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Elucidation of Mechanisms of Ceftazidime Resistance among Clinical Isolates of *Pseudomonas aeruginosa* by Using Genomic Data

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Ceftazidime is one of the few cephalosporins with activity against *Pseudomonas aeruginosa*. Using whole-genome comparative analysis, we set out to determine the prevalent mechanism(s) of resistance to ceftazidime (CAZ) using a set of 181 clinical isolates. These isolates represented various multilocus sequence types that consisted of both ceftazidime-susceptible and -resistant populations. A presumptive resistance mechanism against ceftazidime was identified in 88% of the nonsusceptible isolates using this approach.

Pseudomonas aeruginosa is an opportunistic pathogen associated with numerous nosocomial infections, where β-lactam antibiotics remain key in treatment (1, 2). One of the major antimicrobials used to fight *P. aeruginosa* infections is ceftazidime (CAZ), a well-known cephalosporin that acts primarily as a penicillin-binding protein 3 (PBP3) inhibitor (3, 4).

A significant proportion of ceftazidime-resistant isolates arise through the horizontal acquisition of β-lactamases or altered expression of the chromosomal drug-inducible wide-spectrum class C β-lactamase AmpC (reviewed in reference 5). The overproduction of AmpC can result from mutations affecting the peptidoglycan (PG) recycling process, where accumulation of cell wall intermediates ultimately induces *ampC* overexpression (5).

We focused our study on a panel of 181 clinical *P. aeruginosa* isolates, where comparative analysis between multiple isolates belonging to the same multilocus sequence types (MLSTs) allowed for identification of chromosomal gene variants unique to the ceftazidime-resistant population (6).

The initial MIC to ceftazidime was determined using frozen plates (Thermo Scientific) following the Clinical and Laboratory Standards Institute guidelines (7, 8). Of the 181 isolates in the analysis set, 99 (55%) were resistant to ceftazidime (MIC, ≥16 μg/ml), and 82 were susceptible (MIC, ≤8 μg/ml) (Table 1; see Table S1 in the supplemental material).

Genomic analysis (see Table S1 in the supplemental material) was performed using CLC Genomic Workbench 7.0.4 (CLCBio). Unique sequence variants exclusive to the resistant population of each MLST group were flagged for further analysis as outlined in the summary column of Table S1. To account for resistance in the 99 ceftazidime-resistant isolates in a parsimonious manner, we followed a triage process—accounting first for resistance-inducing β-lactamases, second for mechanisms allowing for derepression of *ampC*, and third for other candidate causes of resistance.

Forty-six isolates had β-lactamases that have been reported to hydrolyze ceftazidime (Table 1). Analysis of the *ampC* regulon and additional cephalosporin targets was also completed for these isolates (see Table S1 in the supplemental material); however, resistance was attributed primarily to the presence of the β-lactamases, as clinically, detection of such an element would rule out treatment with ceftazidime. It was apparent that certain MLST lineages were enriched in β-lactamases, particularly sequence type 111 (ST111) and ST233, which were the predominant carriers of bla_{VIM-2}. This is consistent with previous reports that these lineages represent global disseminators of the class B metallo-β-lactamases (9–12).

Comparative analysis of the *ampC* regulons from the remaining 53 ceftazidime-resistant isolates identified mutations in *ampR*, *ampD*, and *dacB* (Table 1; see Table S1 in the supplemental material). In the global transcriptional regulator *ampR*, which directly controls expression of the intrinsic β-lactamase *ampC*, unique amino acid changes were identified in five isolates of the resistant population. Of these isolates, three (AZPAE14890, AZPAE14909, and AZPAE15058) had a mutation that resulted in the D135N amino acid change. This mutation has previously been reported to affect the regulatory function of *ampR*, leading to derepression of *ampC* (13). Two of these isolates were obtained in France and were of different STs (ST175 and ST235) and 1 was from Spain (ST175), suggesting this mutation can be independently acquired by an isolate rather than being unique to a single lineage. Two other isolates (both ST319) had a G154R variation in AmpR. This change occurs within a region that has been purported to interact with the permease, AmpG (14). Reverse transcription-PCR (RT-PCR) analysis revealed a >20-fold increase in *ampC* expression in the presence of ceftazidime from these two isolates. As no other changes in the *ampC* regulon were apparent in these isolates, the increased expression level was attributed to the change in AmpR.

The most common sequence variations identified within the *ampC* regulon were located in the 1,6-anhydro-*N*-acetylmuramyl-[scapi]l-alanine amidase, AmpD, with 32 isolates having unique sequence changes. Mutations of *ampD* were easily identified in 14/32 isolates as they introduced early stop codons, frame-

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TABLE 1 Summary of β-lactamase content and AmpC regulon polymorphisms identified in *P. aeruginosa* isolates by MLST

MLST	No. of isolates: Total	Susceptible to CAZ (MIC, <8 μg/ml)	Nonsusceptible to CAZ (MIC, >16 μg/ml)	Total with acquired β-lactamase	Total with acquired β-lactamase attributed to mediation of nonsusceptibility to CAZ	No. of PBP3 polymorphisms (target modification)	Polymorphism(s) identified among regulators of *ampC* expression (*n*)
111	24	8	16	19	8 (VIM-2)		AmpD (6), DacB (2)
116	4	3	1	0	0		
155	4	3	1	1	0		
167	3	1	2	2	2 (IMP-15)		
175	9	4	5	0	0		AmpD (3), AmpR (2)
179	7	4	3	2	2 (PER-1)		
233	8	1	7	7	7 (VIM-2)		
235	37	11	26	23	14 (1 GES-19, 1 GES-9, 1 IMP-4, 1 VIM-11 and OXA-17, 1 OXA-19, 1 OXA-11, 1 OXA-74 and PER-1, 1 VIM-4, 3 KPC-2, 1 VIM-1, 1 VIM-2, 1 OXA-17)	1	AmpD (8), AmpR (1)
244	9	5	4	3	1 (OXA-2 [Y158S])		AmpD (2)
277	6	1	5	4	4 (SPM-1)		AmpD (1)
298	6	4	2	3	0		AmpD (2)
308	12	5	7	3	3 (1 GES-7, 2 VIM-2)		AmpD (2)
309	4	2	2	2	2 (1 GES-19, 1 VIM-2)		
313	5	3	2	0	0		AmpD (2)
316	2	1	1	1	1 (GES-9)		
319	5	3	2	0	0		AmpR (2)
348	8	5	3	2	1 (GES-1)		AmpD (2)
357	3	1	2	1	1 (VIM-5)		AmpD (1)
395	10	9	1	0	0		AmpD (1)
446	2	1	1	0	0		
500	3	2	1	0	0		
560	3	1	2	0	0		AmpD (2)
569	2	1	1	0	0	1	
606	2	1	1	0	0		
1714	3	2	1	0	0		
Total	181	82	99	73	46	2	39

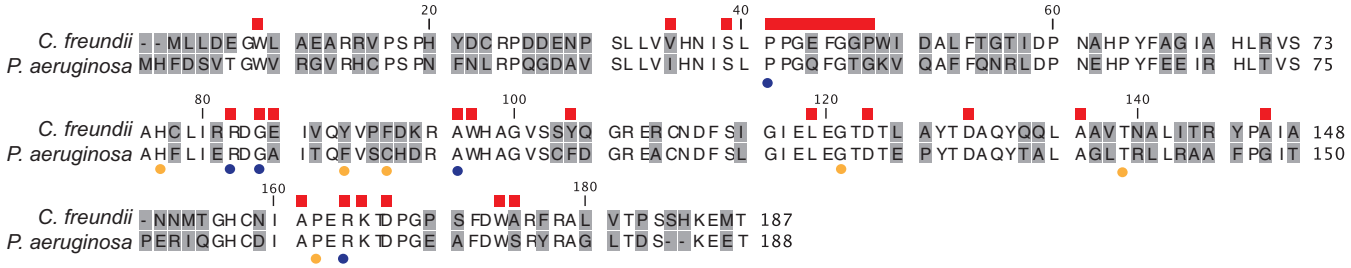

FIG 1 Changes identified within AmpD responsible for derepression of *ampC*. Alignment of the protein sequence of *Citrobacter freundii* and *P. aeruginosa* PAO1 for comparative purposes. Red boxes above the sequence of *C. freundii* denote residues identified from structural studies and mutational analysis as being important in the activity and functionality of AmpD (15, 31). Blue dots below indicate residues that had changes exclusive to the ceftazidime-resistant population and which previously have been shown to be important in increasing *ampC* expression in other *Enterobacteriaceae* (16). Yellow dots indicate newly identified changes that were exclusive to the ceftazidime-resistant *P. aeruginosa* population.

shifts, or in-frame deletions known to result in inactivation of *ampD*. The remaining isolates had unique amino acid variations within AmpD. These differences were aligned with the sequence of AmpD from *Citrobacter freundii*, for which a structure (PDB accession no. 1J3G) has been determined (15), to deduce changes that may affect the activity of this enzyme (Fig. 1). Of the 18 *P. aeruginosa* isolates with unique variations, 6 were deduced to affect the activity of this enzyme in *C. freundii* (15). Another 6/18 isolates had changes that have been experimentally noted to affect the activity of AmpD in multiple *Enterobacteriaceae* (16). Additional unique sequence variants were identified among the remaining 6 isolates (Fig. 1), and RT-PCR was used to determine the impact of these variations on the level of *ampC* expression (Table 2). Briefly, isolates were grown to the mid-log phase in Mueller-Hinton broth II (MHB II) at 37°C with shaking (200 rpm). The culture was split into two aliquots, where one was exposed to 1/2 the respective MIC of ceftazidime for 15 min and the other was treated as an unexposed control. Samples were treated with RNAprotect cell reagent (Qiagen), and RNA was prepared using a Maxwell 16 LEV simplyRNA purification kit (Promega). A total of

TABLE 2 Summary of AmpD variants identified among the clinical population of *P. aeruginosa*[a]

Variant type	Isolate	MLST	MIC of CAZ (μg/ml)	Allelic variation[b]	Δ^{-CT}: Relative to PAO1[c]	Δ^{-CT}: Induced/uninduced ratio[d]
Control	PAO1		1		1.00	1.91
With structurally important residue identified in *C. freundii* crystal structure	AZPAE14403	175	16	P41S (39)	0.56	3.69
	AZPAE14892	313	64	P41S (39)	3.61	7.52
	AZPAE14860	308	32	A96T (94)	54.16	1.13
	AZPAE15054	298	16	A96T (94)	49.38	6.06
	AZPAE14886	111	16	R164S (161)	0.05	4.18
	AZPAE14983	111	16	R164S (161)	0.18	5.94
With structurally important residue identified in *Enterobacteriaceae*	AZPAE14394	175	64	R82C (80)	3.96	70.67
	AZPAE15006	235	16	G84D (82)	3.50	6.82
	AZPAE14842	235	16	G84D (82)	24.18	2.01
	AZPAE14422	235	32	G84D (82)	5.24	2.63
	AZPAE14979	235	32	G84D (82)	28.56	4.82
	AZPAE14843	235	32	G84D (82)	13.99	6.35
With unique changes identified among isolates	AZPAE14722	175	32	H77Y (75)	2.33	16.00
	AZPAE14730	235	64	F89S (87)	3.96	12.45
	AZPAE15015	235	32	C92Y (90)	6.60	2.00
	AZPAE14987	298	32	G121R (119)	12.26	1.47
	AZPAE15035	560	16	T139A (137)	1.26	2.29
	AZPAE14710	235	32	P162L (159)	0.69	2.28

[a] Presented are mutations that have been shown to be important based upon the *C. freundii* structure, those identified to be important from the study of other *Enterobacteriaceae*, and those changes that were unique and identified among isolates in this study. RT-PCR values for *ampC* are provided for these isolates to confirm overexpression of the intrinsic β-lactamase.

[b] The positions listed are numbered according to the sequence of the AmpD from *P. aeruginosa* PAO1. Positions provided in parentheses are for the corresponding position in AmpD of *C. freundii*.

[c] Results are representative of 3 independent experiments. The Δ^{-CT} ratio is calculated relative to the RT-PCR result for the housekeeping gene *rpsL*. The values listed represent the ratio of the Δ^{-CT} of the isolate in MHB II relative to that of strain PAO1.

[d] The values listed represent the ratio of the Δ^{-CT} of the isolate in MHB II in the presence of 1/2 the MIC of ceftazidime for 15 min in the log phase compared to that in MHB II at the same point in time.

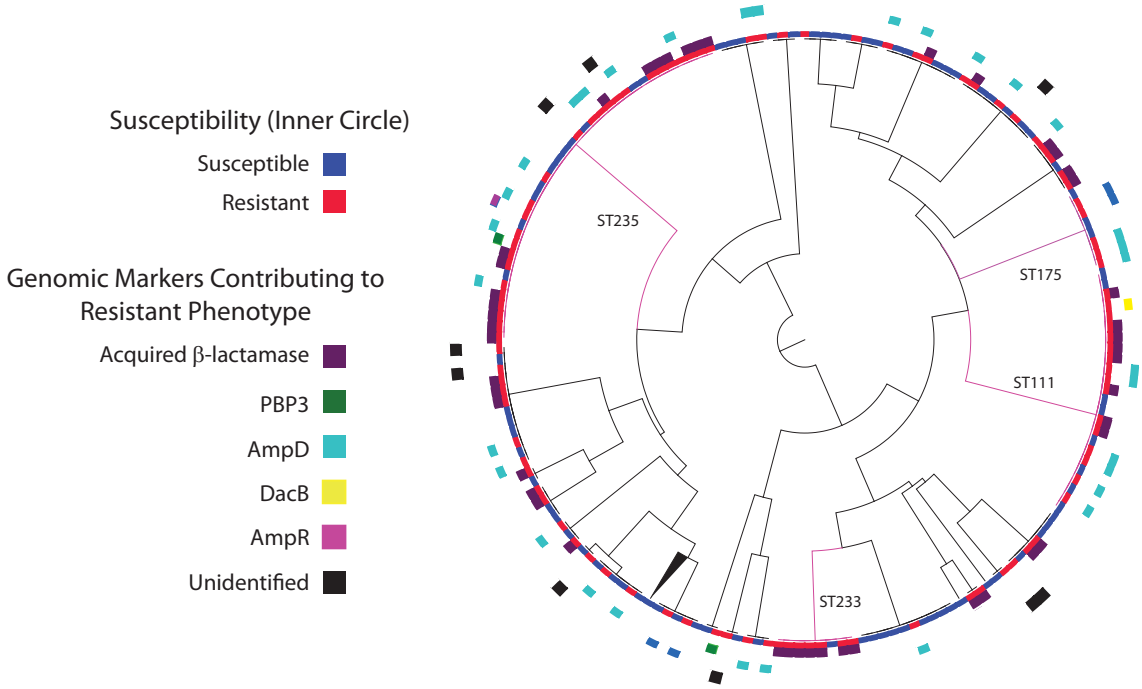

FIG 2 Summary of resistance mechanisms among ceftazidime-resistant clinical isolates of *P. aeruginosa* by MLST. Global lineages are highlighted on the MLST tree in pink and labeled accordingly. The 12 isolates for which a genomic marker for resistance could not be determined are highlighted on the outer circle with black boxes.

5 ng RNA was used in an RT-PCR assay using a QuantiTect SYBR green RT-PCR kit (Qiagen) with a Bio-Rad CFX96 instrument. The level of expression of *rpsL* was used for normalization, and the relative quantification of *ampC* expression was performed using a comparative threshold cycle (C_T) method. The oligonucleotides used to quantify transcript expression for *ampC* and *rpsL* were obtained from previous publications (17, 18).

Isolates were examined for constitutive expression of *ampC* in MHB II and derepressed *ampC* expression in the presence of sub-MICs of ceftazidime (Table 2). Elevated constitutive expression (>4-fold) of *ampC* relative to the sensitive control strain PAO1 was evident in 8/18 isolates, and elevated induced expression was present in the remainder.

Additional analysis of the data set identified two isolates of the ST111 lineage (AZPAE14727 and AZPAE14728) with the same unique variation in the *dacB* gene. DacB is a nonessential low-molecular-weight PBP that is involved in maintaining PG composition and mediates β-lactam resistance through increased expression of AmpC and the two-component system, BrlAB, also known to mediate resistance (19, 20). RT-PCR of *ampC* expression from these isolates also indicated derepression of *ampC* (*ampC*/*rpsL* ratio of >10-fold; *ampC* expression relative to PAO1, 4.82-fold).

No unique changes to the *ampR*/*ampC* promoter region were identified in the resistant population, and examination of additional genes of the *amp* regulon did not identify variants unique to the ceftazidime-resistant population (see Table S1 in the supplemental material). It is interesting to note that the comparative analysis of alleles by MLST grouping showed almost identical sequence profiles, with the exception of genes encoding the lytic transglycosylases, which were quite diverse. Further studies on the structure of these enzymes and the effect of changes in the mature protein are needed to understand the genetic diversity and potential impact of these changes in the lytic transglycosylases.

Mutations within or near the active sites of the essential PBPs (PBP3, PBP1a, and PBP1b) may mediate decreased susceptibility to ceftazidime in *P. aeruginosa* (21). Unique changes to the PBP3 sequence were identified in two of the clinical isolates (AZPAE13850 and AZPAE12156). Both had the same PBP3 mutation resulting in the change of R504C. This residue is part of an important hinge region of the PBP (22) and may cause interference with ceftazidime binding. These isolates were of different STs from India and the United States, strongly indicating independent acquisition of this amino acid variation. Examination of PBP1a and PBP1b, which can both be inhibited by ceftazidime at high concentrations (4), did not identify any sequence variations unique to the resistant population.

Overexpression of efflux components has also been implicated in reduced susceptibility to ceftazidime (23, 24). Although 3 (AZPAE12150, AZPAE13876, and AZAPE14872) of the remaining 12 ceftazidime-resistant isolates did have mutations in efflux regulatory components (*nalD* and *mexZ*), examination of the whole population showed that they were not exclusive to the ceftazidime-resistant population. However, it is likely that these mutations contribute to the overall resistance or reduced susceptibility of the organisms.

Additional alleles associated with resistance, including genes identified in studies with transposon libraries (25), mutator-associated genes (26), and quorum sensing genes (26, 27), were also evaluated (see Table S2 in the supplemental material). Analysis of these alleles did not reveal any variants that were unique to the resistant isolates (data not shown).

The 12 isolates and all other strains belonging to the same STs

were mapped to the reference strain *P. aeruginosa* PAO1 in an attempt to identify common polymorphisms unique to this population; however, no single target gene was identified from this analysis. In part, this may be due to the small number of isolates spread across a diverse genetic background. Additionally, this is not unexpected as resistance can occur singularly or in a multifaceted manner through direct target changes, expression-level changes of numerous factors, as well as changes to membrane permeability, to name but a few. Indeed, 6 of these 12 isolates had a MIC to ceftazidime (16 μg/ml) 1 doubling dilution higher than the nonsusceptible breakpoint. This level of elevation could easily be due to the combinatorial changes in several factors, as opposed to a single predominant factor.

It may also be prudent to consider the pathogenic/disease association of the isolates. Two of the isolates for which a mechanism of resistance was not clearly defined were highly resistant to ceftazidime (MIC, 128 μg/ml) and were collected from cystic fibrosis (CF) patients. Isolates associated with CF are often multidrug resistant due to phenotypic traits that change and develop with adaptation to the lung environment (28). For example, the overproduction of alginate may affect the susceptibility of an isolate as it provides another barrier to antibiotic entry (29). A mutation in *mucA*, an anti-sigma factor that controls alginate production (30), was identified in isolate AZPAE12416 and may be one of many contributory factors to resistance of this isolate.

Using a comparative genomic approach with alleles previously associated with ceftazidime resistance in *P. aeruginosa*, we were able to identify the probable factor(s) mediating resistance in 88% of the 99 resistant isolates in our data set (Fig. 2). This type of analysis provides a real depiction of the probable mutations that are mediating resistance among a relevant population and is invaluable in aiding our understanding of resistance mechanisms and designing new antimicrobials that evade these pathways.

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