "orphan" methyltransferases (**Table S8**). No CRISPR-Cas
216 systems were identified within these elements.

217

218 ## Discussion

219 We have set out to comprehensively identify the CEGs among all bacterial genomes
220 deposited in NCBI and the CEG-bearing ICE sequences. Our study considerably expands
221 the knowledge of the repertoire of CEGs-bearing ICEs. We uncovered more than 100
222 putative ICEs that may be involved in HGT of CEGs amongst bacterial genomes. To
223 expand our predictions, we also used the CONJscan module of Macsyfinder to trace the
224 MPF families likely involved in HGT.

225 Even though CEGs might rapidly spread worldwide, local selection is likely required for
226 them to reach fixation, as can be seen for the clonal expansion of *K. pneumoniae* ST258
227 harboring *bla*$_{KPC-2/3}$ [27, 28]. The scenario observed for the acquisition of the most
228 important CEGs by ICEs (Tn*4401* for *bla*$_{KPC-2/3}$, ISs for *bla*$_{NDM-1}$ and *bla*$_{SPM-1}$ and class I
229 integrons for *bla*$_{IMP}$ and *bla*$_{VIM}$) resembles that of plasmids [3] and provide additional
230 support to the notion that the line separating these elements is blurred [7–9]. We now
231 show that besides plasmids, this promiscuous repertoire of integrons and transposons

232 frequently targets ICEs of different MPF families. Surprisingly, we noted that *bla*$_{SPM-1}$ was
233 unable to be detected beyond the same clonal lineage [26]. Indeed, all hits were
234 identified in *P. aeruginosa* ST277 strains from Brazil and within a MPF$_T$ ICE family,
235 indicating that these ICEs are only transferring vertically and/or horizontally within this
236 ST. Understanding the limitations on HGT of this family of ICE may be translatable to
237 other, more transferable, ICEs and could underpin a control strategy to prevent the
238 spread of these elements in the future.

239 CEGs are rarely identified in Firmicutes. Local selection and enriched metagenome might
240 have also contributed to the ICE encoding NDM-1 observed in a *B. subtilis* from a highly
241 polluted marine environment [32]. Despite the works on ICEs in Gram positive bacteria
242 [33, 34], this first report of a CEG-bearing ICE in ubiquitous and resilient bacterial groups
243 is of remarkable epidemiological interest as they may represent an as yet unappreciated
244 vector for CEGs within ICEs.

245 In addition to AR, ICEs can be involved in other adaptive traits such as carbon-source
246 utilization, symbiosis, restriction-modification and bacteriocin synthesis [9]. Since
247 bacteria commonly inhabit highly competitive environments, the production of toxins
248 (such as bacteriocins) confers a selective advantage to the host [35]. Class III bacteriocins
249 – bacteriolysins - consists of high-molecular-weight and heat-labile toxins [36]. We
250 speculate that the presence of these toxins within the ICEs of five *K. pneumoniae* ST258
251 strains can promote ICE stability by preferentially selecting for cells harboring the ICE.
252 Restriction-modification systems protect a bacterial host against the invasion of foreign
253 DNA through the action of a restriction enzyme that cuts DNA lacking the epigenetically
254 methylated DNA modified by the methyltransferase [37]. The presence of these systems

255 within ICEs may also allow their stable maintenance through postsegregational killing.
256 This has already been demonstrated for plasmids [38]. Additionally, these systems may
257 also prevent further infection of the bacterial host by another ICE or MGE lacking the
258 methylation.

259 One caveat of our studies is that these results do not yet expose the complete set of
260 CEG-bearing ICEs present in all bacteria. There is an inherent bias in the number of times
261 we detect a particular CEG in certain pathogen genomes as some are over represented
262 in the database compared to others. It is possible that certain observations will flatten
263 out as more genomes are analyzed. Less than 10% of the bacterial genomes currently
264 present on NCBI are complete. This is a major drawback since ICEfinder can only detect
265 ICEs within complete genomes or contigs with or without directed repeats as the tRNA-
266 distal boundaries, meaning that if the ICE was integrated into the 3' or 5' ends of protein-
267 encoding genes and/or split into several contigs, this tool may miss the element. Even
268 though ICEfinder uses the profile Hidden Markov Models (HMM) for signature proteins
269 coded by ICEs (**Table S2**), we identified putative plasmid hits among our data (**Table S3)**.
270 Also, ICEfinder may not provide the accurate boundaries of ICEs. Indeed, the relaxase
271 and the T4SS encoded by ICEs resemble those of plasmids [6, 8, 12]. Plus, it is possible
272 that ICEs and plasmids have swapped conjugation modules throughout their
273 evolutionary history [6]. We believe that a more thorough exploration of this issue,
274 especially regarding the precise delimitation of ICEs, will be an important further step
275 toward an improved understanding of the contribution of these elements to bacterial
276 adaptation and evolution of AR.

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

283

284 **Conflicts of interest**

285 The authors declare that there are no conflicts of interest.

286

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290

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