

1

2 **Mutation-driven evolution of *Pseudomonas aeruginosa* in the presence of either**
 3 **ceftazidime or ceftazidime/avibactam**

4 Fernando Sanz-García, Sara Hernando-Amado, José Luis Martínez*

5 Centro Nacional de Biotecnología, CSIC, 28049 Madrid, Spain.

6 *Corresponding author. jlmtnz@cnb.csic.es

7 Phone 34 5854542

8 Fax: 3458584506

9

10 ***Running title:*** Ceftazidime/avibactam *P. aeruginosa* resistant mutants

11

12 ABSTRACT

13 Ceftazidime/avibactam is a combination of beta-lactam/beta-lactamases inhibitor, which
 14 use is restricted to some clinical cases including cystic fibrosis patients infected with
 15 multidrug resistant *Pseudomonas aeruginosa*, in which mutation is the main driver of
 16 resistance. This study aims to predict the mechanisms of mutation-driven resistance that
 17 are selected for when *P. aeruginosa* is challenged with either ceftazidime or
 18 ceftazidime/avibactam. For this purpose, *P. aeruginosa* PA14 was submitted to
 19 experimental evolution in the absence of antibiotics and in the presence of increasing
 20 concentrations of ceftazidime or ceftazidime/avibactam for 30 consecutive days. Final
 21 populations were analysed by whole-genome sequencing. All evolved populations
 22 reached similar levels of ceftazidime resistance. Besides, all of them were more
 23 susceptible to amikacin and produced pyomelanin. A first event in the evolution was the
 24 selection of large chromosomal deletions containing *hmgA* (involved in pyomelanin
 25 production), *galU* (involved in β -lactams resistance) and *mexXY-oprM* (involved in
 26 aminoglycoside resistance). Besides mutations in *mpl* and *dacB* that regulate β -
 27 lactamase expression, mutations related to MexAB-OprM overexpression were
 28 prevalent. Ceftazidime/avibactam challenge selected mutants in the putative efflux
 29 pump *PA14_45890-45910* and in a two-component system (*PA14_45870-45880*), likely
 30 regulating its expression. All populations produce pyomelanin and were more
 31 susceptible to aminoglycosides likely due to the selection of large chromosomal
 32 deletions. Since pyomelanin-producing mutants, presenting similar deletions are
 33 regularly isolated from infections, the potential aminoglycosides hyper-susceptibility and
 34 reduced β -lactams susceptibility of pyomelanin-producing *P. aeruginosa* should be
 35 taken into consideration for treating infections by these isolates.

36 INTRODUCTION

37 *Pseudomonas aeruginosa* is an opportunistic pathogen widely distributed in nature (1),
 38 which is a major cause of lung and airway infections in hospitalized patients, as well as
 39 chronic infections in patients with cystic fibrosis (CF) and chronic obstructive
 40 pulmonary disease (2, 3). This bacterial species presents a characteristic low
 41 susceptibility to antibiotics, including β -lactams, which is mainly the consequence of its
 42 low permeability and the presence in its genome of different intrinsic resistance genes,
 43 including those encoding multidrug (MDR) efflux pumps (4) and beta-lactamases. In
 44 addition, an increasing number of *P. aeruginosa* isolates has acquired several resistance
 45 genes through horizontal gene transfer (HGT), including different classes of
 46 carbapenemases. Finally, *P. aeruginosa* is able to develop resistance through mutation,
 47 particularly when causing chronic infections, to nearly any available antibiotic. Under
 48 this situation, the emergence and spread of MDR resistant global clones is of special
 49 concern (5).

50 The use of β -lactam/ β -lactamase inhibitor combinations, such as amoxicillin/clavulanic
 51 acid or ceftolozane/tazobactam, has proven to be effective against class A β -lactamases
 52 (which include narrow and extended-spectrum β -lactamases and some
 53 carbapenemases); whereas effective combinations against class B, C (extended
 54 spectrum cephalosporinases) and D β -lactamases (6-8) have not been available until
 55 recently. One of them is the ceftazidime/avibactam combination, whose use was
 56 approved in 2015 by the FDA (9).

57 Avibactam, formerly known as NXL104, belongs to a new class of β -lactamase
 58 inhibitors, the diazabicyclooctanes (10). This inhibitor has a potent activity against most
 59 Class A, Class C and some Class D β -lactamases (11). Avibactam has been mainly used

60 for restoring the activity of the third generation cephalosporin ceftazidime (12). Thus
61 far, it has been used for the treatment of patients with complicated urinary tract
62 infections, including pyelonephritis; and community-acquired intra-abdominal
63 infections, usually in combination with metronidazole (13). Besides, future studies are
64 likely to expand the use of ceftazidime/avibactam to include other cases, such as cystic
65 fibrosis patients with MDR resistant *P. aeruginosa* infections (13).

66 Given the fact that this treatment is currently reserved for patients who have no
67 alternative therapeutic options, a judicious use of antibiotic stewardship should be
68 applied in order to prevent the incidence of drug resistance. Nevertheless, and although
69 there are numerous studies on the activity of ceftazidime/avibactam against pathogens
70 resistant to other antibiotics (14-17), analysis for predicting potential mechanisms of
71 resistance to this antimicrobial combination are still scarce.

72 In the present study, experimental evolution followed by whole-genome sequencing
73 (WGS) was used to examine the evolutionary trajectories taken by *P. aeruginosa*
74 towards resistance against the combination ceftazidime/avibactam compared to the ones
75 followed in absence of avibactam. This may throw light upon the different mechanisms
76 of resistance that are selected for in *P. aeruginosa* when its β -lactamase activity is
77 inhibited by the presence of this novel inhibitor. In addition, the present work may
78 allow us to elucidate whether the presence of avibactam modifies the resistance level
79 acquired by the bacterial populations in comparison to the one developed when
80 ceftazidime is used alone. Thus, these results may give rise to strategies for predicting,
81 managing, and eventually reducing resistance to ceftazidime/avibactam. This is widely
82 important, as this treatment is strictly restricted to few clinical cases in which resistant
83 strains would be of major concern.

84 MATERIALS AND METHODS

85 Growth conditions and antibiotic susceptibility assays

86 Unless otherwise stated, bacteria were grown in Luria Bertani (LB) Broth at 37°C with
 87 shaking at 250 rpm. The susceptibility to tigecycline, tetracycline, aztreonam,
 88 ceftazidime, imipenem, meropenem, ciprofloxacin, levofloxacin, norfloxacin,
 89 tobramycin, streptomycin, amikacin, gentamycin, colistin, polymyxin B,
 90 chloramphenicol, fosfomycin and erythromycin was determined by disk diffusion in
 91 Mueller Hinton Agar (MHA) (Sigma) at 37°C. For a set of antibiotics, MICs were
 92 determined using E-test strips (MIC Test Strip, Liofilchem®). MICs of ceftazidime and
 93 ceftazidime/avibactam were determined in LB by double dilution in microtiter plates.

94 Experimental evolution procedure

95 Twelve bacterial populations from a stock *P. aeruginosa* PA14 culture (four controls
 96 without antibiotic, four populations challenged with ceftazidime, and four populations
 97 challenged with ceftazidime/avibactam) were grown in parallel in LB for 30
 98 consecutive days. Each day, the cultures were diluted (1/250) in fresh LB. The
 99 concentrations of ceftazidime used for selection were increased over the evolution
 100 experiment from the concentration that hinders the growth of *P. aeruginosa* PA14 under
 101 these culture conditions (4 µg/ml) up to 128 µg/ml, doubling them every 5 days. The
 102 avibactam concentration was maintained constant, as used in clinical tests (18), at 4
 103 µg/ml. In some occasions, the cultures did not grow when antibiotic concentration
 104 increased, in which case, the selection was kept at the concentration that allows growth.
 105 Every five days, samples from each culture were preserved at -80 °C for further
 106 research.

107 **Whole-genome sequencing (WGS)**

108 Gnome® DNA kit (MP Biomedicals) was used to extract genomic DNA. WGS was
109 performed by Sistemas Genómicos S.L. The quality of the extracted material was
110 analysed via a 4200 Tape Station, High Sensitivity assay and the DNA concentration
111 ascertained by real-time PCR using a LightCycler 480 device (Roche). Libraries were
112 obtained without amplification following Illumina protocols and were pair-end
113 sequenced (100 x 2) in an Illumina HiSeq 2500 sequencer. The average number of reads
114 per sample was 7,178,870, which represents a 200x coverage, on average.

115 **Bioinformatics analysis of WGS and confirmation of genetic changes**

116 Mutations in the evolved populations were identified using CLC Genomics Workbench
117 9.0 (QIAGEN). *P. aeruginosa* UCBPP-PA14 reference chromosome (NC_008463.1)
118 was used to align the reads obtained from WGS data (previously trimmed). Sanger
119 sequencing was used to verify and to settle the order of appearance of the putative
120 mutations found via WGS (Table S1). Thirty-two pairs of primers, which amplified
121 200-400 base pair regions containing each genetic modification, were designed (Table
122 S2). After PCR amplification, the corresponding amplicons were purified using the
123 QIAquick PCR Purification Kit (QIAGEN) and sequenced at GATC Biotech.

124 **RESULTS**

125 **Stepwise evolution of *P. aeruginosa* towards ceftazidime and** 126 **ceftazidime/avibactam resistance**

127 To determine the potential evolutionary trajectories that can lead to either ceftazidime or
128 ceftazidime/avibactam resistance, four biological replicates were allowed to evolve in
129 parallel in each of the following conditions (Figure 1): under selective pressure with

130 ceftazidime (populations 1-4), ceftazidime/avibactam (populations 5-8), and in the
 131 absence of any selective pressure (populations 9-12). The susceptibility of each
 132 population to the selecting antibiotic was determined every 5 days by E-test. However,
 133 after 20 days of evolution, MICs reached the highest limits of the E-test strips and MICs
 134 were again determined, for each evolutionary step, by double dilution (Table S3).
 135 Stepwise evolutionary trajectories were observed for both treatments, in which the
 136 selected populations reached quite similar levels of resistance (Figure 2). These results
 137 suggest that avibactam inhibition may not be a guarantee of impeding *P. aeruginosa* to
 138 acquire high-level ceftazidime resistance. An increase in the MIC of an antibiotic after
 139 experimental evolution does not necessarily imply that antibiotic-resistant mutants have
 140 been selected for: resistance may have arisen due to a phenotypic (inducible) adaptation
 141 to the presence of ceftazidime rather than to mutations (19-21). To address this
 142 possibility, the evolved populations were cultured in the absence of selection pressure
 143 (three sequential passages on LB) and the MICs again determined. These were found
 144 not to vary, indicating that the observed modifications were due to the selection of
 145 stable mutants.

146 **Cross-resistance and collateral sensitivity of the evolved populations**

147 Taking into consideration the few therapeutic options for patients submitted to
 148 ceftazidime/avibactam therapy, knowing whether or not acquisition of resistance to this
 149 combination might alter the susceptibility to other antibiotics is of crucial importance.
 150 To that end, the susceptibility to a range of representative antibiotics was tested by disk
 151 diffusion assay (Table S4). From these results, a set of antibiotics was chosen for
 152 determining their MICs against the different evolved populations. Every evolved
 153 replicate showed altered susceptibility to antimicrobials belonging to different structural

154 families (Table 1), implying that at least some resistance mutations were not
 155 ceftazidime- or ceftazidime/avibactam-specific. All populations evolved in the presence
 156 of either ceftazidime or ceftazidime/avibactam presented decreased susceptibility to
 157 other β -lactams, to chloramphenicol and to erythromycin and they were more
 158 susceptible to fosfomycin and to amikacin. Notably, while populations evolving in the
 159 presence of ceftazidime were less susceptible to tetracycline and did not present changes
 160 in the susceptibility to tigecycline, populations evolved in the presence of
 161 ceftazidime/avibactam were hyper-susceptible to both antibiotics.

162 **Analysis of mutations associated with the acquisition of resistance**

163 To know the genetic events associated with the acquisition of resistance in the evolved
 164 populations, the genomes of each, as well as that of the original PA14 strain, were
 165 sequenced on the last day of the experiment. Table 2 encompasses the resulting mutated
 166 genes and their functional significance, whereas Table S1 shows the locations of all 40
 167 genetic changes that were unveiled and were not present in control populations evolving
 168 in the absence of antibiotics. A total of 37 single nucleotide variants (SNVs) and 3
 169 multi-nucleotide variants (MNVs; deletions and substitutions of various nucleotides)
 170 were found, 36 located in genes and 4 in intergenic regions. Most mutations located in
 171 genes resulted in amino acid alterations, stop codons or frameshifts. In addition, all the
 172 populations evolved in the presence of antibiotics contained large chromosomal
 173 deletions (55 to 443 kbp) representing from 0,88% to 7,09% of the *P. aeruginosa* PA14
 174 genome. Five different deletions were selected, they all presenting a 55 kbp common
 175 region (Figure 3).

176 To verify the presence and the order of appearance of the genetic changes identified by
177 WGS, the regions holding these mutations were amplified using specific
178 oligonucleotides (Table S2) and the amplicons Sanger-sequenced in each evolutionary
179 step (Figure 4). Regarding the large chromosomal deletions, primers located at the
180 flanking sequences were used to verify their presence. In all cases, these analyses
181 confirmed the information obtained from WGS.

182 **Common mutations selected upon either ceftazidime or ceftazidime/avibactam** 183 **selection pressure**

184 Upon one day of experimental evolution, all *P. aeruginosa* PA14 cultures challenged
185 with antibiotic produced a brown pigment, which appeared to be pyomelanin, whose
186 accumulation is normally due to the lack of homogentisate 1,2-dioxygenase activity
187 provided by the enzyme HmgA (22). All the chromosomal large deletions selected
188 during evolution presented *hmgA* (Figure 3). In addition, the deletions included as well
189 *galU* (involved in LPS biosynthesis), whose inactivation reduces ceftazidime
190 susceptibility (23); and the MDR efflux pump *mexXY-oprM*, which contributes to
191 aminoglycoside resistance in *P. aeruginosa* (24). Deletion of the latter is likely the
192 cause of the observed amikacin hyper-susceptibility of all evolved populations (Table
193 1).

194 Another common element in both evolutions is *nalD*, which encodes a secondary
195 repressor of MexAB-OprM (25, 26). Three out of eight replicates showed the same
196 T11N change, which has been previously found in XDR *P. aeruginosa* high-risk clones
197 (27) that overexpress MexAB-OprM. Notably, four replicates (including the three
198 presenting mutations in *nalD*) also presented mutations in *mexB*, indicating this efflux
199 system to be a relevant element in the acquisition of resistance. Two other elements that

are selected for in both treatments are *ftsI* and *clpA*. The first encodes PBP3, the target of different β -lactams (28, 29), which has been already found to be mutated in numerous resistant *P. aeruginosa* isolates. Indeed, the mutations R504C/H found in populations 1 and 5 are also present among isolates from widespread nosocomial *P. aeruginosa* clones (29-31). *clpA* encodes for an intracellular protease involved in different aspects of *P. aeruginosa* physiology, in addition to aztreonam resistance (28, 32).

Mutations selected by ceftazidime

In addition to the observed mutations in *nalD*, which would allow *mexAB-oprM* overexpression, we found as well mutations that should lead to the overexpression of this system in the populations evolving under ceftazidime challenge. Two populations carried mutants in *mexR*, which encodes a local repressor of *mexAB-oprM* expression. Another population presented a mutation upstream *mexA* that might prevent the interaction of NalD with its operator (25), thus allowing *mexAB-oprM* overexpression.

Other mutations specifically selected for by ceftazidime were found in *mpl* and *dacB*. The proteins encoded by these genes are involved in the recycling of peptidoglycan muropeptides. Besides, they control the activity of AmpR and consequently the level of AmpC expression (23, 33), which is known to be a main element in *P. aeruginosa* resistance to β -lactams. Interestingly, *mpl* V124G (replicate 2, Table S1) has been found before in a clinical isolate (*P. aeruginosa* NCGM1984). These finding, along with the *ftsI* and *nalD* mutations aforementioned, validate our experimental evolution approach as a valuable predictive model for the *in vivo* selection of antibiotic resistance.

222 Finally, mutations at *orfN*, *pitA*, *infB*, *grpE*, *clpP* and *dnaK* were selected for in
 223 populations challenged with ceftazidime. *orfN* codes for a putative glycosyl transferase
 224 of type A flagellins (34). Mutations on this gene have been found in ciprofloxacin *P.*
 225 *aeruginosa* resistant strains (35), and also in *P. aeruginosa* populations submitted to
 226 tigecycline and tobramycin experimental evolutions (Sanz-García *et al.* submitted).
 227 *pitA* encodes a phosphate transporter, *infB* the translation initiation factor IF-2 and
 228 *dnaK*, *grpE* and *clpP* encode proteins involved in regulatory gene networks involved in
 229 response to stress. None of them has been previously related to ceftazidime resistance,
 230 excepting *dnaK*, whose inactivation leads to stronger susceptibility to various
 231 antimicrobials in *Escherichia coli* (36).

232 **Mutations selected by ceftazidime/avibactam**

233 The challenge with ceftazidime/avibactam selected mutants in a predicted efflux pump
 234 (*PA14_45890-45910*), as well as in the two-components system (TCS) encoded by the
 235 operon *PA14_45870-45880*, likely regulating its expression. Previous studies have
 236 shown this efflux pump to be involved in *P. aeruginosa* intrinsic resistance (37) and
 237 susceptibility to carbapenems (38). Regarding the substrate recognition profile this
 238 pump might display, it is remarkable that populations 5 and 7, which present the
 239 aforementioned mutations, show a much lower susceptibility to imipenem than any
 240 other replicate (Table 1), suggesting this pump to have certain specificity to
 241 carbapenems.

242 Other mutations that were selected upon ceftazidime/avibactam treatment were found in
 243 *pepA*, *spoT*, *dnaJ* and *flgF*. *pepA* encodes for a protease necessary for *P. aeruginosa*
 244 cytotoxicity, virulence and, consequently, lung infection (39, 40). Although its
 245 implication in antibiotic resistance has not been studied in detail, it has been reported

246 that its inactivation confers meropenem resistance in *P. aeruginosa* (41). Moreover,
247 *pepA* mutants are selected in the presence of aztreonam (28). SpoT has been related to
248 piperacillin resistance (42); while DnaJ, a chaperone protein and FlgF, a flagellar basal
249 body rod protein (43), have been reported to modify the susceptibility of *E. coli* to a
250 range of antibiotics when they are inactivated (36).

251 The other mutations that were selected for in populations under ceftazidime/avibactam
252 challenge; namely those occurring in *ctpA*, an essential gene for the transition between
253 acute and chronic *P. aeruginosa* infection (44), *pcm*, that encodes for a L-isoaspartate
254 carboxylmethyltransferase type II that participates in protein repair and degradation and
255 *glnD*, which is implicated in N₂ metabolism, (45) have not been reported to be involved
256 in antibiotic resistance.

257 Discussion

258 The use of β -lactamase inhibitors has re-emerged as a fruitful strategy for fighting
259 infections by MDR bacteria. Among them, ceftazidime/avibactam can be a useful
260 combination for treating infections by different organisms, including *P. aeruginosa*. The
261 analysis of the mechanisms of resistance to previous β -lactam/ β -lactamase inhibitor
262 combinations as amoxicillin/clavulanate have shown that the main mechanisms selected
263 along their use have been increased expression or mutation of pre-existing β -lactamases
264 and acquisition of new ones by HGT (46-50). *P. aeruginosa* has already acquired
265 different carbapenemases that might be important elements in ceftazidime/avibactam
266 resistance. In addition, resistance can be achieved through mutations, particularly in the
267 case of *P. aeruginosa* causing chronic infections. To identify potential mutations
268 involved in the acquisition of either ceftazidime or ceftazidime/avibactam resistance,
269 bacterial populations were submitted to increasing selective concentrations of these

antimicrobials. In both cases, the first event in the evolution seems to be the deletion of large regions of *P. aeruginosa* chromosome that comprise, among several other genes, *hmgA*, *galU* and *mexXY*. A similar situation has been previously reported in other *P. aeruginosa* experimental evolution assays in the presence of β -lactams, such as piperacillin (42) and meropenem (51). Additionally, pyomelanin-producing mutants are regularly isolated from infections; up to 13% of CF patients harbour pyomelanin-producing mutants (52), likely because the production of pyomelanin increases resistance to oxidative stress and persistence in chronic lung infections (22). Recent work has also shown that these mutations can be selected to prevent bacteriophage predation (53). Notably, melanogenic clinical isolates of *P. aeruginosa* present large chromosomal deletions, similar to those reported in the present work (54). Our results then support that ceftazidime selects for these genome deletions, and the presence of avibactam cannot prevent them from happening. It might be possible that deletions are the consequence of increased recombination triggered by the presence of the antibiotic. However, the fact that *P. aeruginosa* evolving in the presence of ciprofloxacin do not produce pyomelanin (a marker of these deletions) and that pyomelanin-producing mutants are selected when a *recA* *P. aeruginosa* defective strain is challenged with either ceftazidime or ceftazidime/avibactam (data not shown), goes against this possibility. Besides the already known effect of the lack of *galU* on the susceptibility to β -lactams, the absence of other genes located in the deletion, such as *mexXY*, may affect *P. aeruginosa* susceptibility to antibiotics. Deletion of this pump is likely the cause of the observed hyper-susceptibility to amikacin of the evolved populations. In addition, it might have an indirect effect on the decreased susceptibility to beta-lactams, particularly in the case of those strains carrying mutations in the repressors of *mexAB-oprM*.

294 MexAB-OprM is an important determinant of intrinsic *P. aeruginosa* resistance to
 295 different antibiotics, including β -lactams (24). Further, mutants overexpressing this
 296 efflux pump are regularly isolated from infections and it has been shown its expression
 297 to be prevalent among resistant *P. aeruginosa* clinical isolates (55-58). MexAB and
 298 MexXY share the outer membrane protein OprM, which produces antagonistic
 299 interactions when both systems are expressed (51, 59). Hence, MexXY-OprM
 300 elimination might favour the efficiency of β -lactams efflux, reducing the competition of
 301 both efflux pumps for OprM.

302 Important elements in the acquisition of ceftazidime resistance are efflux pumps,
 303 particularly MexAB-OprM, since mutations either in the elements regulating its
 304 expression or in the efflux pump itself were found in six out of eight evolved
 305 populations, whereas the two remaining populations harboured mutants in the putative
 306 *PA14_45890-45910* and in its potential TCS regulator. While the substrates of MexAB-
 307 OprM are known and include β -lactams, the substrates of *PA14_4590-45910* are
 308 unknown. Nevertheless, it is remarkable that populations presenting mutations on this
 309 determinant display a much lower susceptibility to imipenem than any other replicate,
 310 suggesting this pump to have certain specificity to β -lactams.

311 Mutations in elements involved in the regulation of AmpC expression were selected
 312 when just ceftazidime was used for selection and not in the presence of
 313 ceftazidime/avibactam. This suggests that, at least in the *P. aeruginosa* PA14
 314 background, the efficient inhibition by avibactam of intrinsic β -lactamases, preclude the
 315 emergence of mechanisms based on their overexpression and other mechanisms,
 316 including the above mentioned large deletions and modifications in the activity of efflux
 317 pumps are preferentially selected. This does not necessarily mean that resistance to

318 ceftazidime/avibactam cannot be associated to changes in the activity of AmpC,
319 particularly if the challenged isolate is already resistant to ceftazidime. Indeed,
320 avibactam resistant mutants presenting changes in the avibactam binding pocket of
321 AmpC are selected *in vitro* at low frequency from Amp C-overexpressing ceftazidime
322 resistant *P. aeruginosa* isolates (60).

323 Although most of the mutants here reported have been previously associated to be
324 involved in antibiotic resistance, it is still possible that some of the mutations might be
325 selected for compensating the fitness costs associated with the acquisition of resistance.
326 This might be the case of *ctpA*, *pcm* or the mutations at structural elements of efflux
327 pumps that were selected after mutations in the regulators of their expression. For the
328 latter, it might also be possible that these mutations increase the capability of extruding
329 the antibiotic substrates, as described for AcrB (61). The fact that in all evolved
330 populations mutants in efflux pumps are selected, provides an explanation of the cross-
331 resistance phenotype observed in all resistant strains. This situation might be of
332 concern, since both ceftazidime and ceftazidime/avibactam might select for resistance to
333 other antibiotics, at least along chronic infections in which mutation is the main cause of
334 acquisition of resistance.

335 *P. aeruginosa* evolution in chronic infections frequently involves large genome
336 deletions (62), usually linked to the production of pyomelanin (54). Whether these
337 deletions are selected by antibiotic treatment or are just the consequence of the
338 adaptation to the environment of the lungs of the CF patient remains to be established.
339 However, this evolution provides a link between antibiotic resistance and virulence for
340 this relevant pathogen. In any case, and given that deletions containing *galU* and *hmgA*
341 appear to be a first step on the evolution towards ceftazidime/avibactam resistance,

pyomelanin production could be considered as a marker in the selection of the antibiotic of choice for treating *P. aeruginosa* infections. Both *in vitro* work, including the results here shown, and the analysis of clinical pyomelanin-producers, have shown that these isolates are hyper-susceptible to aminoglycosides probably because the deletions they present include *mexXY*. It would then be judicious using aminoglycosides, and not β -lactams for treating infections by pyomelanin-producing *P. aeruginosa*.

Acknowledgments

Work in our laboratory is supported by grants from the Instituto de Salud Carlos III (Spanish Network for Research on Infectious Diseases [RD16/0016/0011]), from the Spanish Ministry of Economy and Competitiveness (BIO2017-83128-R) and from the Autonomous Community of Madrid (B2017/BMD-3691). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. FSG is the recipient of a FPU fellowship.

REFERENCES

1. Silby MW, Winstanley C, Godfrey SA, Levy SB, Jackson RW. 2011. *Pseudomonas* genomes: diverse and adaptable. *FEMS Microbiol Rev* 35:652-80.
2. Martinez-Solano L, Macia MD, Fajardo A, Oliver A, Martinez JL. 2008. Chronic *Pseudomonas aeruginosa* infection in chronic obstructive pulmonary disease. *Clinical Infectious Diseases* 47:1526-33.
3. Talwalkar JS, Murray TS. 2016. The Approach to *Pseudomonas aeruginosa* in Cystic Fibrosis. *Clin Chest Med* 37:69-81.
4. Vila J, Martínez JL. 2008. Clinical impact of the over-expression of efflux pump in nonfermentative Gram-negative bacilli, development of efflux pump inhibitors. *Current drug targets* 9:797-807.
5. Oliver A, Mulet X, Lopez-Causape C, Juan C. 2015. The increasing threat of *Pseudomonas aeruginosa* high-risk clones. *Drug Resist Updat* 21-22:41-59.
6. Tzouveleakis LS, Markogiannakis A, Psychogiou M, Tassios PT, Daikos GL. 2012. Carbapenemases in *Klebsiella pneumoniae* and other Enterobacteriaceae: an evolving crisis of global dimensions. *Clin Microbiol Rev* 25:682-707.
7. Woodford N, Turton JF, Livermore DM. 2011. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. *FEMS Microbiol Rev* 35:736-55.
8. Buynak JD. 2006. Understanding the longevity of the beta-lactam antibiotics and of antibiotic/beta-lactamase inhibitor combinations. *Biochem Pharmacol* 71:930-40.

9. Liscio JL, Mahoney MV, Hirsch EB. 2015. Ceftolozane/tazobactam and ceftazidime/avibactam: two novel beta-lactam/beta-lactamase inhibitor combination agents for the treatment of resistant Gram-negative bacterial infections. *Int J Antimicrob Agents* 46:266-71.
10. Coleman K. 2011. Diazabicyclooctanes (DBOs): a potent new class of non-beta-lactam beta-lactamase inhibitors. *Curr Opin Microbiol* 14:550-5.
11. Aktas Z, Kayacan C, Oncul O. 2012. In vitro activity of avibactam (NXL104) in combination with beta-lactams against Gram-negative bacteria, including OXA-48 beta-lactamase-producing *Klebsiella pneumoniae*. *Int J Antimicrob Agents* 39:86-9.
12. Hayes MV, Orr DC. 1983. Mode of action of ceftazidime: affinity for the penicillin-binding proteins of *Escherichia coli* K12, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *J Antimicrob Chemother* 12:119-26.
13. Hidalgo JA, Vinluan CM, Antony N. 2016. Ceftazidime/avibactam: a novel cephalosporin/nonbeta-lactam beta-lactamase inhibitor for the treatment of complicated urinary tract infections and complicated intra-abdominal infections. *Drug Des Devel Ther* 10:2379-86.
14. Pitart C, Marco F, Keating TA, Nichols WW, Vila J. 2015. Activity of ceftazidime-avibactam against fluoroquinolone-resistant Enterobacteriaceae and *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 59:3059-65.
15. Calvopina K, Hinchliffe P, Brem J, Heesom KJ, Johnson S, Cain R, Lohans CT, Fishwick CWG, Schofield CJ, Spencer J, Avison MB. 2017. Structural/mechanistic insights into the efficacy of nonclassical beta-lactamase inhibitors against extensively drug resistant *Stenotrophomonas maltophilia* clinical isolates. *Mol Microbiol* 106:492-504.
16. Lopez-Hernandez I, Alonso N, Fernandez-Martinez M, Zamorano L, Rivera A, Oliver A, Conejo MC, Martinez-Martinez L, Navarro F, Pascual A. 2017. Activity of ceftazidime-avibactam against multidrug-resistance Enterobacteriaceae expressing combined mechanisms of resistance. *Enferm Infecc Microbiol Clin* 35:499-504.
17. Fraile-Ribot PA, Cabot G, Mulet X, Perianez L, Martin-Pena ML, Juan C, Perez JL, Oliver A. 2017. Mechanisms leading to in vivo ceftolozane/tazobactam resistance development during the treatment of infections caused by MDR *Pseudomonas aeruginosa*. *J Antimicrob Chemother* doi:10.1093/jac/dkx424.
18. Zhanel GG, Lawson CD, Adam H, Schweizer F, Zelenitsky S, Lagace-Wiens PR, Denisuk A, Rubinstein E, Gin AS, Hoban DJ, Lynch JP, 3rd, Karlowsky JA. 2013. Ceftazidime-avibactam: a novel cephalosporin/beta-lactamase inhibitor combination. *Drugs* 73:159-77.
19. Levin BR, Rozen DE. 2006. Non-inherited antibiotic resistance. *Nat Rev Microbiol* 4:556-62.
20. Martinez JL, Rojo F. 2011. Metabolic regulation of antibiotic resistance. *FEMS Microbiol Rev* 35:768-89.
21. Martinez JL, Fajardo A, Garmendia L, Hernandez A, Linares JF, Martinez-Solano L, Sanchez MB. 2009. A global view of antibiotic resistance. *FEMS Microbiol Rev* 33:44-65.
22. Rodriguez-Rojas A, Mena A, Martin S, Borrell N, Oliver A, Blazquez J. 2009. Inactivation of the hmgA gene of *Pseudomonas aeruginosa* leads to pyomelanin hyperproduction, stress resistance and increased persistence in chronic lung infection. *Microbiology* 155:1050-7.
23. Alvarez-Ortega C, Wiegand I, Olivares J, Hancock RE, Martinez JL. 2010. Genetic determinants involved in the susceptibility of *Pseudomonas aeruginosa* to beta-lactam antibiotics. *Antimicrob Agents Chemother* 54:4159-67.
24. Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. 2000. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 44:3322-7.

25. Morita Y, Cao L, Gould VC, Avison MB, Poole K. 2006. *nalD* encodes a second repressor of the *mexAB-oprM* multidrug efflux operon of *Pseudomonas aeruginosa*. J Bacteriol 188:8649-54.

26. Sobel ML, Hocquet D, Cao L, Plesiat P, Poole K. 2005. Mutations in PA3574 (*nalD*) lead to increased MexAB-OprM expression and multidrug resistance in laboratory and clinical isolates of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 49:1782-6.

27. Cabot G, Ocampo-Sosa AA, Dominguez MA, Gago JF, Juan C, Tubau F, Rodriguez C, Moya B, Pena C, Martinez-Martinez L, Oliver A, Spanish Network for Research in Infectious D. 2012. Genetic markers of widespread extensively drug-resistant *Pseudomonas aeruginosa* high-risk clones. Antimicrob Agents Chemother 56:6349-57.

28. Jorth P, McLean K, Ratjen A, Secor PR, Bautista GE, Ravishankar S, Rezayat A, Garudathri J, Harrison JJ, Harwood RA, Penewit K, Waalkes A, Singh PK, Salipante SJ. 2017. Evolved Aztreonam Resistance Is Multifactorial and Can Produce Hypervirulence in *Pseudomonas aeruginosa*. MBio 8.

29. Cabot G, Lopez-Causape C, Ocampo-Sosa AA, Sommer LM, Dominguez MA, Zamorano L, Juan C, Tubau F, Rodriguez C, Moya B, Pena C, Martinez-Martinez L, Plesiat P, Oliver A. 2016. Deciphering the Resistome of the Widespread *Pseudomonas aeruginosa* Sequence Type 175 International High-Risk Clone through Whole-Genome Sequencing. Antimicrob Agents Chemother 60:7415-7423.

30. Lopez-Causape C, Sommer LM, Cabot G, Rubio R, Ocampo-Sosa AA, Johansen HK, Figuerola J, Canton R, Kidd TJ, Molin S, Oliver A. 2017. Evolution of the *Pseudomonas aeruginosa* mutational resistome in an international Cystic Fibrosis clone. Sci Rep 7:5555.

31. Kos VN, Deraspe M, McLaughlin RE, Whiteaker JD, Roy PH, Alm RA, Corbeil J, Gardner H. 2015. The resistome of *Pseudomonas aeruginosa* in relationship to phenotypic susceptibility. Antimicrob Agents Chemother 59:427-36.

32. Fernandez L, Breidenstein EB, Song D, Hancock RE. 2012. Role of intracellular proteases in the antibiotic resistance, motility, and biofilm formation of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 56:1128-32.

33. Aguilera Rossi CG, Gomez-Puertas P, Ayala Serrano JA. 2016. In vivo functional and molecular characterization of the Penicillin-Binding Protein 4 (DacB) of *Pseudomonas aeruginosa*. BMC Microbiol 16:234.

34. Schirm M, Arora SK, Verma A, Vinogradov E, Thibault P, Ramphal R, Logan SM. 2004. Structural and genetic characterization of glycosylation of type a flagellin in *Pseudomonas aeruginosa*. J Bacteriol 186:2523-31.

35. Wong A, Rodrigue N, Kassen R. 2012. Genomics of adaptation during experimental evolution of the opportunistic pathogen *Pseudomonas aeruginosa*. PLoS Genet 8:e1002928.

36. Liu A, Tran L, Becket E, Lee K, Chinn L, Park E, Tran K, Miller JH. 2010. Antibiotic sensitivity profiles determined with an *Escherichia coli* gene knockout collection: generating an antibiotic bar code. Antimicrob Agents Chemother 54:1393-403.

37. Fajardo A, Martinez-Martin N, Mercadillo M, Galan JC, Ghysels B, Matthijs S, Cornelis P, Wiehlmann L, Tummler B, Baquero F, Martinez JL. 2008. The neglected intrinsic resistome of bacterial pathogens. PLoS ONE 3:e1619.

38. Kohler T, Michea-Hamzehpour M, Epp SF, Pechere JC. 1999. Carbapenem activities against *Pseudomonas aeruginosa*: respective contributions of OprD and efflux systems. Antimicrob Agents Chemother 43:424-7.

39. Hauser AR, Kang PJ, Engel JN. 1998. *PepA*, a secreted protein of *Pseudomonas aeruginosa*, is necessary for cytotoxicity and virulence. Mol Microbiol 27:807-18.

472 40. Potvin E, Lehoux DE, Kukavica-Ibrulj I, Richard KL, Sanschagrin F, Lau GW, Levesque RC.
473 2003. In vivo functional genomics of *Pseudomonas aeruginosa* for high-throughput screening
474 of new virulence factors and antibacterial targets. *Environ Microbiol* 5:1294-308.

475 41. Isabella VM, Campbell AJ, Manchester J, Sylvester M, Nayar AS, Ferguson KE, Tommasi
476 R, Miller AA. 2015. Toward the rational design of carbapenem uptake in *Pseudomonas*
477 *aeruginosa*. *Chem Biol* 22:535-547.

478 42. Yen P, Papin JA. 2017. History of antibiotic adaptation influences microbial
479 evolutionary dynamics during subsequent treatment. *PLoS Biol* 15:e2001586.

480 43. Homma M, Kutsukake K, Hasebe M, Iino T, Macnab RM. 1990. FlgB, FlgC, FlgF and FlgG.
481 A family of structurally related proteins in the flagellar basal body of *Salmonella typhimurium*.
482 *J Mol Biol* 211:465-77.

483 44. Seo J, Darwin AJ. 2013. The *Pseudomonas aeruginosa* periplasmic protease CtpA can
484 affect systems that impact its ability to mount both acute and chronic infections. *Infect Immun*
485 81:4561-70.

486 45. Contreras A, Drummond M, Bali A, Blanco G, Garcia E, Bush G, Kennedy C, Merrick M.
487 1991. The product of the nitrogen fixation regulatory gene *nfrX* of *Azotobacter vinelandii* is
488 functionally and structurally homologous to the uridylyltransferase encoded by *glnD* in enteric
489 bacteria. *J Bacteriol* 173:7741-9.

490 46. Reading C, Cole M. 1977. Clavulanic acid: a beta-lactamase-inhibiting beta-lactam from
491 *Streptomyces clavuligerus*. *Antimicrob Agents Chemother* 11:852-7.

492 47. Baquero F, Reig M. 1989. Mechanisms of antimicrobial resistance in anaerobic
493 bacteria: the predictive approach. *Scand J Infect Dis Suppl* 62:25-8.

494 48. Blazquez J, Baquero MR, Canton R, Alos I, Baquero F. 1993. Characterization of a new
495 TEM-type beta-lactamase resistant to clavulanate, sulbactam, and tazobactam in a clinical
496 isolate of *Escherichia coli*. *Antimicrob Agents Chemother* 37:2059-63.

497 49. Canton R, Gonzalez-Alba JM, Galan JC. 2012. CTX-M Enzymes: Origin and Diffusion.
498 *Front Microbiol* 3:110.

499 50. Toussaint KA, Gallagher JC. 2015. beta-lactam/beta-lactamase inhibitor combinations:
500 from then to now. *Ann Pharmacother* 49:86-98.

501 51. Cabot G, Zamorano L, Moya B, Juan C, Navas A, Blazquez J, Oliver A. 2016. Evolution of
502 *Pseudomonas aeruginosa* Antimicrobial Resistance and Fitness under Low and High Mutation
503 Rates. *Antimicrob Agents Chemother* 60:1767-78.

504 52. Mayer-Hamblett N, Rosenfeld M, Gibson RL, Ramsey BW, Kulasekara HD, Retsch-
505 Bogart GZ, Morgan W, Wolter DJ, Pope CE, Houston LS, Kulasekara BR, Khan U, Burns JL, Miller
506 SI, Hoffman LR. 2014. *Pseudomonas aeruginosa* in vitro phenotypes distinguish cystic fibrosis
507 infection stages and outcomes. *Am J Respir Crit Care Med* 190:289-97.

508 53. Shen M, Zhang H, Shen W, Zou Z, Lu S, Li G, He X, Agnello M, Shi W, Hu F, Le S. 2018.
509 *Pseudomonas aeruginosa* MutL promotes large chromosomal deletions through non-
510 homologous end joining to prevent bacteriophage predation. *Nucleic Acids Research* 46:4505-
511 4514.

512 54. Hocquet D, Petitjean M, Rohmer L, Valot B, Kulasekara HD, Bedel E, Bertrand X, Plesiat
513 P, Kohler T, Pantel A, Jacobs MA, Hoffman LR, Miller SI. 2016. Pyomelanin-producing
514 *Pseudomonas aeruginosa* selected during chronic infections have a large chromosomal
515 deletion which confers resistance to pyocins. *Environ Microbiol* 18:3482-3493.

516 55. Chalhoub H, Saenz Y, Rodriguez-Villalobos H, Denis O, Kahl BC, Tulkens PM, Van
517 Bambeke F, Pan YP, Xu YH, Wang ZX, Fang YP, Shen JL, Riou M, Avrain L, Carbonnelle S, El
518 Garch F, Pirnay JP, De Vos D, Plesiat P, Tulkens PM, Van Bambeke F, Castanheira M, Mills JC,
519 Farrell DJ, Jones RN. High-level resistance to meropenem in clinical isolates of *Pseudomonas*
520 *aeruginosa* in the absence of carbapenemases: role of active efflux and porin alterations

521 Overexpression of MexAB-OprM efflux pump in carbapenem-resistant *Pseudomonas*
522 *aeruginosa*

523 Increase of efflux-mediated resistance in *Pseudomonas aeruginosa* during antibiotic treatment
524 in patients suffering from nosocomial pneumonia

525 Mutation-driven beta-lactam resistance mechanisms among contemporary ceftazidime-
526 nonsusceptible *Pseudomonas aeruginosa* isolates from U.S. hospitals.

527 56. Pan YP, Xu YH, Wang ZX, Fang YP, Shen JL. Overexpression of MexAB-OprM efflux
528 pump in carbapenem-resistant *Pseudomonas aeruginosa*.

529 57. Riou M, Avrain L, Carboneille S, El Garch F, Pirnay JP, De Vos D, Plesiat P, Tulkens PM,
530 Van Bambeke F. Increase of efflux-mediated resistance in *Pseudomonas aeruginosa* during
531 antibiotic treatment in patients suffering from nosocomial pneumonia.

532 58. Castanheira M, Mills JC, Farrell DJ, Jones RN. Mutation-driven beta-lactam resistance
533 mechanisms among contemporary ceftazidime-nonsusceptible *Pseudomonas aeruginosa*
534 isolates from U.S. hospitals.

535 59. Mulet X, Moya B, Juan C, Macia MD, Perez JL, Blazquez J, Oliver A. 2011. Antagonistic
536 interactions of *Pseudomonas aeruginosa* antibiotic resistance mechanisms in planktonic but
537 not biofilm growth. *Antimicrob Agents Chemother* 55:4560-8.

538 60. Lahiri SD, Walkup GK, Whiteaker JD, Palmer T, McCormack K, Tanudra MA, Nash TJ,
539 Thresher J, Johnstone MR, Hajec L, Livchak S, McLaughlin RE, Alm RA. 2015. Selection and
540 molecular characterization of ceftazidime/avibactam-resistant mutants in *Pseudomonas*
541 *aeruginosa* strains containing derepressed AmpC. *J Antimicrob Chemother* 70:1650-8.

542 61. Blair JM, Bavro VN, Ricci V, Modi N, Cacciottolo P, Kleinekathfer U, Ruggerone P, Vargiu
543 AV, Baylay AJ, Smith HE, Brandon Y, Galloway D, Piddock LJ. 2015. AcrB drug-binding pocket
544 substitution confers clinically relevant resistance and altered substrate specificity. *Proc Natl*
545 *Acad Sci U S A* 112:3511-6.

546 62. Rau MH, Marvig RL, Ehrlich GD, Molin S, Jelsbak L. 2012. Deletion and acquisition of
547 genomic content during early stage adaptation of *Pseudomonas aeruginosa* to a human host
548 environment. *Environ Microbiol* 14:2200-11.

549 63. Alvarez-Ortega C, Wiegand I, Olivares J, Hancock RE, Martinez JL. 2011. The intrinsic
550 resistome of *Pseudomonas aeruginosa* to beta-lactams. *Virulence* 2:144-6.

551

Table 1 | MICs (µg/ml) of antibiotics of different structural families in the populations evolved in the presence of either cefatzidime or ceftazidime/avibactam.

Replicate	Tgc	Tet	Atm	Ipm	Mer	Caz	Cza	Nor	Ak	C	E	F
PA14	2	12	1.5	0.5	0.19	1	1	0.25	1.5	24	32	16
1	2	32	≥256	8	32	≥256	≥256	1.5	1	≥256	≥256	4
2	1.5	24	≥256	4	32	≥256	≥256	1	0.5	≥256	≥256	1.5
3	2	16	≥256	2	32	≥256	≥256	1	0.75	≥256	≥256	4
4	2	16	≥256	3	32	≥256	≥256	1	0.5	≥256	≥256	2
5	0.75	4	≥256	32	32	≥256	≥256	0.25	0.75	≥256	≥256	3
6	0.5	4	≥256	3	16	≥256	≥256	0.25	1	≥256	≥256	4
7	0.38	4	96	32	32	≥256	≥256	0.25	2	≥256	≥256	6
8	0.75	8	≥256	4	32	≥256	≥256	0.75	0.75	≥256	≥256	4
9	3	12	1.5	0.75	0.25	1	1	0.25	1.5	24	32	24
10	3	12	1.5	0.5	0.19	1	1	0.25	1.5	24	32	24
11	3	12	1	0.75	0.25	1	1	0.25	1.5	24	32	16
12	3	16	1	0.75	0.25	1	1	0.38	1.5	24	32	12

556 Populations challenged with ceftazidime: 1-4, ceftazidime/avibactam: 5-8, and controls:
 557 9-12. Tgc: tigecycline, tet: tetracycline, atm: aztreonam, caz: ceftazidime, cza:
 558 ceftazidime+avibactam, ipm: imipenem, mer: meropenem, nor: norfloxacin, ak:
 559 amikacin, c: chloramphenicol, e: erythromycin, f: fosfomycin.

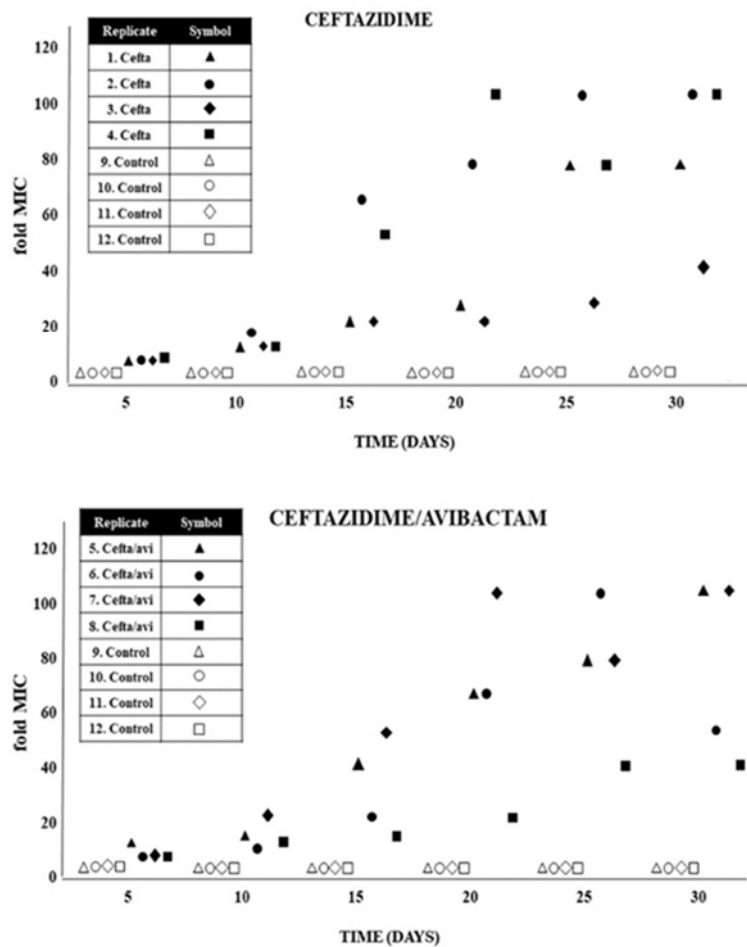
560 **Table 2 | Mutated genes in ceftazidime and ceftazidime/avibactam evolved populations.**

Treatment	Gene name	Functional classification	Replicates	Previous described effect in antibiotics' susceptibility	References
BOTH TREATMENTS	<i>nalD</i>	Efflux regulation	4, 6, 8	Quinolones, macrolides, tetracyclines, chloramphenicol, β -lactams	(25-27)
	<i>mexB</i>	Efflux component	1, 3, 6, 8	Quinolones, macrolides, tetracyclines, chloramphenicol, β -lactams	(24)
	<i>ftsI</i>	Penicillin binding protein	1, 5, 8	β -lactams	(28-31)
	<i>clpA</i>	Protease activity	1, 5	Aztreonam	(28)
	<i>mexR</i>	Efflux regulation	1, 3	Quinolones, macrolides, tetracyclines, chloramphenicol, β -lactams	(24)
	Upstream <i>mexA</i>	Efflux regulation	2	Quinolones, macrolides, tetracyclines, chloramphenicol, β -lactams	(24)
	<i>mpl</i>	Peptidoglycan metabolism (β -lactamase)	2, 4	β -lactams	(23, 63)

CEFTAZIDIME (1-4)	<i>orfN</i>	Flagellin glycolysation	2	Ciprofloxacin, tigecycline, tobramycin	(35) (Sanz et al. submitted)
	<i>infB</i>	Translation factor	2	-	-
	<i>pitA</i>	Phosphate transport	2, 4	-	-
	<i>grpE</i>	Heat shock protein	3	-	-
	<i>clpP</i>	Protease activity	3	-	-
	<i>dnaK</i>	Chaperone	3	Several antibiotics	(36)
	<i>dacB</i>	Penicillin binding protein	4	β-lactams	(33)
	<i>dnaJ</i>	Chaperone	5	Triclosan	(36)
	<i>pepA</i>	Protease activity	6	Meropenem, aztreonam	(28, 41)
	<i>ctpA</i>	Protease activity	6	-	-
CEFTAZIDIME + AVIBACTAM(5-8)	<i>glnD</i>	N ₂ metabolism	6	Aminoglycosides, cephradine	(36)
	<i>flgF</i>	Flagellar component	6	Cephradine, cefoxitin, chloramphenicol	(36)
	<i>pcm</i>	Protein repair/degradation	8	-	-

<i>spoT</i>	Stringent response	8	Piperacillin	(42)
<i>PA14_45870</i>	TCS sensor	5	Carbapenems	(37, 38)
<i>PA14_45880</i>	TCS regulator	7	Carbapenems	(37, 38)
<i>PA14_45890</i>	Efflux component	5, 7	Carbapenems	(37, 38)

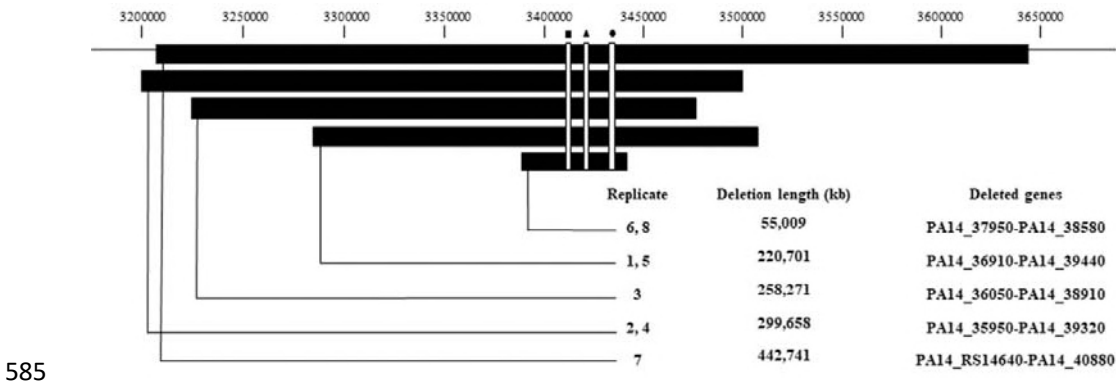
561 The table shows previously described effects in the susceptibility to antibiotics of mutations in the genes that appear to be mutated in the
562 experimental evolution.



575

576 **Figure 2 | Evolution of *P. aeruginosa* PA14 under ceftazidime and**
577 **ceftazidime/avibactam selective pressure.** Graphs show the rise of the MICs over the
578 evolution period ($MIC_{population\ X}/MIC_{PA14}$), being $MIC_{PA14} = 4\ \mu g/ml$ and $X = 1-12$, from
579 the beginning of the experiment to high levels of resistance (doubling the antibiotic
580 concentration every 5 days). The values were obtained by liquid MIC determination in a
581 p96 well plate (Table S3), because the detection limit of the ceftazidime E-test is 256
582 $\mu g/ml$, limiting the assessment of resistance levels from day 20 to the end of the
583 experiment.

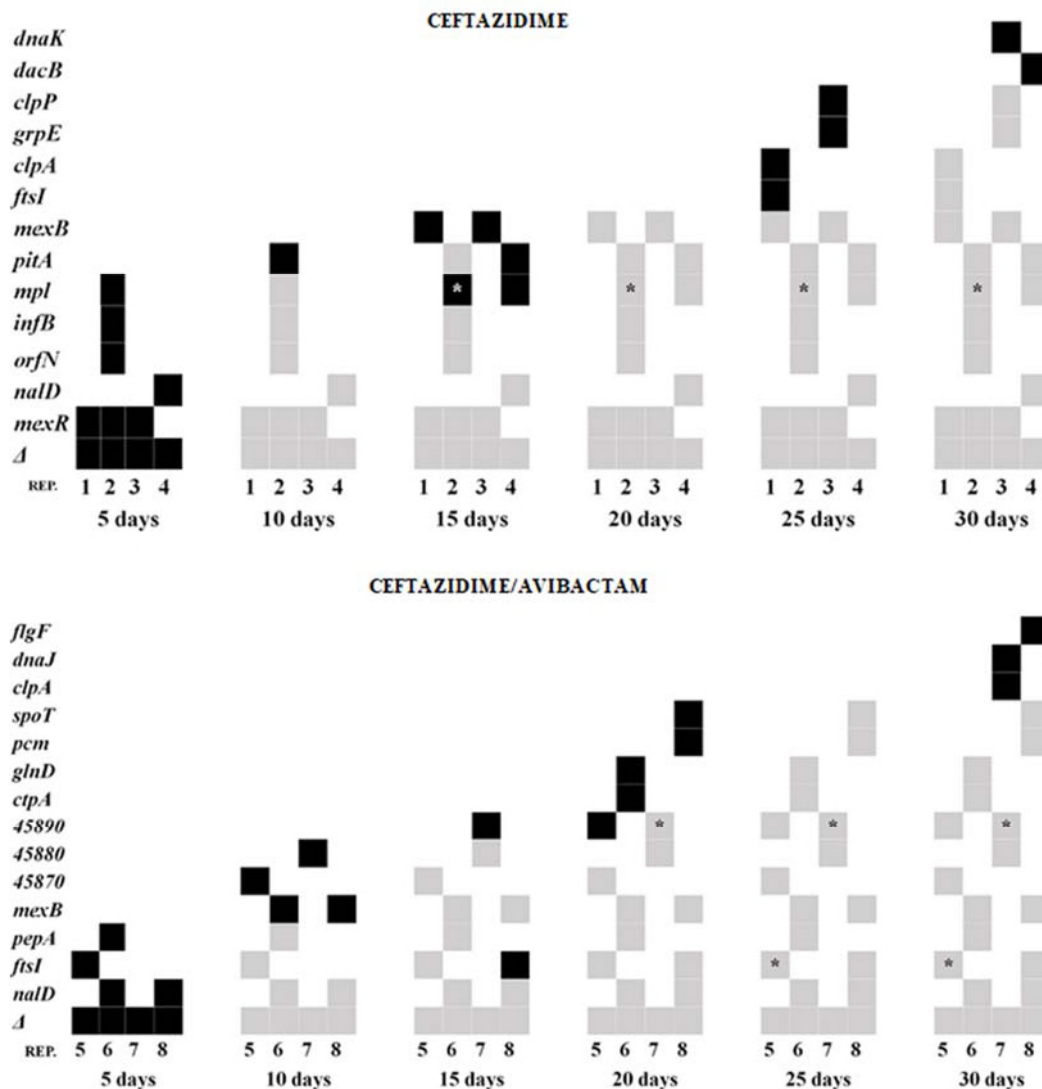
584



585

586 **Figure 3 | Large deletions present in all ceftazidime and ceftazidime/avibactam**
587 **evolved populations since the first day of the experimental evolution.** The figure
588 indicates the length of the deletions and the deleted genes in each replicate, as well as
589 their genome localization, which corresponds with *P. aeruginosa* UCBPP-PA14
590 reference chromosome (NC_008463.1). Black square: *mexXY*, black triangle: *galU*,
591 black circle: *hmgA*.

592



593

594 **Figure 4 | Order of appearance of genetic changes.** Ceftazidime and
595 ceftazidime/avibactam resistance mutations appearance during the evolution process, as
596 determined by PCR amplifications of known SNVs/MNVs in evolved populations. The
597 *mexR* mutation in ceftazidime population 2 actually indicates that this mutation
598 occurred in the intergenic region between *mexR* and *mexA*. The “*” refers to a second
599 mutation in a gene that mutated before. We cannot ditch that other mutations may have
600 appeared over the 30 days evolution process. “Δ”: large deletion. Rep: replicate. Black
601 square: the mutation appeared in this evolutionary step. Grey square: the mutation
602 appeared in a previous evolutionary step.

603 **Supplementary Table S1 | Genetic modifications detected in ceftazidime and**
604 **ceftazidime/avibactam evolved *P. aeruginosa* PA14 populations.**

605

Gene	Replicate	Mutation	Localization	Aa Change
BOTH TREATMENTS				
<i>nalD</i>	4, 6, 8	G-->T	32	Thr11Asn
	1	C-->G	1126	Leu376Val
<i>mexB</i>	3	T-->G	2300	Val767Gly
	6	C-->T	1345	Leu449Phe
	8	C-->T	1693	Pro565Ser
	1	G-->A	1510	Arg504Cys
<i>ftsI</i>		C-->T	1567	Val523Met
	5	C-->T	1511	Arg504His
	8	C-->T	851	Arg284Gln
<i>clpA</i>	1	Del-A	515	His172fs
	5	A-->G	1634	Tyr545Cys
CEFTAZIDIME TREATMENT				
	1	Ins-A	80-81	Glu27fs
<i>mexR</i>	3	Del-AAT	126-128	Leu43del
	2	Del-CGGCGGCTT	1305-1313	Phe438_Gly440 del
<i>mpl</i>		T-->G	371	Val124Gly
	4	T-->G	416	Val139Gly
<i>orfN</i>	2	Ins-G	138-139	Val50fs
<i>infB</i>	2	C-->T	2407	Val803Ile
	2	A-->C	133	Thr45Pro
<i>pitA</i>	4	A-->C	367	Thr123Pro
<i>grpE</i>	3	Del-G	39	Glu14fs
<i>clpP</i>	3	Del-C	365	Gly122fs
<i>dnaK</i>	3	G-->A	1115	Ala372Val
<i>dacB</i>	4	C-->T	343	Gly115Ser
CEFTAZIDIME/AVIBACTAM TREATMENT				
<i>dnaJ</i>	5	G-->A	1081	Pro361Ser

<i>pepA</i>	6	C-->T	1439	Gly480Asp
<i>ctpA</i>	6	G-->A	971	Ser324Asn
<i>glnD</i>	6	Ins-G	2466-2467	Asp823fs
<i>flgF</i>	6	C-->A	676	Glu226*
<i>pcm</i>	8	Ins-G	530-531	Arg180fs
<i>spoT</i>	8	T-->C	931	Phe311Leu
<i>PA14_45870</i>	5	Del-21 pb	712-732	Glu238fs
<i>PA14_45880</i>	7	C-->A	159	Met53Ile
		G-->A	1001	Ser334Leu
<i>PA14_45890</i>	7	G-->A	836	Pro279Leu
	5	G-->A	830	Ala277Val

INTERGENIC MUTATIONS

Between <i>PA14_45410</i> and <i>PA14_45430</i>	10	C-->T	4044801	-
Between 16S ribosomal RNA and <i>PA14_70920</i>	12	T-->C	6317713	-
Between <i>grpE</i> and <i>recN</i>	1	T-->C	5621761	-
Between <i>mexR</i> and <i>mexA</i>	2	C-->T	486683	-

606

607 Nucleotide location of the mutations and associated amino acid changes. Genetic
608 modifications in intergenic regions are also included, and their locations referred to the
609 nucleotide position in *P. aeruginosa* UCBPP-PA14 reference chromosome
610 (NC_008463.1). Fs: frameshift. *: stop codon. Del: deletion. Ins: insertion.

611 **Supplementary Table S2 | Primers used to verify nucleotide modifications detected in whole-genome sequencing.**

612

Gene	Genetic change	Localization	Primer fw (5'-3')	Primer rv (5'-3')
<i>nalD</i>	C-->A	32	SNVs/MNVs AATACTTCGAGTCCGCCC	TTGAGCATCTCGTTGAACAG
	C-->G	1126		
<i>mexB</i>	C-->T	1345	ATCCTCCTCGTGTTCCCTG	CAGGTATTCGCGCATC
	C-->T	1693		
	T-->G	2300	ACGAACCGCAGTACAAGCTG	CGCTCCAGCTTCGGCGAACC
<i>ftsI</i>	C-->T	1510		
	G-->A	1567	AACAAGTGGTCGAGGCCAG	TGCTGTTCCGGTGGCCGT
	G-->A	1511		
	G-->A	851	CTGCGCAACGCTCTGCT	AATTGCGCGATACGTCGCGA
	Del-A	515	TCAAGCAGCAGAGCATCGC	CTACCAGCAGCGGGTTGTT
<i>clpA</i>	A-->G	1634	TCGTTCTCTTCGCCGGT	CGTTGTTGTCCGTCAGGGT
	Ins-A	80-81		
<i>mexR</i>	Del-AAT	126-128	ATGAACTACCCCGTGAAT	AAGCTGCGCTGGTCGCT
	T-->G	371	GAGCATGTCCTGAACAAGGG	CGGTAATGGACGAACTTCGA
	T-->G	416		
<i>mpl</i>	Del-CGGCGGCTT	1305-1313	AACCTGGGCTGGGACCT	TCACTCGGCCAGCGCCGC

<i>orfN</i>	Ins-G	138-139	ATGGACGTTCCCAATGCCCCG	CCGCCAGAATCAGCAAAACC
<i>infB</i>	C-->T	2407	ATCTGCGGGAGAACATCCT	CCTTCTCGAACACTTCGATC
<i>pitA</i>	A-->C	133	ATGTTTCGATCTTTTCAGCGG	CGCGATGTCGATCGCCTT
	A-->C	367		
<i>grpE</i>	Del-G	39	GAACCGTGGAAAGCCGTCCC	AACCTGTGCGCCTTCTCGAC
<i>clpP</i>	Del-C	365	TTGCTGTTCTGAGGCTGA	TTCAGACGCTCCTTGATGAA
<i>dnaK</i>	G-->A	1115	TGAAGGATGCCGGCCTGGAT	TCTTGTGGACGCTGAAGA
<i>dacB</i>	C-->T	343	ACCTATGCCGCCCTGGAAA	CGTCGTTGAATACCGGCAA
<i>dnaJ</i>	G-->A	1081	AAGCTGTTCCGCCTGCGCG	ATAAAACCACTCCACGCG
<i>PA14_45870</i>	Del-21 pb	712-732	TCGAGAACAAGCTGCTGCAT	CGGTACTGGTCAGCATCT
<i>pepA</i>	C-->T	1439	CTGTTTCGACGAATACCAGGA	TCACTTGGCCC GTTCCAG
<i>ctpA</i>	G-->A	971	AGGAAGTGGTCAAGGCGCT	TGGATGGAGCGCCCCGTT
<i>glnD</i>	Ins-G	2466-2467	ACTCCATCGGCAACAACCC	ACAGCTGCTCGACCAGGGC
<i>flgF</i>	C-->A	676	TGAAGCAGATGGAGAAGGG	TTAGCTGATTTGCAAAACCC
<i>PA14_45880</i>	C-->A	159	TCGAGGACGAGGCGAAGA	CGAGGAAGGAGAACGGCTTGA
<i>pcm</i>	Ins-G	530-531	TTCGTTGGGGCGATGGCTG	TACCGAGTCGAGTACCTGG

<i>spoT</i>	T-->C	931	TCAACGAGATCATGGACGTC	CTCGTCGTTGGACTTGTACA
	G-->A	1001		
<i>PA14_45890</i>	G-->A	836	GCCAACGACCAGTTGTTCAA	ACCGTAGCGACGATCAGGGT
	G-->A	830		

LARGE DELETIONS

Del 1	Del 55009 bp	3387426-3442435	ATGCAACACAGCCAAGTGT CTGTTCGAGATCGTCGAAGG	TCAGAACGGTTTGGTCGGCA TTCGAACTCTTTCTCCTGTA
Del 2	Del 220701 bp	3288650-3509351	ATGCAACACAGCCAAGTGT CTGTTCGAGATCGTCGAAGG	TCAGAACGGTTTGGTCGGCA TTCGAACTCTTTCTCCTGTA
Del 3	Del 258271 bp	3210297-3468568	GCTTCACCGGTTTCGCTGAAG CTGTTCGAGATCGTCGAAGG	TCAGTCAATCGCCGCCCCG TTCGAACTCTTTCTCCTGTA
Del 4	Del 299648 bp	3200274-3499932	GCTTCACCGGTTTCGCTGAAG CTGTTCGAGATCGTCGAAGG	TCAGTCAATCGCCGCCCCG TTCGAACTCTTTCTCCTGTA
Del 5	Del 442741 bp	3203830-3646571	GCTTCACCGGTTTCGCTGAAG GTGGGCAGCATCTACCTGATT	TCAGTCAATCGCCGCCCCG TGATGAGTTCCGGCGCCTT

613

614 The locations of SNVs/MNVs are referred to the specific gene, whereas the large deletions locations remit to the nucleotide position in *P.*
615 *aeruginosa* UCBPP-PA14 reference chromosome (NC_008463.1)

Supplementary Table S3 | MIC values (µg/ml) obtained by double dilution for the population replicates during selective pressure with either ceftazidime or ceftazidime/avibactam.

Treatment	Replicate	5 d	10 d	15 d	20 d	25 d	30 d
CEFTAZIDIME	1	20	40	75	100	300	300
	2	20	60	250	300	400	400
	3	20	40	75	100	150	150
	4	25	40	100	400	300	400
CEFTAZIDIME + AVIBACTAM	5	40	50	150	250	300	400
	6	15	30	75	250	400	200
	7	20	80	200	400	300	400
	8	15	40	50	75	150	150
CONTROLS	9	4	4	4	4	4	4
	10	4	4	4	4	4	4
	11	4	4	4	4	4	4
	12	4	4	4	4	4	4

620

621 The table shows the liquid MIC values against ceftazidime for each replicate population
622 every five days (the antibiotic concentration was doubled every 5 days). The grey boxes
623 indicate the steps in which these particular replicates were unable to grow. In these
624 situations, the previous ceftazidime concentrations (8MIC in populations 6, 8; and
625 16MIC in populations 3, 7) were maintained for the rest of the experiment.

Supplementary Table S4 | Disk diffusion tests with antibiotics of distinct structural families in the ceftazidime and ceftazidime/avibactam final evolved populations.

Sample	Tgc	Tet	Atm	Ipm	Mer	Caz	Cip	Lev	Nor	Tob	S	Ak	Cm	Cs	PB	C	E	F
PA14 wt	14	16	26	30	21	27	36	31	32	26	22	25	31	17	14	18	13	14
CONTROLS																		
9	12	14	28	30	19	28	37	28	32	22	14/21	23	28	17	14	19	13	13
10	13	15	27	31	17	28	37	29	32	23	20	24	30	17	14	19	12	14
11	13	15	28	32	18	28	38	30	34	24	21	25	29	17	14	18	12	15
12	14	15	28	32	23	28	36	27	32	24	20	25	30	17	14	17	12	16
CEFTAZIDIME																		
1	13	12	0	19	0	0	30	21	26	26	26	29	32	19	15	0	0	27
2	15	12	0	19	0	0	33	26	28	29	29	31	35	19	16	0	0	33
3	15	10	0	25	0	0	31	24	28	25	24	28	32	19	16	0	0	27
4	15	12	0	22	0	0	33	24	28	27	28	31	36	19	16	0	0	31
CEFTAZIDIME + AVIBACTAM																		
5	19	19	0	10	0	0	33	30	31	24	23	28	31	17	15	0	6	27
6	20	22	7	24	0	0	34	30	33	25	22	29	34	18	16	0	0	26
7	23	22	0	9	0	0	38	32	34	23	22	26	33	19	16	0	0	25
8	21	19	0	24	0	0	33	29	28	26	24	30	34	19	16	0	0	27

The table presents the diameters of halo (mm) in the disk diffusion assays for each replicate population at the end of the experimental evolution. The antibiotics in which halos differed were selected for an E-test assay (Table 1). Indeed, in some cases we chose for the E-test only one or two representative cases of a particular structural family, in order to avoid redundancy. Double inhibition halos are shown as two values separated by a slash (X/X). Tgc: tigecycline, tet: tetracycline, atm: aztreonam, ipm: imipenem, mer: meropenem, caz: ceftazidime, cip: ciprofloxacin, lev: levofloxacin, nor: norfloxacin, tob: tobramycin, s: streptomycin, ak: amikacin, cm: gentamycin, cs: colistin, pb: polymyxin B, c: chloramphenicol, e: erythromycin, f: fosfomycin.