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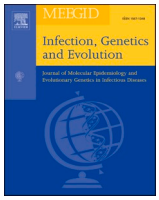

Research paper

Genomic insights of two *Acinetobacter* non-*baumannii* strains with uncommon mechanisms of resistance leading to cefiderocol resistance

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ABSTRACT

The emergence of antimicrobial resistance in *Acinetobacter* species poses a significant clinical challenge, particularly in non-*baumannii* species, which are often overlooked in healthcare settings. In this study, we characterized two *Acinetobacter* clinical isolates, AMA204 and AMA207—identified as *A. junii* and *A. haemolyticus*, respectively—which exhibit uncommon resistance mechanisms that enable survival in the presence of cefiderocol, regardless of their initial minimum inhibitory concentration values. Whole-genome sequencing and comparative genomic analyses were performed to investigate the genetic determinants associated with their resistance profiles. Antimicrobial susceptibility testing confirmed multidrug resistance, with both isolates harboring key β-lactamase genes, including $bla_{\text{OXA-58}}$, and $bla_{\text{NDM-1}}$ in AMA204, and $bla_{\text{OXA-58}}$ and $bla_{\text{PER-2}}$ in AMA207. Phylogenomic analyses revealed genetic relatedness to geographically diverse isolates, suggesting possible evolutionary trends and transmission dynamics. Additionally, iron uptake systems were analysed, highlighting potential mechanisms contributing to cefiderocol resistance together with the presence of listed β-lactamase. This study underscores the clinical relevance of non-*baumannii Acinetobacter* species in antimicrobial resistance and emphasizes the need for continued surveillance and novel therapeutic strategies to combat these emerging threats.

1. Introduction

Acinetobacter, a genus within the *Moraxellaceae* family of Gammaproteobacteria, encompasses approximately a hundred species (https://lpsn.dsmz.de/genus/acinetobacter). Among these, *Acinetobacter junii* and *A. haemolyticus* are known to cause human infections. *A. junii* has been identified as the causative agent of various clinical conditions such as bacteremia, septicemia, corneal ulcer, urinary tract infection,

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necrotizing fasciitis, cellulitis, cholangitis, peritonitis (Aguilar-Vera et al., 2024; Khim et al., 2022; Abo-Zed et al., 2020).

Both *A. junii* and *A. haemolyticus* are related to *A. baumannii*, which is notorious for causing difficult to treat infections in humans due to its resistant to most antibiotics and its ability to persist in clinical settings (Castro-Jaimes et al., 2020; Bai et al., 2020). Like *A. baumannii*, *A. junii* and *A. haemolyticus* exhibit natural resistance to a broad spectrum of commercially available antibiotics. However, unlike *A. baumannii*, *A. junii* and *A. haemolyticus* have been relatively understudied despite recent reports indicating their growing prevalence in human infections. They are often dismissed as contaminants rather than recognized as true pathogens (Aguilar-Vera et al., 2024; Elhosseiny and Attia, 2018). Carbapenem-resistant *A. baumannii* (CRAB) has been classified as a critical priority by the World Health Organization (WHO) in 2019 and has been included in the recently published list in 2024.

Only a limited number of drugs remain active against carbapenem resistant carbapenem-resistant (CR) *Acinetobacter* strains, including cefiderocol and sulbactam/durlobactam (Karruli et al., 2023; Huband et al., 2023). Cefiderocol, a novel siderophore cephalosporine approved by the FDA 2019, is used to treat infections caused by various Gram-negative bacteria, including carbapenem-resistant *Acinetobacter* strains (Sato and Yamawaki, 2019). However, there have been reports of emerging resistance to these new drugs, particularly in *A. baumannii* (Malik et al., 2020; Huang et al., 2024; Strateva and Peykov, 2024).

This study aims to perform an in-depth genomic characterization of two non-*baumannii Acinetobacter* clinical isolates (AMA204 and AMA207) identified as *A. junii* and *A. haemolyticus*, respectively, that exhibited resistance or concentration-dependent attenuation of cefiderocol activity. By combining phenotypic and genomic analyses, we intend to reveal the potential mechanisms driving the antimicrobial resistance seen in the strains.

2. Materials and methods

2.1. Bacterial isolates

In 2016, *A. junii* AMA204 was isolated from a catheter specimen collected from a 45-year-old female patient in Argentina. Similarly, *A. haemolyticus* AMA207 was isolated in 2014 from a skin and soft tissue infection in a 5-year-old patient, also in Argentina (Table 1). The strains were cultured on CLED medium, identified using MALDI-TOF mass spectrometry, and further confirmed by genomic analysis (Almuzara et al., 2015).

2.2. Genome sequencing

Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen Germantown, MD, USA) following the manufacturer's instructions. Whole genome sequencing was carried out using an Illumina NovaSeq X Plus sequencer in one or more multiplexed shared-flow-cell runs, producing 2x151bp paired-end reads (SeqCenter). Demultiplexing, quality control and adapter trimming was performed with bcl-convert (v4.2.4) (SeqCenter). Quality control of sequencing was performed using the FASTQ software. *De novo* assembly and quality assessment were done with the SPAdes and the QUAST software, respectively (Bankevich et al., 2012; Gurevich et al., 2013).

2.3. Comparative genome analysis

Genome annotation of both strains was performed using the PROKKA software (Seemann, 2014). The ortholog functional assignment was done using EggNOG v2.0 (default parameter) (Cantalapiedra et al., 2021). The taxonomy assignment was performed by pairwise Average Nucleotide Identity (ANI) using reference genome of *Acinetobacter* genus and JSpeciesWS software (parameter: default, ANI% > 96) (Richter et al., 2016). The tRNAscan-SE and Infernal software were used for tRNA and ncRNA prediction (Lowe and Eddy, 1997). The Multilocus sequence typing (MLST) profile using Pasteur scheme was determined using MLST scripts (https://github.com/tseemann/mlst, accessed on March 24). (Page et al., 2015a) The antimicrobial resistance genes (ARG) were identified using CARD database *via* RGI (e-value $<10^{-6}$, Amino Acid Identity >80 %, Coverage >80 %) (Alcock et al., 2020). Identification of virulence factors was carried out using BLASTp and the database VFDB (Virulence Factor Database) (e-value $<10^{-6}$, Amino Acid Identity >30 %, Coverage >70 %) (Chen et al., 2012). The K and OC loci were identified with the Kaptive software using the default parameters (Wyres et al., 2020). The high-affinity iron-uptake locus was identified using BLASTp (e-value $<10^{-6}$, Amino Acid Identity >30 %, Coverage >70 %). Nucleotide sequences of each iron-uptake system were taken from Antunes et al. (Antunes et al., 2011a). Insertion sequences were determined using BLASTp and the ISFinder database (e-value$<10^{-6}$, Amino Acid Identity >30 %, Coverage >70 %) (Siguier, 2006). The prophages were predicted using the PHASTEST Software using the default parameters (Arndt et al., 2016). The presence of plasmids of different groups was carried out by rep and mob homology analysis (e-value $<10^{-6}$) (Mindlin et al., 2020).

Pan-genome analysis and the identification of core-genes were done by the ROARY package using default parameter for each species (Page et al., 2015a). We included in the analysis, 70 *A. haemolyticus* and 111 *A. junii* sequences genomes available in the GenBank (Downloaded July 20th,2025) (Table S1). Genome sequences recovered from metagenomic sequencing and categorized as atypical according to the GenBank database were excluded. Core genome phylogeny analysis was performed using the maximum likelihood method implemented in IQ-TREE2 with default parameters (Page et al., 2015b). The substitution genetic model was predicted by ModelFinder software using default parameter (Page et al., 2015b). SNPs were extracted using the snp-sites software with the default parameters (Page et al., 2016). The genes that were unique to each genome were extracted from "gene_presence_absence.csv" of Roary output (Sitto and Battistuzzi, 2020).

bla_{OXA-58} and bla_{NDM-1} genetic context was validated by PCR reaction using specific primers to amplify the genetic structure shown in Fig. 4 (ΔISAba3: TTAGAACCCATTTAAAGTGTC, OXA58_AMA207: GTAAAATCTTTGTCCCATGC, ISAba3: TTAGACTGTAGCTAAATCTCG, ISAba125: GTCATACCATCATCTTAACTTTG).

2.4. Antibiotic susceptibility assays

The antimicrobial resistance profile was determined by disk diffusion according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (CLSI CLSI M100-ED29, 2020). Broth microdilution with iron-depleted cation-adjusted Mueller-Hinton broth (ID-CAMHB, BD Difco) was used as the reference method for cefiderocol susceptibility testing. The iron depletion was generated following the

Table 1
Genomic features and metadata of *A. junii* AMA 204 and *A. haemolyticus* AMA 207.

Strain	Taxon	Genome Size	GC Content (%)	Contig Number	N_{50}	Gender	Age	Country of isolation	Year of isolation	Source
AMA204	*A. junii*	3,519,508	38.71	147	44,595	Female	45	Argentina	2016	BSI
AMA207	*A. haemolyticus*	3,689,522	39.39	60	227,906	Male	5	Argentina	2014	SSTI

BSI: blood stream infection, SSTI: skin and soft tissue infection.

EUCAST recommendation. All procedures were performed in triplicates following the Clinical and Laboratory Standards Institute (CLSI) (CLSI CLSI M100-ED29, 2020) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines European Committee on Antimicrobial Susceptibility Testing (EUCAST), Clinical breakpoints v15.0 (EUCAST, 2025) (https://www.eucast.org/clinical_breakpoints). The results were interpreted with CLSI guidelines, except for colistin and tigecycline, in which cases European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Food and Drug Administration (FDA) recommendations were used, respectively. The CLSI, EUCAST, and FDA publish guidelines for antimicrobial susceptibility testing (AST) that provide recommendations for testing and interpreting the susceptibility of microorganisms to antimicrobial agents. These guidelines include recommendations for standardized methods, quality control procedures (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* ATCC 700603), and interpretive criteria for AST.

2.5. Cefiderocol static concentration time-kill assay

Static concentration time-kill (SCTK) assays were performed, as previously described, to determine bacterial killing kinetics in ID-CAMHB, in the absence and presence of cefiderocol against AMA204 and AMA207 (Mezcord et al., 2023a). Cefiderocol killing activity was evaluated at clinically achievable concentrations (0.5, 1, 4 and 8 μg/mL) (Katsube et al., 2019) against an initial inoculum of 5×10^6 CFU/mL. Serial samples (1 mL) obtained at different timepoints (0, 1, 2, 4, 6, 8 and 24-h) following the addition of cefiderocol were diluted with phosphate buffered saline (PBS, pH 7.4), and 50 μL of the appropriate bacterial dilution were spirally plated on CA-MHA plates using an automated spiral plater (easySpiral, interscience, Saint Nom la Breteche, France) and incubated at 37 °C. After a 16-20 h incubation, bacteria were quantified using a ProtoCOL automated colony counter (Symbiosis, Cambridge, UK). Treatment efficacy was calculated as the percentage reduction in the area under the $\log_{10}$CFU/mL time curve (AUC_CFU) relative to the growth control using GraphPad (version 8.0.2, GraphPad Software, Boston, Massachusetts, USA, www.graphpad.com).

2.6. Data availability

The Whole Genome Shotgun project has been deposited at GenBank with accession numbers JBMVIY000000000-and JBMVIZ000000000 for AMA204 and AMA207, respectively. The Genbank files of prokka annotation, PHASTEST prediction file, pan-matrix, core-gene concatenate alignment, Newick tree and Protein variant cluster files (PirA, PiuA, BauA and CirA) has been deposited in Zenodo repository (Link: https://zenodo.org/records/16749373).

3. Results and discussion

3.1. Genomic features of A. junii AMA204 and A. haemolyticus AMA207 clinical isolates

There is a growing recognition of *Acinetobacter* species other than *A. baumannii* being isolated from clinical specimens. However, these species are frequently overlooked as mere environmental contaminants and thus are often regarded as having limited clinical relevance (Turton et al., 2010). In this study, we performed genotypic characterization of two non-*baumannii Acinetobacter* clinical isolates that demonstrated resistance to carbapenems. For *A. junii* AMA204, the total length of the genome assembly was 3,519,508 bp and the estimated GC content was 38.7 % (Table 1). While for *A. haemolyticus* AMA207 the length of the genome assembly was 3,689,522 bp, and the estimated GC content was 39,4 % (Table 1). The whole genome sequences of both strains were of good quality (50× coverage, Q score > 30). The assembly quality was evaluated using the QUAST software and indicated 147 contigs for AMA204 and 60 contigs for AMA207. The N50 of AMA204 and AMA207 were 44,595 and 227,906, respectively (Table 1).

Mobile genetic elements, including plasmids, insertion sequences (IS), and prophages, were identified in both AMA204 and AMA207 genomes. PHASTEST analysis predicted four putative prophage sequences in AMA204 and one in AMA207. Based on the PHASTEST completeness score criteria defined as intact (>90), questionable (70–90), and incomplete (<70), three intact and one questionable prophage were identified in AMA204, while a single intact prophage was detected in AMA207 (Table S1).A total of 23 and 18 insertion sequences were identified in AMA204 and AMA207, respectively (Table S1), indicating a higher degree of genomic plasticity in AMA204. IS elements are known to play key roles in genome rearrangements, regulation of gene expression, and mobilization of resistance genes in *A. baumannii* (Fournier et al., 2006; Hamidian and Hall, 2018).

Plasmid content was inferred through the detection of *rep* and *mob* genes. A single R3-like plasmid (NODE_9) was identified in AMA204, while no plasmids were detected in AMA207. The R3 plasmid type has been reported to carry the $bla_{\text{OXA-58}}$ gene in various clinical isolates of *A. baumannii* (Poirel et al., 2005; Bertini et al., 2010). Although AMA204 contains a R3-like plasmid, the $bla_{\text{OXA-58}}$ gene was not located on the same contig as the identified *rep* gene. Further validation through plasmid reconstruction or long-read sequencing will be necessary to confirm whether $bla_{\text{OXA-58}}$ is indeed plasmid-borne in AMA204.

These findings suggest a greater mobilome complexity in AMA204 compared to AMA207, which may contribute to its adaptive potential and horizontal gene transfer capacity. The presence of multiple intact prophages and a higher number of IS elements in AMA204 supports the hypothesis of increased genome flexibility, a feature frequently associated with multidrug-resistant and epidemic clones of *A. baumannii* (Touchon et al., 2014). Such mobile elements may facilitate the acquisition and dissemination of antimicrobial resistance genes, underscoring their clinical relevance.

Although the presence and function of various virulence factors have been extensively studied in *A. baumannii* (Ramirez et al., 2019), much less is known about the virulence potential of other *Acinetobacter spp.* (Schramm et al., 2019). Using the VFDB (Virulence Factor Database), we identified 203 and 237 putative virulence factor-encoding genes in AMA204 and AMA207, respectively (Table S1). These genes were associated with multiple virulence-related functions, including type IV and type VI secretion systems, motility, and biofilm formation.

Notably, AMA207 harbored an additional cluster of virulence genes putatively involved in siderophore production. These genes showed homology to the acinetobactin biosynthetic cluster, with amino acid identity ranging from 55 % to 92 % compared to the well-characterized acinetobactin locus of the *A. baumannii* strain ACICU. The presence of such a cluster in AMA207 suggests a potential enhanced ability for iron acquisition, which is a known virulence determinant in *A. baumannii* and other pathogens (Antunes et al., 2011b; Sheldon and Skaar, 2020).

The identification of siderophore-related genes in AMA207 is particularly relevant given the increasing recognition of *A. haemolyticus* and *A. junii* as emerging opportunistic pathogens in clinical settings (Peleg et al., 2008). However, the functional role of these siderophore systems in non-*baumannii Acinetobacter* species remains poorly understood. Further experimental studies are warranted to determine whether these genes are expressed and functional, and how they contribute to virulence and survival in iron-limited environments.

These findings highlight the genomic virulence potential of *Acinetobacter* species beyond *A. baumannii* and underscore the need for expanded research on the pathogenic mechanisms of lesser-studied species.

3.2. Phylogenomic analysis of A. junii AMA204 and A. haemolyticus AMA207 isolates

A core-genome phylogenetic analysis was performed for each of the strains, AMA204 and AMA207. This analysis included 1114 core genes

aligned for *A. haemolyticus* and 1408 core genes aligned for *A. junii.* The resulting phylogenetic trees highlight the extensive genetic diversity of *A. junii* and *A. haemolyticus* genomes collected from various countries, including Argentina, China, and others, underscoring the global distribution and adaptability of these species. AMA204 is closely related to strains originating from Russia, as indicated by their tight phylogenetic clustering (Fig. 1), which suggests the presence of shared traits, such as antimicrobial resistance mechanisms or adaptive strategies where a temporal trend is evident, with more recent isolates (2016–2022) clustering together, suggesting ongoing evolutionary changes, including the potential emergence of novel resistance traits or adaptations to healthcare environments.

From the phylogenetic analysis of *A. haemolyticus*, the AMA207 strain was found within a clade that included isolates recovered from Argentina, China, and the USA (Fig. 2). A temporal trend was observed within the AMA207 clade, with most isolates being recovered in 2017. AMA207, recovered in 2014, may represent an ancestral strain of the clone recovered in 2017 within this clade (Fig. 2).

The comparison emphasizes substantial genetic diversity within this species, with some clades being more region-specific, while others exhibit a wider geographic distribution. This diversity may be linked to differences in environmental pressures, hospital settings, or antibiotic usage patterns.

3.3. Antimicrobial resistance profile of A. junii AMA204 and A. haemolyticus AMA 207 clinical isolates

A total of seven antimicrobial resistance genes (ARGs) were identified in AMA204, while 13 ARGs were found in AMA207 (Table 2 A). The AMA204 strain harbors $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-58}}$ β-lactamases genes, as well as, genes conferring resistance to aminoglycosides, *aph(3′)-VIa (*amikacin) and *aac(3)-IId (*gentamicin and tobramycin), plus *sul2* (sulfamethoxazole), *mph(E)* and *msr(E) (*macrolides). The AMA207 strain contains a broader resistome, including $bla_{\text{PER-2}}$, $bla_{\text{OXA-58}}$, $bla_{\text{OXA-214}}$, $bla_{\text{TEM-1B}}$ (confers resistance to penicillins and cephalosporins), *cmlB1* (chloramphenicol), *tet(B)* and *tet(D)* (tetracyclines.), *aph(3″)-Ib, aph(6)-Id, aph(3′)-VIa, aac(3)-IIa*, and *aac(6′)-Ig* (aminoglycosides, such as gentamicin, tobramycin, and amikacin), *mph(E)* and *msr(E)* (macrolides, including erythromycin and azithromycin). These genes contribute to the strain's multidrug-resistant phenotype, posing significant concerns in clinical settings.

Phenotypically, both isolates exhibited resistance to several antibiotics (Table 2B), but important differences were noted. Despite AMA207 carrying *tet(B)* and *tet(D)*, it remained susceptible to minocycline. This could be due to differential gene expression or the limited activity of these genes against specific tetracyclines like minocycline, as previously reported in *Acinetobacter* species.

AMA204 was resistant to trimethoprim-sulfamethoxazole (STX) and cefiderocol, with a cefiderocol MIC of 16 mg/L. In contrast, AMA207 demonstrated a lower cefiderocol MIC of 0.5 mg/L.

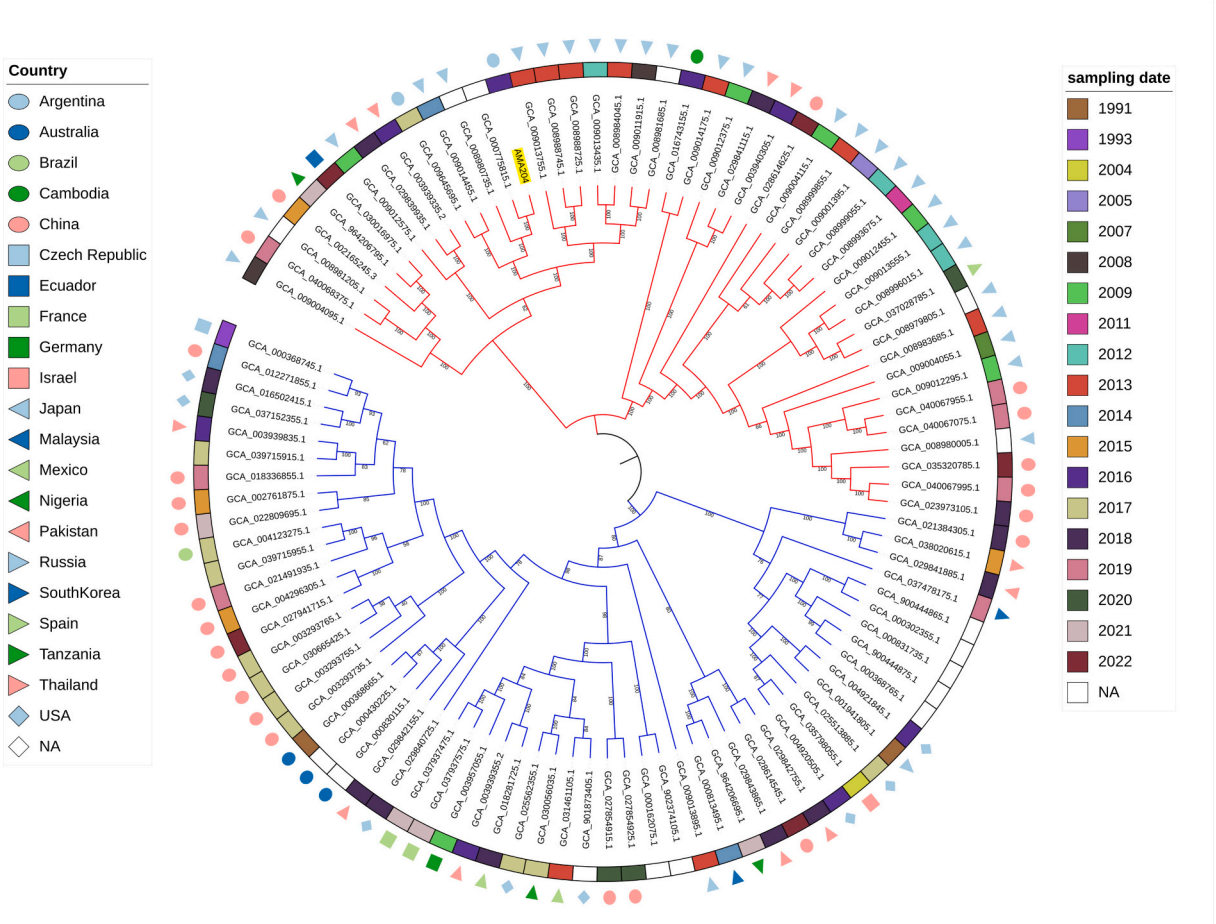


Fig. 1. Core-phylogeny of AMA 204 and 111 *A. junii* genomes. The figure displays the maximum likelihood phylogeny of 112 *A. junii* sequences. The bootstrap method was used as a supporting method (1000 iterations). The molecular substitution model was GTR. The tree representation was carried out by iTOL. The symbols and colors represent the year and country of origin of each genome. The yellow highlight indicates the phylogenetic cluster that it found the AMA204 genome. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

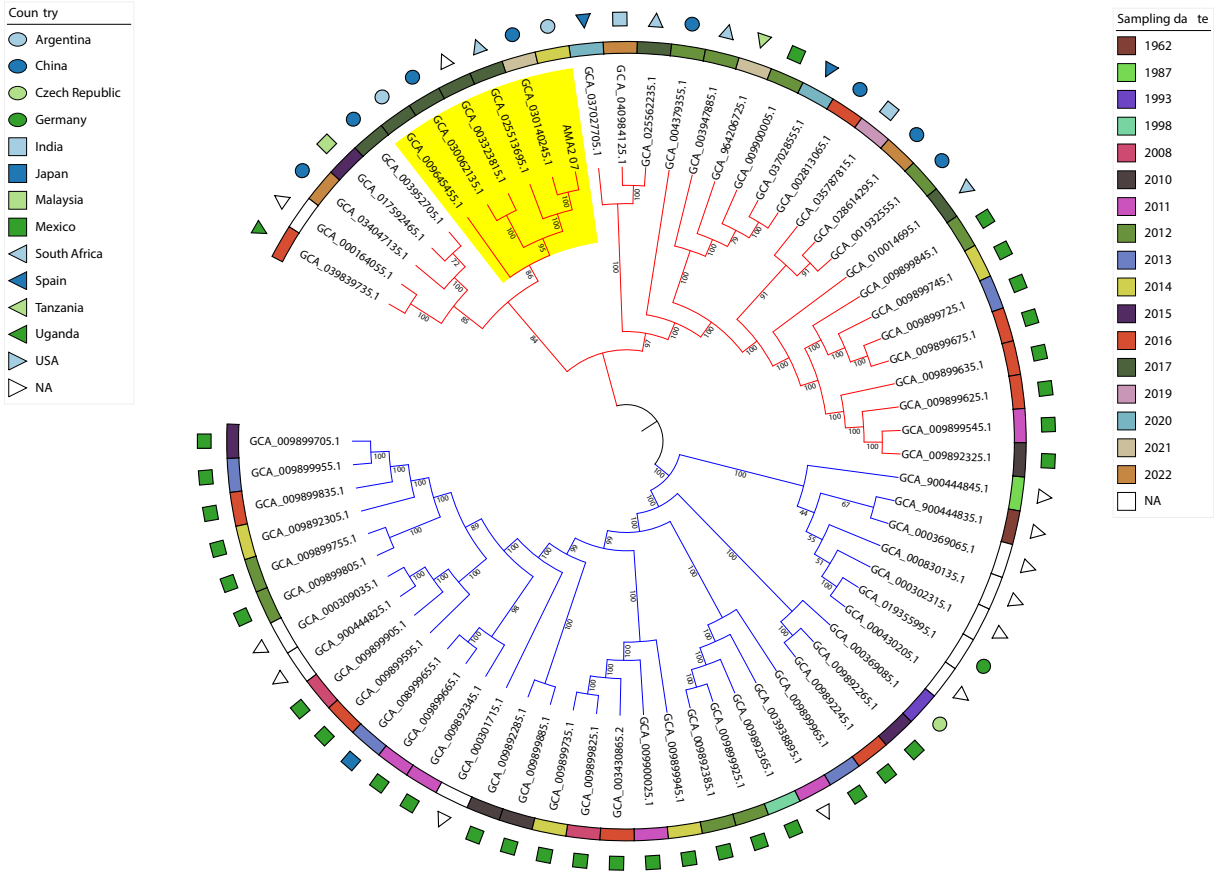


Fig. 2. Core-phylogeny of AMA 207 and 70 *A. haemolyticus* genomes. The figure displays the maximum likelihood phylogeny of 71 *A. haemolyticus* sequences. The bootstrap method was used as a supporting method (1000 iterations). The molecular substitution model was GTR. The tree representation was carried out by iTOL. The symbols and colors represent the year and country of origin of each genome. The yellow highlight indicates the phylogenetic cluster that it found the AMA207 genome. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A. junii, is less commonly associated with high-level resistance compared to other *Acinetobacter* species like *A. baumannii*. Regeen et al. (2014), reported the coexistence of $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-58}}$ genes in a strain of *A. junii* (Regeen et al., 2014a). The coexistence is significant, as it complicates treatment options and suggests that these resistance mechanisms could spread to other bacterial species or become widespread in *A. junii*.

Regarding aminoglycosides, AMA204 encoded two aminoglycoside resistance genes (*aph(3′)-VIa* and *aac(3)-IId*), while AMA207 encoded five (*aph(3″)-Ib*, *aph(6)-Id*, *aph(3′)-VIa*, *aac(3)-IIa*, and *aac(6′)-Ig*), this genetic difference correlated with slightly broader phenotypic resistance in AMA207, which showed higher MICs for several aminoglycosides compared to AMA204 (Table 2).

The 2020 Spanish nationwide surveillance study provides a comprehensive analysis of the resistance profiles and genomic characteristics of *A. junii* and *A. haemolyticus*, along with other species within the genus. The findings indicate that both *A. junii* and *A. haemolyticus* exhibit substantial levels of antimicrobial resistance. While *A. baumannii* frequently often garners attention due to its carbapenem resistance, these other *Acinetobacter* species also demonstrate notable resistance across a range of antibiotics. Both species display variable susceptibility to antimicrobial agents; specifically, certain isolates of *A. junii* and *A. haemolyticus* remain susceptible to colistin and tigecycline. However, resistance to commonly used antibiotics, including carbapenems, cephalosporins, and aminoglycosides, is prevalent. A key factor contributing to the resistance profiles of both species is the presence of specific beta-lactamases, particularly extended-spectrum beta-lactamases (ESBLs) (Lasarte-Monterrubio et al., n.d.).

Recently, we investigated the impact of β-lactamase inhibitors on the efficacy of cefiderocol against carbapenem-resistant *Acinetobacter* species (Mezcord et al., 2023b). The study evaluated the synergistic potential of combining cefiderocol with β-lactamase inhibitors to enhance its antimicrobial activity. The results demonstrated that certain inhibitors, especially those targeting serine β-lactamases, can improve cefiderocol's activity by mitigating resistance mechanisms. However, MBL inhibitors showed limited effectiveness due to their inability to neutralize all resistance determinants (Mezcord et al., 2023b).

A distinct genetic context for the $bla_{\text{OXA-58}}$ gene was observed between AMA204 and AMA207, with AMA207 lacking four genes encoding proteins of unknown function (*hyp*). Previous studies have described the Δ*ISAba3*-$bla_{\text{OXA-58}}$-*ISAba3* genetic structure (Matos et al., 2019). In our analysis, this structure was identified in AMA204. However, genome sequencing revealed that the structure appeared incomplete in AMA207. To further investigate the presence of the Δ*ISAba3*-$bla_{\text{OXA-58}}$-*ISAba3* arrangement, we performed PCR using specific primers. The complete genetic structure was not detected in AMA207.

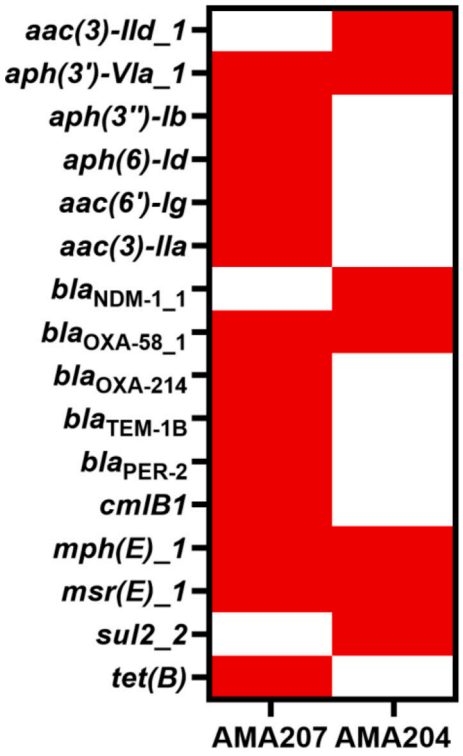
The genetic context of $bla_{\text{NDM-1}}$ appeared incomplete in AMA204 based on genome sequencing data. To verify the presence of an insertion sequence upstream of the gene, we performed PCR using primers specific for the detection of the *ISAba125*-$bla_{\text{NDM-1}}$ arrangement. The PCR results confirmed the presence of this genetic structure in AMA204, consistent with previous reports in clinical *Acinetobacter* isolates (Nordmann et al., 2011).

Additionally, the genetic context of the $bla_{\text{PER-1}}$ gene in AMA207 was identified, flanked by the insertion sequence ISPa13 and the *yibF* gene (Fig. 3).

This is the first report of the genetic context of $bla_{\text{OXA-58}}$ and $bla_{\text{PER-2}}$ in *A. haemolyticus* AMA207. The coexistence of $bla_{\text{OXA-58}}$ and $bla_{\text{NDM-1}}$ in

Table 2
A) Antimicrobial resistance genotypic prediction of AMA 204 and AMA 207 strains. Red indicates presence and white indicates absence. B) *A. junii* AMA204 and *A. haemolyticus* AMA207 antimicrobial susceptibility testing.

A) Antimicrobial resistance genotypic prediction of AMA 204 and AMA 207 strains. Red indicates presence and white indicates absence



B) Antibiotic susceptibility in *Acinetobacter junii* AMA204 and *Acinetobacter haemolyticus* AMA207.

	Diameters of Inhibition Zones(mm)/Minimum Inhibitory Concentrations (MICs)	
	AMA204	**AMA207**
Ampicillin/sulbactam (AMS)	13 (I)	17 (I)
Trimethoprim-sulfamethoxazole (TMS)	6 (R)	17 (S)
Amikacin (AK)	10 (R)	8 (R)
Cefepime (FEP)	6 (R)	8 (R)
Ceftazidime (CAZ)	6 (R)	6 (R)
Ciprofloxacin (CIP)	29 (S)	28 (S)
Imipenem (IMP)	6 (R)	14 (R)
Meropenem (MEM)	6 (R)	17 (I)
Gentamicin (GEN)	11 (R)	6 (R)
Tigecycline (TIG)	29	23
Minocycline (MIN)	33 (S)	24 (S)
Colistin (COL)	15	12
Cefiderocol (FDC)		

ND: not determined. S: susceptible, R: resistant diameters of inhibition zones of antibiogram plates performed according to CLSI. The experiments were repeated at least three times for each strain. The results were interpreted with CLSI guidelines.

A. junnii has been previously reported (Regeen et al., 2014b). Our findings, along with the geographical distribution of previously reported bla_{OXA-58} in *A. junii* and *A. haemolyticus*, (Fig. S1) suggest a potential global widespread of this antibiotic resistance determinants in these *Acinetobacter* species, although few reports have been documented (Fig. S1). Further genomic sequencing of *A. junii* and *A. haemolyticus* isolates is essential to understand the geographical extent of these observations, and whether this constitutes a global trend.

3.4. Iron uptake system in A. junii AMA204 and A. haemolyticus AMA 207

In human hosts, free iron is scarce, as it is primarily bound to proteins like haemoglobin, transferrin, and lactoferrin. Consequently, bacteria produce siderophores to bind and solubilize iron, making it available for various metabolic processes and influencing their pathogenicity (Arora et al., 2013). The siderophore acinetoferrin was characterized by Okujo et al. in *A. haemolyticus* (Okujo et al., 1994). The *acbABCD* and *actBCAD* operons, consisting of eight consecutive genes, are involved in the biosynthesis and transport of acinetoferrin (Funahashi et al., 2013). *A. junii* also produces an active siderophore in response to iron scarcity (Arora et al., 2013).

In *A. baumannii*, the genes *pirA, piuA, cirA*, and *bauA* encode TonB-dependent receptors (TBDRs) crucial for iron acquisition, a vital process for bacterial survival and pathogenicity. PiuA and PirA are TBDRs facilitate the uptake of siderophores—molecules that bind and transport

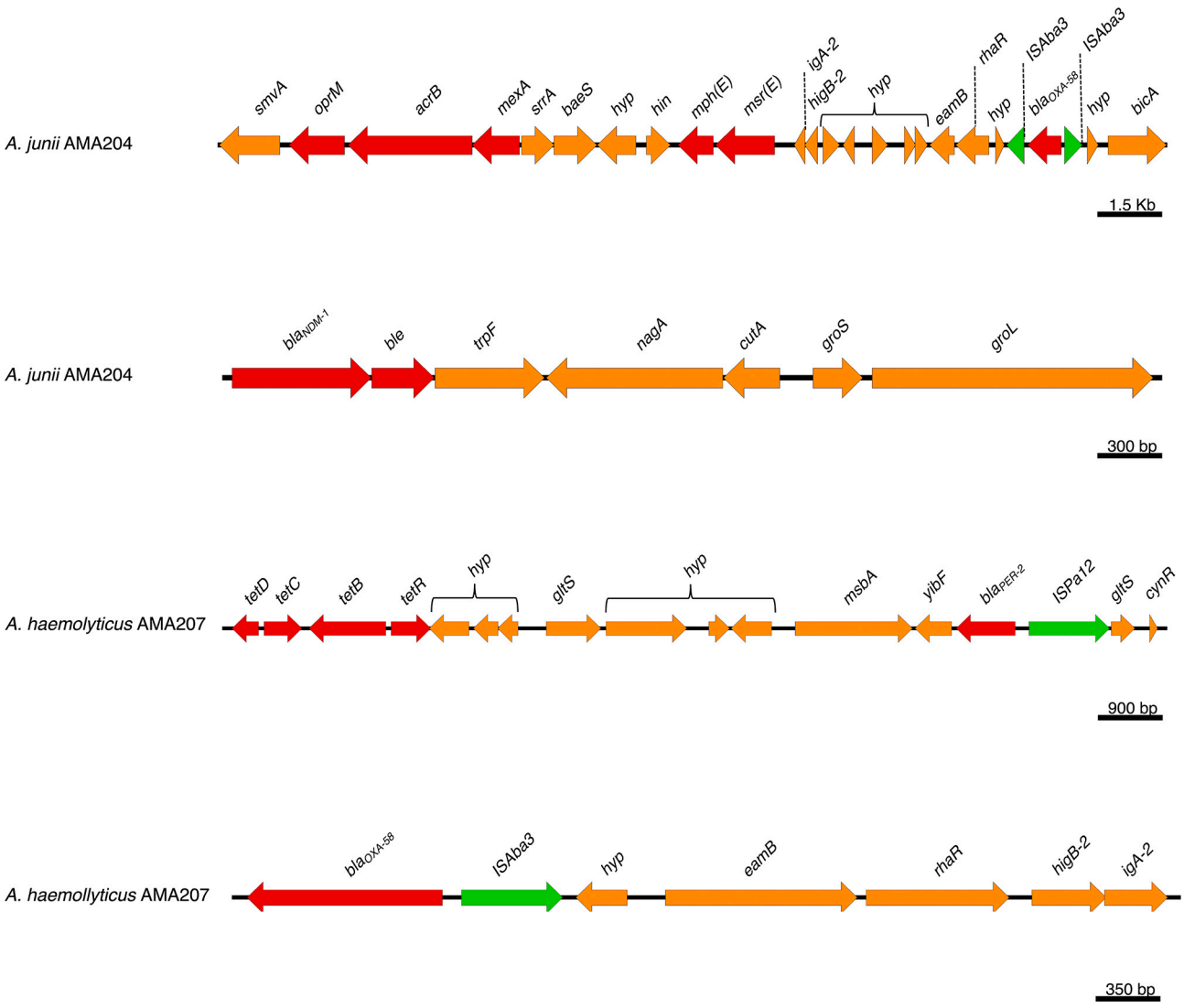


Fig. 3. Genetic context of bla_{OXA-58}, bla_{NDM-1}, and bla_{PER-2}. Green and red arrows indicate insertion sequences and antimicrobial resistance genes, respectively, while orange arrows represent other genes with known or unknown functions. Genes labeled as "hyp" correspond to those with an unknown function. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

iron into the bacterial cell. Deletion of either *piuA* or *pirA* in *A. baumannii* leads to a 4- to 8-fold decrease in susceptibility to siderophore-drug conjugates such as BAL30072 and MC-1, whereas overexpression in *Pseudomonas aeruginosa* increases susceptibility by 4- to 32-fold (Moynié et al., 2017). BauA is another TBDR in *A. baumannii*, serving as the receptor for the siderophore acinetobactin (Zimbler et al., 2009). *bauA* expression is upregulated under iron-depleted conditions, enhancing the bacterium's ability to acquire iron from its environment, playing a significant role in the pathogen's virulence and survival within the host (Nishimura et al., 2022). CirA is a TBDR associated with the uptake of catecholate-type siderophores. While its role in *A. baumannii* is less well-characterized compared to PiuA, PirA, and BauA, it is believed to contribute to iron acquisition and may influence susceptibility to siderophore-antibiotic conjugates (Le et al., 2022).

To explore the conservation of these receptors, we aligned the amino acid sequences of PirA, PiuA, CirA, and BauA from the clinical isolates AMA204 and AMA207 with those from *A. baumannii* AB5075 (Jacobs et al., 2014) (Fig. S2). The alignment revealed that AMA204 had lower identity percentages relative to AB5075 for PirA (27.2 %) and PiuA (26.1 %), compared to AMA207 which showed higher identity for these receptors (48.5 % and 47.8 %, respectively). CirA displayed intermediate conservation, with 48.8 % identity in AMA204 and 52.1 % in AMA207, whereas BauA showed the highest sequence conservation among the proteins analysed, with 56.3 % identity for AMA204 and 60.4 % for AMA207. Beyond the percentage data, qualitative analysis of the alignments provided further insights. For PirA, AB5075 shows significant sequence divergence compared to AMA204, with no sequence alignment observed in several regions, suggesting possible functional divergence or even loss of this receptor's role in AMA204.

In contrast, AMA207 shares more conserved regions with AB5075, particularly in ligand-binding and TonB-dependent receptor motifs, although distinct differences remain in several functional domains. Regarding PiuA, AB5075 exhibits conserved sequences with AMA207 in regions linked to siderophore binding and transport, while AMA204 shows the least alignment, reinforcing the hypothesis of species- or strain-specific adaptations in iron acquisition strategies.

For CirA, all three strains exhibit conserved motifs essential for catecholate-type siderophore uptake, though sequence variations, particularly in N-terminal and loop regions, may affect receptor regulation or interactions with distinct siderophores. Finally, BauA shows the highest conservation, especially between AB5075 and AMA207, with conserved siderophore binding domains suggesting a similar role in acinetobactin-mediated iron uptake across species, albeit with minor species-specific adaptations.

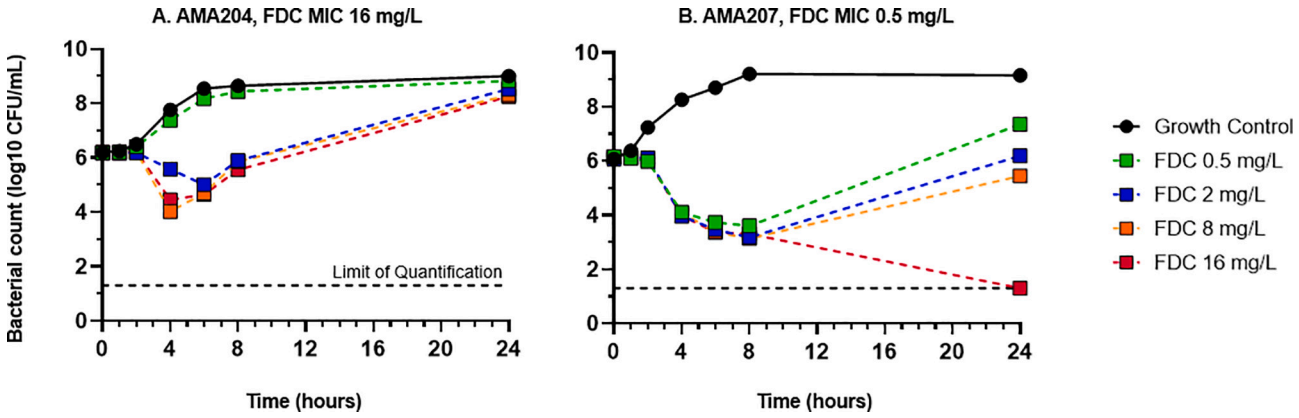


Fig. 4. Static concentration time-kill studies evaluating the killing kinetics of cefiderocol (FDC) monotherapy (0.5, 1, 4 and 8 mg/L) against an initial inoculum of 5 × 106 CFU/mL of A. junii AMA204 (A) and A. haemolyticus AMA207 (B) clinical isolates over 24 h of incubation at 37 °C.

Furthermore, we analysed the presence or absence of the iron acquisition proteins PirA, PiuA, CirA, and BauA in *A. junii* and *A. haemolyticus* genomes available in the NCBI database using tblastn. Among the 112 *A. junii* genomes analysed, the presence of PirA, PiuA, CirA, and BauA was detected in 85 (75.89 %), 77 (69.64 %), 62 (55.36 %), and 101 (90.18 %) genomes, respectively. In contrast, among 71 *A. haemolyticus* genomes, these genes were detected at lower frequencies: PirA in 2 (2.82 %), PiuA in 18 (25.35 %), CirA in 50 (70.42 %), and BauA in 17 (23.94 %) genomes (Table S2).

Sequence analysis revealed substantial protein diversity, with 45, 31, 22, and 44 variants identified for PirA, PiuA, CirA, and BauA, respectively, in *A. junii.* In *A. haemolyticus,* 1, 11, 14, and 6 variants were identified for these same proteins (Table S2, https://zenodo.org/records/16749373). Despite this variability, the amino acid identity among variants ranged from 98 % to 100 %, suggesting a high degree of conservation within each species.

These findings highlight notable differences in the distribution of iron uptake systems between *A. junii* and *A. haemolyticus*. Iron acquisition is a critical virulence mechanism in *Acinetobacter spp.*, allowing pathogens to survive in the iron-limited environment of the host (Antunes et al., 2011b). The *bauA* gene, in particular, encodes a TonB-dependent receptor for acinetobactin—a siderophore essential for iron acquisition in *A. baumannii*—and has been associated with enhanced virulence and survival in host tissues (Penwell et al., 2012). The high prevalence and diversity of BauA in *A. junii* suggest that similar iron uptake strategies may contribute to its opportunistic pathogenicity. In contrast, the limited presence of PirA and BauA in *A. haemolyticus* genomes could reflect distinct ecological niches or iron acquisition strategies.

Further studies are needed to elucidate the functional roles of these proteins in non-*baumannii Acinetobacter* species and to determine whether they represent viable targets for novel therapeutics or vaccine development.

3.5. Pharmacodynamic activity of cefiderocol against AMA204 and AMA207

In the absence of cefiderocol, AMA204 and AMA207 showed a similar growth pattern in ID-CAMHB, reaching a maximum carrying capacity of ~9 $\log_{10}$ CFU/mL after 8 h (Fig. 4). Change in bacterial burden ($\log_{10}$ CFU/mL) over time is illustrated in Fig. 4, with a lower limit of detection of 1.3 $\log_{10}$ CFU/mL. For both isolates, cefiderocol showed a concentration dependent killing activity. For AMA204, cefiderocol-resistant, NDM-producing strain, cefiderocol 0.5 mg/L did not show significant killing activity with a 2.5 % reduction in AUC_CFU. With cefiderocol 2, 8 and 16 mg/L, an early killing activity reaching a reduction of around 4 $\log_{10}$ CFU/mL by 4 h was followed by regrowth similar to growth control, leading to 20–24 % reduction in AUC_CFU (Table S3).

Resistance to cefiderocol has been associated with a range of mechanisms, including the production of β-lactamases—particularly NDM, KPC, AmpC variants, OXA-427, and PER- and SHV-type ESBLs—as well as mutations in porins, siderophore receptors, and alterations in PBP-3. Among Gram-negative bacteria, the predominant resistance mechanisms appear to involve the co-expression of multiple β-lactamases combined with reduced membrane permeability (Choby et al., 2021; Kayama et al., 2024; Kohira et al., 2020). NDM expression contributes to cefiderocol resistance through additional pathways, such as increased copy numbers of the bla_{NDM} gene and mutations in siderophore receptor genes like *pirA* and *piuA* (Nurjadi et al., 2022; Lan et al., 2022). In the case of the NDM-producing strain AMA204, no sequence similarity was observed with the *pirA* gene, and only minimal alignment was found with *piuA*, suggesting possible functional divergence or even a loss of receptor activity (see Section 2.4 for details). Mutations in the PiuA and PirA proteins have been linked to reduced susceptibility to cefiderocol (Viñes et al., 2025), a pattern also observed in the AMA204 strain (Fig. 4A). Genomic analyses of cefiderocol-resistant *A. baumannii* clinical isolates have further implicated the absence of *piuA*, either alone or in conjunction with reduced *pirA* expression, as contributing factors to cefiderocol resistance (Malik et al., 2020; Yamano et al., 2022)).

In the cefiderocol-susceptible strain AMA207, a strong bactericidal effect was observed, achieving ~4 $\log_{10}$ CFU/mL reduction by 8 h. However, this effect was sustained only at the highest tested cefiderocol concentration of 16 mg/L. AMA207 carries the PER-2 β-lactamases, and PER-like β-lactamases have been associated with reduced cefiderocol susceptibility in CRAB (Seifert et al., 2023). Poirel et al. also reported a link between cefiderocol resistance and the presence of PER-like β-lactamases (Poirel et al., 2021).

In AMA207, reduced cefiderocol activity at lower concentrations is likely due to hydrolysis of cefiderocol by the PER-2 enzyme (Fig. 4B). Therefore, while AMA207 meets the susceptibility threshold by MIC testing, the dynamic bactericidal activity is affected at submaximal concentrations, suggesting a narrow therapeutic window. This highlights a potential limitation of relying solely on static MIC values for clinical interpretation, particularly in strains harboring PER-type enzymes. The increased activity at higher cefiderocol concentrations may be attributed to the presence of functional TonB-dependent receptor (TBDR) genes, *piuA* and *pirA* (see Section 3.4), which are essential for siderophore-mediated iron uptake and facilitate the transport of cefiderocol into bacterial cells.

4. Conclusions

This study focuses on the sequencing and analyzing the genomes of AMA204 and AMA207 to identify key genetic characteristics such as gene content, antibiotic resistance determinants, and potential virulence factors. The analysis offers a thorough overview of the genomic differences between these two strains, providing insights into their biological functions and pathogenic potential. By detailing the genomic features of these isolates, the study aims to deepen our understanding of their clinical relevance and potential treatment challenges.

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Data available statement

The data that supports the findings of this study are available in the supplementary material of this article.

Ethics statement

Not applicable.

Informed consent

Not applicable.

CRediT authorship contribution statement

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Declaration of competing interest

The authors declare no conflicts of interest.

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None.

Data availability

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

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