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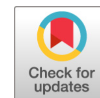
MECHANISMS OF RESISTANCE



Genomic Analysis Identifies Novel *Pseudomonas aeruginosa* Resistance Genes under Selection during Inhaled Aztreonam Therapy *In Vivo*

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ABSTRACT Inhaled aztreonam is increasingly used for chronic *Pseudomonas aeruginosa* suppression in patients with cystic fibrosis (CF), but the potential for that organism to evolve aztreonam resistance remains incompletely explored. Here, we performed genomic analysis of clonally related pre- and posttreatment CF clinical isolate pairs to identify genes that are under positive selection during aztreonam therapy *in vivo*. We identified 16 frequently mutated genes associated with aztreonam resistance, the most prevalent being *ftsI* and *ampC*, and 13 of which increased aztreonam resistance when introduced as single gene transposon mutants. Several previously implicated aztreonam resistance genes were found to be under positive selection in clinical isolates even in the absence of inhaled aztreonam exposure, indicating that other selective pressures in the cystic fibrosis airway can promote aztreonam resistance. Given its potential to confer plasmid-mediated resistance, we further characterized mutant *ampC* alleles and performed artificial evolution of *ampC* for maximal activity against aztreonam. We found that naturally occurring *ampC* mutants conferred variably increased resistance to aztreonam (2- to 64-fold) and other β-lactam agents but that its maximal evolutionary capacity for hydrolyzing aztreonam was considerably higher (512- to 1,024-fold increases) and was achieved while maintaining or increasing resistance to other drugs. These studies implicate novel chromosomal aztreonam resistance determinants while highlighting that different mutations are favored during selection *in vivo* and *in vitro*, show that *ampC* has a high maximal potential to hydrolyze aztreonam, and provide an approach to disambiguate mutations promoting specific resistance phenotypes from those more generally increasing bacterial fitness *in vivo*.

KEYWORDS aztreonam, *Pseudomonas aeruginosa*, *ampC*, antibiotic resistance, β-lactamase, cystic fibrosis, genome analysis, selection

Chronic lung infections remain a serious source of morbidity and mortality for patients with cystic fibrosis (CF), and significant efforts have focused on controlling the incipient pulmonary bacterial populations present in affected individuals (1, 2). Aztreonam lysine, a fully synthetic β-lactam antibiotic, is one of several inhaled drugs that have been developed for the suppression of chronic *Pseudomonas aeruginosa* infections in CF patients. Like other inhaled antibiotic formulations, aztreonam lysine is able to reach high concentrations within the patient airway with only minimal systemic absorption (3, 4). Since its approval in 2010 (5), inhaled aztreonam has become widely used as therapy for CF patients residing in the United States, with nearly half of eligible individuals currently being prescribed that drug in monthly treatment cycles (6), often alternating with inhaled tobramycin (7, 8).

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Although inhaled aztreonam is widely administered, the potential for *P. aeruginosa* to develop and maintain resistance to that antibiotic over the course of treatment is still incompletely explored. Several clinical trials have reported that *P. aeruginosa* can evolve modest, dose-dependent, and seemingly transient increases in aztreonam resistance following inhaled therapy (9–11), suggesting that the organism has inherent potential to combat the drug (12). Accordingly, chromosomal changes affecting aztreonam resistance in *P. aeruginosa* have been previously described, including those that promote active drug efflux through overexpression of the *mexAB-OprM* system (13–15), alteration of *ftsI* (PBP3) and consequent disruption of drug binding (16), and mutational overexpression (17) or coding sequence alteration (12, 18) of the chromosomal *ampC* β-lactamase. To more comprehensively explore the scope of aztreonam resistance mutations possible in *P. aeruginosa*, we recently employed *in vitro* selection to identify multiple known and previously unknown genes associated with increased drug resistance (12). Genes recurrently altered during aztreonam selection *in vitro* were also identified in a small cohort of clinical isolates from patients treated with the antibiotic (12). Nevertheless, mutations in several genes recurrently altered during artificial selection were not observed in clinical isolates, suggesting that they may incur unacceptable fitness costs *in vivo*, and conversely, several clinical isolates lacked mutations in any candidate genes identified during selection, indicating the existence of additional relevant pathways (12).

Here, we sought to better understand the factors most relevant to *P. aeruginosa* aztreonam resistance *in vivo* by cataloging genes under positive selection during inhaled aztreonam therapy in CF patients. We performed whole-genome sequencing and analysis of clonally related sequentially collected clinical isolate pairs obtained from the sputa of CF patients who were either treated with inhaled aztreonam or were not exposed to the drug. This approach identifies genes known to be involved in aztreonam resistance, including *ftsI* and *ampC*, while newly implicating factors with resistance contributions that we subsequently confirmed experimentally. We also observed differences between mutations recovered during *in vivo* and *in vitro* selection, likely reflecting differential fitness costs present under those conditions. As a secondary aim, given its strong signature of positive selection and its potential to be mobilized to plasmids (19, 20), we characterized resistance capabilities of mutant *ampC* alleles from aztreonam-resistant isolates and performed *in vitro* evolution of the gene to more fully investigate its existing and maximal evolutionary potential to hydrolyze aztreonam.

RESULTS

Clonal *P. aeruginosa* isolates demonstrate various levels of aztreonam resistance after inhaled antibiotic therapy *in vivo*. We identified 64 pairs of clonally related *P. aeruginosa* isolates for genomic analysis that were obtained through the AIR-CF5 clinical trial, a 5-year observational study designed to monitor *P. aeruginosa* aztreonam susceptibility in CF patients treated with inhaled drug (21). We selected isolate pairs exhibiting at least a 4-fold difference in aztreonam MIC, regardless of whether they originated from the aztreonam-exposure or nonexposure group, leaving a final count of 60 isolate pairs (Table 1). Each pair originated from a different patient, 48 of whom underwent inhaled aztreonam therapy during the study and 12 who never received any formulation of that drug.

The average time between collection of paired isolates was 1.3 years (range, 0.75 to 2.22 years), and whole-genome sequencing subsequently revealed pairs to be distinguished by a range of 14 to 2,379 genomic variants (single nucleotide polymorphisms and indels). Two paired isolates from patients with no previous aztreonam exposure and three paired isolates from the aztreonam exposure group (7.8% of total) carried *mutS* frameshift variants that were consistent with a hypermutator phenotype (22) (see Data Set S1 in the supplemental material). Isolates from patients treated with inhaled aztreonam had an average fold difference in MIC (239.5-fold) and absolute aztreonam MIC (381 μg/ml) that were significantly ($P = 0.007$ and $P = 0.004$, respectively, 2-tailed *t* test) greater than seen for the group which was not exposed to aztreonam (average

TABLE 1 Paired isolate summary

Isolate pair identifier	No. of yrs between collections	Aztreonam MIC (μg/ml)		Fold increase in MIC	Aztreonam resistance stratification group
		Sensitive isolates	Resistant isolates		
P_1329	1.88	8	512	64	No exposure
P_1266	1.07	8	128	16	No exposure
P_1268	0.86	1	64	64	No exposure
P_1273	1.04	8	64	8	No exposure
P_1279	0.98	1	64	64	No exposure
P_1455	1.92	1	64	64	No exposure
P_1078	1.95	4	32	8	No exposure
P_1468	0.90	4	32	8	No exposure
P_1011	1.00	1	16	16	No exposure
P_1085	1.15	1	16	16	No exposure
P_1099	1.11	2	16	8	No exposure
P_1274	1.01	1	16	16	No exposure
P_1010	2.09	2	32	16	Low resistance
P_1237	1.15	1	32	32	Low resistance
P_1324	0.96	2	32	16	Low resistance
P_1335	1.22	4	32	8	Low resistance
P_1384	1.80	2	32	16	Low resistance
P_1451	1.98	1	32	32	Low resistance
P_1072	1.18	4	16	4	Low resistance
P_1146	1.05	1	16	16	Low resistance
P_1215	0.83	1	16	16	Low resistance
P_1261	0.92	1	16	16	Low resistance
P_1305	1.92	1	16	16	Low resistance
P_1390	1.05	1	16	16	Low resistance
P_1351	1.08	1	256	256	Medium resistance
P_1429	1.01	2	256	128	Medium resistance
P_1494	1.94	8	256	32	Medium resistance
P_1505	0.75	1	256	256	Medium resistance
P_1019	1.04	1	128	128	Medium resistance
P_1022	1.19	2	128	64	Medium resistance
P_1119	2.22	1	128	128	Medium resistance
P_1144	0.99	1	128	128	Medium resistance
P_1257	1.87	1	128	128	Medium resistance
P_1259	1.95	2	128	64	Medium resistance
P_1316	2.04	1	128	128	Medium resistance
P_1323	1.00	8	128	16	Medium resistance
P_1431	1.97	1	128	128	Medium resistance
P_1432	0.91	2	128	64	Medium resistance
P_1440	1.97	4	128	32	Medium resistance
P_1499	1.07	4	128	32	Medium resistance
P_1137	2.04	1	64	64	Medium resistance
P_1197	1.86	1	64	64	Medium resistance
P_1230	1.11	16	64	4	Medium resistance
P_1334	2.03	8	64	8	Medium resistance
P_1342	1.02	4	64	16	Medium resistance
P_1397	1.82	2	64	32	Medium resistance
P_1403	0.90	1	64	64	Medium resistance
P_1437	0.99	8	64	8	Medium resistance
P_1475	1.15	1	64	64	Medium resistance
P_1018	1.11	2	>2,048	>1,024	High resistance
P_1210	0.94	1	>2,048	>2,048	High resistance
P_1411	0.80	1	2,048	2,048	High resistance
P_1439	0.86	4	2,048	512	High resistance
P_1472	1.78	1	2,048	2,048	High resistance
P_1004	0.96	8	1,024	128	High resistance
P_1115	0.86	1	1,024	1,024	High resistance
P_1379	1.06	8	1,024	128	High resistance
P_1038	1.04	16	512	32	High resistance
P_1308	2.03	2	512	256	High resistance
P_1407	0.81	64	512	8	High resistance

29.3-fold difference, 85.3 μg/ml MIC). Half of isolate pairs from patients without inhaled aztreonam exposure had aztreonam MICs greater than 32 μg/ml, despite lacking specific exposure to that drug.

To facilitate the identification of genes having differing contributions to resistance levels and those occurring at different temporal stages as resistance emerged (12), the isolates from aztreonam-exposed patients were stratified based on their quantitative resistance phenotypes: 12 with low-level MICs (≤32 μg/ml), 25 with medium-level MICs between 64 μg/ml and 256 μg/ml, and 11 with highly resistant phenotypes (MICs ≥ 512 μg/ml).

Specific *P. aeruginosa* genes are under positive selection during inhaled aztreonam exposure. We identified *de novo* mutations arising in the antibiotic-resistant member of each isolate pair in order to assess frequently mutated genes that exhibited positive selection associated with aztreonam exposure. We identified 16 candidate resistance genes (Table 2) in one or more groups of isolates as stratified by aztreonam resistance level and for which nonsynonymous mutations were specifically enriched in bacteria that were exposed to aztreonam ($P \leq 0.038$). Multiple alignments were subsequently created for each candidate gene to explore the distribution of amino acid changes observed.

Three genes, *ftsI*, *ampC*, and *gyrA*, carried recurrent missense mutations, consistent with gain-of-function changes.

Penicillin binding protein 3 (PBP3; encoded by *ftsI*), which is the target of aztreonam, was the most frequently mutated target in our study. Mutations were identified in 23/60 (38.33%) isolate pairs and were most prevalent in the high-resistance group (8/11 isolates [72.73%]). We observed 25 unique missense mutations in *ftsI* (Fig. 1A), with affected isolates carrying a range of 1 to 4 mutations each. Two variants (A244T and R504C) were recurrently identified in more than one isolate. Five variants (A244T, R504C, P527S, H394R, and F533L) have been reported in other studies of CF clinical isolates (22, 23), three of which (A244T, R504C, and P527S) also resulted from our previous *in vitro* selection for aztreonam resistance (12). The majority of mutations (18/24) fell within the protein's transpeptidase domain (residues 225 to 579) (24).

Mutations in β-lactamase *ampC* were strongly associated with highly drug-resistant phenotypes, occurring only in isolates exposed to aztreonam having MICs >64 μg/ml. A total of eight unique *de novo ampC* mutations (Q174R, Q152R, Q157R, V239A, G242S, G242_P243insG, D245G, and N347S) were cataloged across 11 different alleles, with a range of 1 to 4 mutations per isolate (Fig. 1B). Three mutations (V239A, G242S, and N347S) occurred in multiple isolates. Only one mutation, Q157R, was previously associated with altered β-lactam substrate specificity in *P. aeruginosa* (25), with the remaining seven being newly reported here.

Recurrent variants in DNA gyrase gene *gyrA* were also common in the isolate collection; however, all of the variants that were identified (T83I, T83A, and D87N) have been shown to cause fluoroquinolone resistance in *P. aeruginosa* (26). This suggests that the association of *gyrA* with aztreonam in our study is likely spurious and reflects uncontrolled differences in other antibiotic exposures that have occurred between patient groups.

Mutations in the remaining 13 genes were sporadic (*mexA*, *oprD*, *opdJ*, *pvdS*, *sbcC*, *ispB*, *PA1866*, *PA2480*, *PA2557*, and *PA4681*) and/or nonsense (*oprD*, *PA4681*, and *ERW19588*), making them more likely to represent loss-of-function changes.

Transposon mutagenesis functionally validates genes associated with aztreonam resistance *in vivo*. Because the majority of genes implicated by our study were likely to be affected by loss-of-function mutations, we initially determined whether a disruption of candidate genes could lead to increased aztreonam resistance. We evaluated aztreonam sensitivities of available transposon mutants in 14 candidate genes and included bidirectional pairs of transposon disruptions where possible (27). Two genes, DNA gyrase subunit *gyrA* and one encoding a hypothetical protein (Q034_02961), were not available as transposon mutants, due to essentiality and absence from the MPAO1 genome, respectively. Due to variability in the measured susceptibilities of several mutants, MIC testing was

TABLE 2 Genes positively selected during aztreonam exposure

			% isolates with nonsynonymous mutations					dN/dS ratio (decimal)					
Locus name	Common gene name	Predicted gene function	No exposure group (n = 12)	Low resistance group (n = 12)	Medium resistance group (n = 25)	High resistance group (n = 11)	All isolates exposed to aztreonam (n = 48)	No exposure group (n = 12)	Low resistance group (n = 12)	Medium resistance group (n = 25)	High resistance group (n = 11)	All isolates exposed to aztreonam (n = 48)	Significance of dN/dS relative to no exposure group (P value)
PA4110	*ampC*	β-Lactamase precursor	0.00	0.00	28.00	63.64	29.17	0/0 (UND[a])	0/0 (UND)	7/0 (UND)	13/0 (UND)	20/0 (UND)	6.20×10^{-5}
ERW19588		Hypothetical protein	0.00	0.00	24.00	18.18	16.67	0/0 (UND)	0/1 (0)	6/0 (UND)	2/0 (UND)	8/1 (8)	1.80×10^{-2}
PA4418	*ftsI*	Penicillin binding protein 3	8.33	16.67	48.00	72.73	45.83	1/0 (UND)	2/2 (1)	18/0 (UND)	15/1 (15)	35/3 (11.67)	9.68×10^{-3}
PA3168	*gyrA*	DNA gyrase subunit A	8.33	50.00	32.00	54.55	41.67	1/0 (UND)	9/1 (9)	9/2 (4.5)	12/1 (12)	30/4 (7.5)	1.98×10^{-2}
PA4569	*ispB*	Octaprenyl diphosphate synthase	0.00	16.67	4.00	36.36	14.58	0/0 (UND)	2/0 (UND)	1/0 (UND)	4/0 (UND)	7/0 (UND)	6.78×10^{-3}
PA0425	*mexA*	Multidrug resistance protein MexA	0.00	16.67	16.00	45.45	22.92	0/0 (UND)	4/0 (UND)	4/0 (UND)	6/0 (UND)	14/0 (UND)	5.02×10^{-4}
PA2420	*opdJ*	Outer membrane porin OpdJ	0.00	8.33	24.00	9.09	16.67	0/0 (UND)	1/1 (1)	7/0 (UND)	1/0 (UND)	9/1 (9)	6.78×10^{-3}
PA0958	*oprD*	Outer membrane porin OprD	8.33	58.33	32.00	27.27	37.50	1/0 (UND)	9/0 (UND)	10/1 (10)	3/0 (UND)	22/1 (22)	1.88×10^{-2}
PA0847		Diguanylate cyclase	0.00	8.33	24.00	0.00	14.58	0/0 (UND)	1/0 (UND)	9/0 (UND)	0/1 (0)	10/1 (10)	3.24×10^{-2}
PA1866		Hypothetical protein	0.00	8.33	28.00	9.09	18.75	0/1 (0)	1/0 (UND)	8/2 (4)	2/1 (2)	11/3 (3.67)	3.82×10^{-2}
PA2480		Probable two-component sensor	0.00	0.00	16.00	36.36	16.67	0/0 (UND)	0/0 (UND)	4/1 (4)	4/0 (UND)	8/1 (8)	6.78×10^{-3}
PA2557		Probable AMP-binding enzyme	0.00	8.33	20.00	36.36	20.83	0/0 (UND)	1/0 (UND)	7/0 (UND)	4/0 (UND)	12/0 (UND)	9.79×10^{-4}
PA4681		Hypothetical protein	0.00	0.00	24.00	18.18	16.67	0/0 (UND)	0/0 (UND)	7/0 (UND)	2/0 (UND)	9/0 (UND)	3.59×10^{-3}
PA1798	*parS*	Two component sensor ParS	0.00	8.33	24.00	27.27	20.83	0/1 (0)	1/2 (0.5)	7/0 (UND)	3/0 (UND)	11/2 (5.5)	1.60×10^{-2}
PA2426	*pvdS*	Sigma factor PvdS	0.00	16.67	28.00	45.45	29.17	0/0 (UND)	2/0 (UND)	9/0 (UND)	6/0 (UND)	17/0 (UND)	6.20×10^{-5}
PA4282	*sbcC*	Probable exonuclease	0.00	8.33	24.00	18.18	18.75	0/0 (UND)	1/0 (UND)	8/1 (8)	2/0 (UND)	11/1 (11)	9.89×10^{-3}

[a]UND, undefined, due to an absence of synonymous mutations.

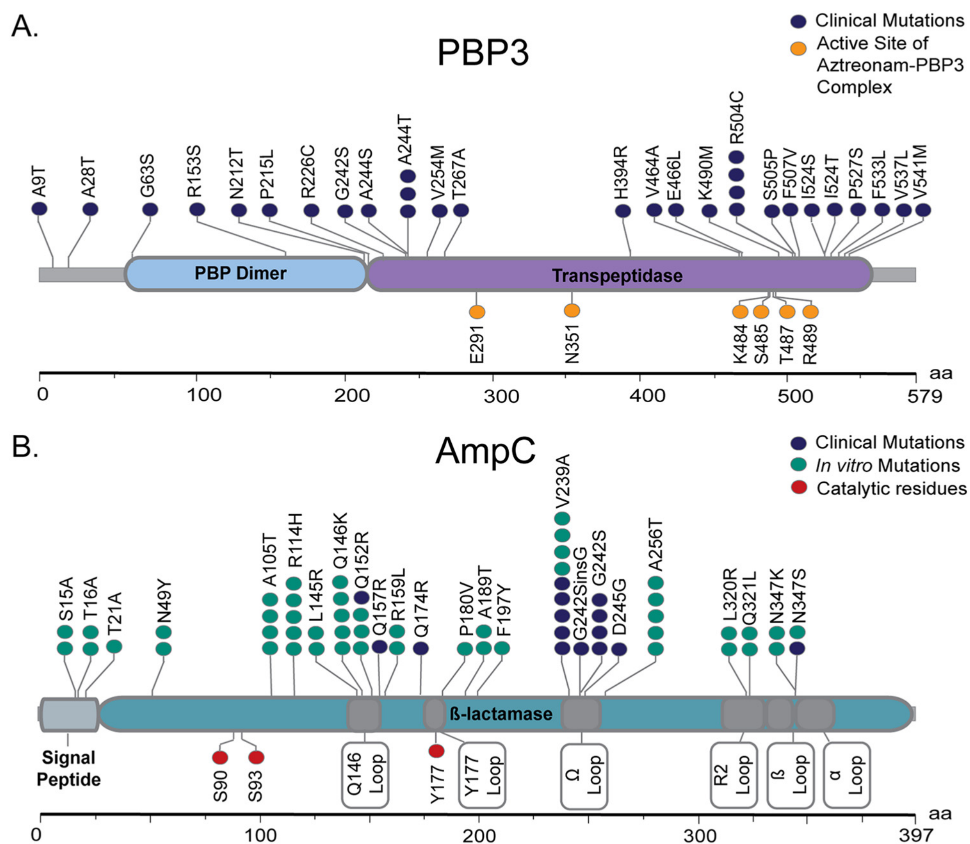

FIG 1 *De novo* mutations in proteins associated with aztreonam resistance. Mutations are displayed as lolliplots for PBP3 (A) and AmpC (B). Amino acid position is indicated along the *x* axis, and protein domains are shown as labeled, colored regions. Subregions in AmpC are indicated by dark gray shading, with labels shown at the bottom. The location and abundance of specific amino acid mutations are indicated by colored circles.

performed in replicates and expressed as the average from at least three separate experiments.

All mutants demonstrated statistically significant (Student's 2-tailed *t* test, $P \leq 0.013$) changes in MIC relative to those for the parental MPAO1 strain and a physiologically neutral transposon control (12) (Table 3). Transposon mutants in 13 of the 14 genes showed increases in MICs from 1.5- to 5.9-fold, while transposon mutants for *mexA* were hypersensitive to aztreonam. Two genes, *sbcC* and *parS*, resulted in measurably dissimilar MICs among two different bidirectional transposon mutants, potentially reflecting polar effects.

Different aztreonam resistance genes are under selection *in vivo* and *in vitro*. We previously identified 15 aztreonam resistance genes that were recurrently mutated in isolates passaged for antibiotic resistance *in vitro* and which were subsequently experimentally validated (12), of which only two (*ampC* and *ftsI*) were also recovered through our present analysis of *in vivo* isolate pairs. Examination of the 13 disparate genes revealed three different reasons for their not being identified as significant in this study (see Table S1 and Data Set S1). *mexR* and *mexT*, relevant to *mexAB-oprM* and *mexEF-oprN* efflux pump function, respectively, were under positive selection *in vivo* in isolates from aztreonam-exposed pateints, but *de novo* mutations did not occur in a large enough fraction of clinical isolates to pass our filtering criteria. Eight other genes (*nalD*, *mexF*, *mpl*, *clpA*, *pgi*, *dacB*, *pepA*, and *PA3206*) were under positive selection in CF clinical isolates regardless of aztreonam exposure, and their mutation was therefore not specific to aztreonam resistance. The last seven genes (*phoQ*, *aroB*, *nalC*, *clpS*, *atpA*, *atpD*, and *orfN*) were not under positive selection in clinical isolates from aztreonam-exposed patients.

TABLE 3 Transposon mutant MICs

Strain	Disrupted locus	Common name of disrupted gene	MIC (μg/ml)		Fold increase over transposon control	Significance of MIC difference relative to transposon control (*P* value)
			Average	SEM		
MPAO1 (parental strain)			1.31	0.13	0.96	7.85×10^{-1}
PW3303 (transposon control)	PA1274		1.36	0.15		
PW5294	PA2557		4.00	0.00	2.93	8.66×10^{-9}
PW5293	PA2557		3.33	0.42	2.44	4.04×10^{-3}
PW5085	PA2426	*pvdS*	4.00	0.00	2.93	8.66×10^{-9}
PW2543	PA0847		3.80	0.55	2.79	1.58×10^{-3}
PW2544	PA0847		4.00	0.00	2.93	8.66×10^{-9}
PW8212	PA4282	*sbcC*	3.82	0.18	2.80	2.42×10^{-9}
PW8213	PA4282	*sbcC*	2.00	0.00	1.47	1.88×10^{-3}
PW5076	PA2420	*opdJ*	4.00	0.00	2.93	8.66×10^{-9}
PW8445	PA4418	*ftsI*	3.14	0.40	2.30	3.60×10^{-3}
PW4274	PA1866		4.00	0.47	2.93	1.64×10^{-4}
PW4275	PA1866		4.36	0.36	3.20	3.20×10^{-6}
PW5165	PA2480		3.43	0.37	2.51	8.23×10^{-4}
PW5164	PA2480		4.00	0.00	2.93	8.66×10^{-9}
PW2742	PA0958	*oprD*	4.00	0.00	2.93	8.66×10^{-9}
PW4165	PA1798	*parS*	4.00	0.47	2.93	1.64×10^{-4}
PW4164	PA1798	*parS*	8.00	0.00	5.87	9.62×10^{-13}
PW7954	PA4110	*ampC*	3.14	0.40	2.30	3.60×10^{-3}
PW7953	PA4110	*ampC*	4.00	0.00	2.93	8.66×10^{-9}
PW1778	PA0425	*mexA*	≤0.25	0.00	0.18	2.54×10^{-5}
PW1779	PA0425	*mexA*	≤0.25	0.00	0.18	2.54×10^{-5}
PW8689	PA4569	*ispB*	2.00	0.00	1.47	1.88×10^{-3}
PW8881	PA4681		4.33	0.80	3.18	1.33×10^{-2}
PW8880	PA4681		4.00	0.00	2.93	8.66×10^{-9}

The differences in resistance genes that were mutated during selection *in vitro* and *in vivo* could reflect disparities in bacterial fitness incurred under those different conditions. To more fully explore whether separate aztreonam resistance mutations are favored *in vitro* and *in vivo*, we performed transposon sequencing (Tn-Seq) (28) of a comprehensive *P. aeruginosa* mutant library that we selected for increased antibiotic resistance. Likely owing to the small changes in aztreonam MIC conferred by individual transposon mutants (12), only 12 mutants were found to be significantly enriched on aztreonam-containing medium relative to the unselected transposon pool (see Table S2). Five of the genes (*mexT*, *mexR*, *nalD*, *dacB*, and *pepA*) were recurrently mutated during *in vitro* passaging for aztreonam resistance in our previous study (12), whereas no genes identified as under positive selection in clinical isolates were recovered as significant by Tn-Seq. These findings are consistent with gene inactivation events conferring the greatest aztreonam resistance *in vitro* having a fitness cost or otherwise being unfavorable *in vivo*.

Chromosomal *ampC* mutations promote aztreonam resistance. To assess the activity of mutant *ampC* alleles identified in aztreonam-selected *P. aeruginosa*, each was cloned into an expression shuttle vector and transformed into *Escherichia coli*. MICs were assessed using aztreonam and other structurally distinct β-lactam antibiotics (Table 4). Ten alleles conferred 2- to 64-fold increases in aztreonam resistance, while one (allele 8) had an MIC equivalent to that for the empty plasmid vector. The alleles conferred variable levels of resistance to the other β-lactam antibiotics tested. None provided increased resistance to meropenem, consistent with the low activity of wild-type *P. aeruginosa ampC* with that substrate (29). One allele (allele 3) conferred a 2-fold increase in imipenem resistance. Five alleles showing increased resistance to aztreonam resulted in decreases in ampicillin resistance, and nine provided attendant gains in ceftazidime resistance. Remarkably, the allele providing the highest levels of aztreonam resistance (allele 6) also conferred the highest levels of resistance to ampicillin, ceftazidime, and cefpirome.

Similar resistance patterns were observed after expression shuttle vectors were transformed into a *P. aeruginosa* background from which chromosomal *ampC* had been

TABLE 4 MICs of *ampC* alleles from clinical isolates

		MIC in μg/ml (fold change relative to WT AmpC) of:											
		E. coli DH5α						Δ*ampC* MPAO1					
ampC allele	AmpC amino acid substitutions[a]	Aztreonam	Ampicillin	Ceftazidime	Cefpirome	Meropenem	Imipenem	Aztreonam	Ampicillin	Ceftazidime	Cefpirome	Meropenem	Imipenem
None	Empty vector	0.0625	2	0.125	0.0078125	0.0625	1	2	64	1	1	4	1
Wild type	None	0.25	64	0.5	0.015625	0.0625	1	16	1,024	16	8	4	4
1	P7S, T105A, **G242S**, **N347S**	1 (4)	32 (0.5)	2 (4)	0.015625 (1)	0.0625 (1)	1 (1)	128 (8)	2,048 (2)	128 (8)	16 (2)	4 (1)	2 (0.5)
2	G27D, R79Q, T105A, **G242S**	0.5 (2)	32 (0.5)	0.5 (1)	0.0078125 (0.5)	0.0625 (1)	1 (1)	64 (4)	2,048 (2)	64 (4)	16 (2)	4 (1)	2 (0.5)
3	T105A, **G242S**	0.5 (2)	16 (0.25)	1 (2)	0.015625 (1)	0.0625 (1)	2 (2)	32 (2)	2,048 (2)	64 (4)	16 (2)	4 (1)	2 (0.5)
4	R79Q, T105A, **Q152R**, **Q157R**, **V239A**, **N347S**	4 (16)	32 (0.5)	4 (8)	0.015625 (1)	0.0625 (1)	0.5 (0.5)	256 (16)	2,048 (2)	256 (16)	32 (4)	4 (1)	4 (1)
5	T105A, **G242_P243insG**	0.5 (2)	32 (0.5)	2 (4)	0.015625 (1)	0.0625 (1)	1 (1)	32 (2)	2,048 (2)	64 (4)	32 (4)	4 (1)	2 (0.5)
6	R79Q, T105A, **V239A**, **D245G**	16 (64)	256 (4)	16 (32)	0.0625 (4)	0.0625 (1)	1 (1)	256 (16)	2,048 (2)	256 (16)	32 (4)	4 (1)	2 (0.5)
7	T105A, L176R, **V239A**	1 (4)	64 (1)	2 (4)	0.015625 (1)	0.0625 (1)	1 (1)	32 (2)	2,048 (2)	64 (4)	16 (2)	4 (1)	2 (0.5)
8	T21A, T105A, **Q174R**, G391A	0.0625 (0.25)	2 (0.03125)	0.125 (0.25)	0.0078125 (0.5)	0.0625 (1)	1 (1)	2 (0.125)	32 (0.03125)	2 (0.125)	1 (0.125)	4 (1)	2 (0.5)
9	R79Q, T105A, **V239A**, P274L	0.5 (2)	64 (1)	1 (2)	0.015625 (1)	0.0625 (1)	1 (1)	32 (2)	2,048 (2)	128 (8)	16 (2)	4 (1)	4 (1)
10	T105A, **V239A**	1 (4)	128 (2)	1 (2)	0.015625 (1)	0.0625 (1)	1 (1)	64 (4)	2,048 (2)	64 (4)	16 (2)	4 (1)	4 (1)
11	R79Q, T105A, **G242S**, V356I	0.5 (2)	64 (1)	1 (2)	0.015625 (1)	0.0625 (1)	1 (1)	32 (2)	1,024 (1)	64 (4)	8 (1)	4 (1)	4 (1)

[a]Boldface font indicates *de novo* mutation.

TABLE 5 MICs of artificially evolved *ampC* alleles

Selection replicate	Allele	AmpC mutations	MIC in μg/ml (fold change from WT control) of:					
			Aztreonam	Ampicillin	Ceftazidime	Cefpirome	Meropenem	Imipenem
None	Wild type		0.125	32	0.25	0.03125	0.0625	1
1	A	S15A, T16A, N49Y, R114H, Q146K, Q152R, F197Y, V239A, A256T, Q321L, N347K	64 (512)	256 (8)	256 (1,024)	0.125 (4)	0.0625 (1)	1 (1)
1	B	S15A, T16A, N49Y, A105T, R114H, L145R, Q146K, Q152R, V239A, A256T, Q321L, N347K	64 (512)	32 (1)	8 (32)	0.0625 (2)	0.0625 (1)	1 (1)
2	A	A105T, R114H, Q146K, Q152R, R159L, V239A, A256T, L320R, Q321L, N347K	64 (512)	128 (4)	32 (125)	0.03125 (1)	0.125 (2)	1 (1)
2	B	T21A, A105T, R114H, Q146K, R159L, A189T, V239A, A256T, L320R	128 (1,024)	512 (9)	64 (256)	0.0625 (2)	0.0625 (1)	1 (1)
3	A	A105T, R114H, L145R, Q146K, P180V, A256T, N347S	64 (512)	64 (2)	16 (64)	0.03125 (1)	0.125 (2)	1 (1)

ablated by transposon mutagenesis (27). Resistance levels for all drugs tested were higher than seen in *E. coli*, indicating greater basal resistance levels in *P. aeruginosa*. Consistent with its performance in *E. coli*, allele 8 conferred no increase in resistance to any of the antibiotics tested. Six alleles provided equivalent or enhanced resistance to ampicillin, ceftazidime, and cefpirome, which was not observed in *E. coli*. These findings indicate that higher resistance levels across multiple drugs can be achieved when expressing mutant *P. aeruginosa ampC* alleles in their species of origin. Somewhat contrarily, relative increases in resistance which were apparent in an *E. coli* background were not seen after transfer to *P. aeruginosa*; however, this may reflect limited ability of 2-fold serial dilution MIC testing to resolve subtle differences between resistance phenotypes occurring at high antibiotic concentrations. All alleles tested exhibited equal or reduced resistance to imipenem relative to the wild type when expressed in *P. aeruginosa*.

Although genomic analysis did not identify regulators of *ampC* expression as being under positive selection during aztreonam therapy, focused analysis of relevant genes (*dacB*, *ampD*, *ampR*, and *mpl*) identified nonsynonymous changes for multiple isolates in both the aztreonam exposure and control treatment groups (Data Set S1). As such, mutational upregulation of *ampC* may contribute to aztreonam resistance in a subset of cases, albeit by processes which are not specifically selected during aztreonam exposure.

Artificial evolution of *ampC* reveals its evolutionary potential for elevated aztreonam hydrolysis. To more fully evaluate the evolutionary potential for *ampC* to confer aztreonam resistance, we lastly performed artificial evolution of that gene using cycles of mutagenic PCR, library cloning in *E. coli*, and selection of resultant populations against increasing concentrations of aztreonam. Bacterial growth at the highest drug concentration was harvested for plasmid and used as the template for the next round of mutagenesis. Three separate evolutionary replicates were generated in parallel. Seven to nine rounds of selection were performed before aztreonam resistance levels plateaued, after which multiple individual colonies were isolated and subjected to formal MIC analysis. Isolates from the same replicates with different aztreonam resistance phenotypes were considered to potentially harbor different alleles, and mutations were catalogued by sequencing.

Evolved populations proved largely homogeneous, with a total of five different alleles carrying a total of 19 different mutations identified. Artificially evolved alleles carried a range of 7 to 11 point mutations (Fig. 1B) and conferred between 512- to 1,024-fold increases in aztreonam resistance relative to the wild-type gene (Table 5). Moreover, all alleles either maintained original resistance levels or demonstrated increased resistance to ampicillin, ceftazidime, imipenem, and/or cefpirome. Alleles 2A and 3A uniquely showed modest 2-fold increases in meropenem resistance.

DISCUSSION

It is challenging to study bacterial adaptation to antibiotics *in vivo* during chronic infection, because the invading organism concurrently accumulates numerous other changes which promote its survival in the host environment (30–32). Moreover, alterations to metabolic networks, including loss of nonessential metabolic functions (30), may result from selection for increased pathogenic fitness but incidentally impact antibiotic susceptibilities (33). Here, we have attempted to disambiguate *de novo* genomic changes in *P. aeruginosa* that are selected in response to inhaled aztreonam therapy from those more generally promoting fitness in the CF patient airway by identifying genes that are specifically under positive selection in patients treated with the drug. This strategy has revealed both known and previously unappreciated genes which promote aztreonam resistance in *P. aeruginosa*.

Sixteen genes, only three of which (*ftsI*, *ampC*, and *mexA*) were previously associated with aztreonam resistance (13–18), were implicated by our study (Table 2). Thirteen of these subsequently tested using transposon mutants to model gene inactivation events were functionally validated as conferring modest but statistically significant elevations in aztreonam resistance (Table 3). These resistance phenotypes were qualitatively consistent with our prior study, where the slight gains in resistance accompanying mutation of individual genes exerted additive effects when occurring multiply in the same strain (12).

Mutations in *ftsI* were most common, reinforcing the idea that altered aztreonam target binding is a frequent mechanism underlying drug resistance (12, 34). *ftsI* encodes the only essential penicillin binding protein in *P. aeruginosa*, and deleterious mutations in the gene result in growth defects (35). Though an increase in aztreonam resistance was seen for an *ftsI* transposon mutant (Table 3), which contained an insertion at the end of the transpeptidase domain and therefore likely imparts a partial loss of function (36), point mutations may confer enhanced resistance while less dramatically impacting cell physiology. Specific, recurrent *ftsI* missense mutations were identified from aztreonam-resistant *P. aeruginosa* isolates in this study (Fig. 1A) and our prior work, suggesting that particular regions of PBP3 affect the resistance phenotype. PBP3 contains two functional domains, the N-terminal (residues 1 to 221) and the C-terminal transpeptidase domains (residues 222 to 579) (36). The vast majority (19 of 25) of *de novo* mutations identified *in vivo*, and all 11 variants recovered from our *in vitro* evolution experiments, occurred within the transpeptidase domain (12), with 9 of them being localized to the enzyme binding pocket surrounding the PBP3 active site (24, 37). The R504C binding pocket mutation was most commonly identified in our study and has been reported in CF isolates with reduced aztreonam susceptibility (12, 22, 23). Variants at that residue have also been linked with resistance to carbapenems (38) and ceftazidime (39). Two additional binding pocket variants (P527T and F533L) have also been observed in CF clinical isolates with reduced aztreonam susceptibility (12, 22, 23). All variants within this domain are likely to influence binding pocket conformation and disrupt aztreonam binding. The newly identified binding pocket mutations (K490M, S505P, F507V, I524S, and I524T) likely affect resistance by the same mechanism. Mutations elsewhere in the transpeptidase domain have been observed in CF isolates, including two specific changes found by our study (A244T and H394R) (23), and may indirectly influence the structure of the binding pocket. The remaining PBP3 mutations (A9T, A28T, G63S, R153S, N212T, and P215L) localized to the N-terminal domain. The N-terminal domain is thought to play an accessory role in folding and stability of the transpeptidase (37), and so mutations within it could similarly influence conformation of the enzyme binding pocket.

Loss-of-function mutations in two membrane porins, OprD and OpdJ, were newly identified as mechanisms promoting aztreonam resistance, likely by decreasing outer membrane permeability to the antibiotic (40). OprD has long been recognized as a porin thought to be exclusive for the entry of carbapenem antibiotics, and loss of OprD expression is accordingly correlated with increased resistance to drugs from that class

without reportedly impacting susceptibilities to other β-lactam drugs (13, 40–43). Our findings contrarily show that OprD loss promotes increased resistance to aztreonam (Table 3), indicating an additional role in aztreonam uptake. OpdJ has been identified as a probable specific outer membrane protein based on weak homology to OprD (44, 45), but the protein has been studied far less extensively and its function has not yet been experimentally elucidated. We have found that OpdJ disruption results in an aztreonam resistance phenotype equivalent to that seen with OprD loss, consistent with the two proteins having similar functional roles.

PvdS is an alternative sigma factor that serves as the major iron starvation factor of *P. aeruginosa* and regulates the expression of at least 26 genes or operons, including virulence factors and genes otherwise unrelated to iron metabolism (46–49). Although our study identifies a link between loss of PvdS activity and elevated aztreonam resistance, disruption of that transcriptional regulator could impart profound changes to metabolism and phenotype, making it difficult to ascertain the specific mechanism by which this phenotype is affected.

The role of MexAB-OprM in the active efflux of aztreonam has been well described (13–15), and we previously observed overexpression of this system in isolates selected for aztreonam resistance *in vitro* secondary to inactivation of negative regulators NalD, MexR, and NalC (12). Although these negative regulators did not harbor *de novo* variants and were not under significant positive selection in clinical isolates from aztreonam-exposed patients, we found that the multidrug efflux membrane subunit *mexA* was. Although loss of MexA resulted in aztreonam hypersensitivity (Table 3), observed mutations were not recurrent or spatially restricted to specific regions of the protein, making them less likely to fit the profile of gain-of-function changes. Though these results appear paradoxical, they are consistent with prior analyses of CF clinical isolates which have shown frequent missense and loss-of-function mutations in MexA (22, 50). Given the correlation of MexAB-OprM overproduction with increased virulence (12), it is possible that inactivating *mexA* mutations reflect selection for attenuation during aztreonam therapy (51) rather than increased resistance to aztreonam itself. Alternatively, it is known that loss of MexA promotes expression of the MexXY-OprM efflux system (22, 52), which has distinct antibiotic specificities and may therefore be selected in response to other antibiotics administered to these patients.

Four genes likely impacting gene regulation were under positive selection during aztreonam exposure. Two separate two-component sensors, *parS* and *PA2480*, were identified. *parS* encodes the sensor kinase of the *P. aeruginosa* ParRS regulatory system. It is reported that induction or mutational activation of this system increases resistance to multiple drugs by affecting *oprD* repression, efflux system activation, and lipopolysaccharide modification (53, 54). Our study indicates that loss of *parS*, rather than its activation, can increase resistance to aztreonam, arguing that it has additional functions in regulating alternative aztreonam resistance pathways. PA2480 is a putative two-component sensor based on protein sequence homology, but its role and regulatory targets are currently undescribed. The remaining two proteins, PA2557, a hypothetical AMP-binding enzyme, and PA0847, a diguanylate cyclase, also have possible roles in regulating gene expression (55, 56). However, without experimentally testing the function of these genes, it is difficult to hypothesize through which pathways they promote aztreonam resistance.

Two genes, *PA1866* and *PA4681*, encode hypothetical proteins without notable homology to better-described factors in *Pseudomonas* or other organisms. As such, the role that these genes may play in antibiotic resistance is opaque but they will serve as interesting targets for future studies. A third gene, *sbcC*, has been well studied in *Escherichia coli*, where it is believed to serve as in the exonuclease cleavage of hairpin DNA (57), which also has an unclear relationship to aztreonam resistance.

We placed particular emphasis on the potential for the final gene, the *ampC* β-lactamase, to confer aztreonam resistance. Although aztreonam is known to be poorly hydrolyzed by *P. aeruginosa* chromosomal *ampC* (58), mutations which increase the enzyme's activity against aztreonam are concerning, because resistance-causing alleles

could be mobilized to plasmids and rapidly disseminated through a population (59). Unexpectedly, but consistent with earlier findings(12), two separate transposon mutants ablating *ampC* function resulted in minor gains in aztreonam resistance, possibly by reducing the metabolic burden of its expression (58). Regardless, more than one-quarter of aztreonam-exposed strains (14 of 48) carried *de novo* mutations in *ampC*, which were entirely absent in *P. aeruginosa* from patients without aztreonam exposure. Multiple recurrent *ampC* mutations were identified in this and our prior study(12), suggesting gain-of-function effects which could impart aztreonam resistance either through increased gene expression (17, 60) or by modifying the structure and function of the enzyme itself (12, 18).

Mutant alleles from clinical isolates harbored 8 unique *de novo* mutations (Fig. 1B), only two of which (Q157R and Q174R) were previously reported (25). It is probable that these variants affect enzyme substrate specificity or hydrolytic activity given their placement relative to the functional domains of *ampC* (61). The catalytic residues of *ampC* comprise Ser90-Ser93-Tyr177 (62), with boundaries of the larger active site defined by the Gln146 loop (residues 143 to 154), Tyr177 loop (residues 176 to 179), Ω-loop (residues 238 to 252), R2-loop (residues 315 to 333), β11 (residues 338 to 346), and α11 (residues 373 to 390) (Fig. 1B) (61). Five of the eight *de novo* mutations (Q152R, V239A, G242S, G242_P243insG, and D245G) mapped within the active site and could directly impact catalytic activity. Prior work has shown that mutations of the Ω-loop, in particular, can impart greater catalytic abilities for specific drugs (61, 63), and half of the observed mutations reside in this domain. The other three mutations (Q157R, Q174R, and N347S) were immediately adjacent to the active site and could plausibly affect its conformation.

In vitro evolution revealed the evolutionary potential of *ampC* to confer high levels of aztreonam resistance while maintaining or increasing activity against other β-lactam antibiotics. Artificially evolved alleles conferred greater levels of aztreonam resistance (512- to 1,024-fold increases) than those from clinical isolates (2- to 12-fold increases) (Table 4 and 5), although the total mutational burden per evolved allele was also correspondingly higher (range of 7 to 11 mutations per allele, versus a maximum of 3 mutations) (Fig. 1B). Three artificially evolved mutations (T21A, V239A, and N347S) were also identified in clinical alleles, suggesting advantages *in vivo* and *in vitro*. Six of the 19 mutations recovered (L145R, Q146K, Q152R, V239A, L320R, and Q321L) occurred within the AmpC active site and likely altered the enzyme's specificity, while four others (R159L, P180V, N347K, and N347S) were immediately adjacent and could exert similar effects. The functional impact of the remaining nine mutations, if any, is presently unclear. Interestingly, three changes (R114H, Q146K, and A256T) that were universally present among the evolved alleles were not seen in clinical isolates, and only one of these (Q146K) mapped to an active site domain.

Surprisingly, relatively few genes identified as under positive selection for aztreonam resistance *in vivo* were previously implicated through *in vitro* selections for resistance to that drug (12), with only *ampC* and *ftsI* being identified across both studies (see Table S1 in the supplemental material). Focused analysis of the resistance genes identified from *in vitro* studies in the paired clinical strains revealed three separate reasons for this discrepancy. First, two genes known to affect resistance by efflux pump regulation were identified as under positive selection in aztreonam exposed isolates *in vivo*, but too few isolates carried mutations in those regulators to meet our selection criteria. Second, some genes that promote aztreonam resistance *in vitro* were not under positive selection in *in vivo* isolates. It is likely that such genes incur unacceptable fitness costs to mutant bacteria within CF patient lungs. Thus, the spectrum of possible aztreonam resistance mutations arising in clinical practice is likely to be more constrained than the constellation of possible mutations recovered during growth in rich media *in vitro*. Although the changes in aztreonam MIC conferred by individual transposon mutants has proven generally small (12), this conclusion is supported by our Tn-seq experiments (Table S2), which exclusively overlap the output of *in vitro* selection. Lastly, some resistance genes identified from *in vitro* passaging were under

positive selection in clinical isolates, but this selection was equivalent whether isolates were exposed to aztreonam or not. This indicates that such genes are generally advantageous to *P. aeruginosa* during CF infection and shows that multiple mutations arising spontaneously during pathogenesis can promote aztreonam resistance even in the absence of that drug. Indeed, up to 64-fold differences in aztreonam resistance were observed in clinical isolate pairs from individuals who were not exposed to inhaled aztreonam therapy, and several genes identified from our study are reported as being highly mutated in isolates from CF patients (50) or to generally contribute to the *P. aeruginosa* resistome (22). Taken together, these findings highlight the importance of examining clinical isolates from antibiotic-treated patients when identifying resistance genes that are relevant *in vivo*.

In summary, leveraging signatures of positive selection specific to bacterial isolates exposed to inhaled aztreonam therapy *in vivo*, this work has ascertained novel resistance determinants underlying that polygenic trait. We identify *ftsI* and *ampC* mutations as being of particular importance and have demonstrated the potential for *ampC* to evolve high-level activity against aztreonam without sacrificing conferred resistance to other β-lactam agents. Nevertheless, we acknowledge several limitations of our study design. The patient groups used for analysis in this study were not perfectly controlled, either with respect to size or clinical characteristics, and systemic antibiotic exposures were not recorded, likely resulting in some spurious associations. For example, the *gyrA* mutations identified in this work are likely to be artifactual, reflecting differences in administration of fluoroquinolone agents between study populations instead of being related to aztreonam exposure. Additionally, the number or courses of aztreonam inhalation therapy in the exposed population were variable, and the timing of isolate collection in relation to drug exposure is unknown, both of which may influence resistance selection pressures at the time of isolate collection. Dedicated investigation of genes found to be mutated in posttherapy strains will be required to characterize their roles in aztreonam resistance. Finally, because we have focused on identifying the most commonly mutated aztreonam resistance factors, there are likely to be additional chromosomal resistance genes that currently remain uncharacterized.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and MIC testing. Clinical *P. aeruginosa* isolates from patient sputa collected during the AIR-CF5 clinical trial (21) (ClinicalTrials identifier NCT01375036) were provided by Gilead Sciences, Inc., as was associated information on antibiotic sensitivities. Per the clinical trial protocol, patients were classified as being exposed to inhaled aztreonam if they received one or more courses of therapy within 12 months prior to respiratory cultures. All patients in the nonexposure group had never been exposed to inhaled aztreonam. A total of 60 paired isolates (120 total isolates) which exhibited 4-fold or greater changes in aztreonam MIC were ultimately selected for analysis.

All individual *P. aeruginosa* transposon mutants (36) were previously described. Electrocompetent *E. coli* DH5α and 10-β were purchased from New England BioLabs. *E. coli* λ-pir (64) was a generous gift from Pradeep Singh at the University of Washington.

All strains were maintained at 37°C in Luria-Bertani (LB) broth under selection with the appropriate antibiotic, if required, for plasmid maintenance.

Liquid MIC determination was performed according to CLSI guidelines (65), except that LB broth was used and plasmid-bearing strains were induced with 3 mM IPTG (isopropyl-β-D-thiogalactopyranoside; Thermo Scientific), where appropriate.

Microbial sequencing. DNA was extracted using a DNeasy UltraClean Microbial DNA Isolation kit (Qiagen), and sequencing libraries were prepared as described previously (66, 67). Sequencing was performed using Illumina NextSeq 500 and MiSeq platforms with 150-bp paired-end chemistries.

Transposon sequence analysis. Transposon mutant pool generation and sequence analysis (Tn-seq) was performed as described previously (28, 36) using aztreonam-containing medium for selection. Briefly, a pool of >110,000 unique IS*lacZ-hah*-tc transposon insertion mutants in the MPAO1 genome were grown on rich medium and cryopreserved in dimethyl sulfoxide (DMSO). The mutant pool was washed in Dulbecco's phosphate-buffered saline (DPBS) and recovered for 90 min in LB prior to testing on aztreonam. Mutants with enhanced aztreonam resistance were selected on 1, 2, and 4 μg/ml aztreonam-LB plates. For each concentration tested, 100 μl of the recovered transposon pool was diluted to approximately 6×10^6 CFU per 10-cm plate and incubated for 15 h. Isolates were then harvested and libraries prepared using the Tn-seq C-tailing method (28) with primers specific for transposon T8. Transposon insertions per gene were normalized based on average gene size, total number of mapped reads per sequencing run, and the read density of the Tn insertion region. After normalization, genes with a >1.5-fold increase in

transposon insertion events detected on each concentration of aztreonam-containing medium relative to the parental control were considered significant.

Identification and analysis of candidate aztreonam resistance genes. To assess the degree of positive selection for each gene, *de novo* genome assemblies of each aztreonam-sensitive isolate were first constructed using ABySS (68) to be used as a reference genome for each clonally related isolate pair. This measure maximized similarity in genome content between isolate pairs. Draft genomes were annotated using PROKKA (69) to provide uniform gene prediction annotation across sequenced strains. Antibiotic-resistant and antibiotic-sensitive isolates of each pair were independently aligned to the *de novo* assembly genome using bwa-mem (v0.7.12) (70), with single nucleotide polymorphisms and indels identified using SAMtools (v1.1) (71) as described elsewhere (66, 67). Variants were annotated with SNPEff (72). Structurally similar gene homologs with 80% or greater identity were grouped using CD-Hit (73), enabling comparison of mutations across patient isolates.

Isolates from the patients in the aztreonam treatment group were stratified according to their level of drug resistance (low resistance, ≤32 μg/ml; medium resistance, 64 μg/ml to 256 μg/ml; high resistance, ≥ 512 μg/ml). To be considered candidate aztreonam resistance determinants, genes needed to satisfy three different requirements. (i) Genes were under positive selection in one or more stratified groups of isolates from aztreonam-exposed patients, defined as the ratio of nonsynonymous to synonymous mutations (dN/dS ratio) of greater than one. (ii) The relative burden of nonsynonymous mutations was significantly higher in aztreonam-treated groups relative to isolates from the nonexposure group. Testing was performed using a Student's 2-tailed *t* test for samples with unequal variance, expressing the relative number of nonsynonymous mutations (the number of nonsynonymous mutations minus the number of synonymous mutations divided by the total number of mutations) to enable testing of comparisons having zero counts for nonsynonymous or synonymous changes. (iii) Genes were mutated in at least 20% of isolates in one or more of the aztreonam drug resistance level stratification groups.

P. aeruginosa de novo gene predictions were assigned names by performing DIAMOND BLAST-p (74) searches using representative sequences from the grouped homologous gene clusters. Multiple sequence alignment of mutated proteins to assess for recurrent mutations was performed using Clustal Omega (75).

Variant detection in specific targeted genes (see Data Set S1 in the supplemental material) was separately performed against the *P. aeruginosa* PAO1 reference genome (GenBank accession AE004091.2) as previously described (12).

***ampC* cloning.** We replaced the *ampR* gene of *E. coli-Pseudomonas* shuttle vector pMMB190 (ATCC) with the gentamicin resistance cassette of pex18GM (76) to avoid possible interference in subsequent aztreonam resistance assays. The gentamicin resistance cassette was amplified using primers F_Gibson_pMMB190_GM (5′-CCGGGGATCCATTTACCG-3′, all oligonucleotides synthesized by IDT) and R_Gibson_pMMB190_GM (5′-AGACGTCAGGTGGCACTTTTC-3′) and was introduced into pMMB190 by Gibson assembly (77) to generate vector pMMB190_GM.

Wild-type or mutant *ampC* genes were PCR amplified from appropriate templates using primers F_Gibson_*ampC*_GM_pMMB190 (5′-GCTCCCGGGGCGGTTTCT-3′) and R_Gibson_*ampC*_GM_pMMB190 (5′-CATAGCCAGGACCGGCGTC-3′) and then inserted downstream of the *lac* promoter of pMMB190_GM by Gibson assembly. Plasmids were transformed into *E. coli* DH5α by electroporation or into *P. aeruginosa* as described elsewhere (64).

***In vitro* evolution and selection of ampC mutants.** To support the construction of high-diversity mutant libraries, we constructed a small high-efficiency cloning vector derived from pUC19 (NEB) and pMMB190_GM. The pUC19 origin of replication was amplified using primers pUC19_ori_expression_F (5′-GCGGTATCATTGCAGCACTGG-3′) and pUC19_ori_expression_R (5′-TGAGCAAAAGGCCAGCAAAAG-3′). The *lacZ* promoter, multiple cloning site, and gentamicin resistance cassette of pMMB190_GM were PCR amplified using primers pMMB_MCS_F (5′-GCCGACATCATAACGGTTC-3′) and pMMB_MCS_R (5′-TTTAA AAGACGTCAGGTGG-3′). The two products were then Gibson assembled to produce vector pUC_MM.

Mutagenic PCR of *ampC* was performed using primer sets for wild-type or mutant *ampC* amplification (F_Gibson_*ampC*_GM_pMMB190 and R_Gibson_*ampC*_GM_pMMB190) with the Diversify PCR Random Mutagenesis kit (Clontech) under conditions to target an average of 4.6 point-mutations per kb (3.9 mutations per gene copy). PCR products were digested with BamHI and EcoRI and then ligated overnight into appropriately digested and calf-intestinal alkaline phosphatase (CIP; NEB)-dephosphorylated pUC_MM. Ligations were purified and transformed into electrocompetent *E. coli* 10-β. After a 1-h recovery in SOC medium (NEB), 5 h of outgrowth was performed in 100 ml LB containing 10 μg/ml gentamicin, with libraries plated before and after growth to allow estimation of effective library size. Selections were performed using one million transformants added to 4 ml LB-aztreonam medium containing 2-fold serial dilutions of aztreonam. After 24 to 36 h of aerobic incubation, cells from the highest concentration of antibiotic with visible growth were harvested. Cryostocks were prepared from a fraction of the culture, while plasmid was extracted from the remainder and used as the template for the subsequent round of mutagenic PCR. This process was continued until resistance levels plateaued over three consecutive rounds. Three replicates of artificial selection were performed independently, in parallel.

Sanger sequencing of cloned *ampC* alleles was performed using primers F_Gibson_*amp-C*_GM_pMMB190, R_Gibson_*ampC*_GM_pMMB190, ampC_SeqNested_F (5′-AGAAGGACCAGGCACAGAT C-3′), and ampC_SeqNested_R (5′-GAACACTTGCTGCTCCATGA-3′).

Data availability. All sequence data from this project are available from the NCBI Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA534096.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00866-19.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 2, PDF file, 1 MB.

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