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* and *Klebsiella pneumoniae*. 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*.

Introduction

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

Besides chromosomal mutations, ciprofloxacin resistance can be mediated by horizontally transferred genes, such as the quinolone resistance (*qnr*) gene [9]. The ciprofloxacin-modifying *crpP* gene induces ATP-dependent phosphorylation of the antibiotic and was originally identified in a plasmid isolated from a *P. aeruginosa* clinical isolate [10]. This plasmid conferred resistance to ciprofloxacin when transferred to *P. aeruginosa* PAO1 strain. The authors also cloned the gene into a shuttle vector and the resulting recombinant plasmid conferred an increased minimum inhibitory concentration (MIC) to the same antibiotic in *Escherichia coli*. Homologous *crpP* genes were also identified in *E. coli* and *Klebsiella pneumoniae* clinical isolates from Mexican hospitals and conferred decreased susceptibility to ciprofloxacin [11,12].

59 Integrative and conjugative elements (ICEs) are mobile elements involved in vertical and
60 horizontal transmission of antibiotic resistance genes (ARGs) and other beneficial genes in
61 bacterial communities [2,13,14]. ICEs can be classified into eight mating-pair formation (MPF)
62 classes based on the classification of the secretion machinery involved in conjugation [15,16].
63 Recently, Ruiz verified that several *CrpP* proteins were encoded next to genes typically present
64 in ICEs [17]. Building on this observation, we set out to trace and characterize the *crpP*-harboring
65 ICEs present in all complete *Pseudomonas* genomes available on NCBI.

66

67 ## Material and methods

68 ### Pangenome and whole-genome analyses

69 All complete *Pseudomonas* genomes (n=577 chromosomes and 163 plasmids) were downloaded
70 from NCBI's Refseq library on the 01/02/2020 using ncbi-genome-download v 0.2.11
71 (https://github.com/kblin/ncbi-genome-download) (**Table S1**). We used mlst v 2.11
72 (https://github.com/tseemann/mlst) to scan the genomes against the PubMLST *Pseudomonas*
73 typing schemes (https://pubmlst.org/) [18]. The genomes were annotated using prokka v1.14.5
74 (https://github.com/tseemann/prokka) [20]. We used the .gff files created by Prokka to
75 calculate the *P. aeruginosa* pangenome in roary v3.13.0 (https://github.com/sanger-
76 pathogens/Roary) [21]. The core genome alignment created by Roary was used as input in
77 fasttree v2.1.10 (http://www.microbesonline.org/fasttree/) [22] to create an approximately-
78 maximum-likelihood phylogenetic tree using a generalized time-reversible (GTR) model of
79 nucleotide evolution. We used the newick file created by FastTree to create a phylogenetic tree
80 in iTOL (https://itol.embl.de/) [23]. We performed functional annotation based on orthology
81 assignments (COGs) of protein files created by prokka using eggNOG-mapper v2 (http://eggnog-
82 mapper.embl.de/) [24].

83

84 ### Mining ICEs in complete genomes

85 We used the ICEfinder standalone version (http://202.120.12.136/ICEfinder/ICEfinder.html) and
86 manual curation to trace putative ICEs. We searched for ARGs on extracted ICEs using amrfinder
87 v 3.6.7 and default parameters (50% minimum coverage of the reference protein and 90%
88 minimum identity) (https://github.com/ncbi/amr/wiki/AMRFinder-database) [25]. Positive ICE
89 hits for *CrpP*-encoding genes were further characterized; we used fastANI v1.3
90 (https://github.com/ParBLiSS/FastANI) [19] to compute whole-genome average nucleotide

91 identity of non-*P. aeruginosa* *crpP*-positive hits, antismash online tool
92 (https://antismash.secondarymetabolites.org/#!/start) [26] to look for secondary metabolite
93 biosynthesis gene clusters, BAGEL (http://bagel4.molgenrug.nl/) [27] to trace bacteriocins,
94 macsyfinder v1.0.5 (https://github.com/gem-pasteur/macsyfinder) and the CRISPRCasFinder
95 online tool (https://crisprcas.i2bc.paris-saclay.fr/) to detect CRISPRs and *cas* genes [28]. We
96 used roary to infer the total *crpP*-harboring ICE content, here referred to as the ICEome.

97

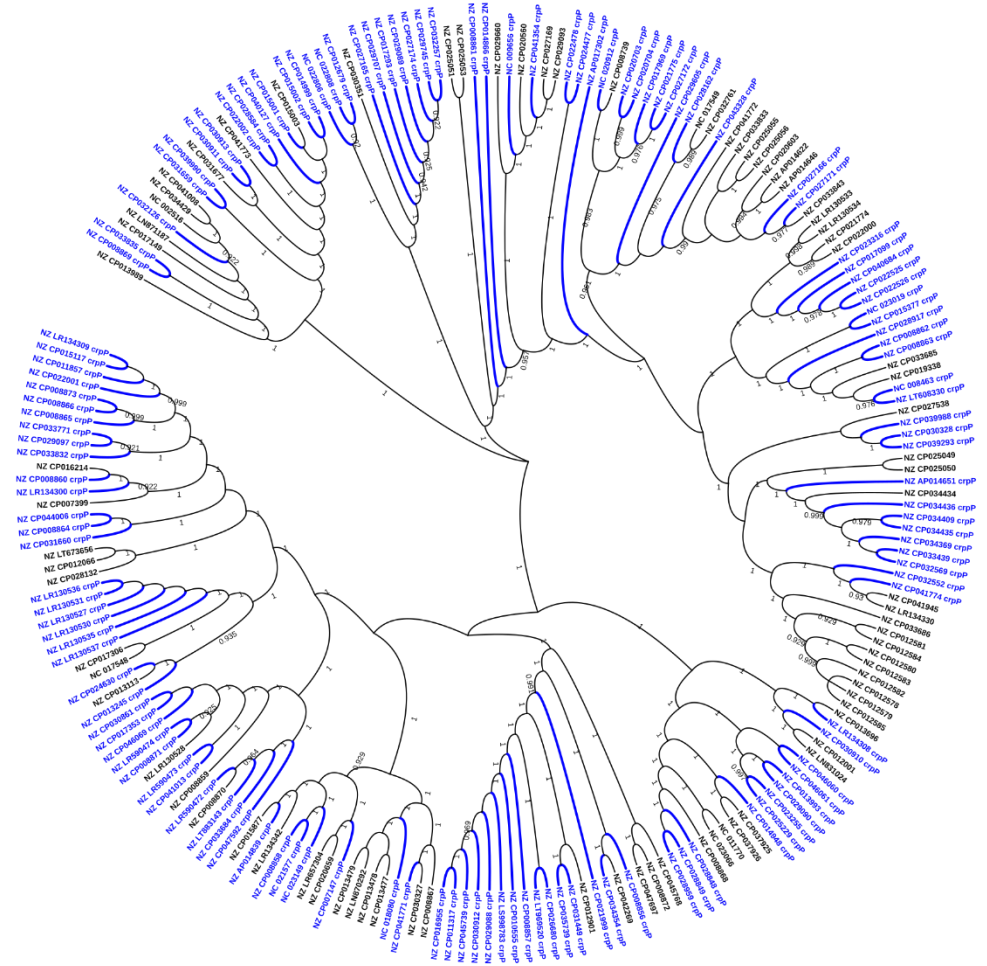
98 **Results**

99 **The *crpP* gene is widespread in *P. aeruginosa***

100 The *CrpP*-encoding gene was identified in 23.1% of the chromosomes (n=133/577, including 131
101 *P. aeruginosa*, 1 *Pseudomonas fluorescens* and 1 *Pseudomonas* sp.) and 1 *P. aeruginosa* plasmid.
102 However, we compared the ANI between non-*P. aeruginosa* hits (the *P. fluorescens* strain
103 NCTC10783 and *Pseudomonas* sp. AK6U strains with accession numbers NZ_LR134300.1 and
104 NZ_CP025229.1, respectively) and the *P. aeruginosa* DSM 50071 type strain (accession number
105 NZ_CP012001.1) and we realized that these strains belong to the *P. aeruginosa* species, as the
106 ANI value is above the 95% cutoff for species delineation (**Table S2**). All *crpP*-hits were only
107 identified in *P. aeruginosa* complete genomes, as so from here on we focused our attention on
108 *P. aeruginosa* genomes. Indeed, the percentage increases if we only consider the *P. aeruginosa*
109 chromosomes (61.9%, n=133/215), meaning that the *crpP* gene is present in more than half of
110 the *P. aeruginosa* complete genomes (**Figure 1**). The GC content of *crpP*-harboring
111 chromosomes varies from 65.6 to 66.5% (**Figure 2**) and the sequence length from 6.3 to 7.5 Mb.
112 We identified several clones (n=52 sequence types), of which the high-risk clone ST111 [29] was
113 the most frequent (**Table S3**).

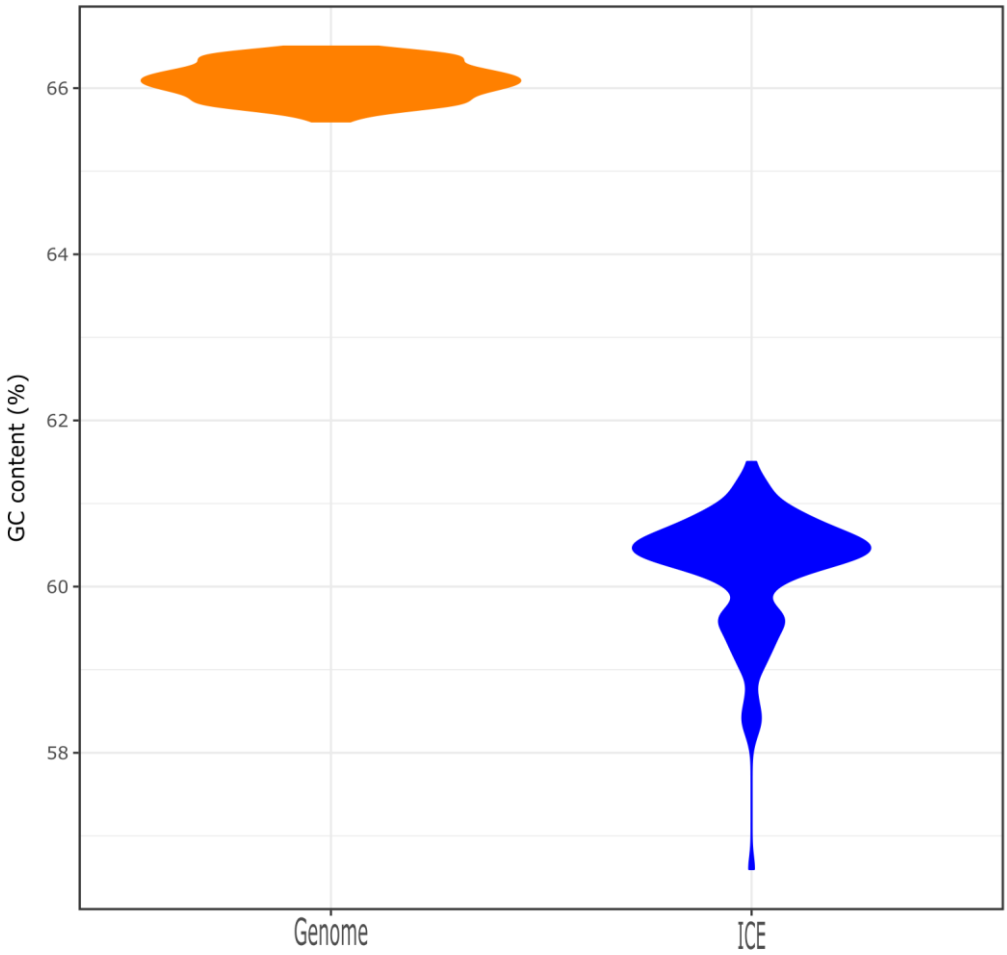
114

Tree scale: 0.01



116 **Figure 1**. Approximately-maximum-likelihood phylogenetic tree base on the core genome
117 alignment of 215 complete *P. aeruginosa* genomes. Branches and labels from *crpP*-positive hits
118 are colored blue and the branches have twice the standard width. Only bootstrap values from
119 0.9 to 1 are displayed. The newick file used to create the tree is available in **Additional file 1**.

120



121

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

124

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

126 A total of 316 putative ICEs were identified among the 133 chromosomes. Each chromosome
127 carries at least one ICE. Most *crpP* genes present in the chromosome were associated with an
128 ICE (besides *P. aeruginosa* strains W60856 and B17932 with the accession numbers
129 NZ_CP008864.2 and NZ_CP034436.1, respectively) with 43.0% of the total putative ICEs

130 (n=136/316) harboring the ciprofloxacin-modifying gene. In *P. aeruginosa* RW109 strain
131 (accession number NZ_LT969520.1, position 5629923-5820312 bp), we identified two *crpP*-
132 harboring ICEs in tandem (**Table S3**). ICE size varied from 81.6 to 145.5 kb, and the GC content
133 from 56.6 to 61.5% (**Figure 2**). Most of the ICEs (n=129/136) ICEs belong to the MPF_G family. For
134 the remaining 7, no MPF family could be determined.

135

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

137 All *crpP*-carrying ICEs identified in this study were integrated at the end of a $tRNA^{Lys}$ gene, that
138 was found alone or in a tRNA cluster with $tRNA^{Pro}$ and $tRNA^{Asn}$. The only exception was observed
139 in the two contiguous ICEs identified in *P. aeruginosa* RW109 strain, where the second ICE was
140 integrated at the end of the first one. The $tRNA^{Lys}$ genes shared the exact same sequence. All
141 ICEs were flanked by 45-bp (5'-TGGTGGGTCGTGTAGGATTCGAACCTACGACCAATTGGTTAAAAG-
142 3') highly conserved direct repeats (DRs). The 136 integrases identified in this study, however,
143 only shared 36.1% amino acid identity.

144

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

146 The total number of genes among the 215 *P. aeruginosa* complete genomes (pangenome) is
147 33570. The core genome (genes present in 99% or more of the genomes) contains 2674 genes
148 (8.0% of all genes, **Figure 3** and **Figures S1**). We also identified 11241 unique genes, which are
149 found in only one strain [30].

150

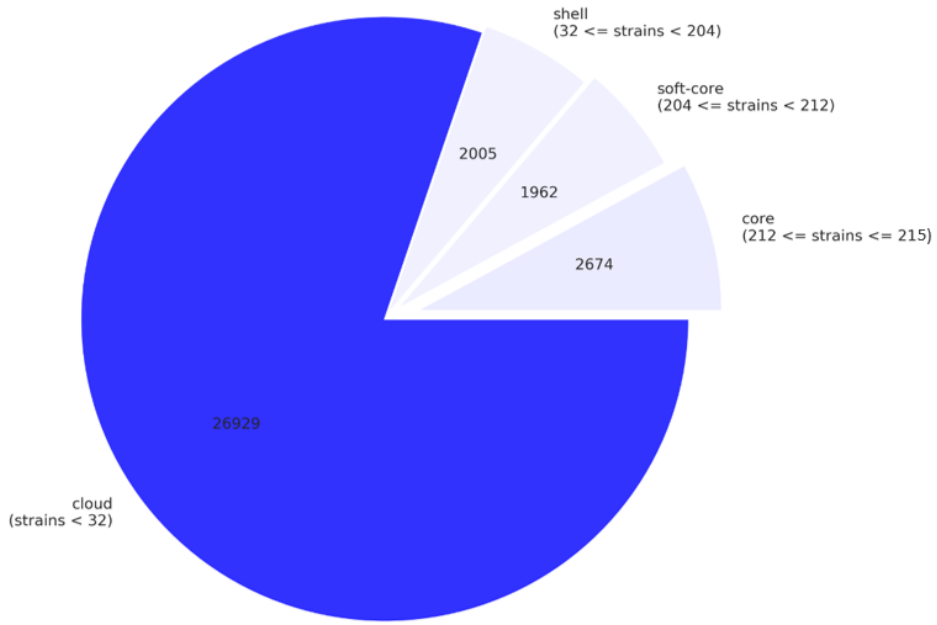


Figure 3 – Pie chart of the breakdown of genes and the number of *P. aeruginosa* genomes they are present in. This figure was created using the contributed Python script roary_plots.py in https://github.com/sanger-pathogens/Roary/blob/master/contrib/roary_plots/roary_plots.py.

The total number of genes in the 136 *crpP*-harboring ICEs (ICEome) is 1193. The core ICEome contains 26 genes (2.2% of all genes). The soft core content (genes present in more or less than 95% and less than 99% of ICEs) includes 11 genes, 129 shell genes (between 15 and 95%) and 1027 cloud genes (between zero and 15%). We identified 451 unique genes among the ICEome.

The *crpP*-carrying ICEs encode other beneficial genes

The most frequently encoded traits on *crpP*-harboring ICEome include replication, transcription, intracellular trafficking, and cell motility (**Figure 4**). However, nearly one-third of the proteins for which a COG category was attributed (30.7%, n=211/687) encode for an unknown function (**Table S4**).

The *crpP* gene encoded for proteins ranging from 39 to 68 amino acid and is present in a single copy, with no flanking mobile units as integrons, insertion sequences and/or transposons or found on the close vicinity of the gene. We also observed a lack of co-resident antibiotic resistance genes (ARGs) within the *crpP*-harboring ICEs. Seven ICEs carry a type I-C CRISPR-Cas system and ten harbor pyocin S5-encoding genes (**Table S3**). The bacteriocins here identified are

171 highly identical, sharing 95% amino acid identity. Besides bacteriocins, no secondary metabolites
172 were identified within the extracted ICEs.

173

174

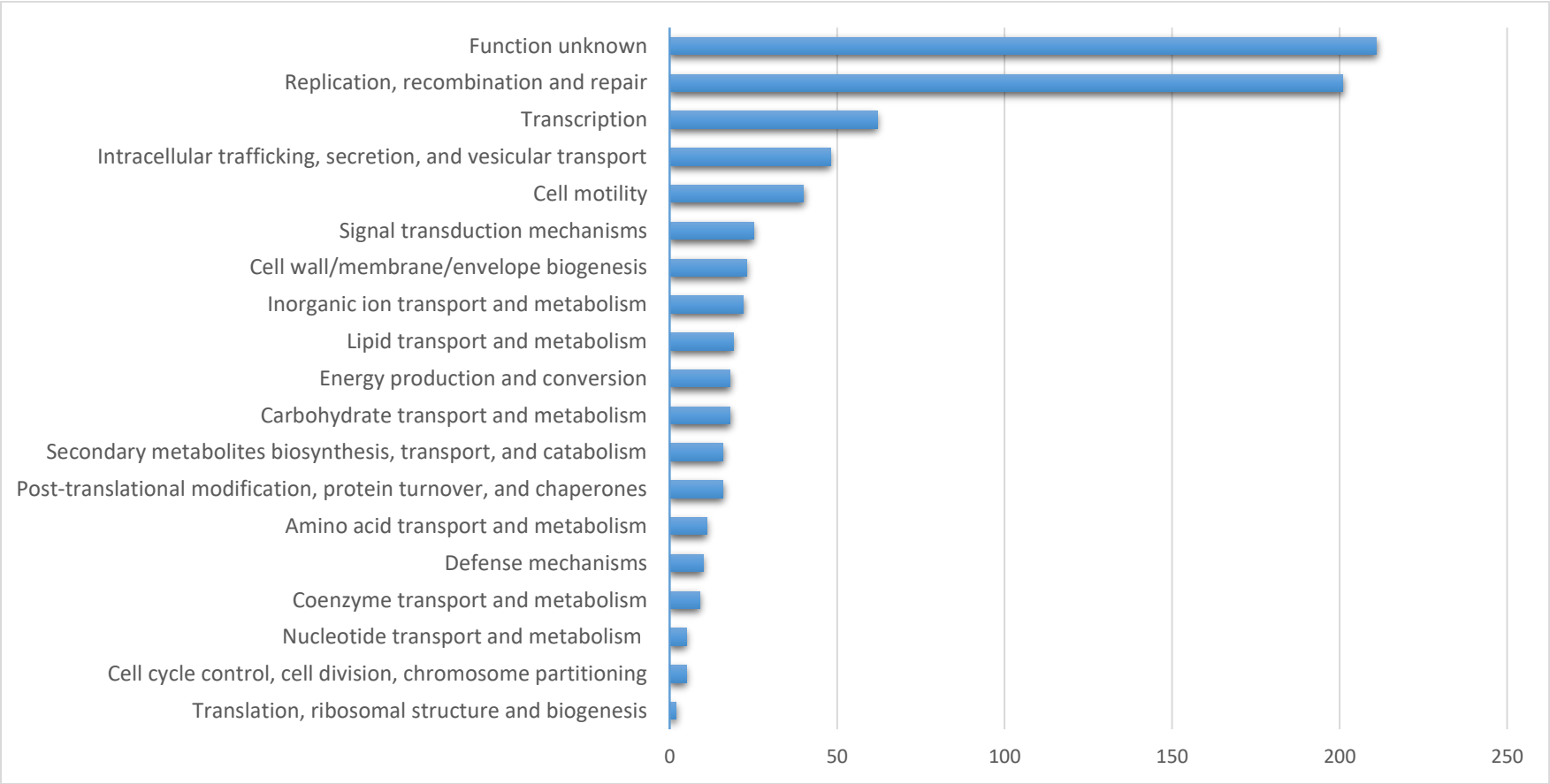


Figure 4. Functional annotation of proteins encoded by the *crpP*-harboring ICEome.

177

178 **Discussion**

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

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

188 As expected for foreign DNA, the GC content of the ICEs is lower than that from the
189 chromosomes. Still, given the high GC content of these mobile elements and the biological
190 barrier for gene acquisition between donor-recipient pairs having >5% difference in GC values
191 [33], we suspect that the ancestor of the *crpP* gene derives from GC-rich taxa.

192 We identified highly conserved DRs that can be used to accurately delimit *crpP*-carrying ICEs in
193 *Pseudomonas*. Interestingly, the exact 45-bp sequence was found on the 2 ICEs from *P.*
194 *fluorescens* and the *Pseudomonas* sp. AK6U strain. Also, even though the *crpP* gene was first
195 reported in a plasmid in *P. aeruginosa* [10], we found a perfect match for the 45-bp DR next to
196 an integrase-encoding gene. The MPF_G class signatures we identified within the boundaries of
197 this plasmid probably means that the plasmid evolved from an ICE, or that the ICE integrated
198 within the plasmid. We only found one match for the DR in this plasmid, meaning that the ICE is
199 degenerated and most likely depends on the plasmid to be mobilized. The same was observed
200 for the *P. aeruginosa crpP*-carrying plasmid detected in this study (accession number
201 NZ_CP030914.1).

202 The *crpP*-carrying ICEs were traced in several clones, taking advantage of the highly conserved
203 attachment site in the $tRNA^{Lys}$ gene. Interestingly, we found in a previous study that MPF_G class
204 ICEs carrying specific ARGs were integrated at the end of $tRNA^{Gly}$ genes [14]. In fact, this class of
205 ICEs is frequently integrated at the end of a tRNA gene [34]. This behavior suggests selection for
206 the maintenance of these non-coding integration sites, and seamless site-specific integration of
207 ICEs in the chromosome will incur in a lower burden to the recipient cell and therefore increase
208 its fitness [2,35]. As so, conferring decreased susceptibility or low levels of ciprofloxacin

209 resistance without incurring in fitness cost can help to explain the high frequency of *crpP*-
210 carrying ICEs observed in this study. Future studies should explore the possible synergistic
211 effects between commonly reported mutations leading to ciprofloxacin resistance and the
212 presence of *crpP*-harboring ICEs.

213 One of the caveats of our study is that we restricted the analyses to complete genomes. Given
214 that the majority of the assemblies present in public databases as NCBI correspond to draft
215 genomes, we probably missed the identification of several *crpP*-carrying ICEs among *P.*
216 *aeruginosa*. However, mobile elements as ICEs tend to be fragmented in genomes sequenced
217 with short-reads technologies, due to the presence of repetitive regions. Also, the identification
218 of ICE fragments within the draft genome would require similar ICE sequences to use as a
219 reference, which would then bias the study. Sequencing bacteria with long-read approaches is
220 increasing, which will improve the accurate delimitation of an ever-growing number of mobile
221 elements.

222

223 **Conclusions**

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

231

232 **Conflict of interests**

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

234

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238

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