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The plethora of resistance mechanisms in *Pseudomonas aeruginosa*: transcriptome analysis reveals a potential role of lipopolysaccharide pathway proteins to novel β-lactam/β-lactamase inhibitor combinations

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ABSTRACT

Objectives: Whole genome and transcriptome analysis of 213 *Pseudomonas aeruginosa* isolates resistant to antipseudomonal β-lactams collected in 30 countries was performed to evaluate resistance mechanisms against these agents.

Methods: Isolates were susceptibility tested by reference broth microdilution. Whole genome and transcriptome sequencing were performed, and data were analysed using open-source tools. A statistical analysis of changes in the expression of >5500 genes was compared to the expression of PAO1.

Results: The high-risk clones ST235 and ST111 were the most prevalent among >90 sequence types (STs). Metallo-β-lactamase (MBLs) genes were detected in 40 isolates. AmpC and MexXY were the most common genes overexpressed in approximately 50% of the 173 isolates that did not carry MBLs. Isolates overexpressing *pmrA* and *pmrB*, the norspermidine production genes *speD2* and *speE2*, and the operon *arnBCADTEF-ugd* were noted among strains resistant to ceftolozane-tazobactam and ceftazidime-avibactam, despite the lack of polymyxin resistance often associated to increased expression of these genes. Overexpression of MuxABC-OpmB, OprG, and OprE proteins were associated with resistance to ceftolozane-tazobactam in addition to the usual genes involved in cephalosporin, monobactam, and carbapenem resistance. Statistical analysis identified discrete mutations in ArmZ, OprD, and AmpC that correlated to antipseudomonal β-lactam resistance.

Conclusions: *P. aeruginosa* resistance mechanisms are complex. This analysis suggests the role of multiple genes in resistance to antipseudomonal β-lactams, including some not commonly described.

1. Introduction

Pseudomonas aeruginosa is a significant cause of serious infections in hospitalized patients [1]. In the United States, the Centers for Disease Control and Prevention (CDC) estimated that 32 600 infections and 2700 deaths among hospitalized patients were caused by *P. aeruginosa* infections during 2017 [2].

The treatment of infections caused by *P. aeruginosa* is challenging because of the limited permeability of pseudomonal cells to many antimicrobial agents and this organism's ability to acquire mutations and resistance genes against agents that otherwise would be active against this species [3–5]. Because of the frequent occurrence of multidrug (MDR)- and carbapenem-resistant *P. aeruginosa* and the limited therapeutic options to treat these infections, the World Health Organization has recognized this species as an important threat to human health. Moreover, MDR and carbapenem-resistant *P. aeruginosa* have been highlighted as an organism for which new therapeutic options are urgently needed [6].

Antipseudomonal β-lactam agents, including β-lactam inhibitor combinations, are widely used for treatment of infections caused by *P. aeruginosa* [5]. Among the resistance mechanisms, the overexpression of the chromosomal cephalosporinase (PDC) affects most

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cephalosporins and, to a lesser extent, imipenem [7]. Overexpression of the PDC gene is controlled by AmpR and AmpD. Isolates with mutations in these regulators can be selected during β-lactam therapy at different rates [7,8].

Another prevalent β-lactam resistance mechanism in *P. aeruginosa* is the decreased expression or ceased production of the specific outer membrane protein OprD [7,9,10]. The deficiency or loss of OprD affects the entrance of the carbapenems into the cell, which is especially true for imipenem [9,11]. Furthermore, the Mex family of resistance nodulation division (RND) tripartate efflux systems is known to increase the extrusion of various compounds from the pseudomonal cell [12]. The overexpression of MexAB-OprM affects most β-lactams, with the exception of imipenem. This efflux pump also extrudes quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, and novobiocin [13]. Our prior investigations demonstrated that in isolates resistant to doripenem, ceftazidime, and ceftazidime-avibactam, the overexpression of MexAB-OprM occurred more often than other efflux systems from the Mex family [9,10,14].

MexCD-OprJ and MexXY extrude most penicillins and cephems, except ceftazidime, among other agents. MexCD-OprJ and MexXY recognize the same non-β-lactam molecules as MexAB-OprM, except MexXY also extrudes aminoglycosides [15]. MexED-OprN is not involved in resistance to β-lactams; this pump's substrates include quinolones, tetracyclines, trimethoprim, and chloramphenicol [16].

Beyond mutation-driven resistance mechanisms, *P. aeruginosa* also can acquire ESBLs, serine-carbapenemases, metallo-β-lactamases (MBLs), and oxacillinases with diverse hydrolytic profiles. More often than not, these resistance mechanisms are combined, generating specific resistance patterns in *P. aeruginosa* isolates.

Identifying these resistance mechanisms in clinical isolates is troublesome. One strategy alone is not sufficient to measure the effect of these combined traits. For example, whole genome sequencing has been used to identify β-lactamase genes and known mutations in several genes, while quantitative methods to detect messenger RNA (mRNA) have been applied to measure the expression of main genes involved in pathways identified to cause β-lactam resistance. Often, these evaluations are limited to a small number of genes because of the cumbersome techniques available. Also, mRNA is generated by the cell regardless of the presence of alterations causing frame-shift mutations of the presence of a premature stop-codon, requiring both methods for a complete evaluation.

In this study, we used a combination of whole genome and whole transcriptome (mRNA) sequencing to analyse the resistance mechanisms against antipseudomonal β-lactam agents among 213 *P. aeruginosa* isolates collected in 55 hospitals located in 30 countries across Europe, Latin America, and the Asia-Pacific region. We initiated the study to evaluate genes that have been previously associated with β-lactam resistance in this species and then expanded our analysis using statistical methods to understand the differential expression of >5500 genes.

2. Materials and methods

2.1. Bacterial isolates

Among the 1909 *P. aeruginosa* isolates collected during 2017 from hospitals located in Europe, Latin America, and Asia-Pacific region that participated in the SENTRY Antimicrobial Surveillance Program, 213 isolates were selected for further analysis. Isolates were randomly selected to be geographically diverse and mimic the distribution of the overall population of isolates that had resistance to the following agents: ceftazidime, cefepime, ceftolozane-tazobactam, meropenem, and piperacillin-tazobactam. These isolates were collected from unique infection episodes from patients of 55 hospitals.

All isolates were identified with matrix-assisted laser desorption ionization-time of flight mass spectrometry using the Bruker Daltonics MALDI Biotyper (Billerica, MA, USA), following the manufacturer's instructions.

2.2. Whole genome sequencing

Total genomic DNA was extracted and used as input material for the library construction. DNA libraries were prepared using the Nextera XT™ library construction protocol and index kit (Illumina, San Diego, CA, USA) and sequenced on a MiSeq Sequencer (Illumina). Sequencing analysis was performed after *de novo* assembly [17], and specific matches were generated for each sample, with the criteria of >94% identity and 40% minimum coverage length to be included in the study. Mutations were considered present when >50% of the sequence reads allowed for base calling. Sequences displaying 100.0% homology with the reference sequences were named according to the reference. Genes with homology <100.0% were called with the suffix "-like" after the gene showing the closest homology. Intrinsic genes were annotated relative to the susceptible reference isolate *P. aeruginosa* ATCC 27853. Annotations were interpreted as one of the following: wild type (sequence identical to ATCC 27853), alterations (single amino acids substitutions relative to ATCC 27853), or disruptions (alterations resulting in the early termination of a gene or an insertion/deletion of ≥3 continuous amino acids).

2.3. Whole transcriptome analysis

Total RNA was extracted and purified from log phase bacterial cultures that displayed a cell density of OD_{600} of 0.3 to 0.5 using the RNeasy Mini Kit in the Qiacube workstation (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Residual DNA was eliminated by treatment with RNAse-free DNase (Promega, Madison, WI, USA). Total RNA sample quality was assessed using the RNA 6000 Pico kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Only preparations with acceptable RNA integrity numbers (RIN) ≥7 and/or that showed no visual degradation were used for experiments. RNA was quantified on a Qubit™ 3.0 Fluorometer (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) using the Qubit RNA HS Assay Kit following the manufacturer protocol.

Up to 2 µg of total RNA were subjected to rRNA depletion using Ribo-Zero® (Gram-Negative) rRNA Removal Kit (Illumina) according to the manufacturer's instructions. Ribo-Zero-treated RNA was purified using the modified RNeasy MinElute option described within the Ribo-Zero protocol and eluted in 14 µL RNase-free water. Eluted samples again were evaluated for quality using the RNA 6000 Pico kit on the Agilent 2100 Bioanalyzer and quantity using the Qubit RNA HS Assay Kit on the Qubit 3.0 Fluorometer. Samples were used that same day for library preparation or stored overnight at −20°C or −80°C for up to 30 days. Whole transcriptome RNA-Seq cDNA library preparation was performed using the TruSeq™ Stranded mRNA Library Prep (Illumina) with eluted Ribo-Zero-treated RNA samples as input material, processed as described above. Library preparation was performed according to the manufacturer's instructions, beginning with the fragmentation of mRNA, as the depletion of rRNA was done instead of the purification step. Fragmentation of mRNA was accomplished by using the entire eluted Ribo-Zero-treated RNA sample (~13 µL) combined with 13 µL of Fragmentation, Prime, Finish Mix (FPF). Sequencing was carried out on MiSeq sequencers using MiSeq Reagent Kit

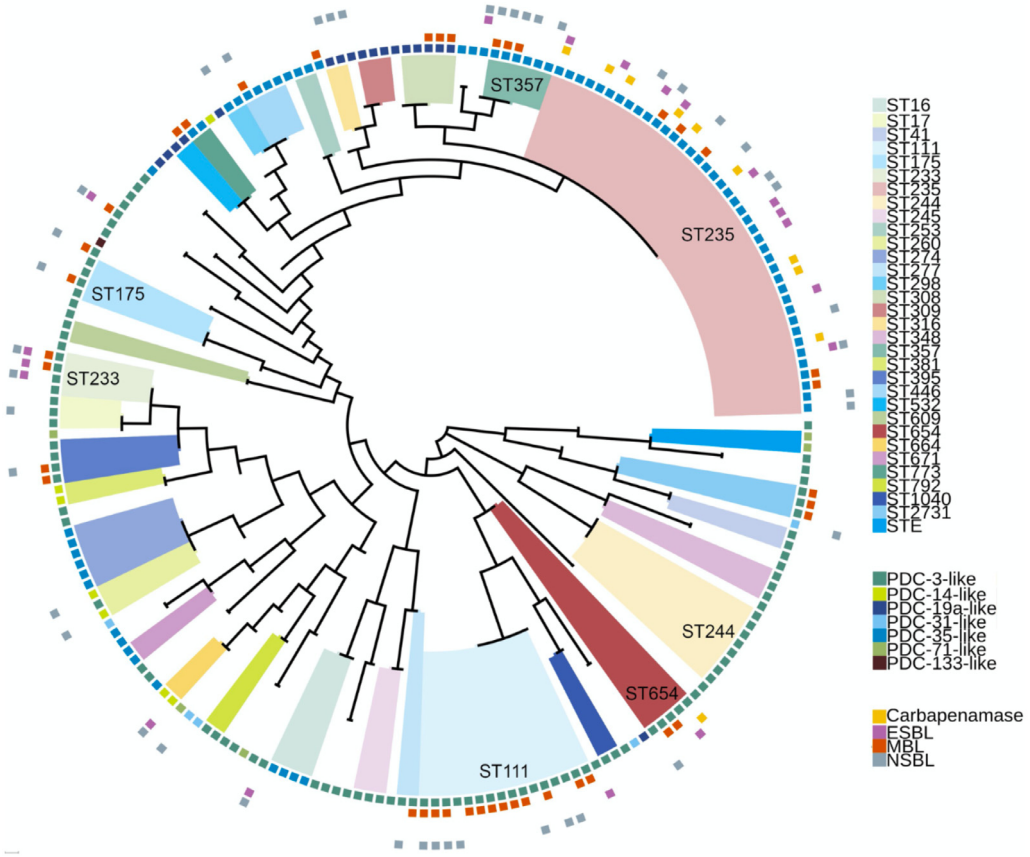

Fig. 1. Occurrence of resistance mechanisms among *P. aeruginosa* and its correlation to resistance to antipseudomonal β-lactams.

v3 (150-cycle). An independently prepared replicate of the control reference isolate (*P. aeruginosa* PAO1) was included with each sequencing run to serve as an internal control for all phases of this experiment.

Differential gene expression was estimated using a proprietary RNA-seq pipeline. First, paired-end reads were trimmed, corrected, and filtered using fastp [25]. Quality-controlled reads were aligned to a *P. aeruginosa* PAO1 reference assembly (ASM676v1) [26], filtered based on alignment scores and assigned to loci to calculate per-gene counts using EDGE-pro [27]. Counts were normalized across samples using trimmed mean of M-values (TMM) normalization [28]. Fold change expression was calculated according to an exact test based on the quantile-adjusted conditional maximum likelihood (qCML) method [29] using edgeR [30]. Synonyms and gene ontology (GO) terms were collected from UniProt [31] to aid in interpretation.

2.4. Statistical analysis

The odds ratios for each gene and β-lactam agent combination were calculated and evaluated using multiple Fisher exact tests (Fig. 1). The Bonferroni correction was used to adjust for multiple comparisons (Table 1). All statistical analysis was completed using R version 4.0.3 and RStudio version 1.2.5033.

3. Results

3.1. Geographic distribution and epidemiology of P. aeruginosa isolates

The 213 *P. aeruginosa* isolates included in this study were recovered from 55 hospitals from 30 countries: 123 isolates from Europe (57.7%), 40 isolates from Latin America (18.8%), and 50 isolates from the Asia-Pacific region (23.5%).

MLST analysis identified 76 defined and 16 novel sequence types (STs), including 32 observed in more than one isolate (Fig. 1). Isolates belonging to ST235 (41/213; 19.2%) were the most commonly found and were observed in 18 countries located in all three regions. ST111 was identified in 16 isolates (7.5%). ST244 was found in eight isolates. ST175 and ST233, highlighted as high-risk clones in the literature, were detected in only four (1.9%) isolates each.

3.2. Acquired β-lactamases

A total of 89 isolates carried acquired β-lactamase genes (Fig. 1). MBLs were observed in 40 (18.8%) isolates. Most MBL-producing isolates carried bla_{VIM-2} (25/40). One isolate carried both genes encoding IMP-62 and NDM-1. Aztreonam inhibited 40.0% of the MBL producers (data not shown), but these isolates displayed resistance to other β-lactam agents tested, including all β-lactamase inhibitor combinations. Because of the ability of MBLs to cause resistance to virtually all agents except aztreonam, the 40 isolates producing MBLs were excluded from further analysis.

Serine-carbapenemase-encoding genes were detected in only 10 isolates (4.7%) and included four bla_{GES-6}, four $bla_{OXA-129}$, and one each of bla_{KPC-2} and bla_{GES-5}. Overall, only 22 (10.3%) isolates carried an ESBL gene. The genes encoding OXA-1/-30, OXA-129, PER-1, PME-1, and OXA-4 were detected alone or in combination in two to four isolates. ESBLs that are usually detected in *P. aeruginosa*, such as GES-1, VEB-1, VEB-9, and others that are less commonly detected, such as CTX-M-2 and SHV-2a, were observed in single isolates.

Table 1
Statistical analysis of gene expression and discrete mutations noted to be significantly more common among *P. aeruginosa* isolates displaying resistance to antipseudomonal β-lactam agents

Gene or protein	Locus tag	Product	Expression or amino acid alteration	Resistance to	Odds ratio	*P* value
oprM	PA0427	RND efflux system outer membrane protein	Overexpression	Ceftazidime	0.3642	0.00329
mexA	PA0425	RND efflux system membrane fusion protein	Overexpression	Ceftazidime-avibactam	4.531725657	0.001253096
speE2	PA4773	alternative spermidine biosynthesis pathway	Overexpression	Ceftazidime-avibactam	4.883138592	0.001420024
speD2	PA4774	alternative spermidine biosynthesis pathway	Overexpression	Ceftazidime-avibactam	4.927957025	0.000733505
pmrA	PA4776	Two-component regulator system response regulator	Overexpression	Ceftazidime-avibactam	6.190897065	0.000371318
pmrB	PA4777	Two-component regulator system signal sensor kinase	Overexpression	Ceftazidime-avibactam	6.619677879	0.0002529
arnA	PA3554	Lipid A modification operon	Overexpression	Ceftazidime-avibactam	6.280349304	0.00193752
arnB	PA3552	Lipid A modification operon	Overexpression	Ceftazidime-avibactam	5.678222326	0.002902985
arnC	PA3553	Lipid A modification operon	Overexpression	Ceftazidime-avibactam	6.280349304	0.00193752
arnD	PA3555	Lipid A modification operon	Overexpression	Ceftazidime-avibactam	7.014294683	0.001244345
arnE	PA3557	Lipid A modification operon	Overexpression	Ceftazidime-avibactam	6.280349304	0.00193752
arnF	PA3558	Lipid A modification operon	Overexpression	Ceftazidime-avibactam	7.637073694	0.00042036
mexZ	PA2020	Repressor of MexXY operon	Overexpression	Ceftolozane-tazobactam	3.1237	0.00468
mexY	PA2018	RND efflux system transporter	Overexpression	Ceftolozane-tazobactam	3.2875	0.00462
arnD	PA3555	Lipid A modification operon	Overexpression	Ceftolozane-tazobactam	4.7636	0.00489
muxC	PA2526	RND component of MuxABC-OpmB efflux pump	Overexpression	Ceftolozane-tazobactam	13.7958	0.00094
oprG	PA4067	Outer membrane protein	Underexpression	Ceftolozane-tazobactam	5.6485	0.00082
oprD	PA0958	Outer membrane protein	Underespression	Imipenem	0.0000	0.00033617
mexY	PA2018	RND efflux system transporter	Overexpression	Imipenem	2.8733	0.000820142
mexZ	PA2020	Repressor of MexXY operon	Overexpression	Imipenem	3.1261	0.000282545
mexX	PA2019	RND efflux system membrane fusion protein	Overexpression	Imipenem	3.1274	0.000265816
mexZ	PA2020	Repressor of MexXY operon	Overexpression	Piperacillin-tazobactam	0.1553	0.00201
ampC	PA4110	Intrinsic β-lactamase	Overexpression	Piperacillin-tazobactam	7.6739	0.00016
oprE	PA0291	Anaerobically induced outer membrane porin	Underexpression	Piperacillin-tazobactam	0.1777	0.00128
ArmZ	PA5471	Anti-repressor of mexZ	H182Q	Aztreonam	2.3880238	0.0073513316
OXA-50	PA5514	Intrinsic β-lactamase	R83K	Aztreonam	0.1354389	0.0011062920
PA5542	PA5542	Intrinsic imipenem β-lactamase	I106V	Aztreonam	3.04095	0.0008553396
AmpR	PA4109	β-lactamase expression regulator	E114A	Ceftazidime-avibactam	3.897091	0.0096600980
ArmZ	PA5471	Anti-repressor of mexZ	L88P	Ceftazidime-avibactam	0.1696251	0.0089972974
AmpO	PA4219	Interacts with ampP	S235T	Ceftazidime	5.662877	0.0004382902
CreD	PA0465	Effector inner membrane protein	V394A	Ceftazidime	0.3280291	0.0012578240
PepA	PA3831	Protease activity	D121G	Ceftazidime	0.2380154	0.0089226190
AmpO	PA4219	Interacts with ampP	S235T	Cefepime	3.177728	0.0081929394
ArmZ	PA5471	Anti-repressor of mexZ	H182Q	Cefepime	2.4365203	0.0066765639
MexB	PA0426	Multidrug efflux pump component	V1042A	Cefepime	4.235591	0.0029732960
MexB	PA0426	Multidrug efflux pump component	S1041E	Cefepime	2.963278	0.0098576010
MexC	PA4599	Multidrug efflux pump component	A31V	Cefepime	3.4676482	0.0065693885
PA5542	PA5542	Intrinsic imipenem β-lactamase	S224T	Cefepime	4.996437	0.0070382691
PepA	PA3831	Protease activity	D121G	Cefepime	0.2041199	0.0042924080
ArmZ	PA5471	Anti-repressor of mexZ	D161G	Imipenem	0.3128004	0.0005019319
ArmZ	PA5471	Anti-repressor of mexZ	H182Q	Imipenem	0.4330186	0.0088589212
CreD	PA0465	Effector inner membrane protein	Q253E	Imipenem	0.1497603	0.0023975180
MexR	PA0424	Efflux regulation	V126E	Imipenem	2.4932523	0.0052998004
OprD	PA0958	Outer membrane protein	G423A	Imipenem	0.10720358	0.0000627649
OprD	PA0958	Outer membrane protein	L359V	Imipenem	0.29335869	0.0002303996
OprD	PA0958	Outer membrane protein	V372M	Imipenem	0.32085531	0.0008412957
OprD	PA0958	Outer membrane protein	D373S	Imipenem	0.32085531	0.0008412957
OprD	PA0958	Outer membrane protein	S374D	Imipenem	0.32085531	0.0008412957
OprD	PA0958	Outer membrane protein	S375N	Imipenem	0.32085531	0.0008412957
OprD	PA0958	Outer membrane protein	S376N	Imipenem	0.32085531	0.0008412957
OprD	PA0958	Outer membrane protein	S377V	Imipenem	0.32085531	0.0008412957
OprD	PA0958	Outer membrane protein	S377_Y378insG	Imipenem	0.32085531	0.0008412957
OprD	PA0958	Outer membrane protein	A379K	Imipenem	0.32085531	0.0008412957
OprD	PA0958	Outer membrane protein	G380N	Imipenem	0.32085531	0.0008412957
OprD	PA0958	Outer membrane protein	L381Y	Imipenem	0.32085531	0.0008412957
OprD	PA0958	Outer membrane protein	L381_Y382insG	Imipenem	0.32085531	0.0008412957
OprD	PA0958	Outer membrane protein	E301Q	Imipenem	0.38385419	0.0036718790
OXA-50	PA5514	Intrinsic β-lactamase	T16A	Imipenem	2.7084858	0.0019968430
PA1435	PA1435	Predicted efflux pump	R384Q	Imipenem	0.3208558	0.0044904320
AmpC	PA4110	Intrinsic β-lactamase	V205L	Imipenem	3.205182	0.0003478860
AmpC	PA4110	Intrinsic β-lactamase	G27D	Imipenem	2.486795	0.0087458920
OprD	PA0958	Outer membrane protein	G423A	Meropenem	0.18033638	0.0010486280
OprD	PA0958	Outer membrane protein	L359V	Meropenem	0.42687561	0.0099836480
AmpC	PA4110	Intrinsic β-lactamase	V205L	Meropenem	2.498518	0.0049183880

Narrow-spectrum β-lactamase genes were more common. These genes were noted in 43 isolates (20.2%). A total of 124/213 isolates (58.2%) did not carry any acquired β-lactamase genes.

The presence of β-lactamases and serine-carbapenemases was more common among isolates resistant to the carbapenems and novel β-lactamase inhibitor combinations: 24% to 35% of the isolates resistant to meropenem, imipenem, ceftazidime-avibactam and ceftolozane-tazobactam had such enzymes (Fig. 2). Similarly, 5.8% of the isolates carried serine-carbapenemases, but 9.4%, 8.1%, and 15.2% of the isolates that were resistant to meropenem, imipenem, and ceftolozane-tazobactam harboured serine-carbapenemases, respectively (Fig. 2). The presence of ESBL

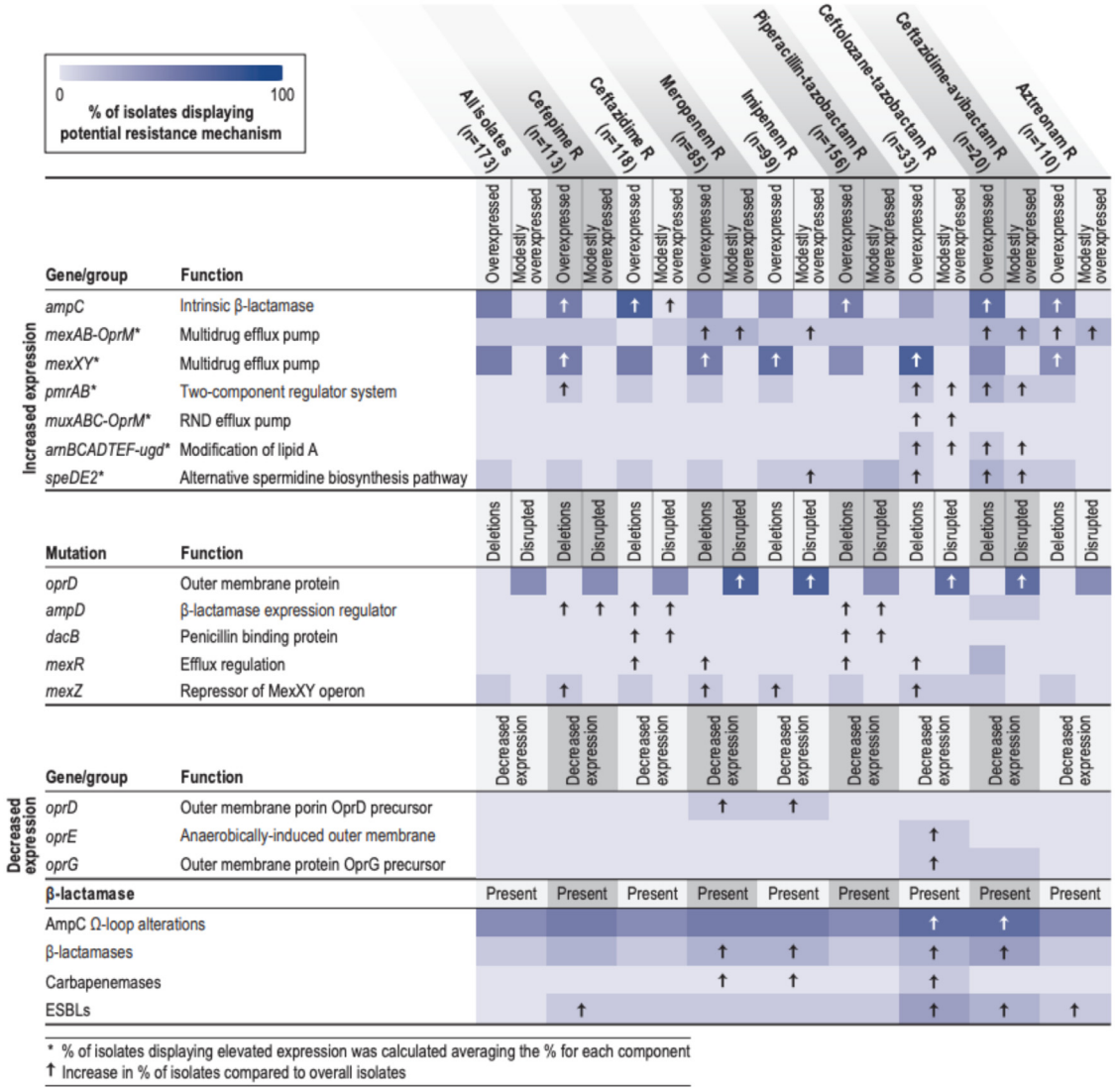

Fig. 2. Summary of β-lactamases among *P. aeruginosa* isolates displaying resistance to antipseudomonal β-lactam agents by MLST.

genes was noted in a higher percentage of cefepime- (14.2%), ceftolozane-tazobactam- (33.3%), ceftazidime-avibactam- (25.0%), and aztreonam-resistant isolates (13.6%) when compared to overall non-MBL isolates (9.3%).

Notably, acquired β-lactamase genes were more often observed within specific STs (Fig. 1). All isolates from ST111 carried acquired β-lactamase genes, including VIM-2, IMP-18, CARB-2, and oxacillinases of diverse hydrolytic profiles. Thirty of 41 ST235 isolates carried acquired β-lactamase genes that were diverse in class and spectrum of hydrolysis. Similarly, ST233 (3/4), ST654 (5/5), and ST357 (5/6) carried transferrable β-lactamase genes of various classes.

3.3. Changes in the Pseudomonas-derived cephalosporinase (PDC)

The evaluated isolates harboured 43 PDC variants, some of which were previously described as expanded-spectrum AmpC cephalosporinases (ESAC) that demonstrated increased hydrolysis to cefepime and imipenem, including PDC-3 and PDC-5 [32]. These variants were noted among 47 and 23 isolates tested, respectively. Alterations in the PDC Ω-loop have been described to cause resistance to ceftazidime-avibactam in *P. aeruginosa* isolates. Two isolates harboured a deletion of seven amino acids (PLRVGPG) at positions 236 to 242, which has been associated with resistance to ceftazidime-avibactam. Other amino acid substitutions in the PDC Ω-loop that have been observed in ceftazidime-avibactam-susceptible isolates also were observed in this study [14].

PDC Ω-loop alterations were noted in higher rates among ceftolozane-tazobactam- (60.6%), ceftazidime-avibactam-resistant isolates (65.0%) when compared overall to non-MBL isolates (49.7%; Fig. 2).

3.4. Gene expression

The effect of increased or decreased expression of 5500 *P. aeruginosa* genes in resistance to antipseudomonal β-lactam agents was evaluated using statistical methods. The percentages of isolates displaying these resistance mechanisms among 173 *P. aeruginosa* isolates that did not harbour MBL genes are summarized in Fig. 2. Among genes commonly associated with β-lactam resistance, *ampC* (>10-fold than the baseline), *mexX*, and *mexY* (>5-fold) were the most common genes overexpressed in the isolates analysed. The overexpression of these genes was detected among 93 (53.8%), 90 (52.0%), and 89 (51.5%) isolates, respectively. Isolates overexpressing *ampC* were more common among *P. aeruginosa* isolates resistant to cefepime (58.4%),

ceftazidime (70.3%), piperacillin-tazobactam (58.3%), ceftazidime-avibactam (60.0%), and aztreonam (57.3%) when compared to the overall isolates (53.8%; Fig. 2).

The genes *mexX* and *mexY* are part of the same operon and will be analysed together herein. Isolates resistant to cefepime (58.0%), meropenem (59.4%), imipenem (63.1%), and ceftolozane-tazobactam (72.7%) displayed higher rates of overexpression of the genes encoding MexXY than the overall isolates (51.7%).

The genes encoding MexAB-OprM efflux system were overexpressed (>5-fold) in 9.3%, 9.4%, and 15.6% of the isolates, respectively, but 13.9% to 16.2% of the isolates had modestly elevated expression (>3-fold) of these genes. Isolates resistant to meropenem (20.0%), ceftazidime-avibactam (28.3%), and aztreonam (17.6%) had higher percentages of MexAB-OprM overexpression compared to the isolates overall (11.6%). Twenty-two isolates that did not carry MBL genes overexpressed or modestly expressed *mexC*, 11 for *mexD* and 12 for *oprJ* (data not shown). These isolates were not present in any of the resistance subsets.

The genes from the two-component regulatory system PmrAB and the alternative spermidine biosynthesis pathway SpeDE2 that were previously associated to β-lactam resistance were either overexpressed or modestly overexpressed among 16/15 for *pmrA/pmrB* and 21/17 for *speD2/speE2* of the *P. aeruginosa* isolates that did not carry MBLs. PmrAB overexpression was more common among isolates resistant to ceftazidime-avibactam (22.5%) and ceftolozane-tazobactam (19.7%) than in the overall non-MBL isolates (9.0%).

Components of the operon *arnBCADTEF-ugd* were modestly overexpressed in 13 to 15 isolates. The operon *arnBCADTEF-ugd* promotes the addition of 4-aminoarabinose molecules to the lipopolysaccharide and contributes to the acquired resistance of polymyxins [18] as well as other agents in *P. aeruginosa*. Interestingly, these isolates were more common among ceftolozane-tazobactam- (10.8%) and ceftazidime-avibactam-resistant (14.3%) groups when compared to overall isolates (5.5%).

Lastly, *muxA, muxB*, and *muxC*, part of the RND efflux pump MuxABC-OpmB, were overexpressed in three to five isolates. Most of these isolates were resistant to ceftolozane-tazobactam (7.6%). Fewer isolates (1.2% to 4.4%) overexpressing *muxA, muxB*, and *muxC* were noted in the groups of isolates resistant to other agents.

Genes for which reduced expression contributes to β-lactam resistance included the outer-membrane proteins *oprD, oprG*, and *oprE*. As expected, meropenem- and imipenem-resistant isolates had a higher proportion (11.8% and 10.1%, respectively) of isolates displaying a decreased expression of oprD. Isolates resistant to ceftolozane-tazobactam had higher rates of isolates with decreased expression of *oprE* (12.2%) and *oprG* (18.2%) when compared to all isolates or other resistant subsets.

3.5. Effect of mutations

The evaluation of missense and nonsense mutations in the genes associated with β-lactam resistance demonstrated that 106 (49.8%) isolates had nonsensical mutations in the OprD gene, and another 14 (6.6%) isolates had deletions. These isolates included 88 isolates that did not carry MBL-encoding genes. OprD disruption was more common among imipenem- (70.7%) and meropenem- (70.6%) resistant isolates. Approximately 65% of the ceftazidime-avibactam-resistant isolates had an OprD disruption, despite the lack of evidence that this is an important resistance mechanism against this combination agent.

Gain of function (GoF) mutations in the *ampR* were noted in 11 isolates, including the alterations D135N and G154R, all of which displayed an overexpression of AmpC. Among the other genes involved in the expression of AmpC, *ampP* deletions were detected among 33 isolates and *ampD* disruptions and alterations were detected among 11 and 10 isolates, respectively. Deletions in *ampR* did not display correlation to any of the resistance groups, but 19, 20, and 21 of the isolates displaying a deletion/disruption in *ampD* were resistant to cefepime, ceftazidime, and piperacillin-tazobactam, respectively (Fig. 2).

Nine isolates had GoF alterations in the penicillin-binding protein (PBP) encoding gene *fstI*. All of these isolates were resistant to meropenem, and all but one were resistant to cefepime and piperacillin-tazobactam. Additionally, three isolates had deletions in *fstI* and were resistant to piperacillin-tazobactam (data not shown). Disruptions and deletions in the PBP gene *dacB* were noted in four and six isolates, respectively. These isolates were resistant to ceftazidime, and nine were also resistant to piperacillin-tazobactam (Fig. 2).

The disruption of efflux system regulators and other related genes were noted. The most common disruption observed was the disruption of *mexZ*, which was identified in 14 (8.1%) isolates. Another 19 isolates had deletions in this gene. Deletions in *mexZ* correlated to resistance to cefepime, meropenem, imipenem, and ceftolozane-tazobactam as well as overexpression of MexXY. Seven isolates had deletions in *mexR*. All of these isolates were resistant to piperacillin-tazobactam, and six were resistant to ceftazidime and/or meropenem.

The statistical analysis of discrete mutations in 33 constitutive genes revealed that the mutations in *armZ* that led to the amino acid alteration H182Q contributed to resistance to aztreonam, cefepime, and imipenem (Table 1). Additionally, the substitutions D161G and L88P affected resistance to imipenem and ceftazidime-avibactam, respectively. OprD substitutions from amino acids 371 to 382 were more likely to occur in isolates resistant to imipenem, and two (G423A and L359V) of these alterations were statistically more frequent among meropenem-resistant isolates. Notably, amino acid substitutions were associated with resistance to cefepime, including AmpO (S235T also for ceftazidime), ArmZ (H182Q), MexB (positions 1041 and 1042), MexC (A31V), PIB-1 (PA5542; S244T), and PepA (D121G also for ceftazidime). Similarly, resistance to aztreonam was more likely in isolates displaying ArmZ (H182Q), OXA-50 (R83K), and PIB-1 (I106V) substitutions. Additional significant mutations are listed in Table 1.

4. Discussion

Resistance to β-lactams in *P. aeruginosa* are caused by an interplay of various mutation-driven resistance mechanisms with or without the acquisition of foreign β-lactamase genes. A combination of DNA sequencing and the measurement of the expression of OprD by quantitative real-time PCR and protein detection have been broadly used to characterize β-lactam resistance in *P. aeruginosa*. This approach is often limited to a set of known genes. In this study, we used a combination of whole genome, transcriptome, and statistical analysis to include more than 5500 genes from the *P. aeruginosa* genome to understand how the expression of genes can contribute to resistance to the β-lactams commonly used to treat serious infections. Our analysis of expression heatmaps included all genes that were expressed $\pm$3-fold compared to *P. aeruginosa* PAO1. This investigation was aided by statistical methods to identify other genes that displayed differential expressions that would be significant within a specific population of resistant isolates. To strengthen our evaluation, we selected clinical isolates tested by reference susceptibility testing methods from various countries and differing β-lactam resistance profiles.

As expected, the overexpression of the AmpC cephalosporinases, the disruption (loss) or reduced expression of OprD, and the overexpression of multiple efflux pumps that have specificity for certain β-lactam agents were detected in many of the tested iso-

lates and were the main resistance mechanisms of older β-lactam agents/combinations.

Among our findings, the most notable conclusion was the effect of the overexpression of the *arnBCADTEF-ugd* operon and PmrAB on resistance to ceftazidime-avibactam. Two component regulatory systems (TCSs) are responsible for the cell response to environmental changes, including nutrition and osmolarity [19,20]. These regulatory systems do not detect the presence of antimicrobial agents but are able to sense stress and thus signal changes that promote a resistant state in the cell [19]. Several of the TCSs are associated with β-lactam resistance. PmrAB seems to be commonly associated with resistance to polymyxins and aminoglycosides [19,21]; however, a few studies demonstrate that changes in these genes have been associated with resistance to β-lactams. Muller et al. [20] demonstrated that *arnBCDTEF-ugd* and *mexXY* operons can downregulate the *oprD* gene through the activation of ParRS. In their experiments, the cascade of events triggered in cells exposed to polymyxins resulted in MDR, including resistance to β-lactam agents. Despite this elegant work by Mueller et al. [20], there were several hypothetical proteins changed by the polymyxin exposure that did not have a defined role. More investigations are needed to better understand the events leading to MDR mediated by TCSs.

Notably, the isolates in this study displaying overexpression of *arnBCDTEF-ugd* and PmrAB were not resistant to colistin. This finding requires further investigation, but the fact that multiple genes within the entire pathway were overexpressed corroborates our results.

The role of SpeDE2 in antimicrobial resistance has been described recently. These genes interact with *arnBCDTEF-ugd* and PmrAB, increasing both the expression of MexXY/OprM and the stress response in the cell. The overexpression of these genes when exposed to ceftazidime-avibactam and ceftolozane-tazobactam requires further investigation, but our data suggest that MexXY is important to ceftolozane-tazobactam resistance and that the overexpression of *oprM* can trigger an increase in *mexA* and *mexB.* This hypothesis remains to be confirmed. Other mechanisms that affect resistance to ceftazidime-avibactam were PDC Ω-loop deletions, which were detected in only two isolates, and the presence of VEB enzymes, which have been reported to cause resistance to ceftazidime-avibactam in *P. aeruginosa* [22].

There are fewer resistance mechanisms deployed by *P. aeruginosa* isolates that affect ceftazidime-avibactam and ceftolozane-tazobactam. The overexpression of PmrAB and the operon *arnBCADTEF-ugd* have been associated with elevated expression of MexXY [23]. This relationship was noted in our sampling among isolates that were resistant to ceftolozane-tazobactam and overexpressed *arnD* and *mexXY*. Resistance to ceftolozane-tazobactam was associated to production of carbapenemases, ESBLs, and MuxABC-OpmB and MexZ mutations. Tazobactam has limited inhibitory activity against some ESBLs and to most carbapenemases detected among the *P. aeruginosa* isolates, allowing the β-lactamases to freely hydrolyse ceftolozane. The overexpression of MexXY has been implicated in resistance to several β-lactams, but no experimental evidence has been generated with ceftolozane. In our observational studies, we noted that several isolates resistant to ceftolozane-tazobactam had elevated MexXY expression [9]. Lastly, Mima et al. demonstrated that the introduction of MuxABC-OpmB in a hypersusceptible *P. aeruginosa* isolates increased MIC values for aztreonam, among other agents [24]. Experimental evidence that this pump alters the MIC results for ceftolozane and/or tazobactam is warranted.

Few therapeutic options are available to treat MDR and carbapenem-resistant *P. aeruginosa*. This species displays high levels of resistance to many antimicrobial agents. Additionally, antipseudomonal β-lactams are still widely used for the treatment of these organisms. Among these agents, ceftolozane-tazobactam and ceftazidime-avibactam are the most active against *P. aeruginosa*, as fewer resistance mechanisms affect these agents. Resistance to these agents can be caused by the overexpression of efflux, but there are several isolates for which the usual resistance mechanisms cannot explain the phenotype. In a previous study, we identified that increased efflux and mutations in chaperones proteins and PBPs are associated with ceftazidime-avibactam resistance in *P. aeruginosa* isolates from U.S. hospitals. In that study, we evaluated gene expression by quantitative-real time PCR against a limited set of genes usually involved in β-lactam resistance. In the present study, in addition to the mutational analysis we previously performed, we expanded the analysis of gene expression to the entire *P. aeruginosa* transcriptome. This approach revealed an increased expression in pathways that have not been associated to resistance to ceftazidime-avibactam and ceftolozane-tazobactam but have been associated to cefepime resistance and resistance to other antimicrobial classes.

Our study did not experimentally confirm the role of novel resistance mechanisms such as overexpression of PmrAB, *arnBCADTEF-ugd* operon, and MuxABC-OpmB. An additional limitation of this study was that we did not create a multifactorial analysis evaluating the effect of combined resistance mechanisms. The resistance mechanisms in *P. aeruginosa* isolates can only be fully understood with more detailed analyses. Hopefully, this study and others will contribute to algorithms designed to detect and evaluate these mechanisms without laborious experiments and help create new therapies.

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Competing interests

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Ethical approval

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