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Cefiderocol activity against planktonic and biofilm forms of β-lactamase-producing *pseudomonas aeruginosa* from people with cystic fibrosis ☆

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Objectives: Chronic *Pseudomonas aeruginosa* infections are a leading cause of acute pulmonary exacerbations in people with cystic fibrosis (pwCF). Intrinsic antibiotic resistance and biofilm formation complicate treatment. This study investigates the genomic diversity and cefiderocol efficacy against planktonic and biofilm-associated forms of *P. aeruginosa* isolates from pwCF.
Methods: Eight *P. aeruginosa* clinical isolates and three laboratory strains underwent whole genome sequencing (WGS). Biofilm formation was assessed through biomass, cell count, metabolic activity, and extracellular DNA (eDNA). The minimum bactericidal concentration (MBC_{90}) and biofilm eradication concentration ($MBEC_{90}$) were also determined.
Results: WGS revealed significant genomic diversity, identifying ten distinct sequence types (STs). Antibiotic susceptibility testing (AST) showed that 10/11 strains were susceptible to cefiderocol, with one isolate (MPA9) displaying resistance linked to the *bla*$_{OXA486}$ gene. Adding the β-lactamase inhibitor avibactam (AVI) restored susceptibility in this resistant strain. Although iron metabolism genes were highly conserved across isolates, MPA9 lacked the *fpvA* iron receptor, potentially contributing to cefiderocol resistance. Biofilm formation significantly increased tolerance to cefiderocol, with an 8-fold rise in $MBEC_{90}$ compared to MBC_{90}.
Conclusion: These findings highlight the genomic diversity and adaptive potential of *P. aeruginosa* in pwCF. Cefiderocol shows promise against planktonic and biofilm-associated *P. aeruginosa*, and combining it with AVI may counteract β-lactamase-mediated resistance.

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1. Introduction

Cystic fibrosis (CF) is one of the most common life-threatening genetic disorders, affecting over 160,000 people worldwide [1,2]. CF is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, which encodes an epithelial anion channel responsible for Cl^- and HCO_3^- transport across epithelial cells [3,4]. Beyond its role in epithelial function, CFTR

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expression and activity in immune cells are critical in controlling bacterial infections [5–7]. More than 1900 CFTR mutations have been identified, with the Phe508del mutation being the most prevalent [8]. Defective CFTR activity, due to Phe508del or other mutations, reduces the hydration of the extracellular environment, thus contributing to progressive multi-organ dysfunction [9].

In the lungs of people with CF (pwCF), defective CFTR causes mucus thickening and impaired mucociliary clearance, which create a favorable environment for chronic colonization by opportunistic pathogens, such as *Pseudomonas aeruginosa, Staphylococcus aureus*, and *Haemophilus influenzae* [10,11]. The gastrointestinal complications include malnutrition, dysmotility, hepatopancreatic biliary disease, and intestinal bacterial overgrowth. These conditions have been linked to growth failure, accelerated disease progression, and an increased risk of future lung transplantation. Moreover, pwCF show skin disorders such as elevated chloride concentration in sweat, increased rates of atopy, drug hypersensitivity reactions, and cutaneous vasculitis than individuals without CF [12]. *P. aeruginosa* is a key pathogen associated with chronic lung infections in pwCF, exacerbated by its ability to form biofilms [13]. Biofilm formation provides a protective matrix that limits antibiotic penetration and enhances antimicrobial resistance [14,15]. This is compounded by *P. aeruginosa*'s intrinsic and acquired resistance mechanisms, including efflux pumps, β-lactamases, and reduced membrane permeability.

Cefiderocol, a novel siderophore cephalosporin, has demonstrated *in vitro* efficacy against multidrug-resistant *P. aeruginosa* strains, particularly those resistant to carbapenems [16]. However, limited data exist on its effectiveness against *P. aeruginosa* isolates from pwCF [17]. This study analyzes eight clinical isolates of *P. aeruginosa* from the sputum of pwCF, along with three laboratory strains. Genotypic characterization using Whole Genome Sequencing (WGS) was conducted to identify key resistance mechanisms and virulence factors. Additionally, the efficacy of cefiderocol against both planktonic and biofilm-associated forms of *P. aeruginosa* was evaluated, focusing on biofilm matrix components such as biomass, extracellular DNA (eDNA), and metabolic activity.

2. Materials and methods

2.1. Experimental plan/clinical protocol

Sputum samples were collected during outpatient clinical visits at the Cystic Fibrosis Regional Reference Center, A.O.U. Policlinico Umberto I, Rome, Italy, from pwCF (≥18 years) homozygous for F508del or heterozygous with one F508del allele.

The outpatient setting minimizes the risk of nosocomial contamination. Sputum samples were transported under controlled conditions and processed within two hours after collection at the Microbiology and Virology Unit, A.O.U. Policlinico Umberto I, Rome, Italy, to ensure optimal microbiological analysis. Written informed consent was obtained from all individuals prior to enrollment. The study was approved by the Ethics Committee Area 5 Lazio (N. 48/ISG/24) and conducted in accordance with ethical guidelines for human research.

2.2. Bacterial strain isolation and susceptibility testing

A single sputum sample was collected from each participant under sterile conditions and plated on complete and selective media to isolate Gram-positive and Gram-negative pathogenic microorganisms for culture studies. Eight *P. aeruginosa* isolates were initially identified by a matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) system (Bruker Daltonik GmbH, Bremen, Germany). The antimicrobial susceptibility was assessed by the BD Phoenix™ automated microbiology system (Becton Dickinson Diagnostic Systems, Sparks, MD, USA). Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (http://www.eucast.org/clinical_breakpoints) [18]. Isolates were cryopreserved and stored at −80 for further studies.

2.3. Whole-genome sequencing

WGS was performed using the Illumina MiSeq instrument (Illumina, Inc., San Diego, CA, USA). Genomic DNA was purified from overnight cultures and resuspended in lysis buffer using the Macherey Nagel (Düren, Germany) DNA extraction kit procedures. Paired-end libraries were generated using the Nextera XT DNA sample preparation kit with the 2 × 151PE protocol (Illumina, Inc.). *De novo* assembly and annotation of Illumina reads were performed as previously described [19]. A SNP-based phylogeny tree was constructed using kSNP3 v3.1 [20]. Singletons identified in the pan-genome analysis were functionally annotated using eggNOG-mapper v2.1.9 [21] to obtain Clusters of Orthologous Groups (COG) functional profiles. Sequence Types (STs) were determined using the PubMLST database [22]. Antibiotic resistance genes were predicted using the Comprehensive Antibiotic Resistance Database (CARD) v3.2.8 and the Resistance Gene Identifier (RGI) tool, with predictions restricted to "perfect" and "strict" matches against high-quality reference sequences, applying a 97% identity cutoff [23]. Metagenome assemblies were then subjected to FeGenie analysis to identify genes related to iron metabolism [24]. Hierarchical clustering was performed based on the presence, absence, and sequence homology of a subset of genes involved in iron uptake and with a potential impact on cefiderocol resistance [19,25,26].

2.4. Cefiderocol minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Antimicrobial susceptibility testing was determined by the reference broth microdilution method using Chelex-treated, iron-depleted, cation-adjusted Mueller-Hinton broth (idCAMHB) for cefiderocol at concentrations ranging from 0.03 to 32 mg/L [16]. The well, containing bacterial suspension without antibiotics, was used as growth control. Details of chelation, determination of iron concentration, quality control testing, and preparation of broth microdilution panels have been described previously [27]. Qualitatively, the MIC was read as the first well in which the reduction of growth corresponds to a button of <1 mm or is replaced by the presence of light haze/faint turbidity, as recommended by the EUCAST. Each isolate was tested in duplicate. For the cefiderocol-resistant strain MPA9, the assay was repeated in triplicate on independent days to confirm reproducibility. In all cases, MIC values were consistent across replicates. Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (http://www.eucast.org/clinical_breakpoints). Viable bacterial counts were quantified by plating serial dilutions of cultures following antibiotic exposure and expressed as CFU/mL. MBC_{90} was defined as the lowest concentration of antibiotic that killed 90% of the bacteria compared to the untreated control.

2.5. Cefiderocol and avibactam synergistic activity

Antimicrobial susceptibility testing was determined by the reference broth microdilution method using idCAMHB for cefiderocol and avibactam (4 μg/mL) [16,28]. The well-containing bacterial suspension (10^6 CFU/mL) without antibiotics was used as growth control. Details of chelation, determination of iron concentration,

quality control testing, and preparation of broth microdilution panels have been described previously [27]. Quantitatively, viable cells were determined by plate counting after antibiotic treatment for the CFU/mL determination. MBC_{90} was defined as the lowest concentration of antibiotic that killed 90% of the bacteria compared to the untreated control.

2.6. Cefiderocol minimum biofilm eradication concentration (MBEC) assays

An overnight bacterial culture grown on a MacConkey plate was used for each experiment to inoculate 2 mL of 0.45% saline solution to 0.5 McFarland turbidity standard (approximately 10^8 CFU/mL). Diluted cell suspensions (approximately 10^5 CFU/mL) were used for biofilm cultures to inoculate a 96-well polystyrene flat-bottom plate with 100 µL of idCAMHB. After 24 h at 37 °C, the wells were rinsed with 0.45% saline solution to remove nonadherent bacteria, and the cells were resuspended in 100 µL of idCAMHB supplemented with serial dilutions of cefiderocol at concentrations ranging from 0.03 to 32 mg/L. The plate was incubated for 20 additional hours at 37 °C, and the well, which contained bacterial suspension (10^6 CFU/mL) without antibiotics, was used as growth control (GC). After 20 h of exposure, the well contents were collected. Each well was washed two times with sterile deionized water, and the cells were resuspended in 100 µL of 0.45% saline solution. Biofilms were scraped thoroughly, and the total number of viable cells was determined by serial dilution and plating on MacConkey plates to estimate the CFU number. *P. aeruginosa* strains PAO1, PA14, and PA27853 were included as standard references to allow reproducibility in the cell counting procedures. The $MBEC_{90}$ levels were identified as the lower concentrations of antibiotics that killed 90% of the bacteria in preformed biofilms compared to the untreated control. $MBEC_{90}/MBC_{90}$-ratios were calculated to assess the biofilm tolerance, which indicates the fold increase in the antimicrobial dose needed to inhibit or kill *P. aeruginosa* cells in biofilm compared to planktonic growth [29].

2.7. Biofilm formation and characterization

Biofilm formation was quantified by assessing biomass using a crystal violet (CV) staining method 24 h post-incubation. Briefly, sterile 96-well polystyrene plates (Corning Inc., Corning, NY, USA) were inoculated with 100 μL of an initial bacterial suspension (1×10^5 CFU/mL) in idCAMHB and incubated at 37 °C for 24 h without shaking. After incubation, the medium was carefully removed, and the wells were washed three times with 100 μL of sterile distilled water to remove planktonic cells. The plates were then air-dried for 45 min, and the adherent biofilm cells were stained with 100 μL of 0.1% CV solution for 15 min. Following staining, excess dye was removed by washing the wells three times with 100 μL of sterile distilled water. The dye incorporated by the biofilm cells was solubilized with 100 μL of pure ethanol. Absorbance was measured spectrophotometrically at 570 nm (OD570) using a Varioskan Lux microplate reader (Thermo Fisher Scientific, Ohio, USA) [29]. Viable cell counts were determined by plate counting to measure CFU/mL. *P. aeruginosa* strains PA14, PAO1, and PA27853 were included as standard references for weak, moderate, and strong biofilm formation, respectively. All experiments were performed in triplicate and independently repeated at least three times.

2.8. Determination of metabolic activity

The metabolic activity of biofilm cells was assessed using resazurin assays, as previously described [30]. Briefly, biofilms were formed by incubating bacterial cultures in idCAMHB at 37 °C for five hours. After incubation, the wells were rinsed with 0.45% saline solution to remove non-adherent cells, and 100 μL of an idCAMHB/resazurin solution was added to each well. The plates were then incubated for an additional 20 h at 37 °C. Metabolic activity was quantified by measuring absorbance at 570 nm every 20 min using a Varioskan Lux microplate reader (Thermo Fisher Scientific, Ohio, USA), and the area under the growth curve (AUG) was calculated. All experiments were conducted in triplicate and repeated at least three times independently.

2.9. Extracellular DNA quantification

Sterile 96-well polystyrene plates (Corning Inc., Corning, NY, USA) were inoculated with 100 μL of an initial bacterial suspension (10^5 CFU/mL) in idCAMHB and incubated at 37 °C for 24 h without shaking. The medium was removed from the wells and washed three times with 100 μL sterile distilled water. Biofilm cultures were resuspended in 100 μL Tris-EDTA (TE) buffer followed by 100 μL freshly made PicoGreen solution (1 μL PicoGreen dye in 199 μL TE buffer). Wells with PicoGreen were incubated for 5 min before measuring the fluorescence intensity (excitation 485 nm/emission 535 nm, 0.1 s) using a Varioskan Lux microplate reader (Thermo Fisher Scientific, Ohio, USA). Lambda DNA (Invitrogen Molecular Probes) generates a standard curve for each run [30]. *P. aeruginosa* strains PAO1, PA14 and PA27853 were included as forming standard references. All experiments were conducted in triplicate and repeated at least three times independently.

3. Results

3.1. Whole-genome analysis (WGS) of P. aeruginosa isolates

Eight clinical isolates were collected from pwCF sputum and subjected to WGS. Sequence data were mapped against *P. aeruginosa* PAO1, PA14, and PA27853 reference strains. The maximum likelihood phylogenetic tree based on single nucleotide polymorphisms (SNPs) shows significant genetic divergence among strains, which were distributed across ten distinct sequence types (STs), except for MPA7 and MPA8, classified as ST262 (Fig. 1A). The pangenome consisted of 8907 genes, of which 56.4% ($n = 5026$) were core genes, 15.5% ($n = 1376$) were shell genes, and 28.1% were cloud genes, including 12.7% ($n = 1132$) singletons, which are genes present in only one genome (Fig. 1B). Functional gene distribution, assessed through the Cluster of Orthologous Groups (COG) categories, revealed notable differences based on singleton sequence variants (Fig. 1C). The Comprehensive Antibiotic Research Database (CARD) identified 72 antimicrobial resistance genes (ARGs) (Fig. 1D). According to the Ambler Classification system, the presence of class C (bla_{PDC}) and D (bla_{OXA}) β-lactamases were highly variable among strains. These findings highlight the genomic variability and functional diversification among *P. aeruginosa* strains, emphasizing their adaptive potential and evolutionary dynamics.

3.2. Antimicrobial susceptibility profiles of P. aeruginosa isolates

Antimicrobial susceptibility testing (AST) showed that 10/11 of *P. aeruginosa* strains were susceptible to cefiderocol according to EUCAST clinical breakpoints (Fig. 2A). Notably, MPA9 was the only strain resistant to cefiderocol with an MIC of 4 mg/L (Fig. 2B). Meropenem demonstrated activity comparable to cefiderocol, while all strains were susceptible to colistin. In contrast, resistance was observed across all strains for levofloxacin, ciprofloxacin, cefepime, ceftazidime, imipenem, and piperacillin/tazobactam (Fig. 2A-B and Supplementary data).

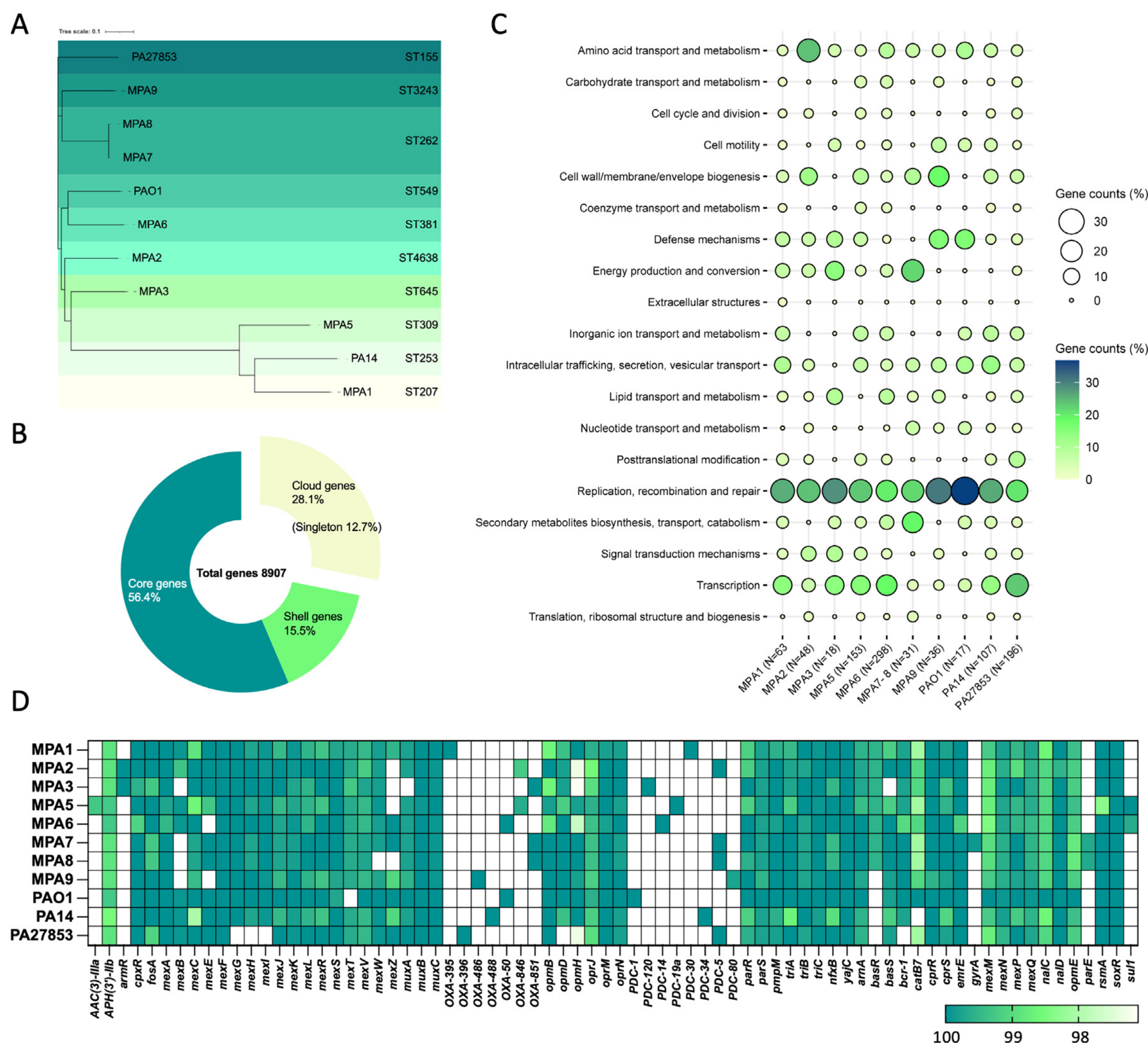

Fig. 1. Comparative genomic analysis of *P. aeruginosa* strains. (A) Maximum-likelihood phylogenetic tree based on single nucleotide polymorphisms (SNPs) of eight *P. aeruginosa* strains isolated from the sputum of patients with cystic fibrosis (pwCF) and three reference strains (PAO1, PA14, and PA27853). (B) Pie chart illustrating the distribution of core, shell, cloud, and singleton genes shared across *P. aeruginosa* strains. (C) Distribution of functional categories based on the Cluster of Orthologous Genes (COGs) identified among the strains. The number above each group represents the absolute count of COGs, with the bubble size reflecting the number of unique genes in each functional category. (D) Heatmap showing the distribution of antibiotic resistance genes (ARGs) across *P. aeruginosa* strains. Shaded cells indicate the presence of ARGs, with intensity correlating with sequence identity, while white cells indicate the absence of the corresponding gene.

WGS identified variability in extended-spectrum class C (bla_{PDC}) and class D (bla_{OXA}) β-lactamase genes. Notably, the MPA9 isolate, resistant to cefiderocol and meropenem, harbored the bla_{OXA486} gene. To assess the impact of β-lactamase inhibition on cefiderocol susceptibility, the β-lactamase inhibitor avibactam (AVI) was included in susceptibility assays. Overall, AVI significantly reduced the MBC_{90} from 0.5 μg/mL to 0.25 μg/mL across the strains tested (Fig. 2C–D). Specifically, in the MPA9 isolate, the MBC_{90} decreased from 4 μg/mL to 2 μg/mL (Supplementary data). Given the absence of the *mexB* gene in the MPA9 isolate, the contribution of the MexAB-OprM efflux pump to cefiderocol resistance in this strain is unlikely.

3.3. Genetic analysis of cefiderocol resistance mechanism

Resistance to cefiderocol and elevated MIC levels can arise through multiple mechanisms, including the production of β-lactamases and mutations affecting iron uptake, mainly when these mechanisms act in combination. To gain insight into the overall iron metabolism potential of *P. aeruginosa* strains, the bioinformatic tool FeGenie was used to identify genes involved in iron acquisition, regulation, and storage. Interestingly, no significant differences were observed in the number and distribution of genes related to iron metabolism across the strains analyzed (Fig. 3A). Genes commonly associated with variable cefiderocol susceptibility were identified in each strain using BLAST and compared against the reference PAO1 genome (Fig. 3B).

Pa27853 and three clinical isolates, including the cefiderocol-resistant MPA9 strain, were found to lack the pyoverdine receptor (*fpvA*) gene (Fig. 3B).

3.4. Biofilm phenotypic characterization in iron-depleted medium growth

Each *P. aeruginosa* strain was phenotypically characterized in id-CAMHB for biomass, biofilm metabolic activity (measured as the area under the growth curve, AUG), extracellular DNA (eDNA), and the number of viable cells in biofilms (CFU/mL) (Fig. 4A-D). The biofilm assay results revealed substantial phenotypic variability across the strains for all the measured parameters. This variation reflects the heterogeneous distribution of STs among the strains. Given the high degree of variability, correlation analy-

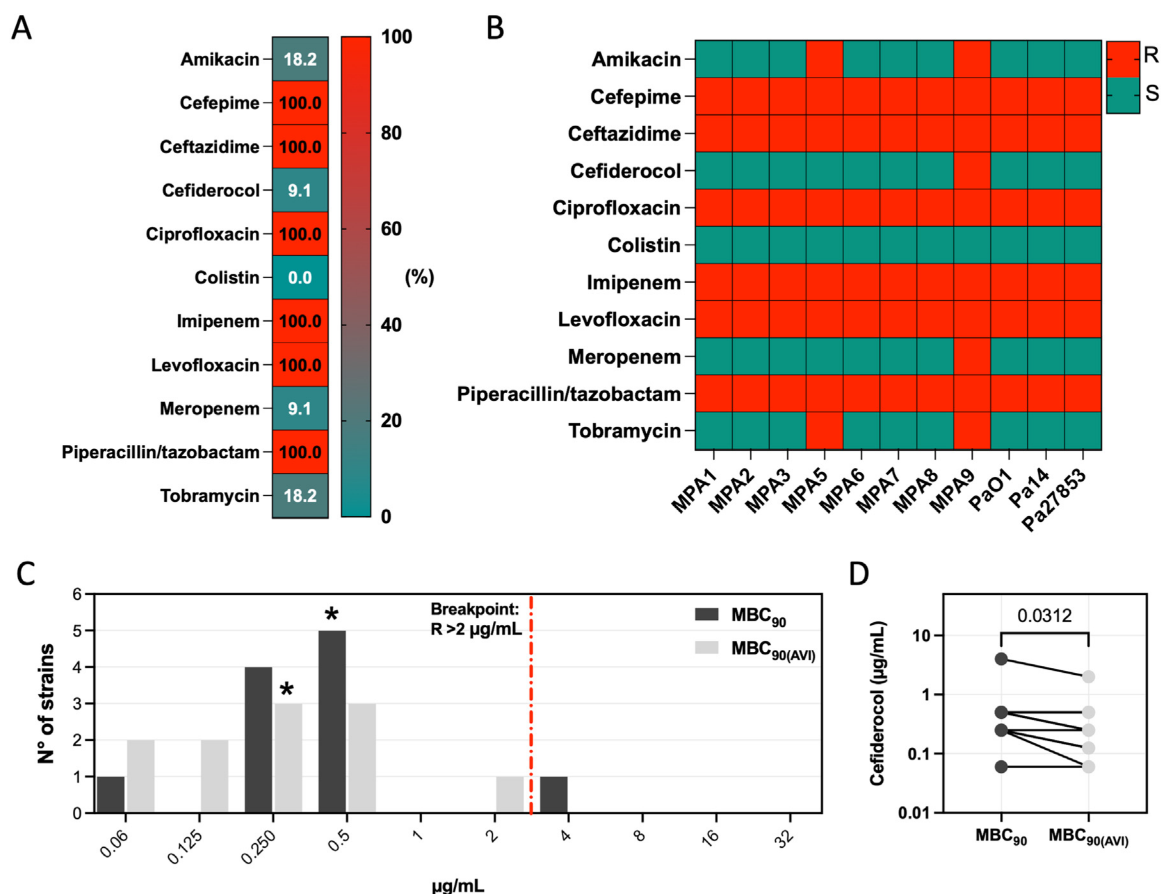

Fig. 2. Cefiderocol activity against *Pseudomonas aeruginosa*. (A) Broth microdilution testing showing the percentage of susceptible (green) and resistant (red) strains to the indicated antimicrobials. (B) Antimicrobial susceptibility testing results for the 11 *P. aeruginosa* isolates. (C) Distribution of cefiderocol Minimum Bactericidal Concentration (MBC_{90}) and MBC_{90} combined with avibactam ($MBC_{90(AVI)}$) for all strains. (D) Comparison of cefiderocol MBC_{90} and $MBC_{90(AVI)}$ for all strains. *indicates the median values.

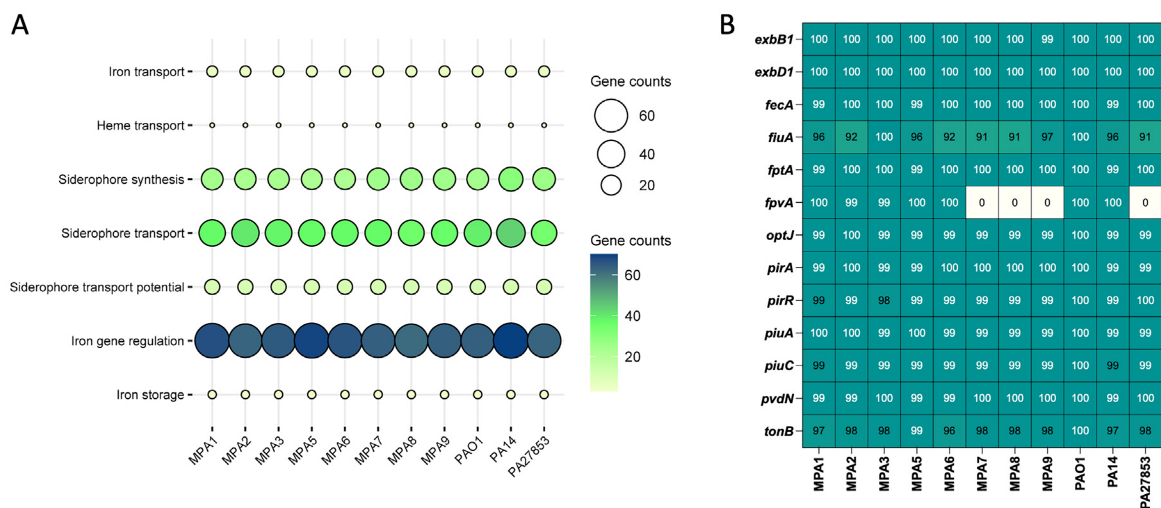

Fig. 3. Iron metabolism across *P. aeruginosa* strains. (A) Distribution of genes related to iron acquisition, regulation, and storage across different *P. aeruginosa* strains. The bubble size represents the number of genes identified, while the color intensity indicates the abundance of specific gene categories. (B) Heatmap displaying the presence (green cells) or absence (white cells) of iron metabolism-related genes, with the percentage of sequence homology to the respective gene in each P. aeruginosa strain. Each row represents a specific gene potentially related to the cefiderocol resistance mechanism, and each column corresponds to a different *P. aeruginosa* strain.

ses were performed to explore whether different biofilm phenotypes covary, suggesting possible coregulation, or vary independently, implying distinct regulatory mechanisms. The results showed significant positive correlations ($P < 0.05$) between most biofilm-associated parameters, indicating a degree of phenotypic co-dependence (Fig. 4E).

3.5. In vitro activity of cefiderocol against P. aeruginosa biofilm

To assess whether biofilm production conferred increased antibiotic resistance to cefiderocol, MBC_{90} and minimum biofilm eradication concentration ($MBEC_{90}$) were compared across the tested *P. aeruginosa* strains. The median $MBEC_{90}$ was 4 μg/mL,

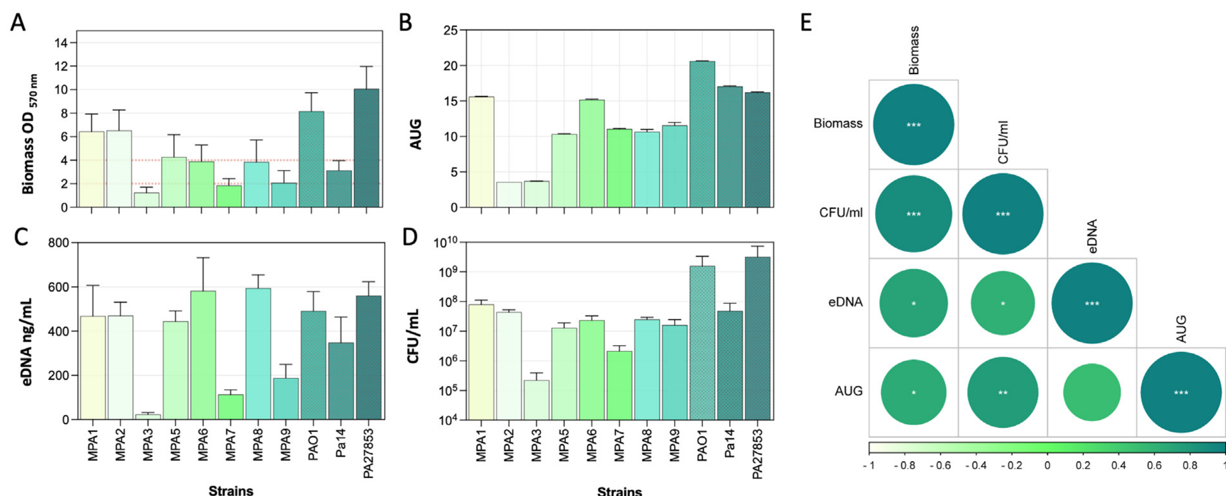

Fig. 4. ***P. aeruginosa*** **strains phenotypic profiles in iron-depleted medium growth**. (A) Biomass formation, (B) metabolic activity measured as the area under the growth curve (AUG), (C) extracellular DNA (eDNA) content, and (D) viable cell counts (CFU/mL) of *P. aeruginosa* strains. Spearman's rank correlation matrix (right panel) illustrates the relationships between biomass, viable cells, eDNA content, and metabolic activity across the strains. Statistical significance of Spearman correlations is indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

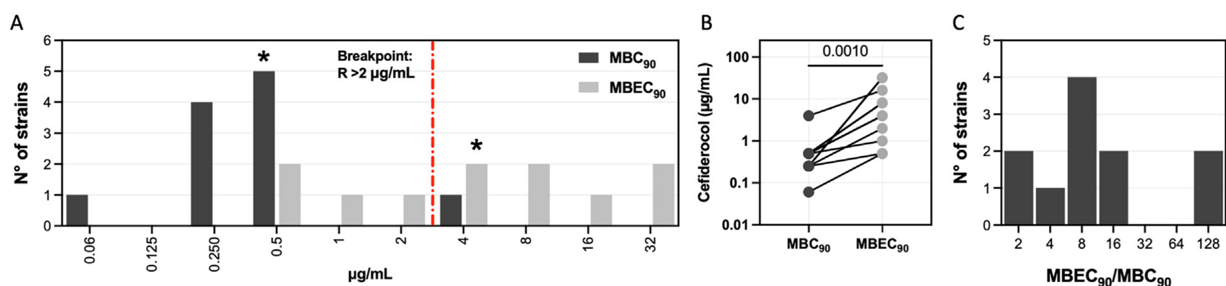

Fig. 5. Cefiderocol activity against *Pseudomonas aeruginosa* biofilm. (A) Comparison of Cefiderocol Minimum Biofilm Eradication Concentration ($MBEC_{90}$) with Minimum Bactericidal Concentration (MBC_{90}). (B) Individual strain analysis comparing MBC_{90} and $MBEC_{90}$ values for cefiderocol. (C) Biofilm tolerance distribution among isolates, expressed as the $MBEC_{90}/MBC_{90}$ ratio for cefiderocol across all strains.*indicates the median values.

higher than the MBC_{90} of 0.5 μg/mL (Fig. 5A). Overall, biofilm formation significantly reduced cefiderocol efficacy ($P = 0.0010$) in all strains tested (Fig. 5B). This was further demonstrated by the $MBEC_{90}/MBC_{90}$ ratio, which quantifies the fold increase in the antimicrobial dose required to inhibit biofilm-embedded cells compared to planktonic cells. The median $MBEC_{90}/MBC_{90}$ ratio was 8-fold, ranging from 2- to 128-fold increases across the strains (Fig. 5C).

4. Discussion

Chronic *P. aeruginosa* infections are one of the most common and fatal causes of acute pulmonary exacerbations in pwCF [31]. Therapeutic interventions are still broadly limited to antibiotic-mediated eradication efforts, which frequently fail in chronic settings due to bacterial adaptations such as biofilm formation and the increasing prevalence of multi-drug resistance [32]. Since *P. aeruginosa* infection remains a concern, with up to 40%–50% of pwCF affected in adulthood [33], more efficient antibiotic approaches are critically required.

Our study investigated the *in vitro* efficacy of cefiderocol against *P. aeruginosa* strains isolated from pwCF sputum in planktonic and biofilm forms. Cefiderocol is a novel siderophore cephalosporin approved for treating urinary tract infections, hospital-acquired pneumonia and ventilator-associated pneumonia caused by Gram-negative bacteria. Since the catechol moiety chelates ferric (Fe-III) iron, cefiderocol penetrates the cell, exploiting the bacterial iron uptake system and circumventing non-specific resistance mechanisms, such as porin loss or hyperactivation of efflux pumps. Once inside the cell, cefiderocol acts like any other cephalosporin, inhibiting penicillin-binding proteins and preventing peptidoglycan cell wall synthesis [33,34].

WGS analysis revealed that *P. aeruginosa* clinical strains were phylogenetically divergent and showed high variability concerning the presence and function of unique genes (singletons). This result reflects the remarkable ability of *P. aeruginosa* to colonize diverse environments, including the lungs of pwCF, either through the acquisition of genetically distinct strains from environmental sources or through adaptation within the host, both supported by the species' extensive genetic versatility [26]. Additionally, the pattern of ARGs mirrored the genetic variability among strains, with β-lactamases bla_{OXA} and bla_{PDC} as the most variable antibiotic-resistance genes. Cefiderocol is highly stable against Class C β-lactams, but its efficacy against Class D oxacillinases varies [35]. This study evaluated the influence of Class D β-lactamases on cefiderocol susceptibility by observing the reduction in MBC_{90} following the addition of AVI, which reversibly inactivates OXA-type enzymes [36]. Notably, the resistance mechanism observed in the MPA9 strain may be associated with the $bla_{OXA-486}$ gene. Previous studies have shown that certain OXA-type β-lactamases, particularly those from the OXA-48 family, can hydrolyze cefiderocol, though their efficiency differs depending on the specific enzyme variant [37]. Adding the β-lactamase inhibitor, AVI significantly reduced the cefiderocol MBC_{90} in MPA9 from 4 μg/mL to 2 μg/mL, partially restoring susceptibility. This finding supports the hypothesis that OXA-486 contributes to cefiderocol resistance, likely

through hydrolysis of the antibiotic, and that β-lactamase inhibition may serve as a potential strategy to mitigate this resistance mechanism. While *P. aeruginosa* strains harboring the $bla_{\text{OXA-486}}$ gene have been reported previously, those isolates remained susceptible to cefiderocol [38]. This finding suggests that the presence of the bla_{OXA486} gene alone may not necessarily confer resistance to cefiderocol, and other resistance mechanisms, such as impaired iron uptake or synergistic β-lactamase activity, may play a more prominent role in mediating resistance. The lack of the *fpvA* gene in MPA9, which encodes the ferripyoverdine receptor involved in iron uptake, may contribute to resistance. The absence of *fpvA* in MPA9 could impair cefiderocol's access to the bacterial cell, effectively limiting its efficacy. This finding is consistent with reports in the literature that link disruptions in iron acquisition systems to cefiderocol resistance, as mutations or the absence of crucial iron transport genes can interfere with the drug's siderophore-mediated entry mechanism [17,39]. Thus, the combination of bla_{OXA486}-mediated β-lactamase production and the absence of *fpvA* likely acts synergistically in MPA9, resulting in the observed high MBC_{90}. This dual mechanism combining β-lactamase hydrolysis and impaired siderophore uptake may represent a barrier to cefiderocol efficacy in certain *P. aeruginosa* strains. In the context of cefiderocol resistance, efflux pumps such as MexAB-OprM are often implicated. However, in the case of the MPA9 isolate, the absence of the *mexB* gene strongly suggests that the MexAB-OprM efflux pump does not contribute to cefiderocol resistance in this strain [39]. While cefiderocol remains a promising option for treating MDR *P. aeruginosa*, these findings highlight the complexity of resistance mechanisms and the need for further exploration of combination therapies, such as cefiderocol with AVI, to mitigate β-lactamase-mediated resistance. In agreement with these results, previous studies have demonstrated significant synergistic effects between cefiderocol and β-lactamase inhibitors such as sulbactam, avibactam, and tazobactam against MDR *Acinetobacter baumannii* and carbapenem-resistant *P. aeruginosa* (CR-Pa) in both *in vitro* and *in vivo* models [40,41]. Specifically, a synergistic interaction between cefiderocol and ceftazidime/avibactam has been observed against carbapenem-resistant Enterobacterales (CRE) and CR-Pa [38]. Moreover, combining cefiderocol with clinically available β-lactamase inhibitors, such as avibactam or tazobactam, has shown enhanced antibacterial activity against CRE, CR-Pa, and carbapenem-resistant *A. baumannii* (CR-Ab) [42,43]. These findings further support the potential of cefiderocol combination therapies to counteract resistance mechanisms, especially in strains producing Class D β-lactamases or exhibiting impaired siderophore uptake. Given our findings that avibactam partially restored cefiderocol susceptibility in bla_{OXA486}-producing *P. aeruginosa*, further studies are warranted to assess the clinical potential of cefiderocol-based combination therapies. Future work will include checkerboard and time-kill analyses to quantify synergy and evaluate the impact of different β-lactamase inhibitors on cefiderocol efficacy in biofilm-associated infections. The biofilm phenotypic characterization revealed substantial variability across pwCF strains regarding biomass, live cell count, eDNA, and metabolic activity. This phenotypic diversity reflects the genetic variability previously described in *P. aeruginosa* strains, further emphasizing the challenge of effectively treating biofilm-associated infections. The significant increase in the $MBEC_{90}$ compared to the MBC_{90} underscores how biofilm formation impairs the efficacy of antibiotics. In our study, the median $MBEC_{90}$ was 4 μg/mL, considerably higher than the MBC_{90} of 0.5 μg/mL, with $MBEC_{90}/MBC_{90}$ ratios ranging from 2-fold to 128-fold. These findings highlight the increased tolerance of *P. aeruginosa* biofilms to cefiderocol, illustrating the difficulty of eradicating biofilm-embedded cells compared to planktonic cells [44].

The observed increase in $MBEC_{90}$ compared to MBC_{90} reinforces the complexity of biofilm-associated resistance, which is likely influenced by multiple factors, including the protective role of the extracellular polymeric substance (EPS) matrix, limited antibiotic diffusion, metabolic dormancy of bacterial subpopulations, and the stabilizing effect of eDNA [15]. These elements collectively contribute to the increased tolerance observed in biofilm-embedded *P. aeruginosa*. Although the tolerance observed for cefiderocol in biofilm-forming strains appears relatively high compared to the breakpoints set by EUCAST, it falls within the range reported by the Clinical and Laboratory Standards Institute (CLSI), where the MIC breakpoint for cefiderocol is set at 8 µg/mL [45].

These findings are clinically relevant in light of *in vivo* data showing that cefiderocol achieves epithelial lining fluid concentrations of 7.63 mg/L at the end of infusion and 10.40 mg/L at 2 h post-infusion in critically ill pneumonia patients [46,47]. While these concentrations exceed the MBC_{90}, they are closer to the $MBEC_{90}$ observed in our biofilm model. This suggests that cefiderocol may be effective against biofilm-associated infections but could require higher or sustained drug exposure to overcome the increased tolerance within biofilms. This highlights the potential need for optimized dosing regimens in treating biofilm-associated *P. aeruginosa* infections, particularly in the context of pneumonia.

The clinical management of chronic infections in pwCF remains challenging. Treatment options for patients with multi-drug-resistant *P. aeruginosa* infections are limited, and the few agents of last resort, such as colistin, are associated with significant side effects, including neurotoxicity and nephrotoxicity. Cefiderocol, with its novel siderophore-mediated mechanism of action, offers a promising alternative, especially in cases where traditional therapies have failed. However, our findings underscore the need for continued vigilance in treating biofilm-associated infections, as biofilm-mediated tolerance can reduce the efficacy of even last-resort antibiotics.

Although our study provides valuable insights into cefiderocol's activity against *P. aeruginosa* biofilms, it has limitations. First, the study was conducted entirely *in vitro*, and the *in vivo* environment may influence bacterial behavior and drug efficacy differently [45]. Additionally, our analysis was limited to a small number of clinical isolates, and the results may not fully represent the genetic and phenotypic diversity of *P. aeruginosa* in broader clinical settings. Although the number of isolates analyzed in this study is limited, including three well-characterized reference strains provides a robust comparative framework, enhancing the reliability of our findings. Future studies should include larger cohorts and investigate *in vivo* models to validate these findings and explore potential therapeutic combinations.

This study highlights the *in vitro* efficacy of cefiderocol against P. aeruginosa isolates from pwCF, particularly in the context of biofilm-associated infections. While cefiderocol shows promising activity, both β-lactamase production (*e.g.,* bla_{OXA486}) and impaired siderophore-mediated iron uptake (*e.g.*, absence of *fpvA*) can significantly reduce its effectiveness. Additionally, the high $MBEC_{90}$ values observed in biofilm-forming strains emphasize the increased tolerance of biofilms compared to planktonic cells, suggesting that biofilm eradication remains a significant clinical challenge.

These findings underscore the need for further investigation into cefiderocol's use in combination therapies, such as with β-lactamase inhibitors, to overcome resistance mechanisms and improve treatment outcomes. Future research should optimize dosing regimens and explore the interplay between bacterial resistance mechanisms to enhance cefiderocol's efficacy in treating chronic and biofilm-associated P. aeruginosa infections.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB81483.

Ethical approval

The study was approved by the Ethics Committee Area 5 Lazio (N. 48/ISG/24) and conducted in accordance with ethical guidelines for human research.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2025.04.010.

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