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MECHANISMS OF RESISTANCE

Mutation-Driven Evolution of *Pseudomonas aeruginosa* in the Presence of either Ceftazidime or Ceftazidime-Avibactam

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ABSTRACT Ceftazidime-avibactam is a combination of β-lactam/β-lactamase inhibitor, the use of which is restricted to some clinical cases, including cystic fibrosis patients infected with multidrug-resistant *Pseudomonas aeruginosa*, in which mutation is the main driver of resistance. This study aims to predict the mechanisms of mutation-driven resistance that are selected for when *P. aeruginosa* is challenged with either ceftazidime or ceftazidime-avibactam. For this purpose, *P. aeruginosa* PA14 was submitted to experimental evolution in the absence of antibiotics and in the presence of increasing concentrations of ceftazidime or ceftazidime-avibactam for 30 consecutive days. Final populations were analyzed by whole-genome sequencing. All evolved populations reached similar levels of ceftazidime resistance. In addition, they were more susceptible to amikacin and produced pyomelanin. A first event in this evolution was the selection of large chromosomal deletions containing *hmgA* (involved in pyomelanin production), *galU* (involved in β-lactams resistance), and *mexXY-oprM* (involved in aminoglycoside resistance). Besides mutations in *mpl* and *dacB* that regulate β-lactamase expression, mutations related to MexAB-OprM overexpression were prevalent. Ceftazidime-avibactam challenge selected mutants in the putative efflux pump *PA14_45890* and *PA14_45910* and in a two-component system (*PA14_45870* and *PA14_45880*), likely regulating its expression. All populations produced pyomelanin and were more susceptible to aminoglycosides, likely due to the selection of large chromosomal deletions. Since pyomelanin-producing mutants presenting similar deletions are regularly isolated from infections, the potential aminoglycoside hypersusceptiblity and reduced β-lactam susceptibility of pyomelanin-producing *P. aeruginosa* should be taken into consideration for treating infections caused by these isolates.

KEYWORDS *Pseudomonas aeruginosa*, avibactam, ceftazidime, mutational studies

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

The use of β-lactam/β-lactamase inhibitor combinations, such as amoxicillin-clavulanic acid or ceftolozane-tazobactam, has been proven to be effective against class

Received 29 June 2018 **Returned for modification** 22 July 2018 **Accepted** 27 July 2018

Accepted manuscript posted online 6 August 2018

Citation Sanz-García F, Hernando-Amado S, Martínez JL. 2018. Mutation-driven evolution of *Pseudomonas aeruginosa* in the presence of either ceftazidime or ceftazidime-avibactam. Antimicrob Agents Chemother 62:e01379-18. https://doi.org/10.1128/AAC.01379-18.

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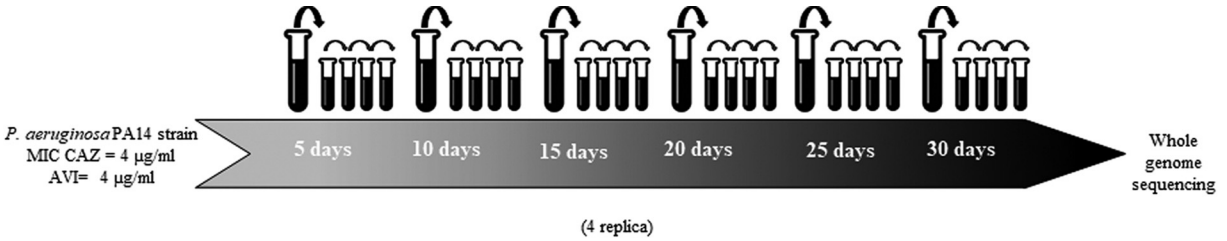

FIG 1 Experimental evolution assay. Eight bacterial cultures of *P. aeruginosa* strain PA14 were challenged with increasing inhibitory concentrations of ceftazidime in Luria Bertani broth (LBB) for 30 consecutive days. The ceftazidime concentration was raised by 2-fold every 5 days, from 4 μg/ml up to 32× MIC. Avibactam was added in combination with ceftazidime in four of these eight populations, at a constant concentration of 4 μg/ml, as this is the value established in clinical tests. Four controls without any selective pressure were also grown in parallel. At the end of the experimental evolution, the genomic DNA of the 12 independent populations was extracted and analyzed by whole-genome sequencing (WGS).

A β-lactamases (which include narrow- and extended-spectrum β-lactamases and some carbapenemases); whereas effective combinations against classes B, C (extended-spectrum cephalosporinases), and D β-lactamases (6–8) have not been available until recently. One of these is the ceftazidime-avibactam combination, the use of which was approved in 2015 by the FDA (9).

Avibactam, formerly known as NXL104, belongs to a new class of β-lactamase inhibitors, the diazabicyclooctanes (10). This inhibitor has a potent activity against most Class A and Class C and some Class D β-lactamases (11). Avibactam has been mainly used for restoring the activity of the third-generation cephalosporin ceftazidime (12). Thus far, it has been used for the treatment of patients with complicated urinary tract infections, including pyelonephritis, and for community-acquired intraabdominal infections, usually in combination with metronidazole (13). In addition, future studies are likely to expand the use of ceftazidime-avibactam to include other cases, such as those of cystic fibrosis patients with MDR *P. aeruginosa* infections (13).

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

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

RESULTS

Stepwise evolution of *P.* aeruginosa toward ceftazidime and ceftazidime-avibactam resistance. To determine the potential evolutionary trajectories that can lead to either ceftazidime or ceftazidime-avibactam resistance, four biological replicates were allowed to evolve in parallel under each of the following conditions (Fig. 1): selective pressure with ceftazidime (populations 1 to 4), selective pressure with ceftazidime-avibactam (populations 5 to 8), and in the absence of any selective pressure (populations 9 to 12). The susceptibility of each population to the selecting antibiotic was determined every 5 days by MIC test strip. However, after 20 days of

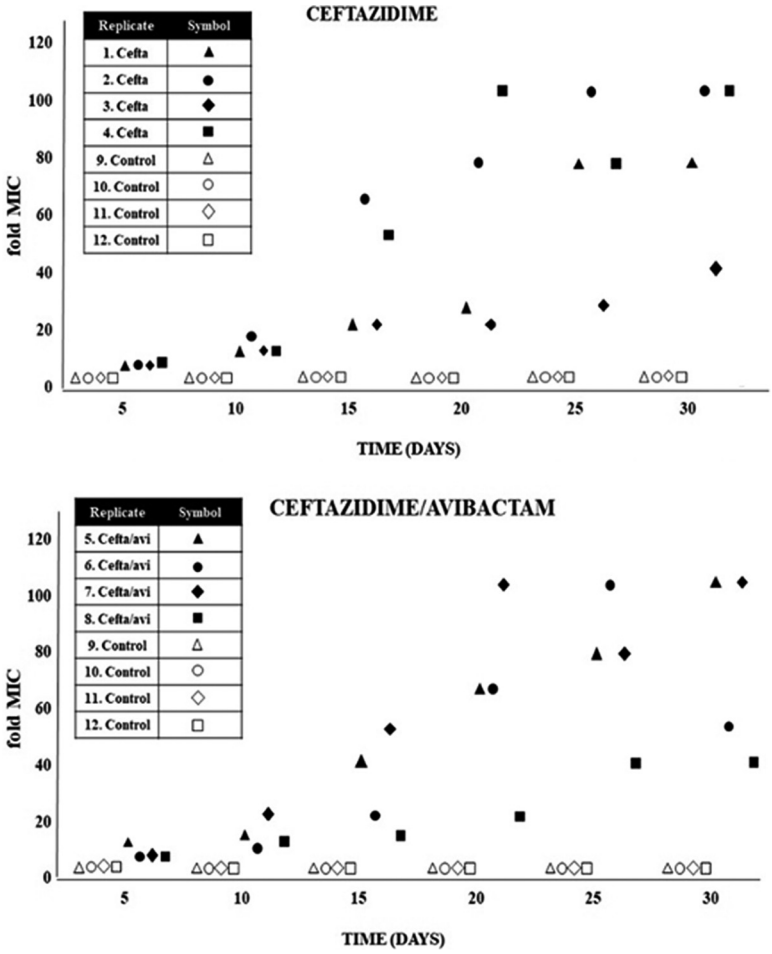

FIG 2 Evolution of *P. aeruginosa* PA14 under ceftazidime and ceftazidime-avibactam selective pressure. Graphs show the increase of the MICs over the evolution period ($MIC_{population\ X}/MIC_{PA14}$, where $MIC_{PA14} = 4\ \mu g/ml$ and X = 1 to 12) from the beginning of the experiment to high levels of resistance (doubling the antibiotic concentration every 5 days). The values were obtained by liquid MIC determination in a 96-well plate (see Table S3 in the supplemental material), because the detection limit of the ceftazidime MIC test strip is 256 $\mu g/ml$, limiting the assessment of resistance levels from day 20 to the end of the experiment.

evolution, MICs reached the highest limits of the MIC test strips and MICs were again determined for each evolutionary step, using a range of antibiotic concentrations (see Table S3 in the supplemental material). Stepwise evolutionary trajectories, in which the selected populations reached quite similar levels of resistance, were observed for both treatments (Fig. 2). These results suggest that avibactam inhibition may not be a guarantee of impeding *P. aeruginosa* from acquiring high-level ceftazidime resistance. An increase in the MIC of an antibiotic after experimental evolution does not necessarily imply that antibiotic-resistant mutants have been selected for; resistance may have arisen due to a phenotypic (inducible) adaptation to the presence of ceftazidime rather than to mutations (18–20). To address this possibility, the evolved populations were cultured in the absence of selection pressure (three sequential passages in Luria-Bertani [LB] broth), and the MICs were again determined. These were found not to vary, indicating that the observed modifications were due to the selection of stable mutants.

Cross-resistance and collateral sensitivity of the evolved populations. Taking into consideration the few therapeutic options for patients submitted to ceftazidime-avibactam therapy, knowing whether or not acquisition of resistance to this combination might alter susceptibility to other antibiotics is of crucial importance. To that end, susceptibility to a range of representative antibiotics was tested by disk diffusion assay

TABLE 1 MICs of antibiotics of different structural families in the populations evolved in the presence of either cefatzidime or ceftazidime-avibactam

Replicate[a]	MIC (μg/ml) for[b]:											
	TGC	TET	ATM	IPM	MEM	CAZ	CZA	NOR	AMK	CHL	ERY	FOF
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

[a]Populations were challenged as follows: 1 to 4, ceftazidime; 5 to 8, ceftazidime-avibactam; and 9 to 12, controls.
[b]TGC, tigecycline; TET, tetracycline; ATM, aztreonam; CAZ, ceftazidime; CZA, ceftazidime-avibactam; IPM, imipenem; MEM, meropenem; NOR, norfloxacin; AMK, amikacin; CHL, chloramphenicol; ERY, erythromycin; FOF, fosfomycin.

(see Table S4 in the supplemental material). From these results, a set of antibiotics was chosen for determining their MICs against the different evolved populations. Every evolved replicate showed altered susceptibility to antimicrobials belonging to different structural families (Table 1), implying that at least some resistance mutations were not ceftazidime or ceftazidime-avibactam specific. All populations evolved in the presence of either ceftazidime or ceftazidime-avibactam presented decreased susceptibility to other β-lactams, chloramphenicol, and erythromycin, and they were more susceptible to fosfomycin and amikacin. Notably, while populations evolved in the presence of ceftazidime were less susceptible to tetracycline and did not present changes in susceptibility to tigecycline, populations evolved in the presence of ceftazidime-avibactam were hypersusceptible to both antibiotics.

Analysis of mutations associated with the acquisition of resistance. To know the genetic events associated with the acquisition of resistance in the evolved populations, the genomes of each population, as well as that of the original PA14 strain, were sequenced on the last day of the experiment. Table 2 encompasses the resulting mutated genes and their functional significance, whereas Table S1 in the supplemental material shows the locations of all 40 genetic changes that were unveiled and were not present in control populations evolving in the absence of antibiotics. A total of 37 single-nucleotide variants (SNVs) and 3 multinucleotide variants (MNVs; deletions and substitutions of various nucleotides) were found, 36 located in genes and 4 in intergenic regions. Most mutations located in genes resulted in amino acid alterations, stop codons, or frameshifts. In addition, all of the populations evolved in the presence of antibiotics contained large chromosomal deletions (55 to 443 kbp), representing from 0.88% to 7.09% of the *P. aeruginosa* PA14 genome. Five different deletions were selected; all presented a 55-kbp common region (Fig. 3).

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

Common mutations selected under either ceftazidime or ceftazidime-avibactam selection pressure. After 1 day of experimental evolution, all *P. aeruginosa* PA14 cultures challenged with antibiotic produced a brown pigment (Fig. 3) that appeared to be pyomelanin, the accumulation of which is normally due to the lack of homogentisate 1,2-dioxygenase activity provided by the enzyme HmgA (21). All of the chromosomal large deletions selected during evolution presented *hmgA* (Fig. 3). In addition,

TABLE 2 Mutated genes in ceftazidime- and ceftazidime-avibactam-evolved populations[a]

Treatment (populations)	Gene name	Functional classification	Replicate(s)	Previously described effect(s) on antibiotic susceptibility	Reference(s) or source
Both treatments	*nalD*	Efflux regulation	4, 6, 8	Quinolones, macrolides, tetracyclines, chloramphenicol, and β-lactams	24–26
	mexB	Efflux component	1, 3, 6, 8	Quinolones, macrolides, tetracyclines, chloramphenicol, and β-lactams	23
	ftsI	Penicillin binding protein	1, 5, 8	β-Lactams	27–30
	clpA	Protease activity	1, 5	Aztreonam	27
Ceftazidime (1–4)	*mexR*	Efflux regulation	1, 3	Quinolones, macrolides, tetracyclines, chloramphenicol, and β-lactams	23
	Upstream *mexA*	Efflux regulation	2	Quinolones, macrolides, tetracyclines, chloramphenicol, and β-lactams	23
	mpl	Peptidoglycan metabolism (β-lactamase)	2, 4	β-Lactams	22, 65
	orfN	Flagellin glycolysation	2	Ciprofloxacin, tigecycline, and tobramycin	34 and F. Sanz-García et al. (submitted for publication)
	infB	Translation factor	2		
	pitA	Phosphate transport	2, 4		
	grpE	Heat shock protein	3		
	clpP	Protease activity	3		
	dnaK	Chaperone	3	Several antibiotics	35
	dacB	Penicillin binding protein	4	β-Lactams	32
Ceftazidime-avibactam (5–8)	*dnaJ*	Chaperone	5	Triclosan	35
	pepA	Protease activity	6	Meropenem and aztreonam	27, 40
	ctpA	Protease activity	6		
	glnD	N_2 metabolism	6	Aminoglycosides and cephradine	35
	flgF	Flagellar component	6	Cephradine, cefoxitin, and chloramphenicol	35
	pcm	Protein repair/degradation	8		
	spoT	Stringent response	8	Piperacillin	41
	PA14_45870	TCS sensor	5	Carbapenems	36, 37
	PA14_45880	TCS regulator	7	Carbapenems	36, 37
	PA14_45890	Efflux component	5, 7	Carbapenems	36, 37

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

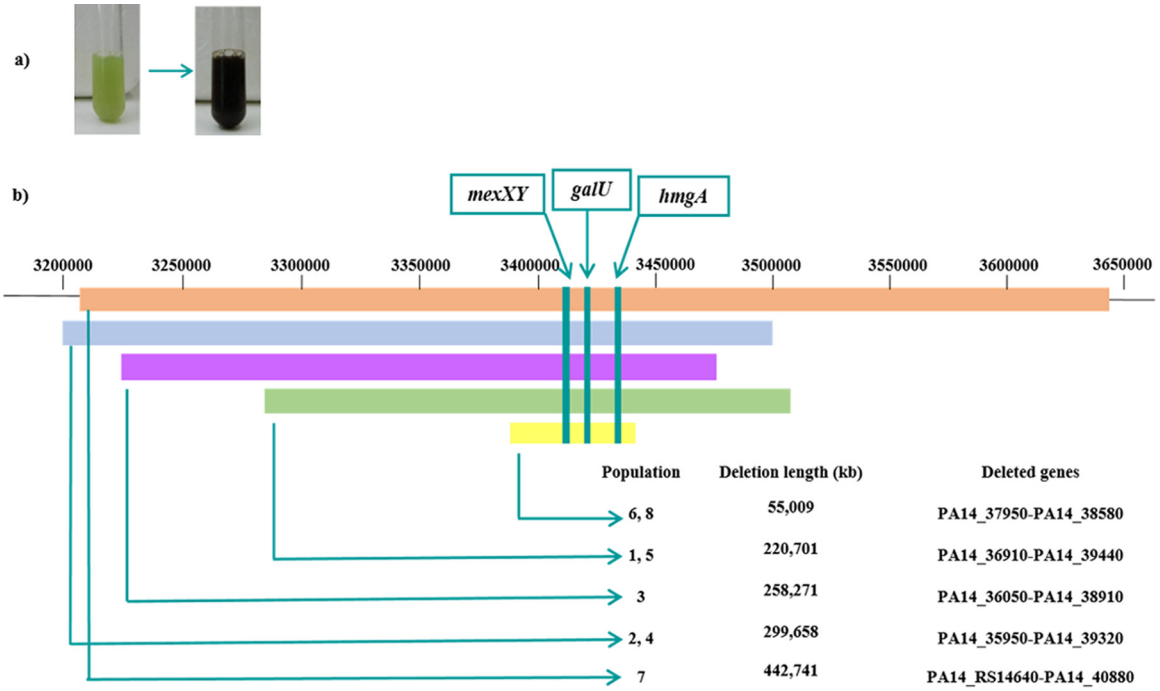

FIG 3 Large deletions present in all ceftazidime- and ceftazidime-avibactam-evolved populations since the first day of the experimental evolution. (a) A first event in the evolution in the presence of the antibiotics was an increase in pyomelanin accumulation due to the loss of *hmgA*, as the consequence of the deletion of large regions of *P. aeruginosa* genome. (b) The length of the deletions and the deleted genes in each replicate, as well as their genome localization, which corresponds with the *P. aeruginosa* UCBPP-PA14 reference chromosome (GenBank accession no. NC_008463).

the deletions also included *galU* (involved in lipopolysaccharide [LPS] biosynthesis), the inactivation of which reduces ceftazidime susceptibility (22), and the MDR efflux pump *mexXY-oprM*, which contributes to aminoglycoside resistance in *P. aeruginosa* (23). Deletion of the latter is likely the cause of the observed amikacin hypersusceptibility of all but one of the evolved populations (Table 1).

Another common element in both evolutions is *nalD*, which encodes a secondary repressor of MexAB-OprM (24, 25). Three out of eight replicates showed the same T11N change, which has been previously found in extensively drug-resistant (XDR) *P. aeruginosa* high-risk clones (26) that overexpress MexAB-OprM. Notably, four replicates (including the three presenting mutations in *nalD*) also presented mutations in *mexB*, indicating this efflux 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 (27, 28), 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 (28–30). *clpA* encodes an intracellular protease involved in different aspects of *P. aeruginosa* physiology, in addition to aztreonam resistance (27, 31).

Mutations selected by ceftazidime. In addition to the observed mutations in *nalD*, which would allow *mexAB-oprM* overexpression, we also found 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 of *mexA* that might prevent the interaction of NalD with its operator (24), 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. In addition, they control the activity of AmpR and consequently the level

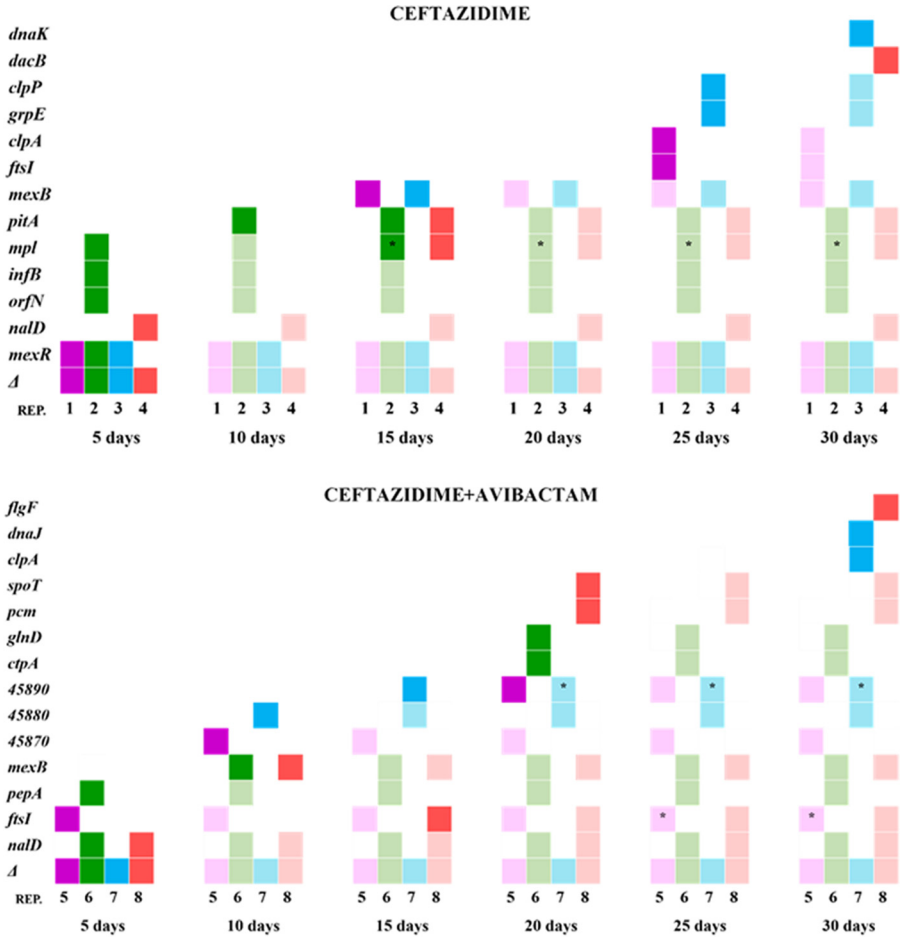

FIG 4 Order of appearance of genetic changes. The appearance of ceftazidime and ceftazidime-avibactam resistance mutations during the evolution process, as determined by PCR amplifications of known SNVs/MNVs in evolved populations. The *mexR* mutation in ceftazidime population 2 actually indicates that this mutation occurred in the intergenic region between *mexR* and *mexA*. An asterisk (*) refers to a second mutation in a gene that mutated previously. We cannot exclude the possibility that other mutations may have appeared over the 30-day evolution process. Δ, large deletion; Rep, replicate; intense-color square, the mutation appeared in this evolutionary step; light-color square, the mutation appeared in a previous evolutionary step.

of AmpC expression (22, 32), which is known to be a main element in *P. aeruginosa* resistance to β-lactams. Interestingly, *mpl* V124G (replicate 2; see Table S1 in the supplemental material) has been found before in a clinical isolate (*P. aeruginosa* NCGM1984). These findings, along with the aforementioned *ftsI* and *nalD* mutations, validate our experimental evolution approach as a valuable predictive model for the *in vivo* selection of antibiotic resistance.

Finally, mutations at *orfN*, *pitA*, *infB*, *grpE*, *clpP*, and *dnaK* were selected for in populations challenged with ceftazidime. *orfN* codes for a putative glycosyl transferase of type A flagellins (33). Mutations on this gene have been found in ciprofloxacin-resistant *P. aeruginosa* strains (34), and also in *P. aeruginosa* populations submitted to tigecycline and tobramycin experimental evolutions (F. Sanz-García, S. Hernando-Amado, and J. L. Martinez, submitted for publication). *pitA* encodes a phosphate transporter, *infB* encodes the translation initiation factor IF-2, and *dnaK*, *grpE*, and *clpP* encode proteins involved in regulatory gene networks involved in response to stress. None of these genes has been previously related to ceftazidime resistance, except for *dnaK*, the inactivation of which leads to stronger susceptibility to various antimicrobials in *Escherichia coli* (35).

Mutations selected by ceftazidime-avibactam. The challenge with ceftazidime-avibactam selected mutants in a predicted efflux pump (*PA14_45890* and *PA14_45910*), as well as in the two-component system (TCS) encoded by the operon *PA14_45870* and *PA14_45880*, likely regulating its expression. Previous studies have shown this efflux pump to be involved in *P. aeruginosa* intrinsic resistance (36) and susceptibility to carbapenems (37). Regarding the substrate recognition profile this pump might display, it is remarkable that populations 5 and 7, which present the aforementioned mutations, show a much lower susceptibility to imipenem than any other replicate (Table 1), suggesting this pump to have certain specificity to carbapenems.

Other mutations that were selected upon ceftazidime-avibactam treatment were found in *pepA*, *spoT*, *dnaJ*, and *flgF*. *pepA* encodes a protease necessary for *P. aeruginosa* cytotoxicity, virulence, and, consequently, lung infection (38, 39). Although its implication in antibiotic resistance has not been studied in detail, it has been reported that its inactivation confers meropenem resistance in *P. aeruginosa* (40). Moreover, *pepA* mutants are selected in the presence of aztreonam (27). SpoT has been related to piperacillin resistance (41), while DnaJ, a chaperone protein, and FlgF, a flagellar basal body rod protein (42), have been reported to modify the susceptibility of *E. coli* to a range of antibiotics when they are inactivated (35).

The other mutations that were selected for in populations under ceftazidime-avibactam challenge, namely those occurring in *ctpA*, an essential gene for the transition between acute and chronic *P. aeruginosa* infection (43), *pcm*, which encodes a L-isoaspartate carboxyl methyltransferase type II that participates in protein repair and degradation, and *glnD*, which is implicated in N_2 metabolism (44), have not been reported to be involved in antibiotic resistance.

Determination of the β-lactamase activity of the evolved populations. The evolution of ceftazidime resistance has been previously investigated in a *P. aeruginosa* PAO1 background (45), and resistance was mainly driven by combinations of mutations leading to greatly enhanced AmpC expression and ceftazidime resistance, a feature that was not so clear in the case of *P. aeruginosa* PA14, an strain in which the level of β-lactamase expression of *ampD* and *dacB* mutants is lower in comparison (46).To ascertain whether or not the overexpression of AmpC is a general outcome in our evolution, the β-lactamase activity of the evolved populations was measured at the endpoint of the experiment. As shown in Fig. 5, populations 2 and 4, which harbor *mpl* and *dacB* mutations, presented a large increase in β-lactamase production. The other populations evolved in the presence of ceftazidime and those evolved in the presence of ceftazidime-avibactam, which do not carry these mutations, also presented an increase in β-lactamase production, although the level reached was lower in comparison.

DISCUSSION

The use of β-lactamase inhibitors has reemerged as a fruitful strategy for fighting infections by MDR bacteria. Among them, ceftazidime-avibactam can be a useful combination for treating infections by different organisms, including *P. aeruginosa*. The analysis of the mechanisms of resistance to previous β-lactam/β-lactamase inhibitor combinations, such as amoxicillin-clavulanate, have shown that the main mechanisms selected by their use have been increased expression or mutation of preexisting β-lactamases and acquisition of new ones by HGT (47–51). *P. aeruginosa* has already acquired different carbapenemases that might be important elements in ceftazidime-avibactam resistance. In addition, resistance can be achieved through mutations, particularly in the case of *P. aeruginosa* causing chronic infections. To identify potential mutations involved in the acquisition of either ceftazidime or ceftazidime-avibactam resistance, 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 the *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

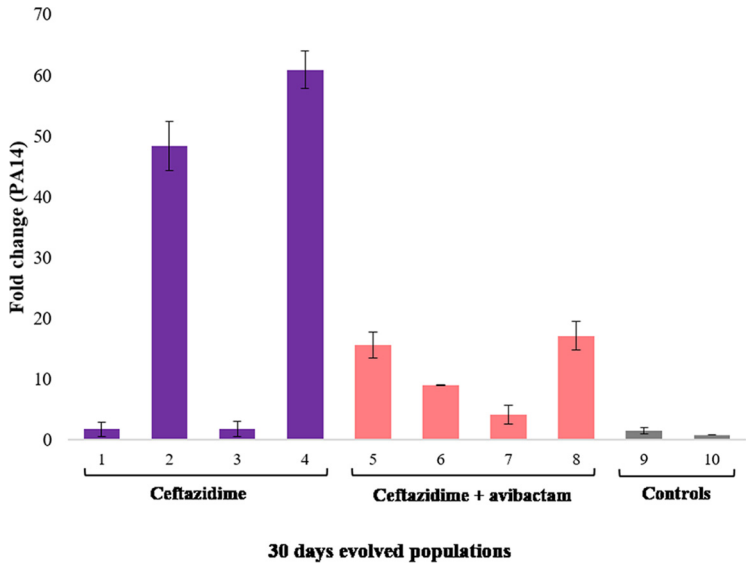

FIG 5 β-Lactamase activity in final evolved populations. The figure shows the relative amount of β-lactamase activity in 30-day ceftazidime- and ceftazidime-avibactam-evolved populations, as well as the activities of two replicates evolved in the absence of antibiotics. Fold changes were estimated with respect to the value given by the *P. aeruginosa* PA14 strain. Error bars indicate standard deviations of the results from two independent experiments.

β-lactams, such as piperacillin (41) and meropenem (45). Additionally, pyomelanin-producing mutants are regularly isolated from infections; up to 13% of CF patients harbor pyomelanin-producing mutants (52), likely because the production of pyomelanin increases resistance to oxidative stress and persistence in chronic lung infections (21). 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 may select 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* PA14 evolved in the presence of ciprofloxacin does not produce pyomelanin (a marker of these deletions) and that pyomelanin-producing mutants are selected when a *P. aeruginosa recA*-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 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 hypersusceptibility to amikacin of the evolved populations. In addition, it might have an indirect effect on the decreased susceptibility to β-lactams, particularly in the case of those strains carrying mutations in the repressors of *mexAB-oprM*. MexAB-OprM is an important determinant of intrinsic *P. aeruginosa* resistance to different antibiotics, including to β-lactams (23). Furthermore, mutants overexpressing this efflux pump are regularly isolated from infections, and its expression has been shown to be prevalent among resistant *P. aeruginosa* clinical isolates (55–58). MexAB and MexXY share the outer membrane protein OprM, which produces antagonistic interactions when both systems are expressed (45, 59). Hence, MexXY-OprM elimination might favor the efficiency of β-lactam efflux, reducing the competition of both efflux pumps for OprM. Besides aminoglycoside hypersusceptibility, the selected mutants also present fosfomycin collateral susceptibility, although the causes of this phenotype are unknown. It has been shown that mutants defective in the *P. aeruginosa* peptidoglycan recycling pathway show a marked increase in fosfomycin susceptibility (60). Although mutations in these elements were not found in

the evolved populations, it is still possible that they might present altered levels of expression, an issue that remains to be explored.

Important elements in the acquisition of ceftazidime resistance include efflux pumps, particularly MexAB-OprM, since mutations in either the elements regulating its expression or in the efflux pump itself were found in six out of eight evolved populations, whereas the two remaining populations harbored mutants in the putative *PA14_45890* and *PA14_45910* operon and in its potential TCS regulator. While the substrates of MexAB-OprM are known and include β-lactams, the substrates of *PA14_4590* and *PA14_45910* are unknown. Nevertheless, it is remarkable that populations presenting mutations on this determinant display a much lower susceptibility to imipenem than does any other replicate, suggesting this pump to have certain specificity to β-lactams, a feature that deserves further work.

Mutations in elements involved in the regulation of AmpC expression were selected when just ceftazidime was used for selection and not in the presence of ceftazidime-avibactam. This suggests that, at least in the *P. aeruginosa* PA14 background, the efficient inhibition by avibactam of intrinsic β-lactamases precludes the emergence of mechanisms based on their overexpression, and other mechanisms, including the above-mentioned large deletions and modifications in the activity of efflux pumps, are preferentially selected. This does not necessarily mean that resistance to ceftazidime-avibactam cannot be associated with changes in the activity of AmpC, particularly if the challenged isolate is already resistant to ceftazidime. Indeed, avibactam-resistant mutants presenting changes in the avibactam binding pocket of AmpC are selected *in vitro* at low frequency from AmpC-overexpressing ceftazidime-resistant *P. aeruginosa* isolates, and the role of mutations in AmpR, the regulator of AmpC expression, in developing ceftazidime-avibactam resistance has been recently explored (61).

Although most of the mutants reported here have been previously associated with antibiotic resistance, it is still possible that some of the mutations might be selected for compensating the fitness costs associated with the acquisition of resistance. This might be the case of *ctpA*, *pcm*, or the mutations at structural elements of efflux pumps that were selected after mutations in the regulators of their expression. For the last, it might also be possible that these mutations increase the capability to extrude the antibiotic substrates, as described for AcrB (62). The fact that in all evolved populations, mutants in efflux pumps are selected, provides an explanation of the cross-resistance phenotype observed in all resistant strains. This situation might be of concern, since both ceftazidime and ceftazidime-avibactam might select for resistance to other antibiotics, at least in chronic infections in which mutation is the main cause of acquisition of resistance.

P. aeruginosa evolution in chronic infections frequently involves large genome deletions (63), which are usually linked to the production of pyomelanin (54). Whether these deletions are selected by antibiotic treatment or are just the consequence of the adaptation to the environment of the lungs of the CF patient remains to be established. However, this evolution provides a link between antibiotic resistance and virulence for this relevant pathogen. In any case, and given that deletions containing *galU* and *hmgA* appear to be a first step in the evolution toward ceftazidime-avibactam resistance, pyomelanin production could be considered 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 usually hypersusceptible to aminoglycosides, probably because the deletions they present include *mexXY*. It would then be judicious to use aminoglycosides and not β-lactams for treating infections by pyomelanin-producing *P. aeruginosa* strains.

MATERIALS AND METHODS

Growth conditions and antibiotic susceptibility assays. Unless otherwise stated, bacteria were grown in Luria Bertani (LB) broth at 37°C with shaking at 250 rpm. The susceptibility to tigecycline, tetracycline, aztreonam, ceftazidime, imipenem, meropenem, ciprofloxacin, levofloxacin, norfloxacin, tobramycin, streptomycin, amikacin, gentamicin, colistin, polymyxin B, chloramphenicol, fosfomycin, and

erythromycin was determined by disk diffusion in Mueller-Hinton agar (MHA) (Sigma) at 37°C. For a set of antibiotics, MICs were determined using MIC test strips (Liofilchem). MICs of ceftazidime and ceftazidime-avibactam were determined in LB broth by pouring into microtiter plates specific antibiotic concentrations in an arithmetic scale spanning from 0.75 to 800 μg/ml.

Experimental evolution procedure. Twelve bacterial populations from a stock *P. aeruginosa* PA14 culture (four controls without antibiotic, four populations challenged with ceftazidime, and four populations challenged with ceftazidime-avibactam) were grown in parallel in LB broth for 30 consecutive days. Each day, the cultures were diluted (1/250) in fresh LB broth. The concentrations of ceftazidime used for selection were increased over the evolution experiment from the concentration that hinders the growth of *P. aeruginosa* PA14 under these culture conditions (4 μg/ml) up to 128 μg/ml, doubling them every 5 days. The avibactam concentration was maintained at a constant 4 μg/ml, as used in clinical tests (64). On some occasions, the cultures did not grow when antibiotic concentration was increased, in which case the selection was kept at the concentration that allowed growth. Every 5 days, samples from each culture were preserved at −80°C for further research.

Whole-genome sequencing (WGS). A Gnome DNA kit (MP Biomedicals) was used to extract genomic DNA. WGS was performed by Sistemas Genómicos S.L. The quality of the extracted material was analyzed via a 4200 TapeStation high-sensitivity assay, and the DNA concentration was ascertained by real-time PCR using a LightCycler 480 device (Roche). Libraries were obtained without amplification, following Illumina protocols, and were pair end sequenced (100 × 2) in an Illumina HiSeq 2500 sequencer. The average number of reads per sample was 7,178,870, which represents a 200× coverage, on average.

Bioinformatics analysis of WGS and confirmation of genetic changes. Mutations in the evolved populations were identified using CLC Genomics Workbench 9.0 (Qiagen). *P. aeruginosa* UCBPP-PA14 reference chromosome (GenBank accession number NC_008463) was used to align the reads obtained from WGS data (previously trimmed). Sanger sequencing was used to verify and to settle the order of appearance of the putative mutations found via WGS (Table S1). Thirty-two pairs of primers, which amplified 200- to 400-bp regions containing each genetic modification, were designed (see Table S2 in the supplemental material). After PCR amplification, the corresponding amplicons were purified using the QIAquick PCR purification kit (Qiagen) and sequenced at GATC Biotech.

Determination of β-lactamase activity. Cells were grown overnight at 37°C and 250 rpm in 20 ml of LB broth. Afterwards, they were harvested by centrifugation (7,000 rpm for 10 min) and resuspended in 500 μl of 0.1 M Na_2HPO_4 (pH 7.4) buffer. Crude protein extracts were prepared by sonication on ice (0.7 Hz) and centrifuged again (13,000 rpm, 15 min). The protein content of each extract was determined using the Bradford protein assay with bovine serum albumin as a standard. The β-lactamase activity was quantified spectrophotometrically by measuring the change in absorbance at 486 nm, using the chromogenic β-lactamase substrate nitrocefin at 500 μg/ml (Oxoid, Basingstoke, United Kingdom) and 0.1 M Na_2HPO_4 (pH 7.4) as the test buffer. The assay was performed using the Infinite M200 plate reader (TECAN) for 2 h at 37°C, with measurements every 2 min.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01379-18.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

ACKNOWLEDGMENTS

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

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