Carbapenemases on the move: it's good to be on ICE

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Running Title: Integrative conjugative elements in *Pseudomonas* spp.

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Abstract

The evolution and spread of antibiotic resistance is often mediated by mobile genetic elements. Integrative and conjugative elements (ICEs) are the most abundant conjugative elements among prokaryotes. However, the contribution of ICEs to horizontal gene transfer of antibiotic resistance has been largely unexplored. Here we report that ICEs belonging to mating-pair formation (MPF) classes G and T are highly prevalent among the opportunistic pathogen *Pseudomonas aeruginosa*, contributing to the spread of carbapenemase-encoding genes (CEGs). Most CEGs of the MPF_G class were encoded within class I integrons, which co-harbour genes conferring resistance to other antibiotics. The majority of the integrons were located within Tn*3*-like and composite transposons. A conserved attachment site could be predicted for the MPF_G class ICEs. MPF_T class ICEs carried the CEGs within composite transposons which were not associated with integrons. The data presented here provides a global snapshot of the different CEG-harbouring ICEs and sheds light on the underappreciated contribution of these elements for the evolution and dissemination of antibiotic resistance on *P. aeruginosa*.

Introduction

Among the non-fermenting Gram-negative bacteria, the *Pseudomonas* genus is the one with the highest number of species [1, 2]. *Pseudomonas aeruginosa*, an opportunistic human pathogen associated with an ever-widening array of life-threatening acute and chronic infections, is the most clinically relevant species within this genus [3–5]. *P. aeruginosa* is one of the CDC "ESKAPE" pathogens – *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa* and *Enterobacter* species –, emphasizing its impact on hospital infections and the ability of this microorganism to "escape" the activity of antibacterial drugs [6]. *P. aeruginosa* can develop resistance to a wide range of antibiotics due to a combination of intrinsic, adaptive, and acquired resistance mechanisms, such as the reduction of its outer membrane permeability, over-expression of constitutive or inducible efflux pumps, overproduction of AmpC cephalosporinase, and the acquisition of antibiotic resistance genes (ARGs) through horizontal gene transfer (HGT) [4, 7, 8]. *P. aeruginosa* has a non-clonal population structure, punctuated by specific sequence types (STs) that are globally disseminated and frequently linked to the dissemination of ARGs [4, 9]. These STs have been designated as high-risk clones, of which major examples are ST111, ST175, ST235 and ST244.

Due to its high importance for human medicine, carbapenems are considered by the World Health Organization (WHO) as Critically-Important Antimicrobials that should be reserved for the treatment of human infections caused by MDR Gram-negative bacteria [10], such as *P. aeruginosa*. Carbapenem-resistant *P. aeruginosa* is in the "critical" category of the WHO's priority list of bacterial pathogens for which research and development into new antibiotics is urgently required [11]. Besides *P. aeruginosa*, carbapenem resistance has been reported in

68 other *Pseudomonas* spp. and is often mediated by the acquisition of carbapenemase-
69 encoding genes (CEGs) [12–14]. Carbapenemases are able to hydrolyse carbapenems and
70 confer resistance to virtually all ß-lactam antibiotics [15]. When it comes to the *Pseudomonas*
71 genus, CEGs are mostly present on class I integrons within the chromosome [4]. Class I
72 integrons are genetic elements that carry ARGs and an integrase gene, which controls
73 integration and excision of genes [16–18] . Mobile genetic elements (MGEs) such as
74 transposons, plasmids and integrative and conjugative elements (ICEs), are responsible for
75 the spread of ARGs [19–23]. The phage-inducible chromosomal islands are a recently reported
76 family of MGEs, but unrelated to the carriage of ARGs [24].

77 Usually, the genes acquired by HGT are integrated in common hotspots in the host's
78 chromosome, comprising a cluster of genes designated by genomic islands (GIs) [19, 25, 26].
79 This broad definition may also encompass other MGEs, such as ICEs and prophages. Although
80 the exact origin of these elements remains unknown, a growing body of evidence shows that
81 phages are one of the likely major ancestors of ICEs [27] [28]. ICEs are self-transmissible
82 mosaic and modular MGEs that combine features of transposons and phages (ICEs can
83 integrate into and excise from the chromosome), and plasmids (ICEs can also exist as circular
84 extrachromosomal elements, replicate autonomously and be transferred by conjugation) [21,
85 25, 29–31]. Integrative and mobilizable elements (IMEs) encode their own integration and
86 excision systems, but take advantage of the conjugation machinery of co-resident conjugative
87 elements to be successfully transferred [32]. ICEs usually replicate as part of the host genome
88 and are vertically inherited, remaining quiescent, and with most mobility genes repressed [33,
89 34]. These elements also encode recombinases related to those in phages and other
90 transposable elements. Conjugation involves three mandatory components: a relaxase
91 (MOB), a T4SS and a type-IV coupling protein (T4CP) [35, 36]. Four mating-pair formation

(MPF) classes cover the T4SS among Proteobacteria: MPF_T, MPF_G, MPF_F and MPF_I [37]. The first is widely disseminated among conjugative plasmids and ICEs, while MPF_F is more prevalent in plasmids of γ-Proteobacteria and MPF_G is found essentially on ICEs. MPF_I is rarely identified. Guglielmini *et al*. constructed a phylogenetic tree of VirB4, a highly conserved ATPase from the T4SS apparatus of different conjugative plasmids and ICEs, and formulated the hypothesis of interchangeable conjugation modules along their evolutionary history [38]. A close interplay between these elements in the ancient clades of the phylogenetic tree was observed, suggesting that plasmids may behave like ICEs and vice-versa, reinforcing the common assumption that the line separating ICEs and conjugative plasmids is blurring [30, 39]. These authors also searched more than 1000 genomes and found that ICEs are present in most bacterial clades and are more prevalent than conjugative plasmids [38]. It was also observed that the larger the genome, the higher the likelihood to harbour a conjugative element at a given moment, which supports the common assumption that bacteria with large genomes are more prone to acquire genes by HGT [40, 41].

Delimiting ICEs in genomic data remains particularly challenging [26]. Some signatures features are frequently observed, such as a sporadic distribution, sequence composition bias, insertion next to or within a tRNA gene, bordering attachment (*att*) sites and over-representation of mobility genes of the type-IV secretion system (T4SS). However, some ICEs present atypical features and may not be detected by these approaches [26, 40]. In *P. aeruginosa*, most ICEs fall into three large families: the ICE*clc*, pKLC102 and Tn*4371*. The PAGI2(C), PAGI3(SG), PAGI-13, PAGI-15 and PAGI-16 were previously described as members of the ICE*clc* family, while the PAPI-1, PAPI-2, PAGI-4 and PAGI-5 were linked to the pKLC102 family [19]. The ICE_{Tn4371} family also represents a large group of ICEs with a common backbone

and which are widely distributed, such as in *P. aeruginosa* UCBPP-PA14, PA7 and PACS171b strains [21]. These ICEs have been frequently implicated in virulence [42, 43].

Previous reports characterized the complete nucleotide sequence of extra-chromosomal genetic elements housing different CEGs in pseudomonads [20, 44–47]; however, the association of CEGs with chromosome-located MGEs has rarely been investigated [48–50]. Taking into consideration that i) in pseudomonads, CEGs are frequently located within the chromosome, ii) ICEs are the most abundant conjugative elements in prokaryotes and iii) ICEs are more frequently identified in large bacterial genomes, such as in pseudomonads, we hypothesize that ICEs may play a key role in the horizontal spread of CEGs. To investigate this hypothesis, we developed an *in silico* approach to explore the association between ICEs and CEGs in pseudomonads.

Methods

Carbapenemases database

Antimicrobial resistance translated sequences were retrieved from the Bacterial Antimicrobial Resistance Reference Gene Database available on NCBI (ftp://ftp.ncbi.nlm.nih.gov/pathogen/Antimicrobial_resistance/AMRFinder/data/2018-04-16.1/). The resulting 4250 proteins were narrowed down to 695 different carbapenemases to create a binary DIAMOND (v. 0.9.21, https://github.com/bbuchfink/diamond) database [51]. Only the sequences presenting ‘carbapenem-hydrolyzing’ or ‘metallo-beta-lactamase’ on fasta-headers were used to build this local database.

Genome collection and blast search

A total of 4565 *Pseudomonas* genomes was downloaded from NCBI (accessed on the 24th of April, 2018). These genomes were blasted against the local carbapenemase database using the following command: 'diamond blastx –d DB.dmnd –o hits.txt --id 100 --subject-cover 100 -f 6 --sensitive'.

Bioinformatic prediction of ICEs and genetic environment analyses

The CEG-harbouring *Pseudomonas* genomes were annotated through Prokka v. 1.12 (https://github.com/tseemann/prokka) [52]. The translated coding sequences were analysed in TXSScan/CONJscan platform to inspect the presence of ICEs (https://galaxy.pasteur.fr/root?tool_id=toolshed.pasteur.fr%2Frepos%2Fodoppelt%2Fconjscan%2FConjScan%2F1.0.2) [37]. All ICEs harbouring CEGs predicted by TXSScan/CONJscan were inspected for direct repeats that define the boundaries of the element. The complete nucleotide sequence in Genbank format of corresponding records was imported into Geneious v. 9.1.8 to help delimiting genomic regions flanking the ICEs [53]. Complete ICE sequences were aligned with EasyFig v. 2.2.2 (http://mjsull.github.io/Easyfig/files.html) [54]. Screening of complete ICEs for ARGs was achieved by ABRicate v. 0.8 (https://github.com/tseemann/abricate). Phage and insertion sequences were inspected through PHASTER (http://phaster.ca/) and ISfinder (https://www-is.biotoul.fr/), respectively [55, 56]. Multiple Antibiotic Resistance Annotator (MARA, http://mara.spokade.com) was used to explore the genetic background of the CEGs [57]. Orthologous assignment and functional annotation of integrase sequences was achieved through EggNOG v. 4.5.1

(http://eggnogdb.embl.de/#/app/home) and InterProScan 5 (https://www.ebi.ac.uk/interpro/search/sequence-search) [58, 59].

Phylogenomics

All CEG-harbouring *P. aeruginosa* genomes were mapped against the *P. aeruginosa* PAO1 reference strain (accession number NC_002516.2), to infer a phylogeny based on the concatenated alignment of high quality single nucleotide polymorphisms (SNP) using CSI Phylogeny and standard settings [60]. The phylogenetic tree was plotted using the iTOL platform (https://itol.embl.de/).

MLST and taxonomic assignment of unidentified species

To predict the sequence type (ST) of the strains harbouring ICEs, the *P. aeruginosa* MLST website (https://pubmlst.org/paeruginosa/) developed by Keith Jolley and hosted at the University of Oxford was used [61]. Taxonomic assignment of unidentified species carrying ICEs was achieved by JSpeciesWS v. 3.0.17 (http://jspecies.ribohost.com/jspeciesws/#home) [62].

Results

A plethora of carbapenemase-encoding genes was identified in a subset of *Pseudomonas* species

From the total *Pseudomonas* genomes analysed (n=4565), 313 CEGs were identified in 297 genomes (**Figure 1** and **Table S1**). As expected, $bla_{\text{VIM-2}}$ represents the majority of the CEGs found among *Pseudomonas* spp., being detected mainly in *P. aeruginosa*, followed by *P. plecoglocissida*, *P. guariconensis*, *P. putida*, *P. stutzeri* and 16 genomes corresponding to unidentified species (**Table S1**). Curiously, some strains presented two CEGs, either presenting a duplication of the same gene, such as $bla_{\text{IMP-34}}$ from NCGM 1900 and NCGM 1984 Japanese isolates, or harbouring different CEGs, such as $bla_{\text{IMP-1}}$ and $bla_{\text{DIM-1}}$ in isolates 97, 130 and 142 recovered in Ghana (**Table S1**, highlighted in red). A wide variety of STs was also observed, including the high-risk clones ST111, ST175 and ST244.

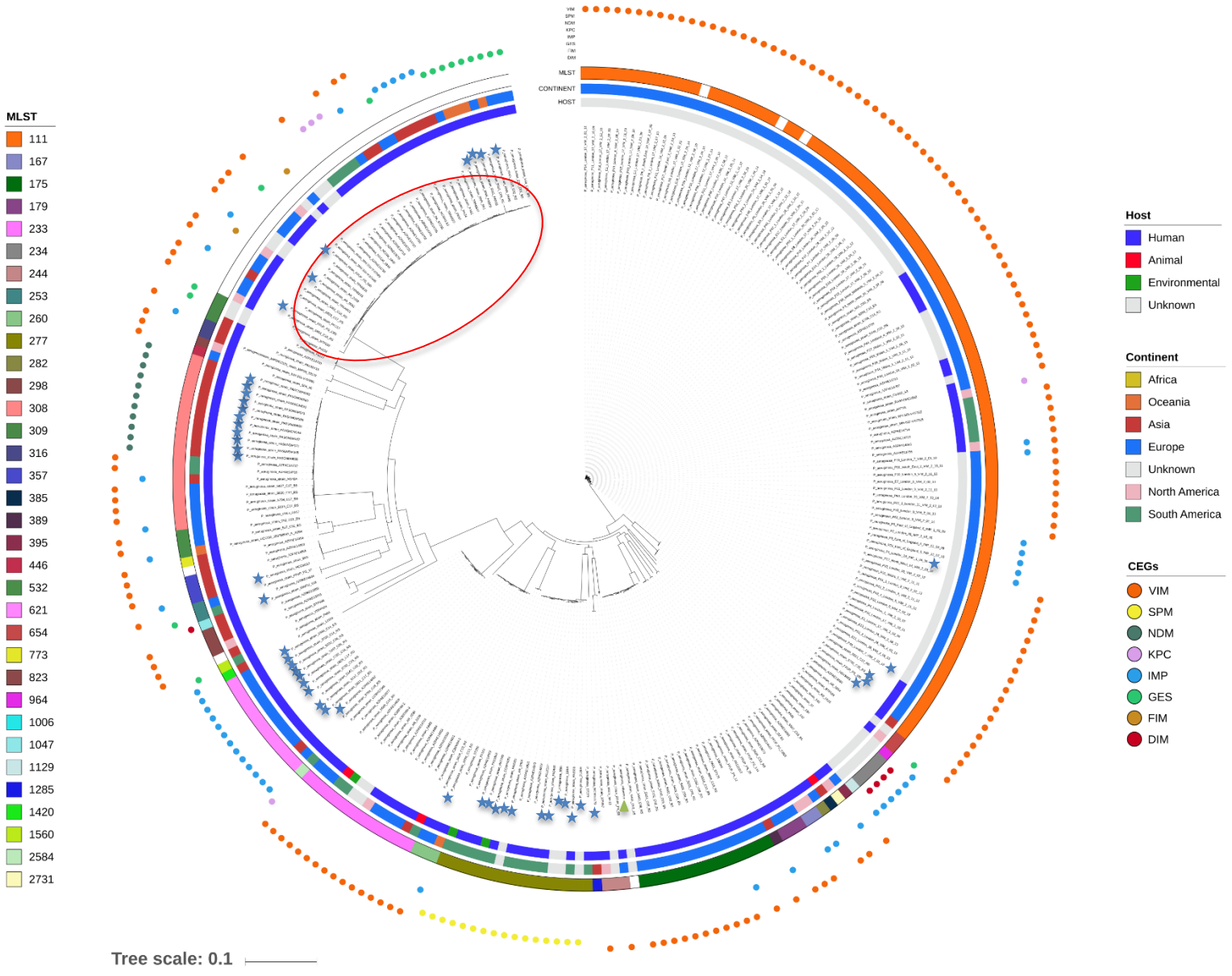
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Figure 1 – Whole-genome phylogeny of the CEG-carrying *P. aeruginosa* isolates. The maximum-likelihood phylogenetic tree was constructed using 146,106 SNPs spanning the whole genome and using the *P. aeruginosa* PAO1 genome (highlighted by a green triangle) as a reference. Multilocus sequence typing (MLST), continent and host data are reported on the outer-most, middle and inner-most circles, respectively. The strains belonging to a double ST profile (ST235/ST2613) are included within the red ellipse. Blue stars point out *P. aeruginosa* strains for which a CEG-harbouring ICE was predicted. The *P. aeruginosa* AR_0356 genome (accession number CP027169.1) was removed from the tree since it corresponds to a strain of which host and origin are unknown. The phylogenetic distance from the tree root to this genome is 1 (calculated with the tree scale). The Newick format file for the original tree is included in the Supplementary information.

Detection of ICEs encoding carbapenemases in *Pseudomonas* spp.

65.5% (205/313, **Table S1**) of the CEG hits are located within small contigs, with a sequence smaller than 20kb in length. The presence of repeated regions, such as those encoding for transposases, tend to split the genome when second-generation sequencing approaches are used. Based on information retrieved from NCBI (accessed on the 24th of May, 2018), the total number of bacterial genomes sequenced at the chromosome/complete genome level is 12,077, while the number of genomes sequenced at the scaffold/contig is much larger (127,231). With this sequencing limitation, we were still able to identify 49 ICEs associated with CEGs (n=20 with complete sequence) among all pseudomonads genomes (**Table 1**, **Table S1 and Figure 1**). When an ICE location was attributed to a CEG located on a small contig, the assumption was based on previously published data, as pointed out on **Table 1**. Besides the

aforementioned ICEs, we also identified a putative MGE within *Pseudomonas* sp. NBRC 111143 strain (**Table S1**). The T4CP-encoding gene was absent from this *bla*$_{IMP-10}$-carrying element, which could be due to contig fragmentation or gene absence. In case the gene is actually missing, this element could still be mobilized by the conjugation machinery of an ICE or conjugative plasmid(s) present in the host, and should be classified as an IME.

The ICEs identified here were all integrated within *P. aeruginosa* genomes (with the exception of the one element identified in *Pseudomonas* sp. PONIH3 genome) and AT-rich when compared to their host's chromosome; the mean GC value for this species is 66.2% according to EZBioCloud (https://www.ezbiocloud.net/taxon?tn=Pseudomonas%20aeruginosa) (**Table 1**).

Table 1. Main characteristics of CEG-carrying ICEs described in this study.

ICE family	Type of integrase[1]	CEG	Nº strains	ST[2]	Country	Isolation source[3]	CONJscan T4SS type[4]	Size range (if complete, kb)[5]	GC range (if complete, %)[6]	CEG within a class I integron	CEG within a transposon	Other ARGs[7]	References
Tn*4371*	Shufflon-specific DNA recombinase Rci and Bacteriophage Hp1-like	*bla*$_{NDM-1}$	11	308	Singapore	Urine, foot wound swab, endotracheal tube aspirate	T	73.7	64.7	No	Yes (IS*CR24*-composite)	Δ*ble*, Δ*bla*$_{PME-1}$	[63], this study
		bla$_{SPM-1}$ (as single or double copy	11	277	Brazil	Urine, bloodstream, tracheal aspirate, catheter tip, NA	T	43.8 – 57.7	64.9 – 65.6	No	Yes (IS*CR4* composite)	None	[64, 65], this study
		bla$_{KPC-2}$ (double copy)	1	NA	USA	Wastewater	T	61.2	59.2	No	Yes (complex transposon)	*bla*$_{SHV-12}$, *qnrB19*	[66], this study

ICE*clc*	Bacteriophage P4	*bla*$_{IMP-13}$	10	621	Italy, India	Urinary tract infection, respiratory sample, blood	G	NA	NA	Yes	Yes (Tn*3*-like)	*aacA4-C329*, *sul1*	[67], this study
		bla$_{GES-5}$	4	235	Australia	Rectal swab, blood culture, hospital ward, hospital gel hand wash	G	92.8	61.9	Yes	Yes (Tn*3*-like)	*aacA4r15*, *gcuE15*, *aphA15*, *sul1*	[48], this study
		bla$_{VIM-2}$	4	111, 235	Portugal, UK	Urine, bronchial aspirate, NA	G	83.4 – 88.9	62.0	Yes	Yes (Tn*3*-like)	*aacC2b*, *aacA7*, *aacC1*, *aacA4-C329*, *sul1*	[50, 68], this study
		bla$_{IMP-1}$	3	111, 357, 1285	Japan, UK	Midstream urine, NA	G	76.2 – 96.4	61.9 – 62.3	Yes	Yes (Tn*3*-like)	Δ*aacA4-C329*, *aadB*, *aacA28*, *aadA1a*, *cmlA9*, *tet(G)*, *sul1*	[68, 69], This study

		bla$_{DIM-1}$	1	1047	Nepal	Urinary catheter	G	88.7	62.8	Yes	Yes (IS*6100* composite)	*dfrB5*, Δ*aacA4-C329*, *rmtF*, *catB12*	This study
		bla$_{GES-6}$	1	235	Portugal	Urine	G	86.6	63.0	Yes	Yes (defective Tn*402*-like)	*aacA7*, *sul1*	[49]
		bla$_{IMP-14}$	1	2613	NA	NA	NA	NA	NA	Yes	Yes (IS*6100* composite within a Tn*3*-like)	*aadB*, *bla*$_{OXA-10-A}$, *aacA4-T329*, *sul1*	This study
		bla$_{VIM-1}$	1	111	Italy	Blood	G	NA	NA	Yes	Yes (Tn*3*-like)	aacA4-C329, *bla*$_{OXA-2}$, *gcu10*, *aadA13*, *sul1*	[67], this study

ARGs, antibiotic resistance genes; ICE, integrative and conjugative element; NA, Not available; ST, sequence type;

[1]NA is shown when no integrase was identified;

[2]NA is shown when the ICE was identified on a species for which no MLST scheme has been developed;

[3]NA is shown when the isolation source was not provided by sequence authors;

[4]NA is shown when no output was obtained by the platform or the conjugative module system was incomplete due to contig fragmentation;

[5, 6]NA is shown when the ICE sequence was incomplete due to contig fragmentation or delimitation of the entire element was not successful;

[7]Representation of total ARGs associated with the same CEG; a given strain harbouring the referred CEG may not present all ARGs here reported; Δ represents incomplete genes.

All ICEs identified here possessed only one tyrosine integrase (**Figure 2**). ICEs belonging to the ICE*clc* family (MPF_G class) carried an integrase belonging to the bacteriophage P4-like family, while ICEs belonging to the ICE_{Tn4371} family (MPF_T class) carried an integrase belonging to shufflon-specific DNA recombinase Rci and Bacteriophage Hp1-like family (**Table 1**).[31] Rci and Hp1-like were only distantly related (13% amino acid identity) to P4-like integrases. Orthologous assignment of these integrases revealed that the former and the later integrases identified were present in more than 100 and 400 proteobacteria species, respectively. While P4-like integrases were more prevalent on γ-proteobacteria, half of the strains carrying Rci and Hp1-like integrases belong to the α-proteobacteria.

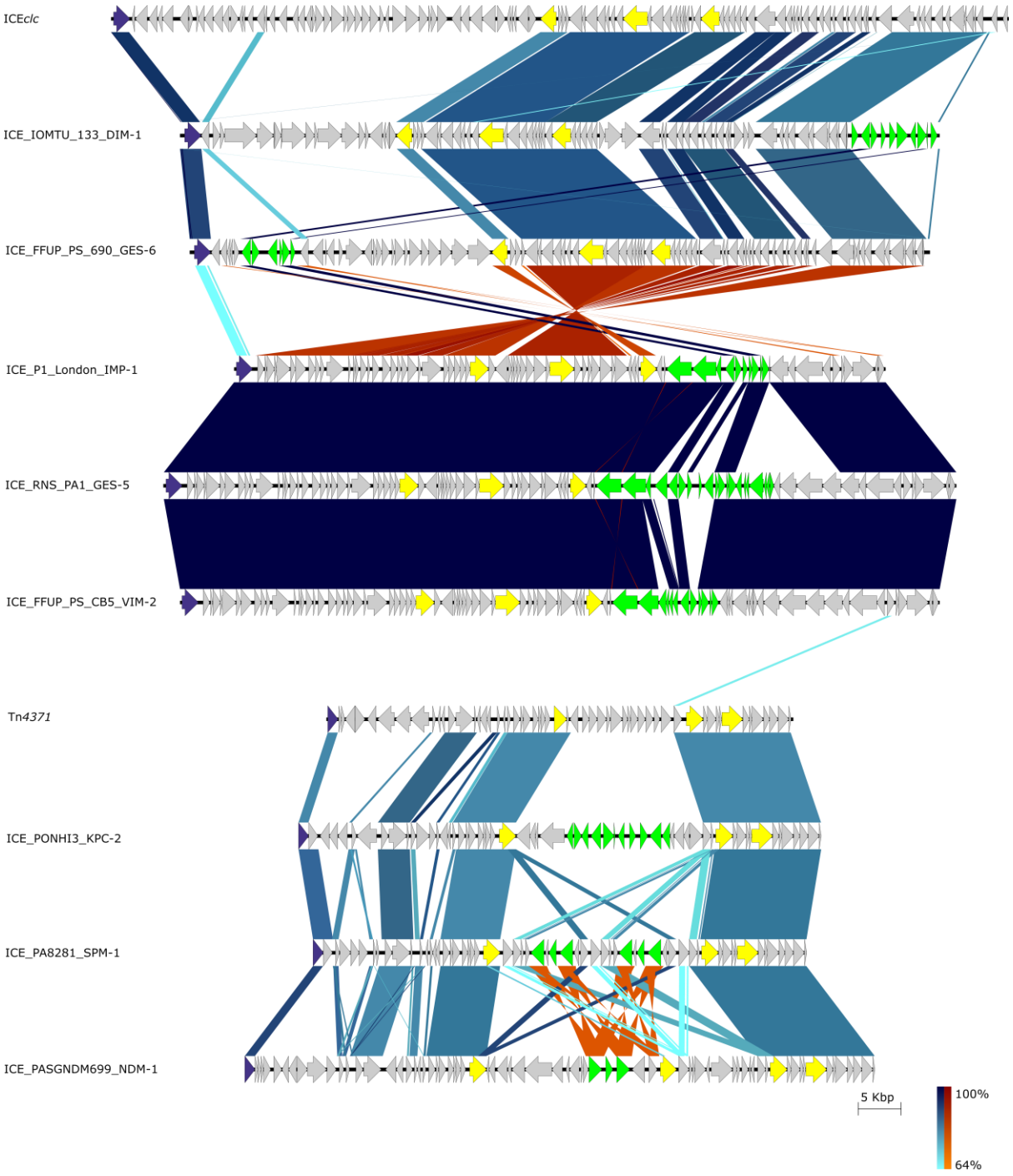

Figure 2 – Blastn comparison among multiple ICEs described in this study. A gradient of blue and red colours is observed for normal and inverted BLAST matches, respectively. Model elements (ICE*clc* for the MPF^{G} and Tn*4371* for the MPF^{T} classes, respectively) were also included for comparison. The arrows and arrowheads point the orientation of the translated coding sequences. In purple are highlighted the integrases, in yellow the mandatory features

of a conjugative system according to Cury *et al*. [40] and in green the transposons harbouring the CEGs. A more detailed view of some of these transposons is displayed in **Figure 3**.

We observed that MPF_G class ICEs tend to integrate next to a single copy of $tRNA^{Gly}$ or a cluster of two $tRNA^{Glu}$ and one $tRNA^{Gly}$ genes, which is in agreement with previous findings [26, 40]. A conserved 8-bp *att* site (5´-CCGCTCCA) flanked all complete ICEs of the MPF_G class identified here (**Table 1**). Notably, most ICEs of this class were adjacent to phages (either at the 5'- or the 3'-end) targeting the same *att* site as the neighbour ICE. No *att* site could be identified for the integration of MPF_T class ICEs. A gene encoding for a catechol 1,2-dioxygenase and a gene encoding for a protein with no described conserved domain were found flanking the bla_{SPM-1}-harbouring ICEs. Regarding the elements carrying bla_{NDM-1}, a gene encoding for a different protein also with no conserved domain identified and a gene encoding for the type III secretion system adenylate cyclase effector ExoY were separated upon insertion of these ICEs. Integration next to hypothetical proteins or tRNA genes was commonly observed.

Carbapenemases are frequently encoded within transposons

CEGs were associated with class I integrons frequently co-harbouring aminoglycoside resistance genes when associated with MPF class G ICEs (**Table 1**). Class I integrons were often associated with a wide array of transposons, such as the Tn*3* superfamily transposons and the IS*6100* composite elements (**Table 1**). MPF_T class ICEs were targeted by more complex elements, such as the composite transposons carrying bla_{SPM-1} and bla_{NDM-1} (**Table 1**). The bla_{NDM-1} gene was identified in Singapore in ICE_{Tn4371}6385 and associated with ST308, as recently reported [63]. The bla_{NDM-1} was flanked by two IS*CR24*-like transposases. bla_{SPM-1} was

linked to ICE$_{Tn4371}$6061, a recently described ICE [64]. Again, the CEG was located within an IS*CR4*-like composite transposon. IS*CR* elements are atypical elements of the IS*91* family which represent a well-recognized system of gene capture and mobilization by a rolling-circle transposition process [21, 70].

Besides previously described *bla*$_{NDM-1}$ and *bla*$_{SPM-1}$ harbouring ICEs, we characterize here new ICE elements of MPF$_G$ and MPF$_T$ classes (**Table 1** and **Figure 3**). The *bla*$_{DIM-1}$-harbouring ICE from IOMTU 133 strain was integrated between the 3'-end of a tRNA^{Gly} gene (IOMTU133_RS11660) and a gene encoding for the R body protein RebB (IOMTU133_RS12085). *bla*$_{DIM-1}$ was first described as a single gene cassette located within a class I integron associated with a 70-kb *Pseudomonas stutzeri* plasmid recovered in the Netherlands [13]. However, the integron carrying *bla*$_{DIM-1}$ in strain IOMTU 133 was unrelated to the one from the *P. stutzeri* plasmid, harbouring genes encoding for aminoglycoside (*aacA4-C329* and *rmtf*), trimethoprim (*dfrB5*) and chloramphenicol (*catB12*) resistance (**Figure 3A**). Direct repeats (DRs) were found flanking the entire IS*6100* composite transposon (5'-TTCGAGTC), indicating the transposition of this element into the ICE element. Besides being identified as a composite transposon, IS*6100* was frequently observed as a single copy at the 3'end of the class I integron (**Figures 3B and 3C**), suggesting that these elements were derived from the In*4* lineage [71]. The *bla*$_{IMP-1}$ from the NCGM257 strain identified in Japan belonged to a different ST (ST357) than the frequently identified ST235 associated with the spread of this CEG in this country [72]. The CEG was also shown to be associated with a novel complex class I integron, co-harbouring *aadB*, *cmlA9* and *tet(G)* genes encoding resistance to aminoglycosides, chloramphenicol and tetracyclines, respectively (**Figure 3B**). This integron was inserted (DRs 5'- GAGTC) within a mercury resistance transposon. This genetic organization was frequently recovered among other ICE-harbouring strains, such as the ones

associated with *bla*$_{GES-5}$, *bla*$_{IMP-13}$ and *bla*$_{IMP-14}$ (**Table 1**). The entire ICE was integrated into the chromosome of NCGM257 strain between the 3'-end of a tRNA^{Gly} gene (PA257_RS24790) and the aforementioned *Pseudomonas* phage Pf1-like element. The new ICE identified on the P1_London_28_IMP_1_04_05 strain presented *bla*$_{IMP-1}$ in a different In*4*-like integron than that observed for the NCGM257 strain, even though both elements were associated with a Tn*3*-like transposon (**Figure 3C**). Unlike most ICEs of the MPF$_G$ class, its integration occurred between a gene encoding for a LysR family transcriptional regulator (AFJ02_RS19410) and a gene encoding for a hypothetical protein (AFJ02_RS19770). Regarding the *bla*$_{KPC-2}$-harbouring *Pseudomonas* sp. PONHI3 strain, a tetra correlation search revealed that this strain was highly similar (Z-score above the 0.999 cut-off) to *Pseudomonas mosselii* SJ10 (accession number NZ_CP009365.1). Average nucleotide identity based on BLAST (ANIb) analysis of these genomes revealed that both strains belong to the same species, since the ANIb value was above the 95% cut-off for species delineation [73]. However, the ANIb value for both strains was below the cut-off when compared with the *P. mosselii* DSM 17497 type train (accession number NZ_JHYW00000000.1), suggesting that both strains may comprise novel species within the *Pseudomonas putida* phylogenetic group [2]. The PONHI3 strain carried a double copy of *bla*$_{KPC-2}$ within an ICE from MPF$_T$ class. A complex genetic environment was found surrounding these genes (**Figure 3D**). This ICE was integrated between a gene encoding for a biopolymer transport protein ExbD/TolR (C3F42_RS18665) and a gene encoding for an alpha/beta hydrolase (C3F42_RS18995).

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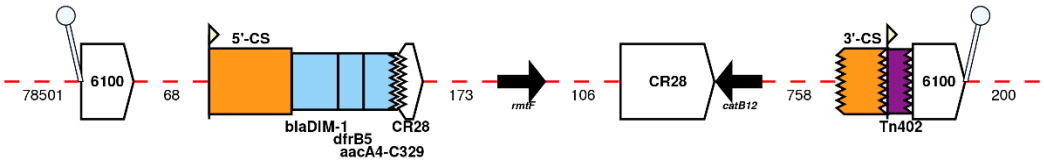

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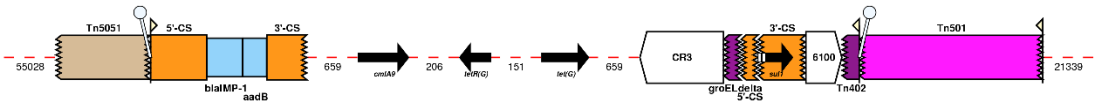

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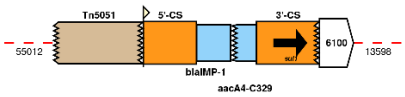

D

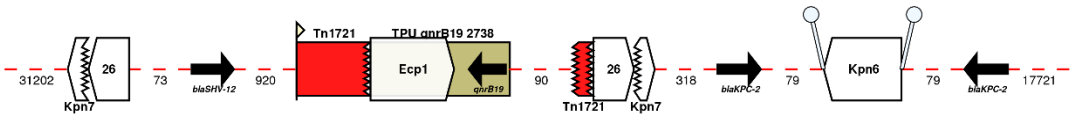

Figure 3. Genetic environment of novel ICEs harbouring *bla*$_{\text{DIM-1}}$ (A), *bla*$_{\text{IMP-1}}$ (B and C) and a double copy of *bla*$_{\text{KPC-2}}$ (D). Arrows indicate the direction of transcription for genes. The dashed part of the arrow indicates which end is missing, for other features the missing end is shown by a zig-zag line. Gene cassettes are shown by pale blue boxes, the conserved sequences (5' and 3'-CS) of integrons as orange boxes and insertion sequences as white block arrows labelled with the IS number/name, with the pointed end indicating the inverted right

335 repeat (IRR). Gaps >50 bp are indicated by dashed red lines and the length in bp given. Unit
336 transposons are shown as boxes of different colors and their IRs are shown as flags, with the
337 flat side at the outer boundary of the transposon. Direct repeats are shown as 'lollipops' of
338 the same color.

339

340 **An atypical GI encoding carbapenemases**

341 Besides ICEs, we also identified an atypical 19.8-kb long GI harbouring *bla*$_{VIM-2}$ in *P. aeruginosa*
342 AZPAE13853 and AZPAE13858 strains from India (**Figure S1**). A similar element was also
343 observed in *P. aeruginosa* BTP038 strain from the USA, with the exception that the Tn*402*-like
344 transposon harbouring *bla*$_{VIM-2}$ was orientated in an inverted position. Five base-pair DRs (5'-
345 CTCTG in AZPAE13853 and AZPAE13858 and 5'-CTGAG in BTP038 strains) were found flanking
346 this transposon structure. Importantly, in these strains the GIs were flanked by identical signal
347 recognition particle RNAs (srpRNAs), indicating a strong site preference for these elements.

348

349 **Discussion**

350 Our results show that *bla*$_{VIM}$ and *bla*$_{IMP}$ are widely disseminated, both geographically and
351 phylogenetically (across *Pseudomonas* spp.). Moreover, and as previously described, *bla*$_{VIM-2}$
352 was the most frequently reported CEG (**Figure 1** and **Table S1**) [4]. On the other hand, *bla*$_{SPM-1}$
353 is still restricted to *P. aeruginosa* and Brazil (or patients who had been previously
354 hospitalized in Brazil) [65]. Even though ST235 has been frequently linked to the
355 dissemination of ARGs, no CEG-harbouring strains belonging to this ST were identified.
356 Curiously, some strains (highlighted on **Figure 1**) belong to a double ST profile

(ST235/ST2613), since the strains carry a double copy with different allele sequences of the house-keeping gene *acsA*, encoding for an acetyl-coenzyme A synthetase. These genes only display 80,3% nucleotide identity. We plan to conduct comparative genomic studies to explore the idiosyncrasies of these double ST profile strains.

Not all CEGs are likely to be geographically and phylogenetically disseminated, but those that are more promiscuous present a serious threat. The geographical distribution of the high-risk clones and the diversity of CEGs propose that the spread of these STs is global and the acquisition of the resistance genes is mainly local [4, 68]. Previous studies suggest that environmental species may pose an important reservoir for the dissemination of clinically relevant carbapenemases, which are vertically amplified upon transfer to *P. aeruginosa* high-risk clones [12, 14]. The high prevalence of these elements among high-risk clones may be partially explained by the genetic capitalism theory, given that a widely disseminated ST should have a greater probability of acquiring new CEGs and to be further selected and amplified due to the high antibiotic pressure in the hospital environment [74]. Other theories support that the high-risk clones have a naturally increased ability to acquire foreign DNA, since these STs appear to have lost the CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR associated proteins) system, which act as an adaptive immune system in prokaryotic cells and protects them from invasion by bacteriophages and plasmids [75–77].

This study underestimates the extent of host range because only ICEs in sequenced genomes were detected. Also, identification of new ICEs could only be achieved in complete genomes or contigs with a sequence length large enough to include the full (nor near complete) sequence of the ICE. As so, it is important to highlight the need to perform third generation

sequencing on CEG-harbouring genomes to avoid fragmentation of the genetic environment surrounding the gene and to provide a wider view of complete supporting ICEs and other MGEs. All ICE elements here identified fulfilled the criteria to be considered conjugative as proposed by Cury *et al*.: a relaxase, a VirB4/TraU, a T4CP and minimum set of MPF type-specific genes [40]. ICEs tend to integrate within the host's chromosome by the action of a tyrosine recombinase, even though some elements may use serine or DDE recombinases instead [30]. Though rare, some elements encode for more than one integrase, most likely resulting from independent integration of different MGEs [40]. Conserved sites are hotspots for ICE integration due to their high conservation among closely related bacteria, and so expanding the host range and be stably maintained after conjugative transfer [78, 79]. ICEs were often integrated next to phages highly similar to the *Pseudomonas* phage Pf1 (NC_001331.1), a class II filamentous bacteriophage belonging to the *Inoviridae* family [77]. Pf1-like phages are widely disseminated among *P. aeruginosa* strains and may have a role in bacterial evolution and virulence [80–82]. Interestingly, no representative of the pKLC102 family was linked to the dissemination of CEGs. This may be explained due to a higher affinity of the transposons carrying the CEGs for hotspots located within representatives of the other two families.

MGEs specifically targeting conserved regions of the genome such as tRNAs are common and this specificity represents an evolutionary strategy whereby the target site of an element is almost guaranteed to be present, due to its essentiality, and very unlikely to change due to biochemical constraints of the gene product. We think a similar situation exists for the elements found between the small srpRNAs described on the atypical GI element here identified and is in contrast to the more permissive nature of target site selection shown for example, by elements of the Tn*916*/Tn*1545* family [83].

Here, we revealed that different Tn*3*-like and composite transposons harbouring a wide array of CEGs were transposed into MPF G and T ICE classes, which were most likely responsible for the dissemination of these genes through HGT and/or clonal expansion of successful *Pseudomonas* clones. This study sheds light on the underappreciated contribution of ICEs for the spread of CEGs among pseudomonads (and potentially further afield). With the ever-growing number of third-generation sequenced genomes and the development of more sophisticated bioinformatics, the real contribution of these ICEs will likely rapidly emerge.

Recently, it was shown that interfering with the transposase-DNA complex architecture of a Tn*916*-like conjugative transposon (also known as ICE) lead to transposition inhibition to a new host [84]. In the future, it would be interesting to determine if the same mechanism is observed for tyrosine recombinases present in ICE*clc* and Tn*4371* derivatives, as well as in other MPF ICE classes, as a potential approach to interfere with the spread of antimicrobial resistance.

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Author contributions

JB, APR, FG and LP designed the study; JB and RLS performed the *in silico* analysis; JB wrote the manuscript. All the authors approved the final manuscript.

Competing interests

None to declare.

References

1. Parte AC. LPSN—list of prokaryotic names with standing in nomenclature. *Nucleic Acids Res* 2014; **42**: D613–D616.

2. Gomila M, Peña A, Mulet M, Lalucat J, García-Valdés E. Phylogenomics and systematics in Pseudomonas. *Front Microbiol* 2015; **6**: 214.

3. Moradali MF, Ghods S, Rehm BHA. Pseudomonas aeruginosa Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. *Front Cell Infect Microbiol* 2017; **7**: 39.

4. Oliver A, Mulet X, López-Causapé C, Juan C. The increasing threat of Pseudomonas aeruginosa high-risk clones. *Drug Resist Updat* 2015; **21**–**22**: 41–59.

5. Juan C, Peña C, Oliver A. Host and Pathogen Biomarkers for Severe Pseudomonas aeruginosa Infections. *J Infect Dis* 2017; **215**: S44–S51.

6. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, et al. Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America. *Clin Infect Dis* 2009; **48**: 1–12.

7. Breidenstein EBM, de la Fuente-Núñez C, Hancock REW. Pseudomonas aeruginosa: all roads lead to resistance. *Trends Microbiol* 2011; **19**: 419–26.

8. Cornaglia G, Giamarellou H, Rossolini GM. Metallo-β-lactamases: a last frontier for β-lactams? *Lancet*

Infect Dis 2011; **11**: 381–93.

9. Kidd TJ, Ritchie SR, Ramsay KA, Grimwood K, Bell SC, Rainey PB. Pseudomonas aeruginosa Exhibits Frequent Recombination, but Only a Limited Association between Genotype and Ecological Setting. *PLoS One* 2012; **7**: e44199.

10. Scientific Opinion on Carbapenem resistance in food animal ecosystems. *EFSA J* 2013; **11**.

11. World Health Organization. GLOBAL PRIORITY LIST OF ANTIBIOTIC-RESISTANT BACTERIA TO GUIDE RESEARCH, DISCOVERY, AND DEVELOPMENT OF NEW ANTIBIOTICS. http://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed. Accessed 11 Aug 2018.

12. Scotta C, Juan C, Cabot G, Oliver A, Lalucat J, Bennasar A, et al. Environmental microbiota represents a natural reservoir for dissemination of clinically relevant metallo-beta-lactamases. *Antimicrob Agents Chemother* 2011; **55**: 5376–9.

13. Poirel L, Rodríguez-Martínez J-M, Al Naiemi N, Debets-Ossenkopp YJ, Nordmann P. Characterization of DIM-1, an integron-encoded metallo-beta-lactamase from a Pseudomonas stutzeri clinical isolate in the Netherlands. *Antimicrob Agents Chemother* 2010; **54**: 2420–4.

14. Juan C, Zamorano L, Mena A, Alberti S, Perez JL, Oliver A. Metallo- -lactamase-producing Pseudomonas putida as a reservoir of multidrug resistance elements that can be transferred to successful Pseudomonas aeruginosa clones. *J Antimicrob Chemother* 2010; **65**: 474–478.

15. Queenan AM, Bush K. Carbapenemases: the versatile beta-lactamases. *Clin Microbiol Rev* 2007; **20**: 440–58, table of contents.

16. Amos GCA, Ploumakis S, Zhang L, Hawkey PM, Gaze WH, Wellington EMH. The widespread dissemination of integrons throughout bacterial communities in a riverine system. *ISME J* 2018; **12**: 681–691.

17. Mazel D. Integrons: agents of bacterial evolution. *Nat Rev Microbiol* 2006; **4**: 608–620.

18. Gaze WH, Zhang L, Abdouslam NA, Hawkey PM, Calvo-Bado L, Royle J, et al. Impacts of anthropogenic

activity on the ecology of class 1 integrons and integron-associated genes in the environment. *ISME J* 2011; **5**: 1253–1261.

19. Kung VL, Ozer EA, Hauser AR. The accessory genome of Pseudomonas aeruginosa. *Microbiol Mol Biol Rev* 2010; **74**: 621–41.

20. Partridge SR, Kwong SM, Firth N, Jensen SO. Mobile Genetic Elements Associated with Antimicrobial Resistance. *Clin Microbiol Rev* 2018; **31**: e00088-17.

21. Toleman MA, Walsh TR. Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. *FEMS Microbiol Rev* 2011; **35**: 912–935.

22. Enault F, Briet A, Bouteille L, Roux S, Sullivan MB, Petit M-A. Phages rarely encode antibiotic resistance genes: a cautionary tale for virome analyses. *ISME J* 2017; **11**: 237–247.

23. San Millan A. Evolution of Plasmid-Mediated Antibiotic Resistance in the Clinical Context. *Trends Microbiol* 2018; **0**.

24. Fillol-Salom A, Martínez-Rubio R, Abdulrahman RF, Chen J, Davies R, Penadés JR. Phage-inducible chromosomal islands are ubiquitous within the bacterial universe. *ISME J* 2018; 1.

25. Bellanger X, Payot S, Leblond-Bourget N, Guédon G. Conjugative and mobilizable genomic islands in bacteria: evolution and diversity. *FEMS Microbiol Rev* 2014; **38**: 720–760.

26. Langille MGI, Hsiao WWL, Brinkman FSL. Detecting genomic islands using bioinformatics approaches. *Nat Rev Microbiol* 2010; **8**: 373–382.

27. Chen J, Novick RP. Phage-mediated intergeneric transfer of toxin genes. *Science* 2009; **323**: 139–41.

28. Tazzyman SJ, Hall AR. Lytic phages obscure the cost of antibiotic resistance in Escherichia coli. *ISME J* 2015; **9**: 809–820.

29. Wozniak RAF, Waldor MK. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nat Rev Microbiol* 2010; **8**: 552–563.

30. Johnson CM, Grossman AD. Integrative and Conjugative Elements (ICEs): What They Do and How They Study. *Annu Rev Genet* 2015; **49**: 577–601.

31. Delavat F, Miyazaki R, Carraro N, Pradervand N, van der Meer JR. The hidden life of integrative and conjugative elements. *FEMS Microbiol Rev* 2017; **41**: 512–537.

32. Guédon G, Libante V, Coluzzi C, Payot S, Leblond-Bourget N. The Obscure World of Integrative and Mobilizable Elements, Highly Widespread Elements that Pirate Bacterial Conjugative Systems. *Genes (Basel)* 2017; **8**: 337.

33. Delavat F, Mitri S, Pelet S, van der Meer JR. Highly variable individual donor cell fates characterize robust horizontal gene transfer of an integrative and conjugative element. *Proc Natl Acad Sci U S A* 2016; **113**: E3375-83.

34. Burrus V. Mechanisms of stabilization of integrative and conjugative elements. *Curr Opin Microbiol* 2017; **38**: 44–50.

35. Smillie C, Garcillán-Barcia MP, Francia MV, Rocha EPC, de la Cruz F. Mobility of plasmids. *Microbiol Mol Biol Rev* 2010; **74**: 434–52.

36. Garcillán-Barcia MP, Alvarado A, de la Cruz F. Identification of bacterial plasmids based on mobility and plasmid population biology. *FEMS Microbiol Rev* 2011; **35**: 936–956.

37. Guglielmini J, Néron B, Abby SS, Garcillán-Barcia MP, de la Cruz F, Rocha EPC. Key components of the eight classes of type IV secretion systems involved in bacterial conjugation or protein secretion. *Nucleic Acids Res* 2014; **42**: 5715–27.

38. Guglielmini J, Quintais L, Garcillán-Barcia MP, de la Cruz F, Rocha EPC. The Repertoire of ICE in Prokaryotes Underscores the Unity, Diversity, and Ubiquity of Conjugation. *PLoS Genet* 2011; **7**: e1002222.

39. Carraro N, Poulin D, Burrus V. Replication and Active Partition of Integrative and Conjugative Elements (ICEs) of the SXT/R391 Family: The Line between ICEs and Conjugative Plasmids Is Getting Thinner. *PLOS Genet* 2015; **11**: e1005298.

40. Cury J, Touchon M, Rocha EPC. Integrative and conjugative elements and their hosts: composition, distribution and organization. *Nucleic Acids Res* 2017; **45**: 8943–8956.

525 41. Baltrus DA. Exploring the costs of horizontal gene transfer. *Trends Ecol Evol* 2013; **28**: 489–495.

526 42. Harrison EM, Carter MEK, Luck S, Ou H-Y, He X, Deng Z, et al. Pathogenicity islands PAPI-1 and PAPI-2
527 contribute individually and synergistically to the virulence of Pseudomonas aeruginosa strain PA14.
528 *Infect Immun* 2010; **78**: 1437–46.

529 43. He J, Baldini RL, Déziel E, Saucier M, Zhang Q, Liberati NT, et al. The broad host range pathogen
530 Pseudomonas aeruginosa strain PA14 carries two pathogenicity islands harboring plant and animal
531 virulence genes. *Proc Natl Acad Sci U S A* 2004; **101**: 2530–5.

532 44. Botelho J, Grosso F, Peixe L. Characterization of the pJB12 Plasmid from Pseudomonas aeruginosa
533 Reveals Tn6352, a Novel Putative Transposon Associated with Mobilization of the blaVIM-2-Harboring
534 In58 Integron. *Antimicrob Agents Chemother* 2017; **61**: e02532-16.

535 45. Botelho J, Grosso F, Quinteira S, Mabrouk A, Peixe L. The complete nucleotide sequence of an IncP-2
536 megaplasmid unveils a mosaic architecture comprising a putative novel blaVIM-2-harbouring
537 transposon in Pseudomonas aeruginosa. *J Antimicrob Chemother* 2017; **72**: 2225–2229.

538 46. San Millan A, Toll-Riera M, Escudero JA, Cantón R, Coque TM, MacLean RC. Sequencing of plasmids
539 pAMBL1 and pAMBL2 from *Pseudomonas aeruginosa* reveals a *bla* $_{VIM-1}$ amplification causing high-level
540 carbapenem resistance. *J Antimicrob Chemother* 2015; **70**: 3000–3003.

541 47. Sun F, Zhou D, Wang Q, Feng J, Feng W, Luo W, et al. Genetic characterization of a novel *bla* $_{DIM-2}$ -
542 carrying megaplasmid p12969-DIM from clinical *Pseudomonas putida*. *J Antimicrob Chemother* 2016;
543 **71**: 909–912.

544 48. Roy Chowdhury P, Scott M, Worden P, Huntington P, Hudson B, Karagiannis T, et al. Genomic islands 1
545 and 2 play key roles in the evolution of extensively drug-resistant ST235 isolates of Pseudomonas
546 aeruginosa. *Open Biol* 2016; **6**.

547 49. Botelho J, Grosso F, Peixe L. Unravelling the genome of a Pseudomonas aeruginosa isolate belonging to
548 the high-risk clone ST235 reveals an integrative conjugative element housing a blaGES-6
549 carbapenemase. *J Antimicrob Chemother* 2018; **73**: 77–83.

550 50. Botelho J, Grosso F, Quinteira S, Brilhante M, Ramos H, Peixe L. Two decades of blaVIM-2-producing

Pseudomonas aeruginosa dissemination: an interplay between mobile genetic elements and successful clones. *J Antimicrob Chemother* 2018; **73**: 873–882.

51. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. *Nat Methods* 2015; **12**: 59–60.

52. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014; **30**: 2068–2069.

53. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 2012; **28**: 1647–9.

54. Sullivan MJ, Petty NK, Beatson SA. Easyfig: a genome comparison visualizer. *Bioinformatics* 2011; **27**: 1009–10.

55. Arndt D, Grant JR, Marcu A, Sajed T, Pon A, Liang Y, et al. PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res* 2016; **44**: W16–W21.

56. Siguier P, Perochon J, Lestrade L, Mahillon J, Chandler M. ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res* 2006; **34**: D32–D36.

57. Partridge SR, Tsafnat G. Automated annotation of mobile antibiotic resistance in Gram-negative bacteria: the Multiple Antibiotic Resistance Annotator (MARA) and database. *J Antimicrob Chemother* 2018; **73**: 883–890.

58. Huerta-Cepas J, Forslund K, Coelho LP, Szklarczyk D, Jensen LJ, von Mering C, et al. Fast Genome-Wide Functional Annotation through Orthology Assignment by eggNOG-Mapper. *Mol Biol Evol* 2017; **34**: 2115–2122.

59. Jones P, Binns D, Chang H-Y, Fraser M, Li W, McAnulla C, et al. InterProScan 5: genome-scale protein function classification. *Bioinformatics* 2014; **30**: 1236–40.

60. Kaas RS, Leekitcharoenphon P, Aarestrup FM, Lund O. Solving the Problem of Comparing Whole Bacterial Genomes across Different Sequencing Platforms. *PLoS One* 2014; **9**: e104984.

61. Jolley KA, Maiden MCJ. BIGSdb: Scalable analysis of bacterial genome variation at the population level.

BMC Bioinformatics 2010; **11**: 595.

62. Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J. JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. *Bioinformatics* 2016; **32**: 929–931.

63. Ding Y, Teo JWP, Drautz-Moses DI, Schuster SC, Givskov M, Yang L. Acquisition of resistance to carbapenem and macrolide-mediated quorum sensing inhibition by Pseudomonas aeruginosa via ICETn43716385. *Commun Biol* 2018; **1**: 57.

64. Fonseca EL, Marin MA, Encinas F, Vicente ACP. Full characterization of the integrative and conjugative element carrying the metallo-β-lactamase *bla* $_{SPM-1}$ and bicyclomycin *bcr1* resistance genes found in the pandemic *Pseudomonas aeruginosa* clone SP/ST277 : Figure 1. *J Antimicrob Chemother* 2015; **70**: 2547–2550.

65. Nascimento APB, Ortiz MF, Martins WMBS, Morais GL, Fehlberg LCC, Almeida LGP, et al. Intraclonal Genome Stability of the Metallo-β-lactamase SPM-1-producing Pseudomonas aeruginosa ST277, an Endemic Clone Disseminated in Brazilian Hospitals. *Front Microbiol* 2016; **7**: 1946.

66. Weingarten RA, Johnson RC, Conlan S, Ramsburg AM, Dekker JP, Lau AF, et al. Genomic Analysis of Hospital Plumbing Reveals Diverse Reservoir of Bacterial Plasmids Conferring Carbapenem Resistance. *MBio* 2018; **9**: e02011-17.

67. Giani T, Arena F, Pollini S, Di Pilato V, D'Andrea MM, Henrici De Angelis L, et al. Italian nationwide survey on Pseudomonas aeruginosa from invasive infections: activity of ceftolozane/tazobactam and comparators, and molecular epidemiology of carbapenemase producers. *J Antimicrob Chemother* 2018; **73**: 664–671.

68. Turton JF, Wright L, Underwood A, Witney AA, Chan Y-T, Al-Shahib A, et al. High-Resolution Analysis by Whole-Genome Sequencing of an International Lineage (Sequence Type 111) of Pseudomonas aeruginosa Associated with Metallo-Carbapenemases in the United Kingdom. *J Clin Microbiol* 2015; **53**: 2622–31.

69. Tada T, Kitao T, Miyoshi-Akiyama T, Kirikae T. Genome sequence of multidrug-resistant Pseudomonas aeruginosa NCGM1179. *J Bacteriol* 2011; **193**: 6397.

602 70. Toleman MA, Bennett PM, Walsh TR. ISCR elements: novel gene-capturing systems of the 21st
603 century? *Microbiol Mol Biol Rev* 2006; **70**: 296–316.

604 71. Partridge SR. Analysis of antibiotic resistance regions in Gram-negative bacteria. *FEMS Microbiol Rev*
605 2011; **35**: 820–855.

606 72. Shimizu W, Kayama S, Kouda S, Ogura Y, Kobayashi K, Shigemoto N, et al. Persistence and epidemic
607 propagation of a Pseudomonas aeruginosa sequence type 235 clone harboring an IS26 composite
608 transposon carrying the blaIMP-1 integron in Hiroshima, Japan, 2005 to 2012. *Antimicrob Agents*
609 *Chemother* 2015; **59**: 2678–87.

610 73. Varghese NJ, Mukherjee S, Ivanova N, Konstantinidis KT, Mavrommatis K, Kyrpides NC, et al. Microbial
611 species delineation using whole genome sequences. *Nucleic Acids Res* 2015; **43**: 6761–71.

612 74. Baquero F. From pieces to patterns: evolutionary engineering in bacterial pathogens. *Nat Rev*
613 *Microbiol* 2004; **2**: 510–518.

614 75. Miyoshi-Akiyama T, Tada T, Ohmagari N, Viet Hung N, Tharavichitkul P, Pokhrel BM, et al. Emergence
615 and Spread of Epidemic Multidrug-Resistant Pseudomonas aeruginosa. *Genome Biol Evol* 2017; **9**:
616 3238–3245.

617 76. Bondy-Denomy J, Davidson AR. To acquire or resist: the complex biological effects of CRISPR-Cas
618 systems. *Trends Microbiol* 2014; **22**: 218–25.

619 77. van Belkum A, Soriaga LB, LaFave MC, Akella S, Veyrieras J-B, Barbu EM, et al. Phylogenetic Distribution
620 of CRISPR-Cas Systems in Antibiotic-Resistant Pseudomonas aeruginosa. *MBio* 2015; **6**: e01796-15.

621 78. Touchon M, Hoede C, Tenaillon O, Barbe V, Baeriswyl S, Bidet P, et al. Organised Genome Dynamics in
622 the Escherichia coli Species Results in Highly Diverse Adaptive Paths. *PLoS Genet* 2009; **5**: e1000344.

623 79. Rocha EPC. The replication-related organization of bacterial genomes. *Microbiology* 2004; **150**: 1609–
624 1627.

625 80. Knezevic P, Voet M, Lavigne R. Prevalence of Pf1-like (pro)phage genetic elements among
626 Pseudomonas aeruginosa isolates. *Virology* 2015; **483**: 64–71.

627 81. Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, et al. Gene expression in
628 Pseudomonas aeruginosa biofilms. *Nature* 2001; **413**: 860–864.

629 82. Secor PR, Michaels LA, Smigiel KS, Rohani MG, Jennings LK, Hisert KB, et al. Filamentous Bacteriophage
630 Produced by Pseudomonas aeruginosa Alters the Inflammatory Response and Promotes Noninvasive
631 Infection In Vivo. *Infect Immun* 2017; **85**: e00648-16.

632 83. Roberts AP, Mullany P. Tn *916* -like genetic elements: a diverse group of modular mobile elements
633 conferring antibiotic resistance. *FEMS Microbiol Rev* 2011; **35**: 856–871.

634 84. Rubio-Cosials A, Schulz EC, Lambertsen L, Smyshlyaev G, Rojas-Cordova C, Forslund K, et al.
635 Transposase-DNA Complex Structures Reveal Mechanisms for Conjugative Transposition of Antibiotic
636 Resistance. *Cell* 2018; **173**: 208–220.e20.

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638