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Bla$_{GES-6}$ producing *Pseudomonas aeruginosa* ST235 is involved in resistance to different β-lactams

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ABSTRACT

Multidrug resistance in *Pseudomonas aeruginosa*, particularly resistance to carbapenem, represents a major challenge for public health. This study investigated resistance mechanisms in three *P. aeruginosa* isolates: HU63 (blaGES-6 carbapenemase-positive), HU141 (carbapenem-resistant without carbapenemase), and PAO1 (control). Genomic analysis revealed distinct sequence types (ST235 for HU63, ST253 for HU141) and chromosomal integration of resistance genes. HU63 harbored diverse resistance mechanisms, including β-lactamases (*bla*$_{GES-6}$, *bla*$_{PDC-35}$, *bla*$_{OXA-488}$) and efflux pumps. Minimum inhibitory concentration assays demonstrated HU63's resistance to all β-lactams tested (meropenem, imipenem-cilastatin, ceftazidime, piperacillin-tazobactam), while HU141 remained susceptible except to cefoxitin and cloxacillin. Time-kill assays revealed tolerance phenotypes, with HU63 showing regrowth after 8–24 h despite initial reductions in bacterial density. Gene expression varied significantlydepending on the antibiotic and the isolate. The HU63 isolate (GES-6 positive) stands out for its marked induction of *bla*$_{GES-6}$ in all the antibiotics tested, contributing to its resistance to carbapenems and broad-spectrum cephalosporins. These expression profiles corroborate the classic molecular mechanisms of resistance: regulation of entry pores (*oprD*), activation of efflux pumps (*mexA*) and production of β-lactamases (*bla*$_{GES-6}$, *ampC*) adapted to each situation. These findings underscore the multifactorial nature of resistance in Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA), combining enzymatic inactivation, efflux, and genetic adaptability. The study emphasizes the urgent need for genomic surveillance to track high-risk clones and develop therapies targeting tolerance mechanisms alongside traditional resistance.

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1. Introduction

Multidrug resistance, including resistance to carbapenems, has become a widely observed characteristic in *Pseudomonas aeruginosa* isolates [1]. This phenomenon arises from the accumulation of multiple resistance determinants in this species, compounded by its limited intrinsic susceptibility to a wide range of therapeutic agents [2].

The antibiotics used to treat *P. aeruginosa* infections are generally broad-spectrum, due to the complexity and intrinsic resistance of this bacterium. Meropenem, a carbapenem, is effective against a wide range of gram-negative bacteria, including *P. aeruginosa* [3]. However, the spread of carbapenemases, such as VIM and GES, has significantly reduced its efficacy in this context [4]. Imipenem, another carbapenem with activity against *P. aeruginosa*, is administered alongside cilastatin, which inhibits its renal degradation and prolongs its therapeutic action [5]. Nonetheless, the production of carbapenemases by the bacterium limits its effectiveness as well [6,7]. Piperacillin, a broad-spectrum penicillin, when combined with tazobactam, a β-lactamase inhibitor, enhances its activity against bacteria that produce β-lactamases [8]. However, this combination is ineffective against carbapenem-resistant *P. aeruginosa* (CRPA) [8,9]. Ceftazidime, a third-generation cephalosporin, demonstrates robust activity against *P. aeruginosa* [10]. Yet, the emergence of extended-spectrum β-lactamases (ESBLs) and carbapenemases in *Pseudomonas* can compromise its efficacy [10,11]. Cefoxitin, a second-generation cephalosporin, exhibits limited activity against *P. aeruginosa* and is generally less effective than third-generation β-lactams or carbapenems in treating infections caused by this bacterium [12,13]. Cloxacillin, a β-lactamase-resistant penicillin, has reduced efficacy against *P. aeruginosa* and is rarely used for treating infections caused by this organism, which possesses multiple mechanisms of resistance to β-lactams [14].

CRPA represents a growing public health threat and is of great concern due to its ability to resist last-line antibiotics. In Portugal, the most frequently identified CRPA variants include VIM-2, IMP-5, and GES-6 [15]. Guyanese extended-spectrum β-lactamase (GES), initially described in a clinical isolate of *Klebsiella pneumoniae* from Guyana, France, is particularly relevant in this context [16]. Several isoforms of the bla_{GES} gene (GES-2, -4, -5, -6, -13, −14, −15, −16, −18, −20, −21, and −24) show a characteristic glycine substitution at position 170 by asparagine or serine, which results in structural changes that impact the resistance profile [17].

Our *in vitro* study began with the isolation of a clinical multidrug-resistant *P. aeruginosa* isolate containing the bla_{GES} gene. The primary objective was to investigate the antibacterial activity against three *P. aeruginosa* isolates, selected to represent the genomic and phenotypic diversity of the species. One isolate carried the blaGES-6 carbapenemase, the second was resistant to carbapenems but did not produce carbapenemase, and the third, PAO1, was used as a control isolate. The efficacy of various β-lactam antibiotics was assessed by determining the minimum inhibitory concentrations (MIC). Additionally, the mechanisms of action of these compounds were explored using time-kill assays. Gene expression related to antibiotic targets was analyzed using real-time PCR (qPCR) at different exposure times and with various antibiotics.

2. Materials and methods

2.1. Bacterial isolates

In the present study, clinical isolates of *P. aeruginosa* were utilized, including the GES-6-producing isolate (HU63) and the non-carbapenemase-producing isolate (HU141). For comparison, the standard *P. aeruginosa* PAO1 isolate, which is widely used in scientific research, served as a reference.

2.2. Evaluation of antibacterial activity

Antibiotic susceptibility testing (AST) was performed on Mueller–Hinton agar (Frilabo, Maia, Portugal) plates using both the standard disk diffusion technique (Kirby-Bauer test) and the Vitek 2 system (bioMérieux, Marcy l'Étoile, France). The Vitek 2 system was primarily employed in the hospital for the rapid identification of antibiotic resistance. Concurrently, the Kirby-Bauer test was carried out in the laboratory to confirm and validate the resistance and susceptibility patterns observed. The AST was conducted following the guidelines established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2022).

The Minimum Inhibitory Concentration (MIC) of the antibiotics meropenem (Ikma Pharmaceuticals, Portugal), imipenem + cilastatin (Ikma Pharmaceuticals, Portugal), ceftazidime (Sigma-Aldrich, St. Louis, MO, USA), cefoxitin (Ikma Pharmaceuticals, Portugal), piperacillin + tazobactam (Fresenius Kabi, Bad Homburg, Germany), and cloxacillin (Sigma-Aldrich, St. Louis, MO, USA) was determined using the microdilution method in Mueller-Hinton broth (Frilabo, Portugal). Isolates were cultured in Mueller-Hinton broth for 24 h at 37 °C, then diluted to a concentration of 5×10^5 cells/mL. These dilutions were subsequently exposed to increasing concentrations of antibiotics (up to 512 μg/mL) in 96-well microtiter plates. The MIC was assessed after a 20 h incubation period at 37 °C, following the standards set by EUCAST. Each isolate was tested in at least three independent replicates.

2.3. Molecular characterization

The hospital of Clermont-Ferrand, France, conducted whole-genome sequencing (WGS) of the isolate HU63. DNA extraction was conducted utilizing the DNeasy UltraClean Microbial kit (Qiagen, Hilden, Germany). Following extraction, libraries were prepared employing the Nextera XT Kit sourced from Illumina (San Diego, CA, USA) and underwent sequencing on the Illumina MiSeq system, generating 2 × 300base pair (bp). Libraries from 1 ng of genomic DNA were prepared using the dual-indexed Nextera XT Illumina library preparation kit before cluster generation and paired-end sequencing (2 × 150 bp) on a NextSeq 550 Illumina platform (Illumina Inc., San Diego, CA).The average depth of mapped reads stood at 98.4X ± 9.9 (mean ± standard deviation), with a minimum of 81-fold coverage, while the average breadth of coverage was 95.1 % ± 1.0 %, referencing the PAO1 genome (NC_002516.2).

The isolate HU141 were sequenced at the National Institute of Health Dr. Ricardo Jorge in Portugal. Genomic DNA extraction was performed using the MagNA Pure 96 instrument (Roche, Mannheim, Germany) and quantified using the Qubit Fluorometric Quantitation (Thermo Fisher Scientific, Waltham, MA).

De novo assembly was performed using INNUca (v 4.2.2-02) [INNUca GitHub: https://github.com/B-UMMI/INNUca], with the options –speciesExpected "*Pseudomonas aeruginosa*" –genomeSizeExpectedMb "6.3" –runKraken. The Kraken2 database utilized was the standard one updated as of 2023-06-05.

2.4. Profiling antibiotic resistance genes

The identification of resistance genes was conducted using established and curated databases, including ResFinder v4.4.3, the Comprehensive Antibiotic Resistance Database (CARD) [18], and BV-BRC v3.32.31a [19]. For ResFinder v4.4.3 [20], the parameters included a 90 % threshold for percentage identity (%ID) and a minimum length of 60 % for chromosomal point mutations and acquired antimicrobial resistance genes. The parameters for CARD and BV-BRC v3.32.31a were utilized in their default settings.

2.5. Time-kill curves

Antibiotic tolerance measurements were conducted using cell death curves. Briefly, each isolate was grown overnight in Mueller-Hinton broth from isolated colonies. The resulting culture was adjusted to an optical density (OD) of 0.08–0.12 at 600 nm. Antibiotics were then added to the bacterial culture at a concentration twice the minimum inhibitory concentration, and the tubes were incubated at 37 °C with shaking. At predetermined time intervals, the number of viable cells was monitored. Colony-forming units (CFUs) were counted using serial dilutions and plating. Survival was calculated as the ratio of the number of colonies at different time points to the initial number of colonies. Each isolate was tested in at least three independent replicates.

2.6. Quantitative reverse transcription PCR (qPCR)

Total RNA extraction was performed using the method described by Atshan et al. [21]. The concentration and quality of RNA were assessed by the A260/A280 ratio using a PowerWave XS2 spectrophotometer (BioTek Instruments, Inc., Winooski, USA) and RNA integrity was confirmed by agarose gel electrophoresis. First-strand cDNA was synthesized from 1,000 ng/μL of total RNA according to the manufacturer's protocol, using an MMLV reverse transcription cDNA synthesis kit (Lucigen, Foster City, USA). The resulting cDNA mixture was diluted at a ratio of 1:10 and stored at −20 °C.

In this study, 5genes were investigated: one housekeeping gene, one associated with MexA efflux pumps and outer membrane porin D, two associated with B-lactamase BlaGES and AmpC, and two housekeeping genes (as shown in Table 1). Specific primers for the bacterial genes were designed based on sequences available in the NCBI database. Primer pairs were prepared using Primer 3 v0.4.0 software, following the criteria of 20–25 bp in size, GC content between 45 % and 60 %, and melting temperature (Tm) around 60–62C.

Gene expression was initially evaluated using semi-quantitative PCR with the Taq PCR Master Mix kit (BIORON, Römerberg, Germany) in a BioRad T100 thermocycler (BioRad, Hercules, USA). Quantitative real-time PCR analyses were conducted using a StepOnePlus system (Applied Biosystems, Foster City, USA). Amplification of each biological sample was performed in triplicate using the ABsolute qPCR SYBR Green mix (Thermo Fisher Scientific, Vilnius, Lithuania), and data were analyzed with the software provided by the equipment. Only the threshold values of the quantification cycle (Ct) were considered, leading to an average Ct with a standard deviation of less than 0.5. The expression values were normalized by the average of reference gene expression, according to Livak [22]. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative mRNA levels of genes [22].

The average PCR efficiency for each gene was determined using standard curves created from tenfold dilutions of the corresponding cDNA mixture, evaluated in triplicate. Efficiency values ranged from 96 % to 108 % for reference and target genes (as shown in Table 1) and were calculated using StepOnePlus Real-Time PCR software (Applied Biosystems, Foster City, USA). No nonspecific products or primer-dimers were detected in melting curve analysis. Therefore, the observed efficiency values are considered acceptable and do not compromise the reliability of the qPCR data. For normalization, one reference gene, *guaA*, were used. Gene expression values were normalized relative to the average expression of these reference genes, following the method proposed by Livak. The relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1
Species-specific genes and primers for PCR and qPCR identification of *P. aeruginosa*.

Gene	Sequence	bp	Tm
***guaA*- F**	AGGTCGGTTCCTCCAAGGTC	372	60
***guaA*- R**	GACGTTGTGGTGCGACTTGA		
***mexA* - F**	ACCTACGAGGCCGACTACCAGA	1152	56
***mexA* - R**	GTTGGTCACCAGGGCGCCTTC		
***oprD* - F**	TCCGCAGGTAGCACTCAGTTC	191	55
***oprD* - R**	AAGCCGGATTCATAGGTGGTG		
bla_{Ges-6} - F	CACGTACTGTGGCTAAAGTCCT	152	60
bla_{Ges-6} - R	ATCTTTAGGAAAACCCGCTCGT		
***ampC*-F**	GATGAAGGCCAATGACATTCCG	576	58
***ampC*-R**	CATGTCGCCGACCTTGTAGTAA		

2.7. Statistical analysis

For variables that showed considerable skewness, data were transformed to the (log (1+x)), and linear modelling on the log scale was performed. Values are represented by mean ± standard deviation (SD) and *t*-test in GraphPad Prism v8, with $p < 0.05$ considered statistically significant.

3. Results

3.1. Genomic diversity between isolates

This study focuses on two *P. aeruginosa* isolates, one containing the β-lactamase GES-6 and the other lacking any carbapenemase, both previously isolated from urinary tract infection samples. Using whole-genome sequencing, we performed a genomic comparison of the two isolates (Table 2). Neither isolate contained plasmids. Isolate HU63 and HU141 have distinct sequence types: HU63 is from ST235 and HU141 is from ST253.

Synteny analysis helps to illustrate the organization of genes around the bla_{GES} gene in different *P. aeruginosa* isolates and assess whether there is gene conservation around this gene across the species (Fig. 1). The region surrounding the bla_{GES} gene (represented by a red arrow in all isolates) shows partial conservation across several selected *P. aeruginosa* isolates. In isolate such as *P. aeruginosa* HU63 and others (4–94, 4–121, 4–92, 1–13, 4–120), there is a well-conserved sequence of genes flanking bla_{GES}, with arrows in green (*Intl1* gene), blue (*aac(6′)-Ic* gene), and orange (transposase), suggesting associated genes or conserved operons.

Isolates such as *P. aeruginosa* PALA50 and PAS8 exhibit greater variation in gene order before and after bla_{GES}, with different colors representing different gene sets. Variations in gene arrangement may also indicate the presence of mobile genetic elements, such as plasmids or transposons, that can carry antibiotic resistance genes like bla_{GES}. The patterns of arrows indicating the direction of gene transcription in some strains appear interrupted or reorganized, particularly in the PALA50 and PAS8 isolates.

3.2. Assessing antibiotic resistance mechanisms in isolate HU63

In Table 3 dataset, the presence of several resistance genes is observed, each acting on different classes of antibiotics through multiple mechanisms. The *emrE* gene, belonging to the methyltransferase family, is associated with aminoglycoside resistance and functions through efflux pumping. YajC, classified as an RND (resistance-nodulation-cell division) efflux pump, confers resistance to a broad spectrum of antibiotics, including fluoroquinolones, cephalosporins, glycylcyclines,

Table 2
Genomic information of the isolates.

Bacteria ID	MLST	Origin	Genome (bp)	RefSeq accession in GenBank database
HU63	235	Urinary tract infection	6,771,287	SAMN40216655
HU141	253	Urinary tract infection	6,983,523	SAMN40216632

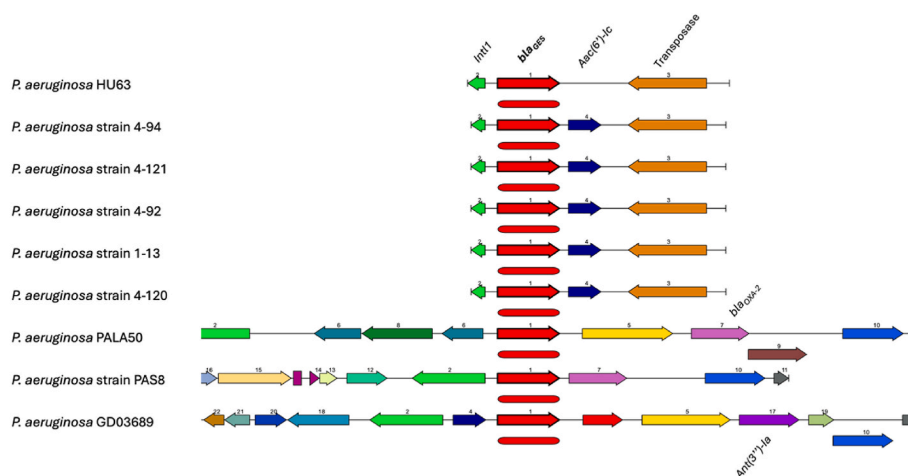

Fig. 1. Synteny analysis of the bla_{GES} gene associated with the system was conducted using the BV-BRC v3.32.31a tool.

Table 3
Resistance gene profile and mechanisms in antibiotic-resistant HU63.

Resistance gene	Gene family	Class of antibiotics	Mechanisms of resistance	Identity (%)
emrE	Metiltransferases	aminoglycoside	antibiotic efflux	100
yajC	RND antibiotic efflux pump	fluoroquinolone antibiotic, cephalosporin, glycylcycline, penicillins, tetracycline antibiotic, oxazolidinone antibiotic, glycopeptide antibiotic, rifamycin antibiotic	antibiotic efflux	100
fosA	fosfomycin thiol transferase	phosphonic acid antibiotic	antibiotic inactivation	100.0
basR	pmr phosphoethanolamine transferase	peptide antibiotic	antibiotic target alteration, antibiotic efflux	99.55
rsmA	RND antibiotic efflux pump	fluoroquinolone antibiotic, diaminopyrimidine antibiotic, phenicol antibiotic	antibiotic efflux	100
soxR	transcriptional regulator of ABC, MFS, and RND antibiotic efflux pump	fluoroquinolones, cephalosporins, glycylcycline antibiotics, penicillins, tetracyclines, rifampicin, amphenicols	Target modification, antibiotic efflux	100
cpxR	RND antibiotic efflux pump	macrolide antibiotic, fluoroquinolone antibiotic, monobactam, aminoglycoside antibiotic, carbapenem, cephalosporin, cephamycin, penicillins, tetracycline antibiotic, peptide antibiotic, aminocoumarin antibiotic, diaminopyrimidine antibiotic, sulfonamide antibiotic, phenicol antibiotic	antibiotic efflux	100
arnA	pmr phosphoethanolamine transferase	peptide antibiotic	antibiotic target alteration	99.55
$\boldsymbol{bla_{PDC\text{-}35}}$	PDC β-lactamase	monobactam, carbapenem, cephalosporin	antibiotic inactivation	100.0
$\boldsymbol{bla_{OXA\text{-}488}}$	OXA β-lactamase, OXA-50-like β-lactamase	penicillins	antibiotic inactivation	100.0
$\boldsymbol{bla_{GES\text{-}6}}$	GES β-lactamase	carbapenem, cephalosporin, penicillins	antibiotic inactivation	100.0
ant(2″)-Ia	ANT(2″)	aminoglycoside antibiotic	antibiotic inactivation	100.0
sul1	sulfonamide resistant sul	sulfonamide antibiotic	antibiotic target replacement	100.0
aph(3′)-IIb	aminoglycoside phosphotransferase	aminoglycoside antibiotic	antibiotic inactivation	98.88
catB7	chloramphenicol acetyltransferase (CAT)	phenicol antibiotic	antibiotic inactivation	98.58
gyrA	fluoroquinolone resistant gyrA T83I	fluoroquinolone antibiotic	antibiotic target alteration	99.78

penams, tetracyclines, oxazolidinones, glycopeptides, and rifamycins. FosA, a fosfomycin thiol transferase, inactivates fosfomycin, a phosphonic acid-based antibiotic.

The *basR* gene, which encodes a *pmr* phosphoethanolamine transferase, contributes to resistance to peptide antibiotics by both target modification and efflux. RsmA, another RND-type efflux pump, interferes with susceptibility to fluoroquinolones, diaminopyrimidines, and phenicols. The transcriptional regulator *soxR*, associated with ABC, MFS, and RND efflux pumps, confers resistance through target modification and efflux mechanisms against fluoroquinolones, cephalosporins, glycylcyclines, penicillins, tetracyclines, rifampicin, and amphenicols.

Similarly, the *cpxR* regulator, another component of RND efflux pumps, is involved in resistance to macrolides, fluoroquinolones, monobactams, aminoglycosides, carbapenems, cephalosporins, cephamycins, penicillins, tetracyclines, peptides, aminocoumarins, diaminopyrimidines, sulfonamides, and phenicols. The *arnA* gene, another pmr phosphoethanolamine transferase, promotes resistance to peptide antibiotics through target modification.

Regarding β-lactamases, $Bla_{PDC\text{-}35}$, a PDC-type enzyme, inactivates monobactams, carbapenems, and cephalosporins. $Bla_{OXA\text{-}488}$, belonging to the OXA family (OXA-50-like subtype), inactivates penicillins. $bla_{GES\text{-}6}$, a GES β-lactamase, confers resistance to carbapenems, cephalosporins, and penicillins.

Other resistance genes include *ant(2″)-Ia* (from the ANT(2″) family), which inactivates aminoglycosides, and *sul1*, a Sul-type sulfonamide resistance gene that modifies the enzyme target to confer sulfonamide resistance. *Aph(3′)-IIb*, responsible for aminoglycoside phosphorylation, also inactivates this class of antibiotics. *catB7*, encoding a

chloramphenicol acetyltransferase (CAT), inactivates phenicols. Finally, the *gyrA* gene, with the T83I mutation, is linked to fluoroquinolone resistance through target modification.

Taken together, these results reveal a wide variety of resistance mechanisms, including enzyme inactivation, target modification, and efflux pumping, that may significantly contribute to the multidrug-resistant phenotype of this isolate.

3.3. Antibacterial activity of β-Lactam antibiotics

Several classes of beta-lactams were tested to evaluate the antimicrobial activity of the isolates. In the penicillin group, piperacillin + tazobactam (antipseudomonal) and cloxacillin (resistant to penicillinase) were included. Among the cephalosporins, cefotetan (2nd generation) and ceftazidime (3rd generation) were tested. For carbapenems, meropenem and imipenem + cilastatin were used. The activity of the different antibiotics varied depending on the isolates (Table 4). As a control, the PAO1 isolate was used, which exhibited the expected profile of resistance to cefoxitin and cloxacillin and susceptibility to other antibiotics. Notably, isolate HU63, carrying the β-lactamase GES-6, showed resistance to all beta-lactams tested. In contrast, isolate HU141 exhibited the expected resistance to cefoxitin and cloxacillin while remaining susceptible to the other antibiotics.

Kinetic analysis of interactions between bacteria and antibiotics over time revealed a bacterial survival strategy distinct from phenotypic resistance. The slow decrease in cell density after the addition of antibiotics, despite concentrations exceeding the MIC, suggests the presence of a tolerant phenotype. This phenotype is characterized by a reduction in cellular activity, enabling survival by avoiding or repairing damage caused by the drug, rather than by expressing classical resistance mechanisms. Time-kill curves were generated for the panel of isolates against different antibiotics, using concentrations 2x MIC, with samples collected at 0h, 4h, 8h, and 24h, and the population response quantified in colony-forming units (CFU) (Fig. 2). Significant differences were observed between the antibiotics and at different time points.

Surprisingly, isolate HU141 demonstrated faster adaptation to antibiotics upon initial contact. Its response to cefoxitin and cloxacillin remained stable over time, while among penicillin's, bacterial growth increased within 8 h. With carbapenems, a reduction in bacterial density was observed after 8 h, followed by a slight increase at 24 h. As for isolate HU63, which harbors the $bla_{GES\text{-}6}$ gene, its response to cefoxitin and cloxacillin was like that observed for HU141, whereas for the other antibiotics, there was a decrease in bacterial density until 8 h, followed by an increase. Interestingly, with carbapenems, particularly meropenem, there was a marked reduction in bacterial density at 8 h, and with imipenem + cilastatin, this reduction occurred within the first hour, followed by additional bacterial growth.

3.4. Variations in antibiotic response and gene expression

Based on the results obtained from antibiotic susceptibility testing of the isolates, different responses to treatment were observed. The GES-6–producing isolate showed greater initial susceptibility to several antibiotics, whereas the other isolate exhibited a more resistant phenotype. These observed variations highlight the importance of further investigating the molecular mechanisms underlying antibiotic resistance. Given the relevance of these mechanisms, differential gene expression, particularly of efflux pump–associated genes, may help explain the variability in antibiotic responses among isolates.

The expression levels of the genes *mexA*, *oprD*, $bla_{GES\text{-}6}$, and *ampC* were assessed in each isolate. The *mexA* gene, which is part of a Resistance–Nodulation–Cell Division (RND) type efflux system, encodes a periplasmic membrane fusion protein that connects the inner membrane pump to the outer membrane channel. The *oprD* gene encodes the outer membrane porin OprD, which functions as a selective channel for the uptake of certain nutrients and antibiotics, particularly carbapenems. The $bla_{GES\text{-}6}$, gene is a variant of the bla_{GES} family, encoding a specific ESBL with activity against various β-lactam antibiotics. Finally, the ampC gene encodes a chromosomally-encoded *AmpC* β-lactamase, naturally present in *P. aeruginosa*, capable of hydrolyzing first- and second-generation cephalosporins as well as other penicillins.

In response to ceftazidime (Fig. 3), the reference isolate HU63 ($p = 0.540225$), HU142 ($p = 0.950231$), and PAO1 ($p = 0.746922$) showed no significant variation in *mexA* expression between 4 h and 8 h. The *oprD* gene exhibited very low expression levels in all isolates at 4 h but showed statistically significant increases at 8 h in HU63 ($p = 0.000019$), HU141 ($p = 0.000138$), and PAO1 ($p = 0.015435$). The $bla_{GES\text{-}6}$ gene was significantly induced in HU63 ($p = 0.0020$), increasing from near-undetectable levels at 4 h to approximately four-fold higher expression at 8 h. The *ampC* gene showed a non-significant increase in expression in HU63 ($p = 0.630235$) and PAO1 ($p = 0.102788$) after 8 h, while in HU141 ($p = 0.874563$), expression remained nearly unchanged.

For treatment with imipenem + cilastatin (Fig. 4), *mexA* expression remained stable in PAO1 at 8 h ($p = 0.756447$). In contrast, HU63 and HU141 showed reduced *mexA* expression ($p = 0.511277$ and $p = 0.299548$, respectively). The *oprD* gene was significantly repressed in HU63 ($p = 0.004733$) and PAO1 ($p = 0.022874$), with a notable decrease in expression after 8 h. In HU141, there was a slight but non-significant reduction ($p = 0.095656$). Induction of $bla_{GES\text{-}6}$occurred exclusively and significantly in HU63 ($p = 0.0492$). The expression of *ampC* remained very low in HU63 under both conditions ($p = 0.086938$), while in HU141 ($p = 0.022293$) and PAO1 ($p = 0.248059$), expression increased at 8 h.

In the case of meropenem treatment (Fig. 5), *mexA* expression increased slightly but not significantly in PAO1 ($p = 0.160497$) at 8 h, whereas HU63 and HU141 showed decreased *mexA* expression during the same interval, though the changes were not statistically significant ($p = 0.541147$ and $p = 0.074194$, respectively). For *oprD*, PAO1 maintained stable expression between 4 and 8 h ($p = 0.249507$). HU63 exhibited a reduction at 8 h ($p = 0.052840$), while HU141 showed an increase in transcript levels ($p = 0.719286$). The expression of $bla_{GES\text{-}6}$ significantly decreased after 8 h ($p = 0.0229$). Regarding *ampC*, PAO1 showed a significant increase in expression from 4 to 8 h ($p = 0.001745$), whereas the increases observed in HU63 ($p = 0.366707$) and HU141 ($p = 0.190842$) were not statistically significant.

Under piperacillin + tazobactam treatment (Fig. 6), an increase in *mexA* expression was observed in HU63 ($p = 0.786357$) and PAO1 ($p = 0.028264$), whereas HU141 ($p = 0.636389$) showed a reduction in *mexA* expression. The *oprD* gene was significantly induced over time in HU63 ($p = 0.004106$), while both HU141 ($p = 0.111132$) and PAO1 ($p = 0.046208$) also showed increased expression. $bla_{GES\text{-}6}$ gene expression decreased significantly over time ($p = 0.0125$) in all isolates at 8 h. Finally, ampC was significantly induced in HU63 ($p = 0.000148$) and

Table 4
Minimum inhibitory concentrations of various β-lactam antibiotics against three *P. aeruginosa* isolates.

Bacteria ID	MIC of meropenem (μg/mL)	MIC of imipenem + cilastatin (μg/mL)	MIC of ceftazidime (μg/mL)	MIC of cefoxitin (μg/mL)	MIC of piperacillin + tazobactam (μg/mL)	MIC of cloxacillin (μg/mL)
HU63	32	16	>128	>128	128	>128
HU141	1	0,5	1	>128	8	>128
PAO1	1	1	0,125	>128	2	>128

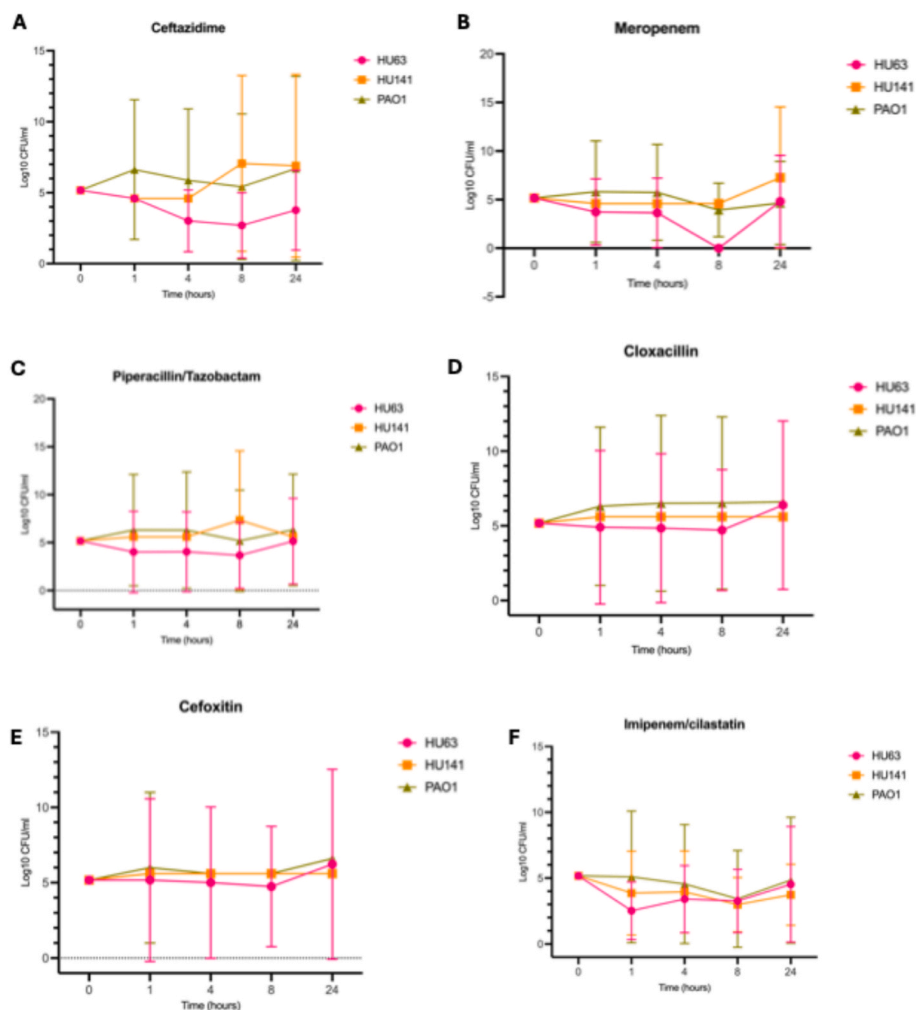

Fig. 2. Time-kill curves for different antibiotics in *P. aeruginosa* isolates.

PAO1 ($p = 0.000006$); although HU141 also showed an increase, it was not statistically significant ($p = 0.250087$).

4. Discussion

In this genomic comparison study of two *P. aeruginosa* isolates from urinary tract infections, the focus is on the detailed analysis of resistance mechanisms and genomic diversity between an isolate carrying the GES-6 β-lactamase (HU63) and another that does not harbor any carbapenemase (HU141). Both isolates were subjected to complete genome sequencing, enabling the identification of striking differences in terms of sequence types and the organization of genes associated with resistance. Isolate HU63 belongs to MLST sequence type 235 (ST235), while HU141 is of ST253, highlighting the intraspecific genetic diversity characteristic of *P. aeruginosa* and underscoring the importance of molecular surveillance to monitor high-risk clones that disseminate resistance mechanisms in hospital environments. These MLST types indicate that although both strains are associated with urinary tract infections, they belong to distinct genetic lineages. ST235, for example, is internationally recognized as a high-risk clone, frequently linked to hospital outbreaks and high antibiotic resistance profiles, making it particularly relevant in clinical settings [15,23–25]. In this study, the isolate HU63 was identified as a member of the ST235 lineage, a globally disseminated high-risk clone of *P. aeruginosa*. This lineage are of particular concern because they frequently harbor chromosomally encoded carbapenemase genes, such as bla_{GES}, bla_{VIM}, and bla_{IMP}, which confer resistance to last-line β-lactam antibiotics. In HU63, $bla_{GES\text{-}6}$ was found integrated into the chromosome, coexisting with other β-lactamases ($bla_{OXA\text{-}488}$ and $bla_{PDC\text{-}35}$), overexpression of the efflux pump gene *mexA*, and downregulation of *oprD*, a porin involved in carbapenem uptake. This combination reflects the classic multifactorial resistance phenotype of high-risk *P. aeruginosa* clones and emphasizes the isolates adaptive capacity under antibiotic pressure. Notably, no plasmids were detected in HU63, suggesting that its resistance determinants are stably maintained in the genome, which may facilitate persistent colonization and transmission within healthcare environments. These findings are in line with previous studies reporting that ST235 isolates often exhibit genomic plasticity and resistance gene integration into mobile elements, such as integrons and transposons, while maintaining stable chromosomal architecture [23].

On the other hand, ST253 is also significant and may exhibit distinct patterns of virulence and resistance, reflecting regional or epidemiological variations [26]. The absence of plasmids in both isolates suggests that the resistance mechanisms are chromosomally encoded, indicating a stability that may favor the persistence of these traits over time, as previously described in studies on the P. *aeruginosa* genome [27,28].

Synteny analysis plays a fundamental role in illustrating the organization of genes around the $bla_{GES\text{-}6}$ gene in different *P. aeruginosa* isolates, enabling the assessment of the genetic conservation of this region across the species. The region surrounding the $bla_{GES\text{-}6}$ gene shows partial conservation in the selected isolates. This analysis revealed consistent patterns as well as notable differences among isolates, reflecting distinct genetic mechanisms that may contribute to the spread of antimicrobial resistance. The presence of *Intl1* indicates a possible

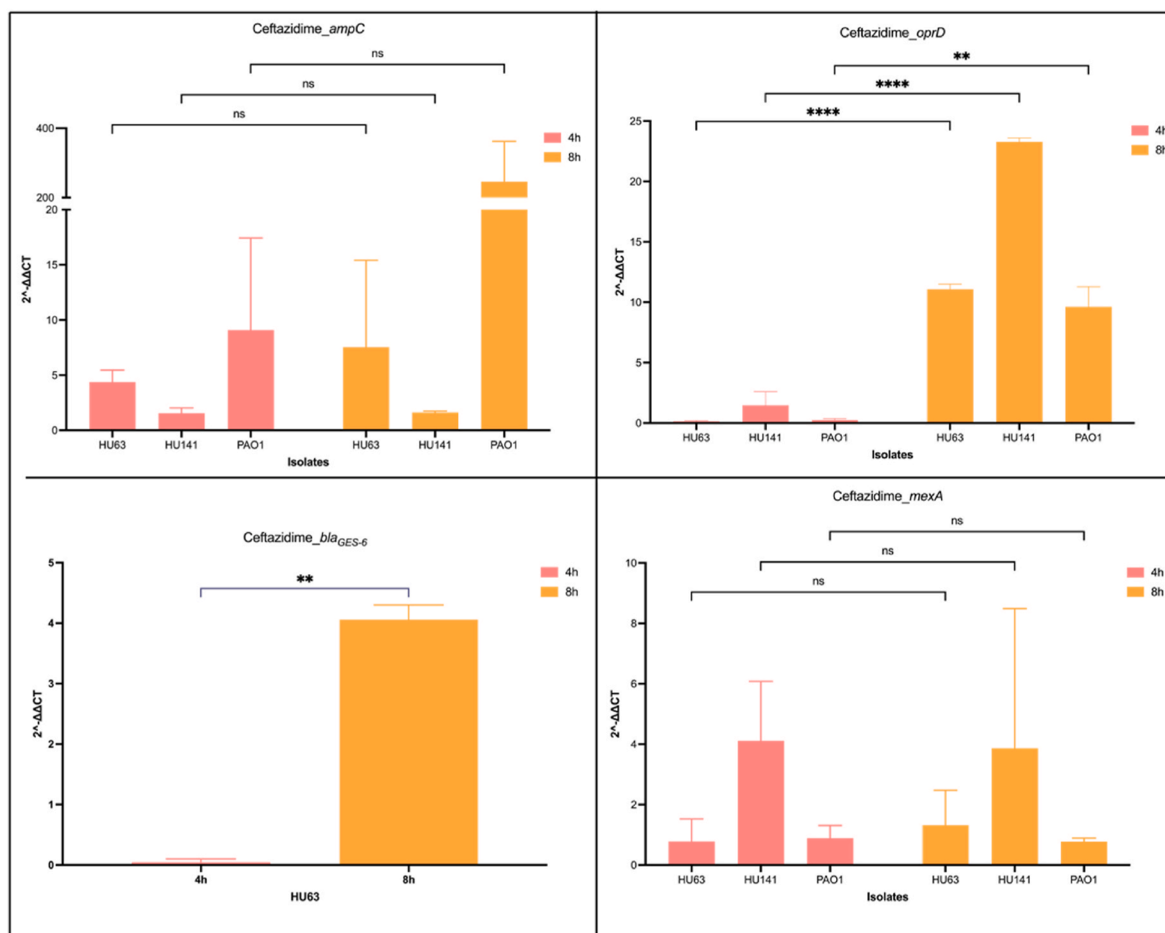

Fig. 3. Relative expression of the genes *mexA*, *oprD*, bla_{GES-6}, and *ampC* in *P. aeruginosa* isolates PAO1 (reference), HU63 (GES-6 producer), and HU141 (non-carbapenemase producer) after exposure to ceftazidime for 4 h (pink bars) and 8 h (orange bars). Bars represent mean ± SD of three independent experiments. Statistical differences are indicated as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and n.s. (not significant).

association with class 1 integrons, which are known to facilitate the capture and transfer of resistance gene cassettes, thereby increasing genomic plasticity in *P. aeruginosa* [29,30].

In contrast, isolates such as *P. aeruginosa* PALA50 and PAS8 exhibit greater variation in the order of genes before and after bla_{GES-6}. This reorganization can be attributed to the presence of mobile genetic elements, such as transposons, which carry antibiotic resistance genes [30,31]. Additionally, strains such as *P. aeruginosa* PAS8 and GD03689 showed significant differences in gene organization around bla_{GES-6}. These structural changes may result from genetic recombination events, suggesting distinct evolutionary adaptations. Reorganization can directly influence the expression of resistance genes and, consequently, the resistance phenotype exhibited by these isolates [30,32]. These results highlight significant variation in the genomic organization around the bla_{GES-6} gene, underscoring the importance of synteny analysis to understand genetic mobility.

Analysis of resistance mechanisms in *P. aeruginosa* reveals that isolate HU63 employs a multifactorial strategy, combining three key processes: enzymatic inactivation of antibiotics, active efflux through efflux systems, and modification of molecular drug targets. Three β-lactamases, Bla_{PDC-35}, a PDC-type enzyme capable of inactivating monobactams, carbapenems, and cephalosporins, $Bla_{OXA-488}$, belonging to the OXA family (specifically the OXA-50-like subtype), which primarily inactivates penicillins, and Bla_{GES-6}, a GES-type β-lactamase with demonstrated activity against carbapenems, cephalosporins, and penicillins. Enzymatic assays confirm that their presence creates a robust barrier to β -lactams. Experimental studies, including enzymatic assays and kinetic analyses, have confirmed that the simultaneous presence of these enzymes creates a robust barrier against beta-lactam antibiotics, severely compromising the efficacy of conventional treatments [28,33].

The antibacterial activity of β-lactam antibiotics was evaluated on different *P. aeruginosa* isolates, including HU63 and HU141, with the PAO1 isolate serving as a control. The results revealed significant variations in susceptibility to the antibiotics tested, reflecting the diversity of resistance mechanisms present in each isolate. Isolate HU63, which carries the bla_{GES-6} gene, exhibited resistance to all beta-lactams tested, including piperacillin-tazobactam, cefotetan, ceftazidime, meropenem, and imipenem-cilastatin. This resistance profile aligns with previous studies that associate GES β-lactamases with the inactivation of a broad spectrum of beta-lactam antibiotics, including carbapenems and third-generation cephalosporins. In contrast, isolate HU141 showed expected resistance to cefoxitin and cloxacillin but remained susceptible to the other antibiotics tested. This pattern suggests the absence of ESBLs or carbapenemases in this isolate, indicating that other resistance mechanisms, such as alterations in membrane permeability or efflux pump activity, may be absent or less active. The control isolate, PAO1, presented the expected resistance profile to cefoxitin and cloxacillin while maintaining susceptibility to other beta-lactam antibiotics.

Kinetic analyses of interactions between bacteria and antibiotics over time revealed distinct survival strategies among the isolates. The slow decrease in cell density after antibiotic exposure, even at concentrations above the MIC suggests the presence of a tolerant phenotype. This phenotype is characterized by reduced cellular activity, allowing bacteria to survive through evasion or repair of drug-induced damage rather

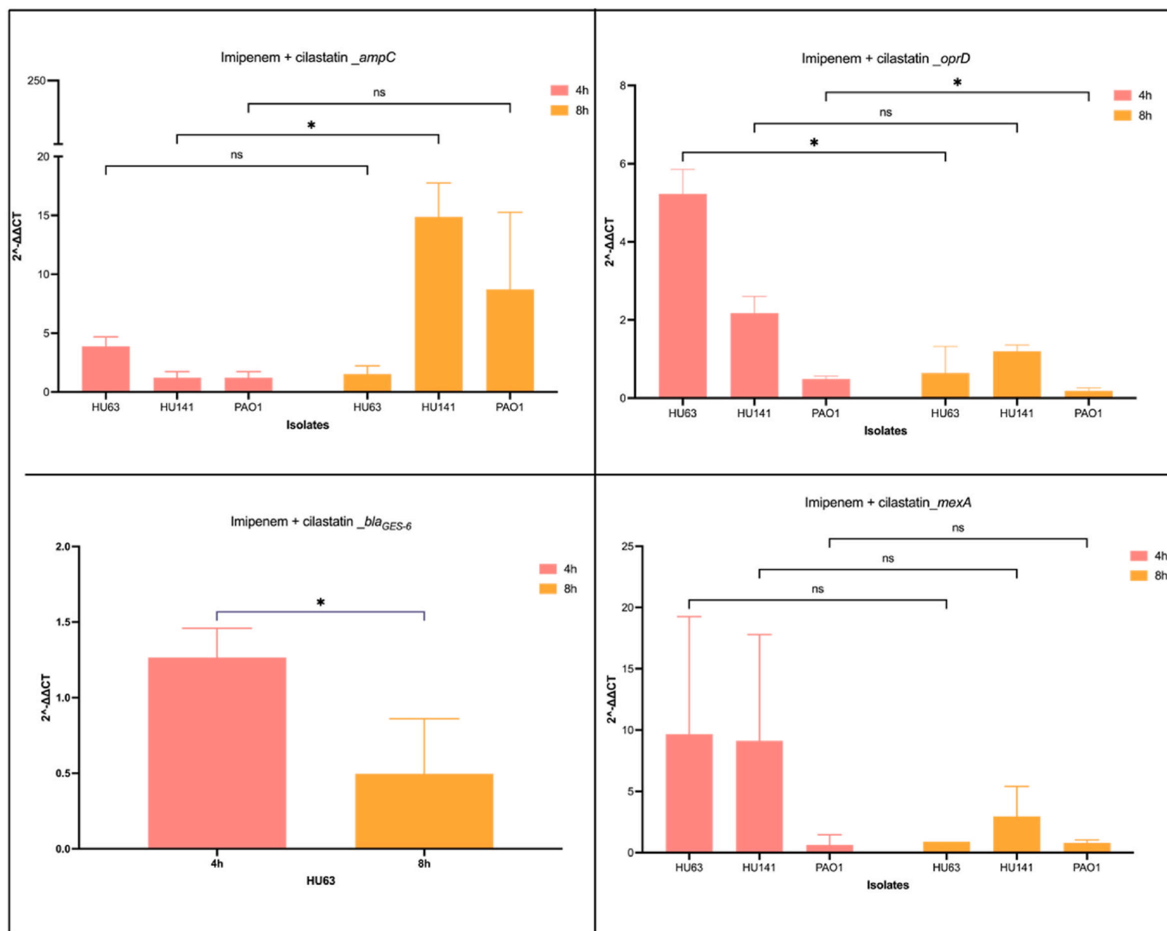

Fig. 4. Relative expression of the genes *mexA, oprD, bla_{GES-6},* and *ampC* in *P. aeruginosa* isolates PAO1, HU63, and HU141 after exposure to imipenem + cilastatin for 4 h (pink bars) and 8 h (orange bars). Bars represent mean ± SD of three independent experiments. Statistical differences are indicated as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and n.s. (not significant).

than by expressing classical resistance mechanisms. The time-kill assays revealed that both HU63 and HU141 exhibited an initial reduction in bacterial load followed by regrowth between 8 and 24 h, indicating a phenotype of tolerance rather than true resistance. In other words, although these strains are initially inhibited by antibiotics (as reflected in MIC testing), they survive long enough to resume growth once drug levels decrease. Antibiotic tolerance refers to the capacity of bacteria to persist under bactericidal antibiotic exposure without a corresponding increase in MIC, contrasting with genetic resistance, which permits active growth at high drug concentrations [34]. Clinically, tolerant isolates are strongly associated with persistent infections and therapeutic failure, as they may "wait out" treatment and subsequently rebound [35]. Consistent with this, HU63, despite harboring *bla_{GES-6}*, showed a marked decrease in CFUs when exposed to imipenem + cilastatin and meropenem up to 8 h, followed by regrowth at 24 h. HU141 exhibited a similar trend under carbapenem exposure, with a reduction at 8 h and slight increase thereafter. These regrowth profiles strongly suggest phenotypic tolerance. Therefore, despite apparent inhibition, the re-emergence of bacterial growth indicates that tolerance contributes significantly to infection persistence, a phenomenon increasingly recognized as a hidden cause of treatment failure in *P. aeruginosa*.

Carbapenems, such as meropenem, are effective against Gram-negative bacteria due to their ability to bind to multiple penicillin-binding proteins (PBPs) and resist hydrolysis by conventional β-lactamases [36]. This explains the rapid decrease in bacterial load in the first h, especially in HU63, which has the *bla_{GES-6}* gene, a β-lactamase with weak activity against carbapenems. However, resistance to these antibiotics can arise through mechanisms such as the production of carbapenemases, although *Bla_{GES-6}* has limited activity against carbapenems, reduction of outer membrane permeability by alterations in porins [37,38], limiting drug entry and activation of efflux pumps that expel the antibiotic from the cell [37,39]. In the case of imipenem-cilastatin, regression after 1 h may be linked to its shorter half-life or greater susceptibility to bacterial enzymes compared to meropenem, allowing residually resistant bacteria to proliferate.

Penicillins are often inactivated by ESBLs or other modifying enzymes. In the case of HU141 and HU63, the stability of the response to cefoxitin and cloxacillin suggests that these bacteria already have intrinsic or acquired resistance mechanisms, such as the production of β-lactamases, which rapidly degrade these antibiotics, allowing bacterial growth [39,40].

The presence of the *bla_{GES-6}* gene in HU63 encodes a β-lactamase with limited activity against carbapenems [36,39], which may explain the more pronounced reduction in bacterial density with meropenem in HU63, since the drug is not efficiently hydrolyzed. However, subsequent regression indicates that other compensatory mechanisms, such as porin alterations or efflux pumps, are activated after initial exposure. The initial rapid reduction followed by regression may reflect faster kinetics of action of imipenem, but also reduced persistence of therapeutic concentrations, allowing resistant subpopulations to survive and multiply. Prolonged exposure to antibiotics selects for resistant mutants [41]. The "return" of growth observed after 24 h suggests that a fraction of the bacterial population has developed resistance, either through mutations or regulation of virulence genes [42–45].

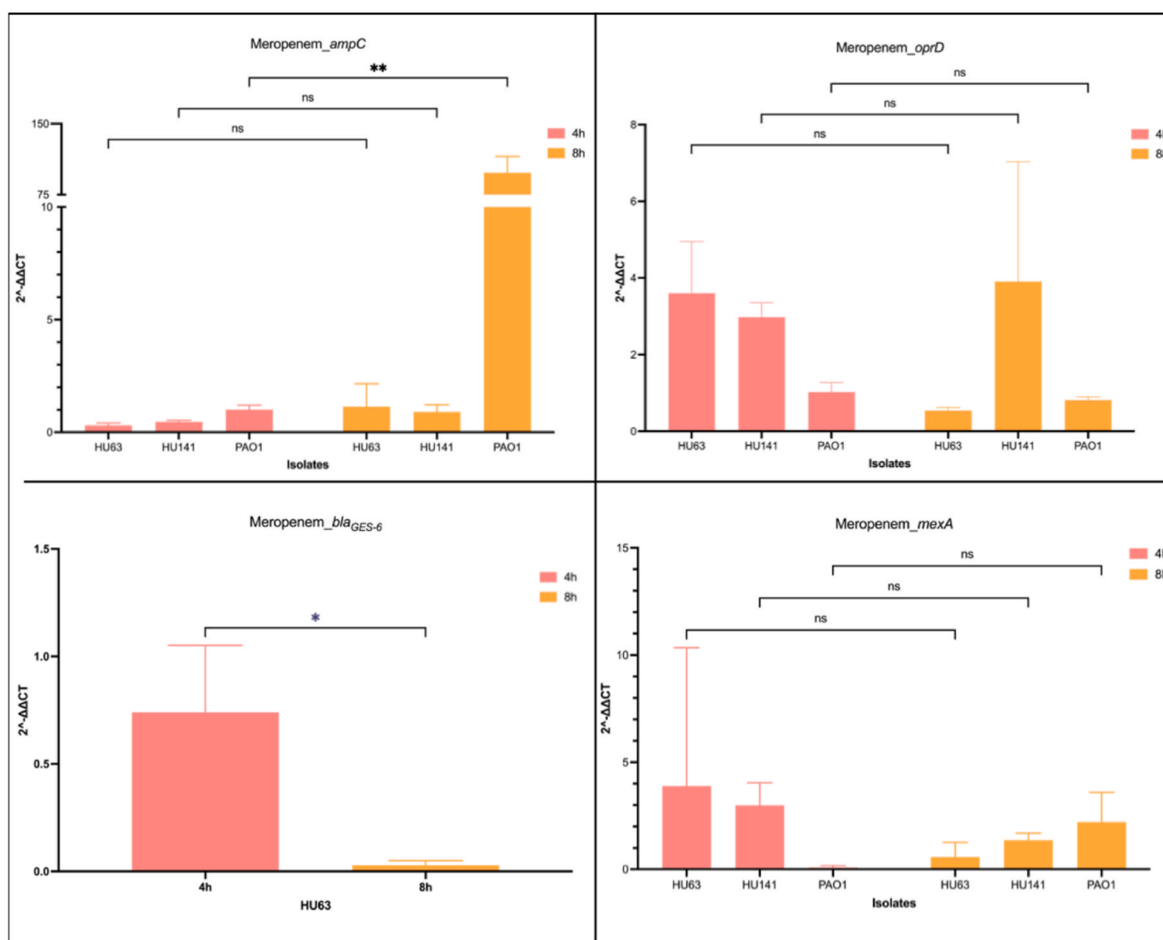

Fig. 5. Relative expression of the genes *mexA*, *oprD*, bla_{GES-6}, and *ampC* in *P. aeruginosa* isolates PAO1, HU63, and HU141 following treatment with meropenem for 4 h (pink bars) and 8 h (orange bars). Bars show mean ± SD of three independent experiments. Statistical significance is indicated as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and n.s. (not significant).

An investigation into carbapenem resistance in *P. aeruginosa* identified the presence of carbapenemase genes, such as bla_{GES-5}, in 5.9 % of the isolates, highlighting the importance of these genes in carbapenem resistance [46]. Additionally, a review on carbapenem resistance in *P. aeruginosa* emphasized that the production of carbapenemases, including GES-type enzymes, is associated with high levels of resistance to multiple antibiotics [47], which may explain the rapid adaptation observed in isolate HU63.

The *mexA* gene, a component of the MexAB-OprM efflux system, exhibited stable expression under ceftazidime treatment but showed a significant reduction in expression in isolates HU63 and HU141 (Supplementary Table 1) following exposure to imipenem combined with cilastatin. This pattern reflects the differential regulation of efflux pumps in response to distinct classes of antibiotics. Previous studies have demonstrated that overexpression of RND systems, such as MexAB-OprM, is directly associated with resistance to β-lactams and quinolones due to the active extrusion of these drugs [48]. The lack of significant changes in expression in the reference strain PAO1 suggests that such strains may maintain more conserved expression profiles, possibly due to the absence of clinically acquired mutations [28].

The *oprD* gene, which encodes the OprD porin (essential for carbapenem uptake), was significantly downregulated in HU63 and PAO1 after treatment with imipenem + cilastatin. This finding supports the hypothesis that carbapenem resistance in *P. aeruginosa* frequently involves *oprD* downregulation [49]. The transient induction of *oprD* in HU63 after 8 h of ceftazidime exposure may reflect an adaptive response to enhance nutrient uptake under stress conditions, although this hypothesis requires functional validation.

bla_{GES-6} is a variant of the GES family of β-lactamases, originally identified as an ESBL but which, in some variants (such as GES-5 and GES-6), has acquired carbapenemase activity due to critical amino acid substitutions, such as the presence of serine or asparagine at position 170 [50]. The expression of bla_{GES-6} was significantly induced in HU63 following ceftazidime exposure (a fourfold increase at 8 h) (Supplementary Table 2), consistent with the established role of ESBLs in the hydrolysis of third-generation cephalosporins [51]. This antibiotic-dependent regulation suggests that the induction of bla_{GES-6} is influenced by specific selective pressures. For instance, β-lactams such as ceftazidime may activate regulatory systems (e.g., AmpC-related pathways or stress response signals) that enhance the transcription of resistance genes, whereas carbapenems may not exert the same inductive effect. However, the observed reduction of bla_{GES-6} under meropenem and piperacillin + tazobactam treatment suggests that the regulation of this enzyme is influenced by the specific type of β-lactam and the selective pressure it imposes. The lack of induction in HU141 further highlights the genetic heterogeneity among clinical isolates, even within the same species [52]. Although bla_{GES-6} is classified as a carbapenemase (Ambler class A), its hydrolytic efficiency against carbapenems is considerably lower than that of other carbapenemases, such as KPC (*K. pneumoniae* carbapenemase) or metallo-β-lactamases (MBLs) like NDM and VIM. Studies have shown that GES-type enzymes with carbapenemase activity exhibit reduced catalytic efficiency (kcat/Km) toward carbapenems, leading to slower hydrolysis rates and, consequently, lower MICs [53].

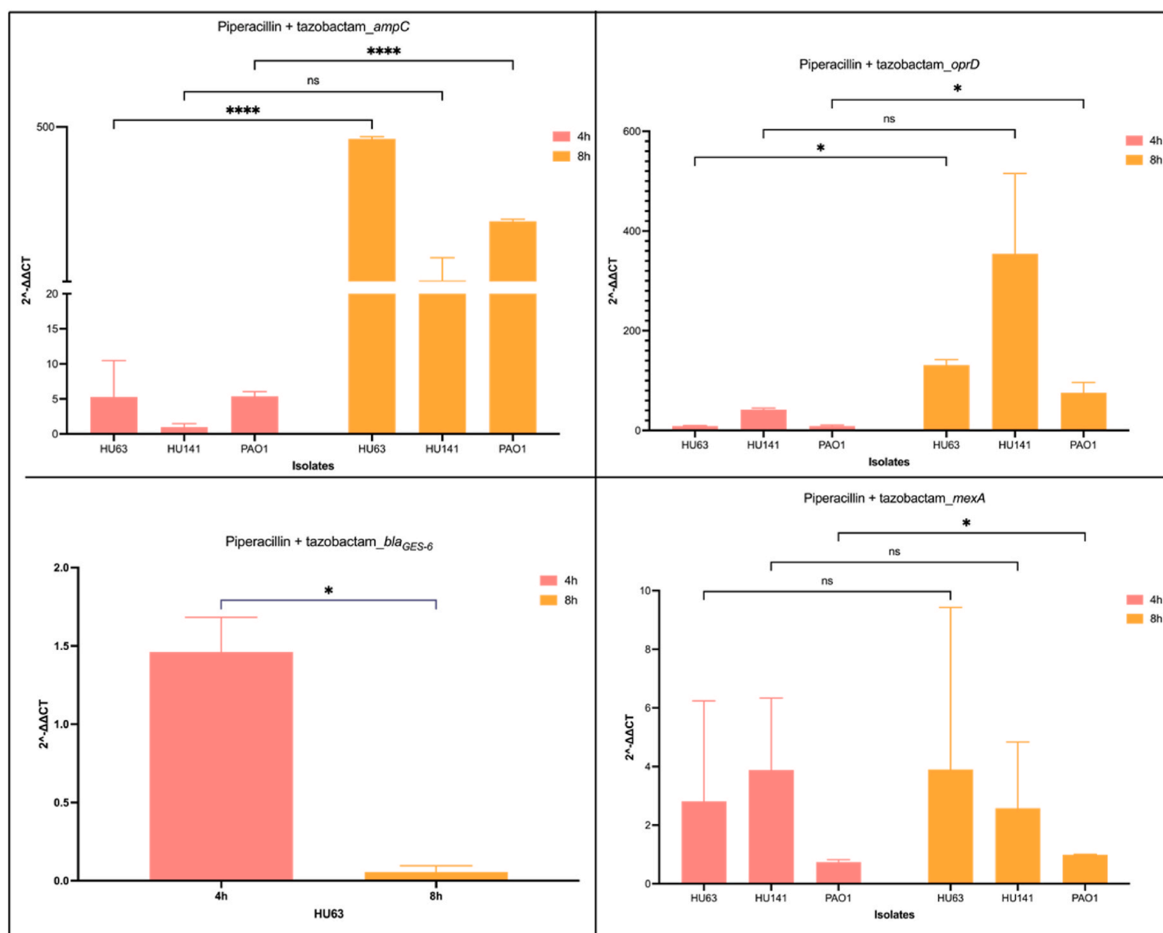

Fig. 6. Relative expression of the genes *mexA*, *oprD*, bla_{GES-6}, and *ampC* in *P. aeruginosa* isolates PAO1, HU63, and HU141 after treatment with piperacillin + tazobactam for 4 h (pink bars) and 8 h (orange bars). Data are shown as mean ± SD from three independent experiments. Statistical significance is denoted by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and n.s. (not significant).

The expression of the *ampC* gene varied depending on the treatment, with significant upregulation observed in PAO1 upon exposure to meropenem and piperacillin + tazobactam. AmpC β-lactamase, which is chromosomally encoded in *P. aeruginosa*, is frequently hyperproduced in response to β-lactam antibiotics, particularly when administered in combination with β-lactamase inhibitors like tazobactam [54]. The low basal expression of *ampC* in HU63 may be attributable to regulatory mutations or compensatory resistance mechanisms, such as overactivity of the MexAB-OprM efflux system.

Although HU63 carries the bla_{GES-6} gene, previous biochemical studies have shown that GES-6 has weak hydrolytic activity against carbapenems, meaning its presence alone does not fully account for the high-level resistance observed [50]. The qPCR results, however, revealed significant downregulation of *oprD* in HU63 after exposure to imipenem + cilastatin, effectively reducing antibiotic influx through this porin channel. Moreover, while the MexAB-OprM efflux system does not actively export imipenem, it is known to efficiently extrude meropenem. Together, these mechanisms act synergistically to raise the effective MIC by lowering intracellular drug concentrations. Prior studies have shown that, in the absence of potent carbapenemases, carbapenem resistance in *P. aeruginosa* often arises from a combination of *oprD* loss and upregulated efflux pumps like MexAB-OprM [55]. In HU63, the observed *oprD* repression (alongside basal efflux activity) likely amplifies the effect of the weak GES-6 carbapenemase, resulting in a clinically resistant phenotype. Therefore, the resistance to carbapenems in HU63 is best explained as a multifactorial process, wherein impaired permeability and active efflux work in concert with enzymatic degradation to sustain high-level resistance [47,53].

5. Conclusions

This study elucidates the complex resistance landscape of carbapenem-resistant *P. aeruginosa*. The blaGES-6-producing isolate HU63 exhibited a multidrug-resistant phenotype driven by β-lactamase production, efflux pump activity, and target mutations, contrasting with HU141's non-carbapenemase-mediated resistance. Genomic divergence between ST235 and ST253 isolates highlights the role of clonal spread in resistance dissemination. Time-kill assays revealed tolerance mechanisms, where bacterial regrowth occurred despite initial antibiotic efficacy, suggesting adaptive survival strategies distinct from genetic resistance. The marked expression of bla_{GES-6} in HU63 under all the β-lactams tested, combined with the dynamic regulation of *oprD* and *mexA*. illustrates the adaptive plasticity of this isolate. However, clinical resistance only manifests when bla_{GES-6} acts synergistically with other mechanisms, such as active drug expulsion (MexAB-OprM) and reduced uptake (OprD repression), corroborating previous studies that emphasize the multifactorial nature of resistance in *P. aeruginosa*. These findings advocate for enhanced genomic surveillance to monitor high-risk clones like ST235 and emphasize the need for novel therapeutic approaches targeting both resistance and tolerance mechanisms.

CRediT authorship contribution statement

Telma de Sousa: Writing – original draft, Methodology,

Investigation, Formal analysis, Data curation, Conceptualization. **Sandro Machado:** Writing – original draft, Methodology, Investigation, Data curation. **Márcia Carvalho:** Writing – review & editing, Validation, Software, Methodology. **Manuela Caniça:** Writing – review & editing, Validation, Resources, Data curation. **Miguel J.N. Ramos:** Writing – review & editing, Software, Formal analysis, Data curation. **Daniela Santos:** Writing – review & editing, Software, Methodology, Data curation. **Racha Beyrouthy:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Richard Bonnet:** Writing – review & editing, Validation, Resources, Data curation. **Michel Hébraud:** Writing – review & editing, Supervision, Formal analysis, Data curation. **João Paulo Gomes:** Writing – review & editing, Validation, Resources, Formal analysis, Data curation. **Gilberto Igrejas:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Data curation. **Patrícia Poeta:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Data curation.

Informed consent statement

Not applicable.

Institutional review board statement

Not applicable.

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Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2025.107962.

Data availability

No data was used for the research described in the article.

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