ICEs are the main reservoirs of the ciprofloxacin-modifying *crpP* gene in *Pseudomonas aeruginosa*

João Botelho[1,2,*], Filipa Grosso[3], Luísa Peixe[3]

[1]Antibiotic Resistance Evolution Group, Max-Planck-Institute for Evolutionary Biology, 24306 Plön, Germany.

[2]Department of Evolutionary Ecology and Genetics, Zoological Institute, Christian-Albrechts-Universität zu Kiel, Am Botanischen Garten 1-9, 24118 Kiel, Germany.

[3]UCIBIO/REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal.

*Corresponding author: João Botelho, Antibiotic Resistance Evolution Group, Max-Planck-Institute for Evolutionary Biology, 24306 Plön, Germany; Department of Evolutionary Ecology and Genetics, Zoological Institute, Christian-Albrechts-Universität zu Kiel, 24118 Kiel, Germany.; e-mail: botelho@evolbio.mpg.de. Luísa Peixe, Laboratório de Microbiologia. Faculdade de Farmácia da Universidade do Porto, Rua Jorge Viterbo Ferreira nº 228, 4050-313 Porto, Portugal; phone: +351 220428500; e-mail: lpeixe@ff.up.pt.

Abstract

The ciprofloxacin-modifying *crpP* gene was recently identified in a plasmid isolated from a clinical *Pseudomonas aeruginosa* clinical isolate. Homologues of this gene were also identified in *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*. We set out to explore the mobile genetic elements involved in the acquisition and spread of this gene in publicly available and complete genomes of *Pseudomonas*. The *crpP* gene was identified only in *P. aeruginosa*, in more than half of the complete chromosomes (61.9%, n=133/215) belonging to 52 sequence types, of which the high-risk clone ST111 was the most frequent. We identified 136 *crpP*-harboring ICEs, with 93.4% belonging to the mating-pair formation G (MPF_G) family. The ICEs were integrated at the end of a $tRNA^{Lys}$ gene and were all flanked by highly conserved 45-bp direct repeats. The core ICEome contains 26 genes (2.2% of all genes), which are present in 99% or more of the *crpP*-harboring ICEs. The most frequently encoded traits on these ICEs include replication, transcription, intracellular trafficking and cell motility. Our work reveals that ICEs are the main vectors promoting the dissemination of the ciprofloxacin-modifying *crpP* gene in *P. aeruginosa*.

Keywords

Integrative and conjugative elements, ciprofloxacin resistance, *crpP*.

Author Notes

43 All supporting data has been provided within the article or through supplementary data
44 files. Supplementary material is available with the online version of this article.

45

46 Abbreviations

47 ARG, antibiotic resistance gene; CrpP, ciprofloxacin resistance protein; ICE, integrative
48 and conjugative element; MPF, mating pair formation; NCBI, National Center for
49 Biotechnology Information.

50

51 Impact Statement

52 A high proportion of *Pseudomonas aeruginosa* clinical isolates are resistant to
53 ciprofloxacin. Resistance to this antibiotic is often mediated by chromosomal mutations,
54 but recently horizontally transferred genes have been identified. We assessed the
55 repartition of the ciprofloxacin-modifying *crpP* gene among *Pseudomonas* genomes and
56 we characterized the mobile elements associated with its acquisition. We found that this
57 gene is prevalent in *P. aeruginosa* and frequently associated with integrative and
58 conjugative elements (ICEs). Importantly, we also identified highly conserved direct
59 repeats that can be used to accurately delimit *crpP*-carrying ICEs in *P. aeruginosa*
60 genomes.

61

62 Data Summary

63 All the bacterial genomes scanned in this study have been deposited previously in the
64 National Center for Biotechnology Information genome database and are listed on the
65 supplementary tables. The newick files used to create the trees in Figures 1 and 4 are
66 deposited on figshare at
67 https://figshare.com/projects/ICEs_are_the_main_reservoirs_of_the_ciprofloxacin-
68 modifying_crpP_gene_in_Pseudomonas_aeruginosa/79308.

69

70 **Introduction**

71 *Pseudomonas aeruginosa* is a frequent cause of severe nosocomial infections and is one
72 of the six ESKAPE pathogens [1,2]. Ciprofloxacin is an antibiotic of the fluoroquinolone
73 class that is active against *P. aeruginosa* infections [3,4]. In this species, a high proportion
74 of clinical isolates are resistant to ciprofloxacin [5,6]. Commonly reported mechanisms
75 of ciprofloxacin resistance include mutations in DNA gyrase and topoisomerase IV-
76 encoding genes *gyrA*, *gyrB*, *parC* and *parE* and efflux pumps regulatory genes as *nalC*
77 and *nfxB* [6–8].

78 Besides chromosomal mutations, ciprofloxacin resistance can be mediated by
79 horizontally transferred genes, such as the quinolone resistance *qnr* gene [9]. The
80 ciprofloxacin-modifying *crpP* gene induces ATP-dependent phosphorylation of the
81 antibiotic and was originally identified in a plasmid isolated from a *P. aeruginosa* clinical
82 isolate [10]. This plasmid conferred resistance to ciprofloxacin when transferred to *P.*
83 *aeruginosa* PAO1 strain. The authors also cloned the gene into a shuttle vector and the
84 resulting recombinant plasmid conferred an increased minimum inhibitory

85 concentration to the same antibiotic in *Escherichia coli*. Homologous *crpP* genes were
86 also identified in *Acinetobacter baumannii* [11] and in *E. coli* and *Klebsiella pneumoniae*
87 clinical isolates from Mexican hospitals and conferred decreased susceptibility to
88 ciprofloxacin [12,13].

89 Integrative and conjugative elements (ICEs) are mobile elements involved in vertical and
90 horizontal transmission of antibiotic resistance genes (ARGs) and other beneficial genes
91 in bacterial communities [2,14,15]. ICEs can be classified into eight mating-pair
92 formation (MPF) classes based on the classification of the secretion machinery involved
93 in conjugation [16,17]. Recently, Ruiz verified that several *CrpP* proteins were encoded
94 next to genes typically present in ICEs [11]. Building on this observation, we set out to
95 trace and characterize the *crpP*-harboring ICEs present in all complete *Pseudomonas*
96 genomes available on NCBI.

97

98 ## Material and methods

99 ### Pangenome and whole-genome analyses

100 All complete *Pseudomonas* genomes (n=577 chromosomes and 163 plasmids) were
101 downloaded from NCBI's Refseq library on the 01/02/2020 using ncbi-genome-
102 download v 0.2.11 (https://github.com/kblin/ncbi-genome-download) (**Table S1**). We
103 used mlst v 2.11 (https://github.com/tseemann/mlst) to scan the genomes against the
104 PubMLST *Pseudomonas* typing schemes (https://pubmlst.org/) [18]. The genomes were
105 annotated using prokka v1.14.5 (https://github.com/tseemann/prokka) [19]. We used
106 the .gff files created by Prokka to calculate the *P. aeruginosa* pangenome in roary

107 v3.13.0 (https://github.com/sanger-pathogens/Roary) [20]. The core genome and
108 ICEome alignments created by Roary was used as input in fasttree v2.1.10
109 (http://www.microbesonline.org/fasttree/) [21] to create an approximately-maximum-
110 likelihood phylogenetic tree using a generalized time-reversible (GTR) model of
111 nucleotide evolution. We used the newick file created by FastTree to create a
112 phylogenetic tree in iTOL (https://itol.embl.de/) [22]. We performed functional
113 annotation based on orthology assignments (COGs) of protein files created by prokka
114 using eggNOG-mapper v2 (http://eggnog-mapper.embl.de/) [23].

115

116 **Mining ICEs in complete genomes**

117 We used the ICEfinder standalone version
118 (http://202.120.12.136/ICEfinder/ICEfinder.html) and manual curation to trace putative
119 ICEs. We searched for ARGs on extracted ICEs using amrfinder v 3.6.7 and default
120 parameters (50% minimum coverage of the reference protein and 90% minimum
121 identity) (https://github.com/ncbi/amr/wiki/AMRFinder-database) [24]. Positive ICE
122 hits for CrpP-encoding genes were further characterized; we used fastANI v1.3
123 (https://github.com/ParBLiSS/FastANI) [25] to compute whole-genome average
124 nucleotide identity of non-*P. aeruginosa crpP*-positive hits, antismash online tool
125 (https://antismash.secondarymetabolites.org/#!/start) [26] to look for secondary
126 metabolite biosynthesis gene clusters, BAGEL (http://bagel4.molgenrug.nl/) [27] to
127 trace bacteriocins, macsyfinder v1.0.5 (https://github.com/gem-pasteur/macsyfinder)
128 and the CRISPRCasFinder online tool (https://crisprcas.i2bc.paris-saclay.fr/) to detect

129 CRISPRs and *cas* genes [28]. We used roary to infer the total *crpP*-harboring ICE content,
130 here referred to as the ICEome.

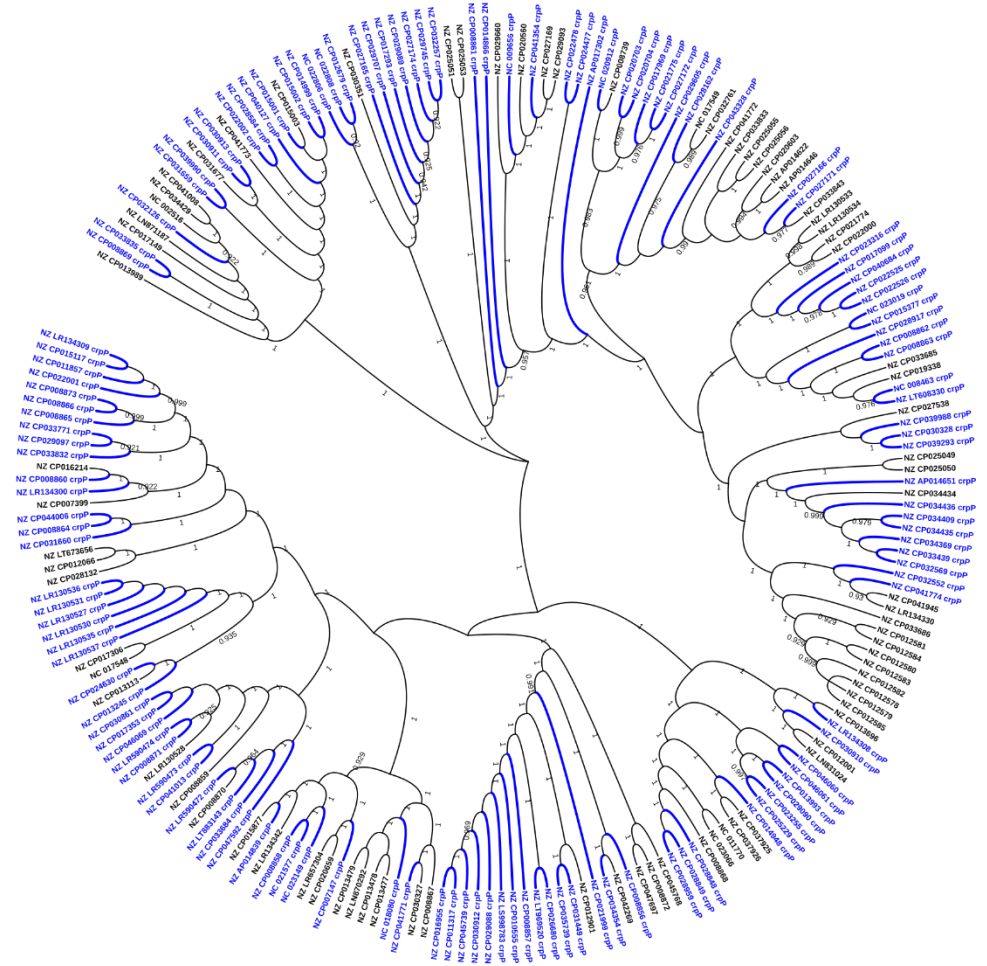
131

132 ## Results

133 ### The *crpP* gene is widespread in *P. aeruginosa*

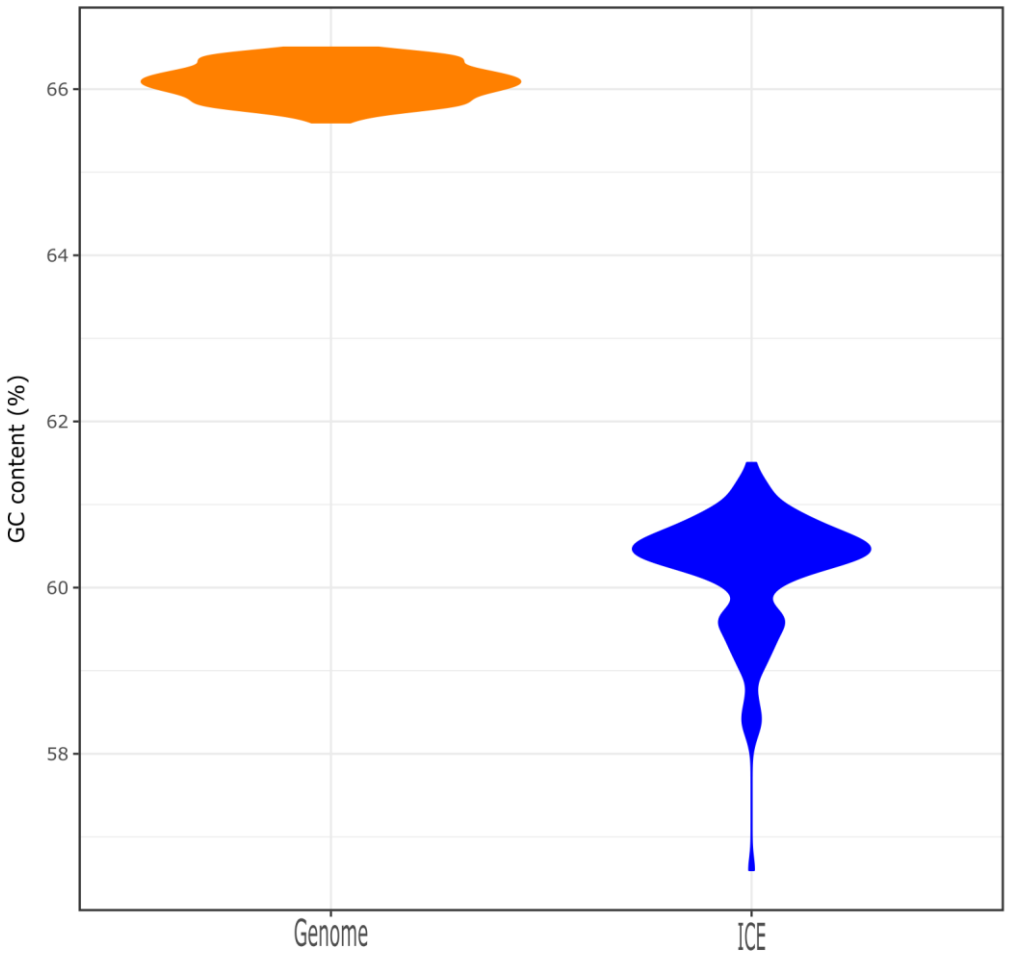
134 The *CrpP*-encoding gene was identified in 23.1% of the chromosomes (n=133/577,
135 including 131 *P. aeruginosa*, 1 *Pseudomonas fluorescens* and 1 *Pseudomonas* sp.) and 1
136 *P. aeruginosa* plasmid (accession number NZ_CP030914.1), which is different than the
137 pUM505 plasmid reported by Chávez-Jacobo and colleagues [10]. However, we
138 compared the ANI between non-*P. aeruginosa* hits (the *P. fluorescens* strain NCTC10783
139 and *Pseudomonas* sp. AK6U strains with accession numbers NZ_LR134300.1 and
140 NZ_CP025229.1, respectively) and the *P. aeruginosa* DSM 50071 type strain (accession
141 number NZ_CP012001.1) and we realized that these strains belong to the *P. aeruginosa*
142 species, as the ANI value is above the 95% cutoff for species delineation (**Table S2**). All
143 *crpP*-hits were only identified in *P. aeruginosa* complete genomes, as so from here on
144 we focused our attention on *P. aeruginosa* genomes. Indeed, the percentage increases
145 if we only consider the *P. aeruginosa* chromosomes (61.9%, n=133/215), meaning that
146 the *crpP* gene is present in more than half of the *P. aeruginosa* complete genomes
147 (**Figure 1**). The GC content of *crpP*-harboring chromosomes varies from 65.6 to 66.5%
148 (**Figure 2**) and the sequence length from 6.3 to 7.5 Mb. We identified several clones
149 (n=52 sequence types), of which the high-risk clone ST111 [29] was the most frequent
150 (**Table S3**).

Tree scale: 0.01



152 **Figure 1**. Approximately-maximum-likelihood phylogenetic tree base on the core
153 genome alignment of 215 complete *P. aeruginosa* genomes. Branches and labels from
154 *crpP*-positive hits are colored blue and the branches have twice the standard width. Only
155 bootstrap values from 0.9 to 1 are displayed.

156



157

158 **Figure 2** – Distribution of the GC content of the chromosomes and ICEs. The violin plots
159 were created using ggplot2.

160

161 **The majority of *crpP*-harboring ICEs belong to the MPF_G family**

162 A total of 316 putative ICEs were identified among the 133 chromosomes. Each
163 chromosome carries at least one ICE. Most *crpP* genes present in the chromosome were
164 associated with an ICE (with the exception of *P. aeruginosa* strains W60856 and B17932
165 with the accession numbers NZ_CP008864.2 and NZ_CP034436.1, respectively) with
166 43.0% of the total putative ICEs (n=136/316) harboring the ciprofloxacin-modifying
167 gene. In *P. aeruginosa* RW109 strain (accession number NZ_LT969520.1, position
168 5629923-5820312 bp), we identified two *crpP*-harboring ICEs in tandem (**Table S3**). ICE
169 size varied from 81.6 to 145.5 kb, and the GC content from 56.6 to 61.5% (**Figure 2**).
170 Most of the ICEs (n=129/136) ICEs belong to the MPF_G family (**Table S4**). For the
171 remaining 7, no MPF family could be determined.

172

173 **The *crpP*-carrying ICEs integrate into a specific hotspot**

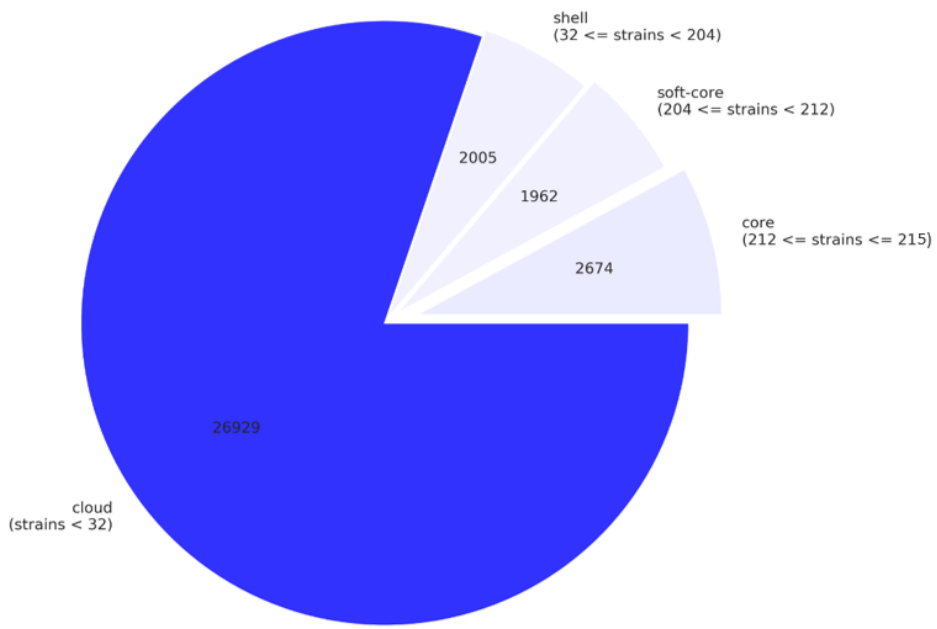
174 All *crpP*-carrying ICEs identified in this study were integrated at the end of a $tRNA^{Lys}$
175 gene, that was found alone or in a tRNA cluster with $tRNA^{Pro}$ and $tRNA^{Asn}$. The only
176 exception was observed in the two contiguous ICEs identified in *P. aeruginosa* RW109
177 strain, where the second ICE was integrated at the end of the first one. The $tRNA^{Lys}$ genes
178 shared the exact same sequence. All ICEs were flanked by 45-bp (5'-
179 TGGTGGGTCGTGTAGGATTCGAACCTACGACCAATTGGTTAAAAG-3') highly conserved
180 direct repeats (DRs). The 136 integrases identified in this study, however, only shared
181 36.1% amino acid identity.

182

183 **The *P. aeruginosa* pangenome and the *crpP*-carrying ICEome**

184 The total number of genes among the 215 *P. aeruginosa* complete genomes
185 (pangenome) is 33570. The core genome (genes present in 99% or more of the genomes)
186 contains 2674 genes (8.0% of all genes, **Figure 3** and **Figures S1**). We also identified
187 11241 unique genes, which are found in only one strain [30].

188



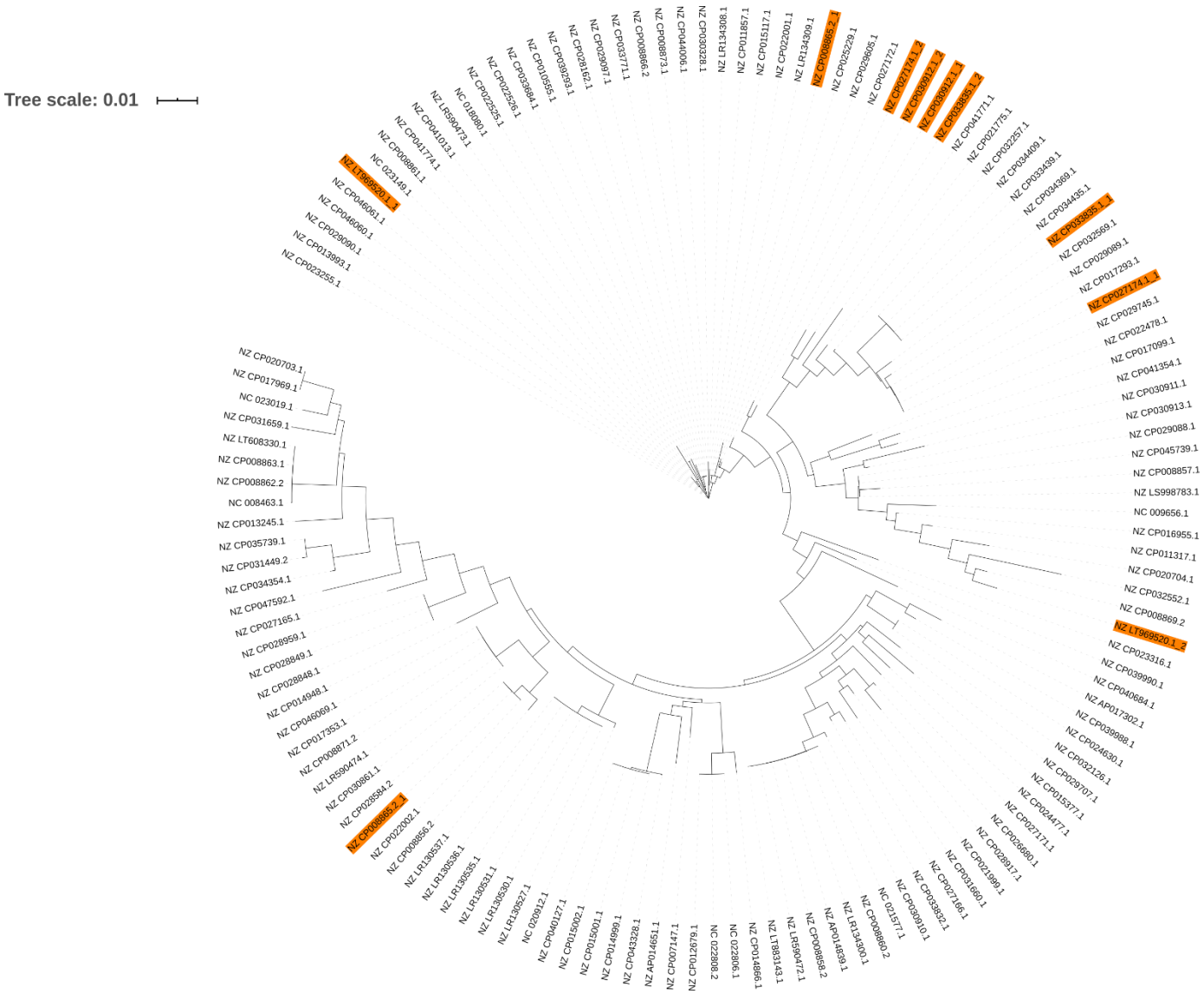
189

190 **Figure 3** – Pie chart of the breakdown of genes and the number of *P. aeruginosa*
191 genomes they are present in. This figure was created using the contributed Python script
192 roary_plots.py in https://github.com/sanger-
193 pathogens/Roary/blob/master/contrib/roary_plots/roary_plots.py.

194

195 The total number of genes in the 136 *crpP*-harboring ICEs (ICEome) is 1193. The core
196 ICEome contains 26 genes (2.2% of all genes). The soft core content (genes present in
197 more or less than 95% and less than 99% of ICEs) includes 11 genes, 129 shell genes
198 (between 15 and 95%) and 1027 cloud genes (between zero and 15%). We identified

199 451 unique genes among the ICEome. These values suggest that *crpP*-harboring ICEs are
200 not clonal, based on the small number of core genes and high number of cloud genes.
201 Phylogenetic analysis of the core ICEome alignment reveals that these ICEs are very
202 diverse and the core genes only share 28.0% nucleotide identity (**Figure 4**).


Tree scale: 0.01
NZ CP008865.2_1
NZ CP025229.1
NZ CP029605.1
NZ CP027172.1
NZ CP027174.1_2
NZ CP030912.1_1
NZ CP030912.1_2
NZ CP033835.1_2
NZ CP041771.1
NZ CP021775.1
NZ CP032257.1
NZ CP034409.1
NZ CP033439.1
NZ CP034369.1
NZ CP034435.1
NZ CP033835.1_1
NZ CP032569.1
NZ CP029089.1
NZ CP017293.1
NZ CP027174.1_1
NZ CP029745.1
NZ CP022478.1
NZ CP017099.1
NZ CP041354.1
NZ CP030911.1
NZ CP030913.1
NZ CP029088.1
NZ CP045739.1
NZ CP008857.1
NZ LS998783.1
NC 009656.1
NZ CP016955.1
NZ CP011317.1
NZ CP020704.1
NZ CP032552.1
NZ CP008869.2
NZ LT969520.1_2
NZ CP023316.1
NZ CP039990.1
NZ CP040684.1
NZ AP017302.1
NZ CP039988.1
NZ CP024630.1
NZ CP032126.1
NZ CP029707.1
NZ CP015377.1
NZ CP024477.1
NZ CP027171.1
NZ CP026680.1
NZ CP028917.1
NZ CP021999.1
NZ CP031660.1
NZ CP027166.1
NZ CP033832.1
NZ CP030910.1
NC 021577.1
NZ CP008860.2
NZ LR134300.1
NZ AP014839.1
NZ CP008858.2
NZ LR590472.1
NZ LT883143.1
NC 022806.1
NC 022808.2
NZ CP014866.1
NZ CP007147.1
NZ CP012679.1
NZ CP014999.1
NZ CP043328.1
NZ AP014651.1
NZ CP015001.1
NZ CP040127.1
NC 020912.1
NZ LR130527.1
NZ LR130530.1
NZ LR130531.1
NZ LR130535.1
NZ LR130536.1
NZ LR130537.1
NZ CP008856.2
NZ CP022002.1
NZ CP008865.2_1
NZ CP028584.2
NZ CP030861.1
NZ LR590474.1
NZ CP008871.2
NZ CP017353.1
NZ CP046069.1
NZ CP014948.1
NZ CP028848.1
NZ CP028849.1
NZ CP028959.1
NZ CP027165.1
NZ CP047592.1
NZ CP034354.1
NZ CP031449.2
NZ CP035739.1
NZ CP013245.1
NC 008463.1
NZ CP008862.2
NZ CP008863.1
NZ LT608330.1
NZ CP031659.1
NC 023019.1
NZ CP017969.1
NZ CP020703.1
NZ CP023255.1
NZ CP013993.1
NZ CP029090.1
NZ CP046060.1
NZ CP046061.1
NZ LT969520.1_1
NC 023149.1
NZ CP008861.1
NZ CP041774.1
NZ CP041013.1
NZ LR590473.1
NC 018080.1
NZ CP022525.1
NZ CP022526.1
NZ CP033684.1
NZ CP010555.1
NZ CP039293.1
NZ CP028162.1
NZ CP029097.1
NZ CP033771.1
NZ CP008866.2
NZ CP008873.1
NZ CP044006.1
NZ CP030328.1
NZ LR134308.1
NZ CP011857.1
NZ CP015117.1
NZ CP022001.1
NZ LR134309.1
NZ CP008865.2_1

204 **Figure 4** – Approximately-maximum-likelihood phylogenetic tree base on the alignment
205 of the 26 core genes identified in the *crpP*-harboring ICEs. Genome accession numbers
206 containing more than one *crpP*-harboring ICE are highlighted in orange.

207

208 **The *crpP*-carrying ICEs encode other beneficial genes**

209 The most frequently encoded traits on *crpP*-harboring ICEome include replication,
210 transcription, intracellular trafficking, and cell motility (**Figure 5**). However, nearly one-
211 third of the proteins for which a COG category was attributed (30.7%, n=211/687)
212 encode for an unknown function (**Table S5**).

213 The *crpP* gene encoded for proteins ranging from 65 to 68 amino acid and is present in
214 a single copy, with no flanking mobile units as integrons, insertion sequences and/or
215 transposons or found on the close vicinity of the gene. The 136 CrpP proteins here
216 identified share 19.7% amino acid identity. We also observed a lack of co-resident
217 antibiotic resistance genes (ARGs) within the *crpP*-harboring ICEs. Seven ICEs carry a
218 type I-C CRISPR-Cas system and ten harbor pyocin S5-encoding genes (**Table S3**). The
219 bacteriocins here identified are highly identical, sharing 95% amino acid identity. Besides
220 bacteriocins, no secondary metabolites were identified within the extracted ICEs.

221

222

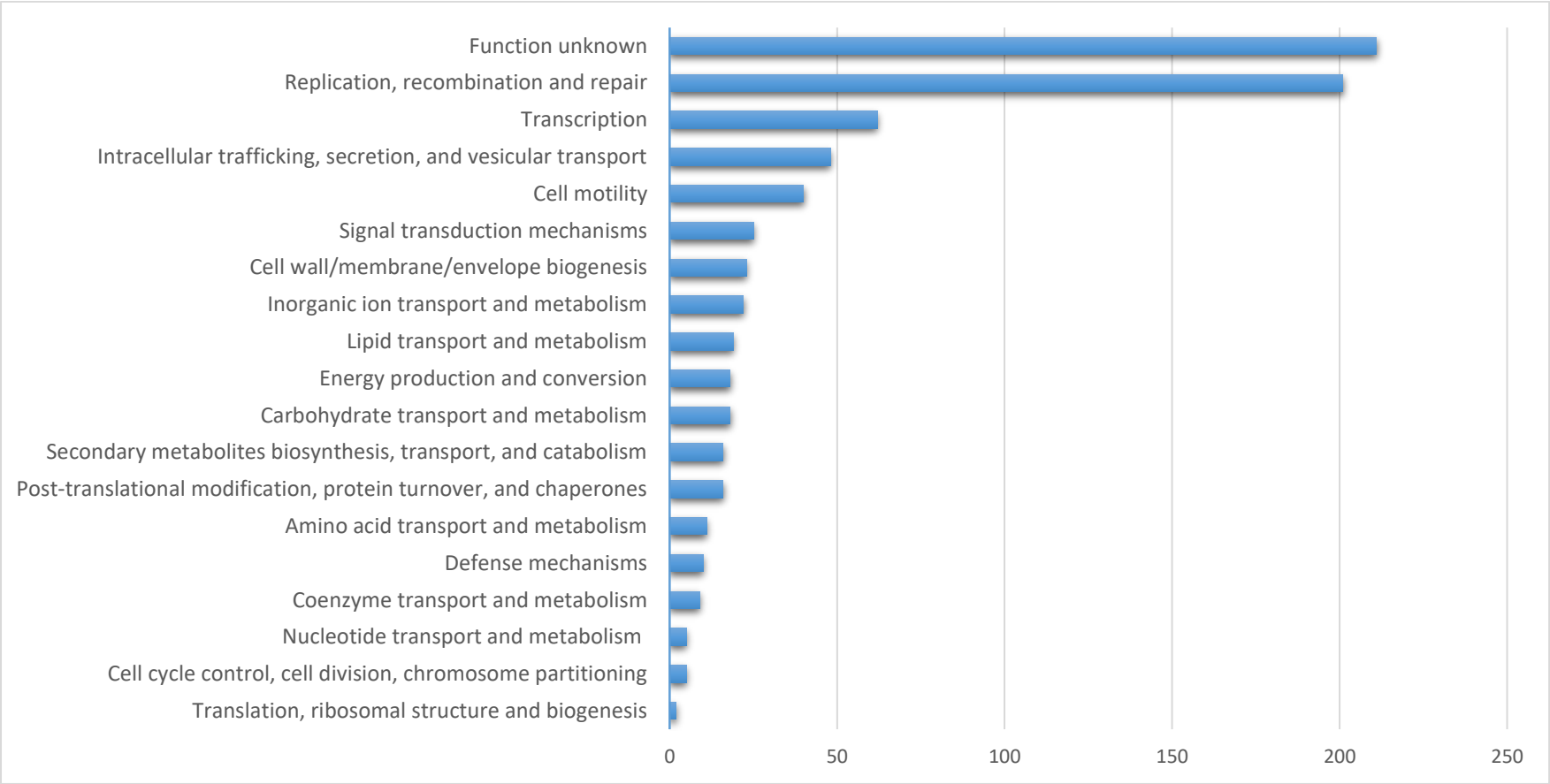


Figure 5. Functional annotation of proteins encoded by the *crpP*-harboring ICEome.

225

226 ## Discussion

227 Our results show that ICEs are the main drivers for the spread of the ciprofloxacin-
228 modifying *crpP* gene in *P. aeruginosa*. A selective pressure exerted by the use of
229 ciprofloxacin to treat *P. aeruginosa* infections may have promoted the dissemination of
230 *crpP*-harboring ICEs among several clones.

231 Curiously, and in opposition to several ARGs, no integrons, insertion sequences or
232 transposons were found on the vicinity of the *crpP* gene. Bacteriocins produced by
233 bacteria are important for niche competition. The pyocin S5 possesses a bactericidal
234 activity against clinical *P. aeruginosa* isolates [31,32]. The presence of these bacteriocins
235 among some *crpP*-harboring ICEs can help to eliminate possible competitors and
236 indirectly enhance ICE maintenance.

237 As expected for foreign DNA, the GC content of the ICEs is lower than that from the
238 chromosomes. Still, given the high GC content of these mobile elements and the
239 biological barrier for gene acquisition between donor-recipient pairs having >5%
240 difference in GC values [33], we suspect that the ancestor of the *crpP* gene derives from
241 GC-rich taxa.

242 We identified highly conserved DRs that can be used to accurately delimit *crpP*-carrying
243 ICEs in *Pseudomonas*. Interestingly, the exact 45-bp sequence was found on the 2 ICEs
244 from *P. fluorescens* and the *Pseudomonas* sp. AK6U strain. Also, even though the *crpP*
245 gene was first reported in a plasmid in *P. aeruginosa* [10], we found a perfect match for
246 the 45-bp DR next to an integrase-encoding gene. We only found one match for the DR

247 in this plasmid, meaning that the ICE is degenerated and most likely depends on the
248 plasmid to be mobilized. The same was observed for the *P. aeruginosa crpP*-carrying
249 plasmid detected in this study (accession number NZ_CP030914.1).

250 The *crpP*-carrying ICEs were traced in several clones, taking advantage of the highly
251 conserved attachment site in the $tRNA^{Lys}$ gene. Interestingly, we found in a previous
252 study that MPF_G class ICEs carrying specific ARGs were integrated at the end of $tRNA^{Gly}$
253 genes [15]. In fact, this class of ICEs is frequently integrated at the end of a tRNA gene
254 [34]. This behavior suggests selection for the maintenance of these non-coding
255 integration sites, and seamless site-specific integration of ICEs in the chromosome will
256 incur in a lower burden to the recipient cell and therefore increase its fitness [2,35]. As
257 so, conferring decreased susceptibility or low levels of ciprofloxacin resistance without
258 incurring in fitness cost can help to explain the high frequency of *crpP*-carrying ICEs
259 observed in this study. Future studies should explore the possible synergistic effects
260 between commonly reported mutations leading to ciprofloxacin resistance and the
261 presence of *crpP*-harboring ICEs.

262 One of the caveats of our study is that we restricted the analyses to complete genomes.
263 Given that the majority of the assemblies present in public databases as NCBI
264 correspond to draft genomes, we probably missed the identification of several *crpP*-
265 carrying ICEs among *P. aeruginosa*. However, mobile elements as ICEs tend to be
266 fragmented in genomes sequenced with short-reads technologies, due to the presence
267 of repetitive regions. Also, the identification of ICE fragments within the draft genome
268 would require similar ICE sequences to use as a reference, which would then bias the

269 study. Sequencing bacteria with long-read approaches is increasing, which will improve
270 the accurate delimitation of an ever-growing number of mobile elements.

271

272 Conclusions

273 Our work demonstrates that ICEs are the main promoters involved in the dissemination
274 of the ciprofloxacin-modifying *crpP* gene among clonally-unrelated *P. aeruginosa*
275 strains. We also identified highly conserved 45-bp DRs than can be used to accurately
276 trace *crpP*-harboring ICEs on *P. aeruginosa* genomes. Future studies should explore the
277 mobile elements involved in the acquisition of the *crpP* homologues identified in species
278 other than *P. aeruginosa* and the possible synergistic effects between commonly
279 reported mutations leading to ciprofloxacin resistance and the presence of *crpP*-carrying
280 ICEs.

281

282 Data bibliography

283 The DNA sequences used in this study are available in NCBI, under the accession
284 numbers provided in supplementary tables S1-S5.

285

286 Funding information

287 This work was supported by the Applied Molecular Biosciences Unit - UCIBIO which is
288 financed by national funds from FCT (UIDB/04378/2020).

Author contributions

J. B., F. G. and L. P. conceptualized the project. J. B. ran the analyses and examined data. J. B. wrote the manuscript. J. B., F. G. and L. P. edited and revised the manuscript. All authors read, commented on and approved the final manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

References

1 Boucher, H.W. *et al.* (2009) Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America. *Clin. Infect. Dis.* 48, 1–12

2 Botelho, J. *et al.* (2019) Antibiotic resistance in *Pseudomonas aeruginosa* – Mechanisms, epidemiology and evolution. *Drug Resist. Updat.* 44, 100640

3 Paulsson, M. *et al.* (2017) Antimicrobial combination treatment including ciprofloxacin decreased the mortality rate of *Pseudomonas aeruginosa* bacteraemia: a retrospective cohort study. *Eur. J. Clin. Microbiol. Infect. Dis.* 36, 1187–1196

4 Klodzińska, S.N. *et al.* (2016) Inhalable antimicrobials for treatment of bacterial biofilm-associated sinusitis in cystic fibrosis patients: Challenges and drug delivery

309 approaches. *Int. J. Mol. Sci.* 17, 1688

310 5 Pitt, T.L. *et al.* (2003) Survey of resistance of *Pseudomonas aeruginosa* from UK
311 patients with cystic fibrosis to six commonly prescribed antimicrobial agents.
312 *Thorax*, 58, 794–796

313 6 Rehman, A. *et al.* (2019) Mechanisms of ciprofloxacin resistance in *Pseudomonas*
314 *aeruginosa*: New approaches to an old problem. *J. Med. Microbiol.* 68, 1–10

315 7 Bruchmann, S. *et al.* (2013) Quantitative contributions of target alteration and
316 decreased drug accumulation to *Pseudomonas aeruginosa* fluoroquinolone
317 resistance. *Antimicrob. Agents Chemother.* 57, 1361–8

318 8 Cantón, R. and Ruiz-Garbajosa, P. (2011) Co-resistance: an opportunity for the
319 bacteria and resistance genes. *Curr. Opin. Pharmacol.* 11, 477–485

320 9 Belotti, P.T. *et al.* (2015) Description of an original integron encompassing bla_{VIM-2},
321 *qnrVC1* and genes encoding bacterial group II intron proteins in *Pseudomonas*
322 *aeruginosa*. *J. Antimicrob. Chemother.* 70, 2237–2240

323 10 Chávez-Jacobo, V.M. *et al.* (2018) CrpP is a novel ciprofloxacin-modifying enzyme
324 encoded by the *Pseudomonas aeruginosa* pUM505 plasmid. *Antimicrob. Agents*
325 *Chemother.* 62, e02629-17

326 11 Ruiz, J. (2019) CrpP, a passenger or a hidden stowaway in the *Pseudomonas*
327 *aeruginosa* genome? *J. Antimicrob. Chemother.* 74, 3397-3399

328 12 Chávez-Jacobo, V.M. *et al.* (2019) Prevalence of the *crpP* gene conferring
329 decreased ciprofloxacin susceptibility in enterobacterial clinical isolates from
330 Mexican hospitals. *J. Antimicrob. Chemother.* 74, 1253–1259

331 13 Pablo, J. *et al.* (2020) Identification of Essential Residues for Ciprofloxacin
332 Resistance of Ciprofloxacin- - Modifying Enzyme (CrpP) of pUM505. *Microbiology*
333 DOI: 10.1099/mic.0.000889

334 14 Partridge, S.R. *et al.* (2018) Mobile Genetic Elements Associated with
335 Antimicrobial Resistance. *Clin. Microbiol. Rev.* 31, e00088-17

336 15 Botelho, J. *et al.* (2018) Carbapenemases on the move: it's good to be on ICEs.
337 *Mob. DNA* 9, 37

338 16 Guglielmini, J. *et al.* (2011) The Repertoire of ICE in Prokaryotes Underscores the
339 Unity, Diversity, and Ubiquity of Conjugation. *PLoS Genet.* 7, e1002222

340 17 Guglielmini, J. *et al.* (2014) Key components of the eight classes of type IV
341 secretion systems involved in bacterial conjugation or protein secretion. *Nucleic*
342 *Acids Res.* 42, 5715–27

343 18 Jolley, K.A. and Maiden, M.C.J. (2010) BIGSdb: Scalable analysis of bacterial
344 genome variation at the population level. *BMC Bioinformatics* 11, 595

345 19 Seemann, T. (2014) Prokka: rapid prokaryotic genome annotation. *Bioinformatics*
346 30, 2068–2069

347 20 Page, A.J. *et al*. (2015) Roary: Rapid Large-Scale Prokaryote Pan Genome Analysis.
348 *Bioinformatics* 31,3691-3

349 21 Price, M.N. *et al* (2009) FastTree: Computing Large Minimum Evolution Trees with
350 Profiles instead of a Distance Matrix. *Mol. Biol. Evol.* 26, 1641-50

351 22 Letunic, I. and Bork, P. (2019) Interactive Tree Of Life (iTOL) v4: recent updates

352 and new developments. *Nucleic Acids Res.* 47, W256–W259

353 23 Huerta-Cepas, J. *et al.* (2019) eggNOG 5.0: a hierarchical, functionally and
354 phylogenetically annotated orthology resource based on 5090 organisms and
355 2502 viruses. *Nucleic Acids Res* 47, D309-D314

356 24 Feldgarden, M. *et al.* (2019) Validating the AMRFinder Tool and Resistance Gene
357 Database by Using Antimicrobial Resistance Genotype-Phenotype Correlations in
358 a Collection of Isolates. *Antimicrob. Agents Chemother.* 63,

359 25 Jain, C. *et al.* (2018) High throughput ANI analysis of 90K prokaryotic genomes
360 reveals clear species boundaries. *Nat. Commun.* 9, 5114

361 26 Blin, K. *et al.* (2019) antiSMASH 5.0: updates to the secondary metabolite genome
362 mining pipeline. *Nucleic Acids Res* 47, W81-W87

363 27 van Heel, A.J. *et al.* (2018) BAGEL4: a user-friendly web server to thoroughly mine
364 RiPPs and bacteriocins. *Nucleic Acids Res.* 46, W278–W281

365 28 Couvin, D. *et al.* (2018) CRISPRCasFinder, an update of CRISRFinder, includes a
366 portable version, enhanced performance and integrates search for Cas proteins.
367 *Nucleic Acids Res.* 46, W246–W251

368 29 Oliver, A. *et al.* (2015) The increasing threat of *Pseudomonas aeruginosa* high-risk
369 clones. *Drug Resist. Updat.* 21–22, 41–59

370 30 Tettelin, H. *et al.* (2005) Genome analysis of multiple pathogenic isolates of
371 *Streptococcus agalactiae*: Implications for the microbial "pan-genome." *Proc.*
372 *Natl. Acad. Sci. U. S. A.* 102, 13950–13955

373 31 Ling, H. *et al.* (2010) A predicted S-type pyocin shows a bactericidal activity against
374 clinical *Pseudomonas aeruginosa* isolates through membrane damage. *FEBS Lett.*
375 584, 3354–3358

376 32 Elfarash, A. *et al.* (2014) Pore-forming pyocin S5 utilizes the FptA ferripyochelin
377 receptor to kill *Pseudomonas aeruginosa*. *Microbiol. (United Kingdom)* 160, 261–
378 269

379 33 Popa, O. *et al.* (2011) Directed networks reveal genomic barriers and DNA repair
380 bypasses to lateral gene transfer among prokaryotes. *Genome Res.* 21, 599–609

381 34 Cury, J. *et al.* (2017) Integrative and conjugative elements and their hosts:
382 composition, distribution and organization. *Nucleic Acids Res.* 45, 8943–8956

383 35 Touchon, M. *et al.* (2014) The chromosomal accommodation and domestication
384 of mobile genetic elements. *Curr. Opin. Microbiol*. 22, 22-9