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Identification of New Targets in Bacterial Genome of Gram-Negative Bacteria

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SUMMARY

Acinetobacter baumannii is one of the most clinically relevant multidrug-resistant (MDR) pathogens responsible for severe hospital-acquired infections, particularly in critically ill patients. Carbapenem resistance in this organism is commonly associated with carbapenem-hydrolysing β -lactamases; however, additional mechanisms, including alterations of antibiotic targets and complex genomic resistance backgrounds, significantly contribute to therapeutic failure. In this context, Cefiderocol (FDC) has emerged as a novel therapeutic option, although heterogeneous microbiological responses have been increasingly reported.

The aim of this work is to investigate the determinants of β -lactam activity and FDC response in clinical *A. baumannii* isolates through an integrated genomic, phenotypic, and biochemical approach, with particular focus on penicillin-binding protein 3 (PBP3), a key antibiotic target whose structural alterations may critically influence β -lactam efficacy and contribute to resistance development.

Five clinical strains obtained from critically ill patients treated with FDC plus ampicillin/sulbactam (AMP/SUL) therapy were analyzed.

Whole-genome sequencing (WGS) was performed to characterize resistome composition, virulence determinants, and mutations affecting functionally relevant genomic regions. Antimicrobial susceptibility testing and time-kill assays were used to evaluate antibiotic activity and combination effects under dynamic conditions. Phenotypic analyses revealed isolate-dependent susceptibility profiles, with partial restoration of FDC activity following sulbactam (SUL) addition in selected strains during early incubation phases, while sustained bactericidal activity was not consistently observed after prolonged exposure.

To investigate the contribution of target modification, penicillin-binding protein 3 (PBP3) variants identified in clinical isolates were cloned, expressed, and purified using a heterologous overexpression system followed by affinity chromatography. Antibiotic–protein interactions were characterized through fluorescence-based bocillin competition assays.

Biochemical experiments demonstrated measurable binding of carbapenems to wild-type PBP3, enabling determination of IC_{50} values, whereas mutant variants carrying K235N and K235N/H370Y substitutions showed markedly reduced or abolished carbapenem interaction. In contrast, limited apparent binding was observed for ampicillin (AMP) and FDC under the experimental conditions employed, suggesting that target engagement may depend on structural or dynamic features not fully reproduced in the in vitro assay system.

Overall, this study demonstrates that antibiotic response in MDR *A. baumannii* results from a multilayer resistance architecture integrating genomic background, phenotypic behavior, and

target-site alterations. These findings emphasize the importance of combining WGS, pharmacodynamic analyses, and biochemical characterization of antibiotic targets to improve interpretation of antimicrobial susceptibility and to support more rational antimicrobial stewardship strategies against difficult-to-treat *A. baumannii* infections.

Keywords: *A. baumannii*, MDR, FDC, PBP3, WGS, phenotypic analyses, time-kill assay, fluorescence-based bocillin competition assays.

List of Abbreviations

A. baumannii — *Acinetobacter baumannii*
AMP — Ampicillin
ATCC — American Type Culture Collection
 β -lactams — Beta-lactam antibiotics
BLI — Beta-Lactamase Inhibitor
BMD — Broth Microdilution
CAZ — Ceftazidime
CDC — Center for Disease Control and Prevention
CRO — Ceftriaxone
CRAB — Carbapenem-Resistant *Acinetobacter baumannii*
CZA — Ceftazidime/Avibactam
FDC — Cefiderocol
ESBL — Extended Spectrum β -lactamase
EUCAST — European Committee on Antimicrobial Susceptibility Testing
ICU — Intensive Care Unit
IPM — Imipenem
KPC — *Klebsiella pneumoniae* Carbapenemase
LB — Luria–Bertani medium
MBL — Metallo- β -lactamase
MDR — Multidrug Resistant
MEM — Meropenem
MIC — Minimum Inhibitory Concentration
NGS — Next-Generation Sequencing
OXA — Oxacillinase
PBP — Penicillin-Binding Protein
PBP3 — Penicillin-Binding Protein 3
PCR — Polymerase Chain Reaction
SUL — Sulbactam
TSA — Tryptic Soy Agar
TSB — Tryptic Soy Broth
VABP — Ventilator-Associated Bacterial Pneumonia
WGS — Whole Genome Sequencing
WHO — World Health Organization

CHAPTER 1

“*Acinetobacter baumannii*: a Comprehensive Introduction to its Pathogenicity and Resistance”

1.1 Introduction: *Acinetobacter baumannii*

Acinetobacter spp. are glucose-non fermentative, non-motile, non-fastidious, catalase-positive, oxidative-negative, aerobic Gram-negative coccobacilli. There are more than 50 species within the *Acinetobacter* genus, but the majority are nonpathogenic environmental organisms¹. Among *Acinetobacter* species, *Acinetobacter baumannii* is the most important member associated with hospital-acquired infections. In particular, *A. baumannii* infections occur in critically ill patients in the Intensive Care Unit (ICU), where the mechanical ventilation systems, urinary catheterization and increasing antibacterial therapies have caused the spreading of this bacterium². Managing this organism represents a challenge, due to its extraordinary genomic plasticity, specifically in response to adverse environmental conditions, but also for its ability to develop antibiotic-resistance³.

The *World Health Organization* (WHO) indicated *A. baumannii* as one of the most serious ESKAPE pathogen (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa* and *Enterobacter species*) because of the increasing prevalence of carbapenem-resistant *A. baumannii* (CRAB): carbapenems are “last-line” antibiotics in clinical practice and the emergence of resistance to these antibiotics determined no therapeutical options. Carbapenems are ineffective against CRAB, leading to significantly higher mortality rates, indeed the last ECDC report underlined a concerning epidemiological situation regarding Multi-Drug Resistant (MDR) *A. baumannii*. Notably, in 2021 the percentages of carbapenem-resistant *Acinetobacter* spp. varied significantly across the European region. According to ECDC data, fewer than 1% of isolates were resistant in three countries (7% of the 45 countries reporting data). In contrast, a high level of resistance (more 50%) was observed in 25 countries (56%). These countries were predominantly located in Southern and Eastern Europe. This alarming distribution highlights substantial geographical disparities in resistance rates and underscore the persistent challenges in managing CRAB infections, especially in regions with high prevalence⁴ (Fig. 1).

In response to this situation, the Centers for Disease Control and Prevention (CDC) has classified MDR *A. baumannii* as a serious public health threat, prompting continuous surveillance and preventive measures. Additionally, WHO has designated CRAB as part of its

critical priority group of pathogens, highlighting the urgent need for research and development of new antimicrobial therapies.

The clinical manifestations of *A. baumannii* are nosocomial pneumoniae and bacteremia. In particular, nosocomial pneumoniae is caused by the endotracheal tube, which is an ideal adhesion surface for *Acinetobacter*, which could develop in severe ventilator-associated bacterial pneumoniae (VABP). Similarly, bloodstream infections start from central venous catheter or due to severe pneumonia. *A. baumannii* pathologies include also urinary tract infections, wound infections, endocarditis and meningitis⁵.

Furthermore, the incidence of community-acquired *A. baumannii* infections is increasing, specifically they occur in warm and humid environments, such as Australia, Oceania and Asia, including China, Taiwan and Thailand⁶.

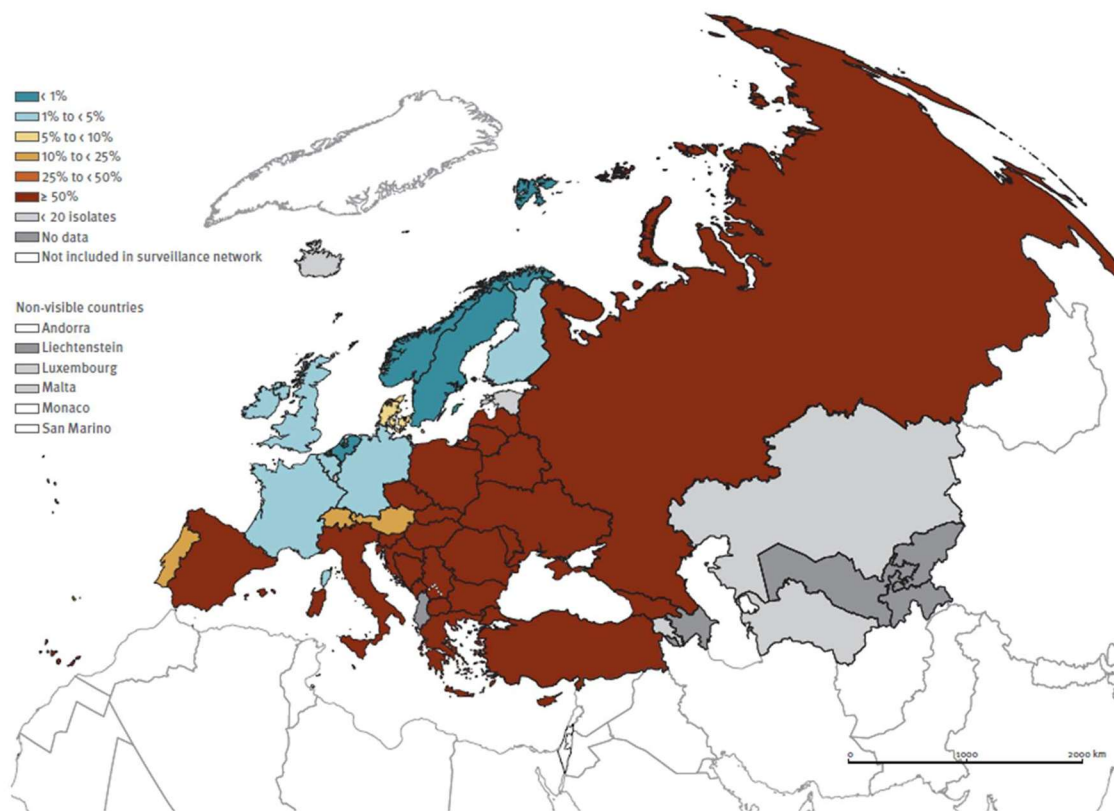


Figure 1. Global distribution of *Acinetobacter baumannii*⁴.

1.2 *A. baumannii* virulence factors

The persistence and dissemination of *A. baumannii* within healthcare settings are largely driven by its exceptional resilience to extreme environmental stressors through the expression of several types of **virulence factors**.

This pathogen is resistant to disinfection and desiccation thanks to its ability to grow in presence of ethanol and salt. The bacterial enzyme **RecA** induces bacterial DNA repair, causing resistance to desiccation and it also prevents *A. baumannii* killing inside macrophages. Period of desiccation and rehydration of this bacterium are linked to an increase of mutations, which activate RecA activity, but this condition leads also to the hypothesis that resistance to desiccation may contribute to the MDR phenotype. Under dry conditions, *A. baumannii* changes its morphology and the cell wall becomes thicker in order to allow persistence on solid surfaces. However, the mechanisms behind desiccation persistence were not investigated and remain to be fully characterized⁷.

Other virulence factors have been identified in *A. baumannii*, including biofilm adherence mechanisms, iron acquisition characteristics, activities of polysaccharide membrane and outer membrane vesicles (OMVs).

- **Biofilm formation**

Regarding biofilm formation - a bacterial community enclosed within an extracellular matrix- it is well established that biofilm plays a key role in the interactions between *A. baumannii* with its host, contributing significantly to medical device-associated infections. It is regarded that bacteria within biofilm communities have an increased tolerance to extracellular stresses. Of particular mention is the expression of **Outer Membrane Protein A (OmpA)**, which is a β -barrel porin and one of the most abundant porin in the outer membrane. *OmpA* is involved in biofilm development and it contributes to adhesion and invasion of host epithelial cells by interacting with fibronectin. It binds also to factor H in human serum which may allow to avoid complement-mediated killing. Furthermore, *OmpA* takes part in the extrusion of antibiotics from the periplasmic space through the outer membrane and couples with inner membrane efflux systems^{8,9}. The expression of **Csu pili** is essential for biofilm formation and maintenance on abiotic surfaces but are not involved in association with biotic surfaces such as human epithelial cells^{10,11,12}.

Concerning cell-to-cell adhesion, *A. baumannii* produces proteins associated with biofilms, such as **Bap** protein, which is secreted via the Type I Secretion System (T1SS) and it was originally identified in *Staphylococcus aureus*¹³.

- ***Glycoconjugates***

Bacterial **glycoconjugates**, including capsular polysaccharides, glycosylated proteins, lipooligosaccharides (LOS), and peptidoglycan, play a fundamental role in *A. baumannii* biology. These molecules mediate interactions with the environment, protect against stress, facilitate immune evasion and contribute to virulence.

Like *Neisseria* and *Campylobacter*, *A. baumannii* lacks an O-antigen, and its lipopolysaccharide is therefore referred to as lipooligosaccharide (LOS). LOS is a major component of the outer membrane leaflet and is essential for viability. Its **lipid A** anchor is the molecular target of colistin, a last-resort antibiotic used against CRAB. However, *A. baumannii* can resist colistin by modifying *lipid A*, most commonly through mutations in the *pmrAB* two-component system, which induce the addition of phosphoethanolamine¹⁴. Additional modifications include galactosamine incorporation and increased hepta-acylation of lipid A. In extreme cases, resistance involves complete LOS loss, a rare adaptation requiring compensatory changes, such as the overexpression of membrane-stabilizing lipoproteins and increased capsule production¹⁵.

The capsular polysaccharide is a major virulence factor, protecting the bacterium from complement-mediated killing. Its absence renders strains avirulent¹⁶. *A. baumannii* also possesses an O-linked protein glycosylation system, which, although not directly involved in immune evasion, is crucial for biofilm formation and virulence. Glycan and capsule biosynthesis share a common pathway beginning with the assembly of lipid-linked oligosaccharides in the cytoplasm, followed by their translocation to the periplasm and processing for capsule formation or protein modification¹⁷. Given the success of capsular polysaccharide-based vaccines in other pathogens, a similar approach was considered for *A. baumannii*. However, the high structural variability of its glycan, often involving rare or species-specific sugars, poses a significant challenge to vaccine development. Furthermore, capsule production can be enhanced by sub-inhibitory antibiotic exposure via the *BfmRS* regulatory system, potentially increasing bacterial virulence under selective pressure.

Despite these complexities, inhibitors targeting glycan biosynthesis represent a promising therapeutic avenue to counteract MDR *A. baumannii* infections¹⁸.

- *Metal acquisition system*

Infecting the human host requires a coordinated response from *A. baumannii*, which not only evades immune defenses— primarily through its capsular polysaccharide— but also adapts its metabolism and nutrient acquisition. Transition metals like iron, manganese, and zinc are essential for life, so hosts have developed complex mechanisms to limit their availability to pathogens, a process known as **nutritional immunity**. A major contributor to *A. baumannii* spreading as a nosocomial pathogen is its ability to scavenge these scarce nutrients in vivo. This is highlighted in studies using high-throughput transposon screens, which show that mutants with disruptions in iron and zinc import or utilization genes are significantly attenuated in virulence models¹⁹.

The primary strategy used by *A. baumannii* to acquire iron involves the secretion of high-affinity iron-chelating molecules known as **siderophores**. The most well-known siderophore produced by *A. baumannii* is **acinetobactin**, a catechol-hydroxamate compound²⁰. Depending on the extracellular pH, acinetobactin can exist in two different forms — oxazoline or isoxazolidinone — capable of binding iron. This structural flexibility allows acinetobactin to function under acidic conditions typically found at infection sites²¹. Acinetobactin is critical for virulence, making its biosynthetic pathway an attractive target for drug development²². Additional siderophores like **fimsbactin A–F** and **baumannoferrin A** and **B** are also used by *A. baumannii*, though the genetic and molecular mechanisms underlying their biosynthesis and transport are not yet fully understood²³.

Zinc, an essential cofactor for many bacterial proteins, is also critical for *A. baumannii* survival. To compete with host systems that sequester zinc — such as through the release of calprotectin — *A. baumannii* has developed strategies to overcome this limitation, including the production of its own zinc-binding proteins. Calprotectin levels remain elevated throughout *A. baumannii* infection in the lungs, intensifying the host's nutritional immunity²⁴. In response, *A. baumannii* utilizes a high-affinity zinc uptake system called **ZnuABC**, which is regulated by the zinc-responsive transcriptional repressor Zur. Under normal zinc conditions, Zur binds upstream of zinc-regulated genes and blocks their transcription. However, when zinc is scarce or calprotectin is present, intracellular zinc levels drop, Zur repression is lifted, and expression of zinc acquisition genes is activated²⁵. A second zinc uptake system, also Zur-regulated, depends on coordination between the metallochaperone ZigA and the histidine utilization (Hut) system,

which imports and releases zinc bound to histidine (His-Zn complexes). Inside the bacterial cell, these His-Zn complexes are converted to a bioavailable form through the combined action of ZigA and the enzyme HutH²⁶. Like siderophores, these zinc uptake systems are attractive targets for antimicrobial development due to their relevance in vivo and the absence of similar systems in eukaryotes.

Recently, it has become clear that micronutrient acquisition and metal metabolism are tightly linked to *A. baumannii* virulence. For example, *A. baumannii* counteracts host-imposed manganese restriction via a dedicated manganese import system and a subsequent urea metabolism pathway. Although the role of urea metabolism is not fully understood, it likely helps manage metabolic buildup under metal-limited conditions²⁷. These findings underscore the importance of micronutrient acquisition systems in *A. baumannii* pathogenesis and highlight them as promising targets for novel antimicrobial strategies.

- ***Outer Membrane Vesicles (OMVs)***

Outer membrane vesicles (OMVs) are spherical structures ranging from 20 to 200 nm in diameter, released from the outer membrane of various Gram-negative pathogenic bacteria²⁸. These vesicles consist of lipopolysaccharides (LPS), outer membrane and periplasmic proteins, phospholipids, and nucleic acids (DNA or RNA). OMVs are recognized as vehicles for delivering bacterial effectors to host cells²⁹. By enabling the simultaneous delivery of multiple virulence factors, OMVs facilitate host-pathogen interactions without requiring direct contact between the bacterial and host cell surfaces³⁰. In *A. baumannii*, many strains are known to secrete OMVs enriched with various virulence factors, including the OmpA, proteases, and phospholipases^{31,32}. These OMVs interact with host cells via lipid rafts and deliver their cargo, triggering cytotoxic effects³³. Purified OMVs from the *A. baumannii* ATCC 19606 strain have been shown to induce a dose-dependent expression of pro-inflammatory cytokine genes in epithelial cells³⁰. Notably, this pro-inflammatory response is significantly reduced when OMVs are treated with protease, suggesting that the membrane-associated proteins are primarily responsible for eliciting innate immune activation³⁰. Supporting this, a study demonstrated that an *A. baumannii* strain producing higher levels of OMVs enriched with virulence factors elicited a stronger innate immune response and showed greater cytotoxicity compared to a strain producing fewer OMVs³⁴.

Given their critical role in *A. baumannii* virulence, OMVs have been explored as potential components of acellular vaccines. Several studies have shown that *A. baumannii* OMVs can induce protective immunity. In a mouse model of systemic sepsis, immunization with OMVs derived from the ATCC 19606 strain conferred protection against both homologous and heterologous *A. baumannii* strains³⁵. Similarly, in a mouse pneumonia model, both active and passive immunization with OMVs significantly reduced bacterial load, inflammatory cell infiltration, and cytokine production³⁶. These findings support the potential use of *A. baumannii* OMVs as an effective acellular vaccine strategy. Interestingly, *A. baumannii* OMVs have also been implicated in the dissemination of antibiotic resistance. They have been shown to mediate horizontal gene transfer, including the spread of the carbapenemase gene *OXA-24*³⁷.

CHAPTER 2

*“Antibiotic Resistance Mechanisms in *A. baumannii*”*

2.1 Introduction

A. baumannii has emerged as a concerning pathogen in healthcare settings, largely due to its remarkable ability to acquire and develop multiple mechanisms of antibiotic resistance. This adaptability allows the organism to survive in hostile environments, persist on abiotic surfaces, and withstand treatment with a wide array of antimicrobial agents. Resistance in *A. baumannii* is multifactorial, involving enzymatic drug inactivation, alteration of target sites (mutation or expression of alternative *PBPs*), reduced membrane permeability, active drug efflux, and biofilm-associated tolerance³⁸. The interplay of these mechanisms not only complicates therapeutic management but also facilitates the rapid dissemination of resistant strains across healthcare networks. Understanding these processes at a molecular level is essential for developing novel treatment strategies and for curbing the spread of multidrug-resistant strains in clinical settings³⁹. Given its prominent role in resistance and its diverse molecular variants, enzymatic inactivation, particularly via β -lactamases, represents one of the most extensively studied and clinically relevant mechanisms in *A. baumannii*.

2.2 *B-lactamases*

The discovery of penicillin in 1929 is widely regarded as a milestone in medical history, and its clinical introduction in the 1940s revolutionized the treatment of bacterial infections. Despite remarkable progress in antimicrobial chemotherapy in the more than 70 years since penicillin was first used, β -lactams remain the cornerstone of the antibacterial armamentarium as the centenary of Fleming’s work approaches⁴⁰. They continue to represent both the most frequently prescribed antibiotic class and the leading one in global sales, underscoring their essential role in treating bacterial diseases. Since their initial introduction, β -lactam antibiotics — like other antimicrobial classes — have undergone continuous development to improve potency, broaden their spectrum of activity, optimize pharmacokinetic and safety profiles, and counter the emergence of resistance⁴¹. Currently, four major β -lactam classes are in clinical use (Fig. 2). Three feature a bicyclic structure: penicillins, in which the four-membered β -lactam ring is fused to a thiazolidine ring (1); cephalosporins, where it is fused to a six-membered dihydrothiazine (2); and carbapenems, characterized by fusion to a five-membered pyrroline

ring (3). The fourth class, monobactams, contains a monocyclic β -lactam structure (4). Although each class was originally discovered as a natural product, all have since undergone extensive structural modification, yielding numerous semi-synthetic derivatives⁴². The antibacterial activity of β -lactams was elucidated by Tipper and Strominger, who demonstrated that their structure mimics the terminal D-Ala–D-Ala moiety of the peptidoglycan stem pentapeptide. In this mimicry, the β -lactam amide and adjacent carboxylate (or sulfonic acid group in monobactams) emulate the peptide bond and terminal carboxylate. The β -lactam ring reacts with the nucleophilic serine residue in *Penicillin-Binding Proteins* (PBPs), leading to irreversible acylation and inhibition of the transpeptidation step of peptidoglycan synthesis⁴³.

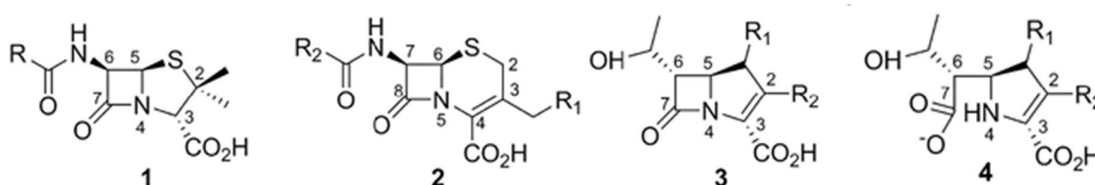


Figure 2. Chemical structures of β -lactam antibiotics: penicillins (1), cephalosporins (2), carbapenems (3), and monobactams (4)⁴⁴.

β -Lactamases can be classified using two principal systems: the Bush–Jacoby–Medeiros scheme, which focuses on functional and substrate-based characteristics⁴⁵, and the Ambler system, which is grounded in amino acid sequence analysis⁴⁶. According to the Ambler classification, β -lactamases are grouped into four categories— A, B, C, and D (Fig. 3) —that not only share distinct sequence signatures but also differ fundamentally in their catalytic mechanisms. A major conceptual division separates the serine β -lactamases (SBLs), comprising classes A, C, and D, from class B enzymes, which form the diverse family of metallo- β -lactamases (MBLs). SBLs contain a conserved *Ser–Xaa–Xaa–Lys* motif and catalyze hydrolysis through a mechanism involving a serine nucleophile and a covalent acyl-enzyme intermediate, reflecting their distant evolutionary relationship to PBPs⁴⁷. In contrast, MBLs rely on one or more zinc ions to activate a water molecule, which directly attacks the β -lactam ring, enabling substrate breakdown without formation of a covalent intermediate. Although the four Ambler classes are broadly distributed among environmental and clinically relevant bacteria, only a limited number of β -lactamase families have achieved widespread global dissemination. These highly successful enzymes are predominantly associated with Gram-negative pathogens responsible for opportunistic infections in hospitalized or immunocompromised patients, such as *Escherichia coli*, *Klebsiella pneumoniae*, and non-fermenting organisms including *Pseudomonas aeruginosa* and *A. baumannii*.

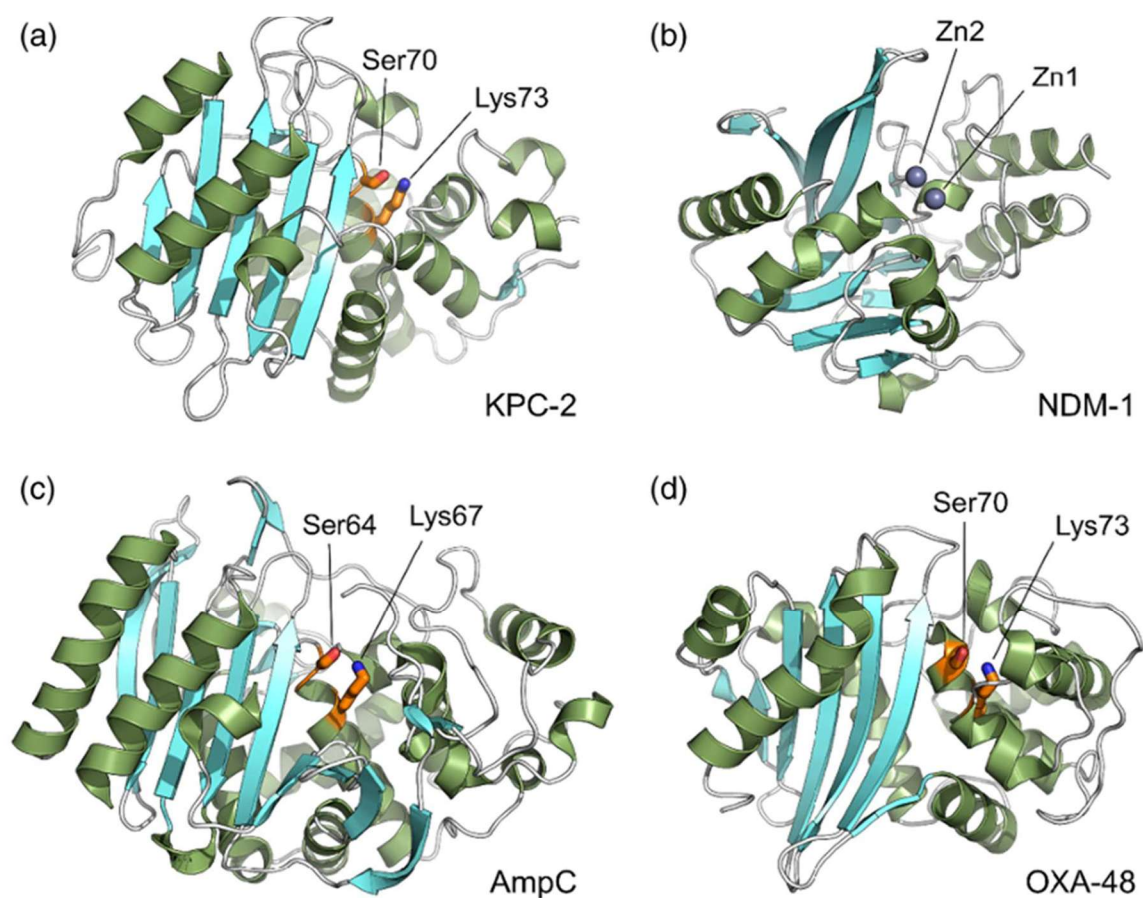


Figure 3. Representative β -lactamases according to the Ambler classification: KPC-2 (a), NDM-1 (b), AmpC (c), and OXA-48 (d)⁴⁴.

Despite considerable sequence diversity among the three serine β -lactamase (SBL) classes, they all share a common catalytic strategy based on an *acylation–deacylation cycle*, similar to that described for serine proteases. In this mechanism, an active-site serine residue is activated by a general base and performs a nucleophilic attack on the carbonyl carbon of the β -lactam amide bond. This step leads to the formation of a tetrahedral oxyanion intermediate, which subsequently collapses to yield a covalent acyl–enzyme species (Fig. 4). The cycle is completed when a water molecule—referred to as the deacylating water—is activated by a general base and hydrolyzes the acyl–enzyme intermediate, releasing the inactivated antibiotic. Although this overarching mechanism is conserved, the Ambler classes differ critically in the identity and functional roles of the residues involved in general base activation, as well as in the detailed interactions that stabilize the reaction intermediates. These distinctions, particularly in classes A and C, have been the subject of ongoing debate and investigation within the field⁴⁴.

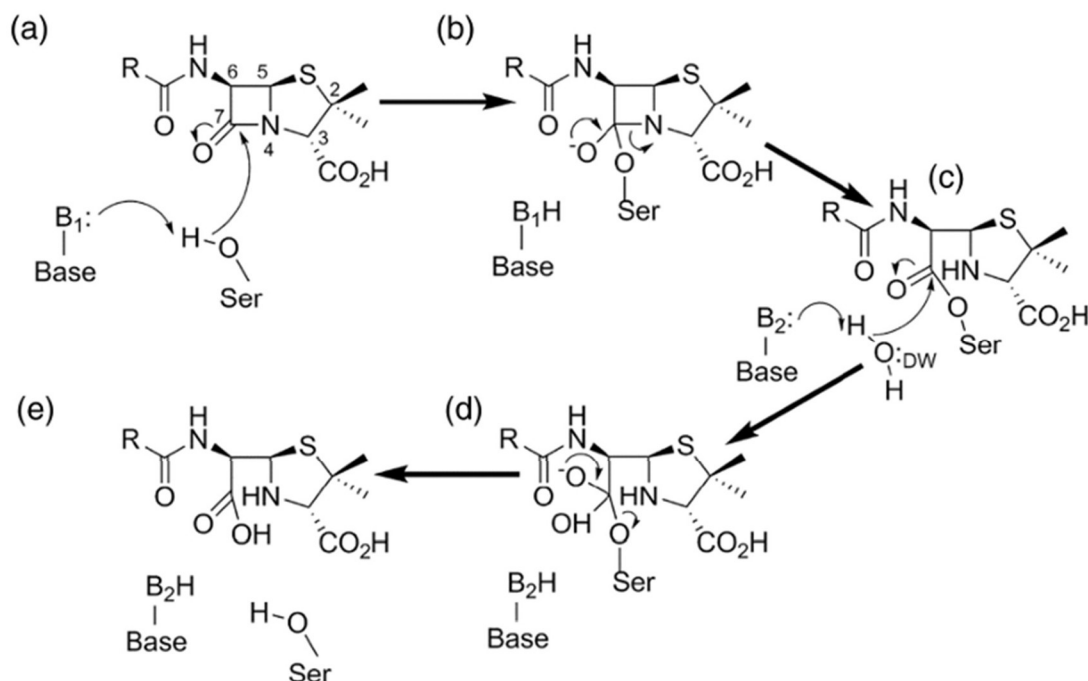


Figure 4. Catalytic mechanism of β -lactamases illustrating the hydrolysis of the β -lactam ring, leading to antibiotic inactivation. Key steps include (A) substrate binding, (B) nucleophilic attack on the β -lactam carbonyl, (C) formation of the acyl-enzyme intermediate, and (D) hydrolysis and (E) release of the inactivated antibiotic⁴⁴.

In this work, particular attention will be devoted to the hydrolytic mechanisms of class C and class D β -lactamases, as these two groups represent the most prevalent and clinically relevant resistance determinants identified in the *A. baumannii* strains analyzed in this study. Their functional characteristics and evolutionary success will be discussed in detail in the following sections.

2.2.1 Class C β -lactamases

Class C β -lactamases are broadly distributed among Gram-negative bacteria, where they are predominantly located on the chromosome. Historically, the class C enzyme from *Escherichia coli* holds particular significance, as it was the first β -lactamase ever described and played a central role in shaping early research in the field. In many clinically relevant opportunistic pathogens, class C enzymes — commonly referred to as *ampC* — are encoded chromosomally but remain largely unexpressed under basal conditions. However, their expression can be strongly upregulated through mutational events or by induction triggered by specific β -lactam antibiotics, resulting in markedly increased production of the enzyme and a corresponding rise in MIC values against otherwise susceptible compounds. Regarding the hydrolytic mechanism of class C β -lactamases, class C β -lactamases catalyze the hydrolysis of β -lactam antibiotics

through a two-step acylation–deacylation process⁴⁸. The reaction begins with the nucleophilic attack of **Ser64**, located in the conserved **SXXK motif**, on the carbonyl carbon of the β -lactam ring, forming a tetrahedral intermediate. Collapse of this intermediate opens the β -lactam ring and generates a covalent acyl–enzyme complex. **Lys67** plays a critical role in this step by acting as a general base, facilitating activation of the serine nucleophile. **Tyr150**, a conserved residue in class C enzymes, assists indirectly by donating a proton to the nitrogen of the β -lactam ring, promoting cleavage of the amide bond. During the **deacylation step**, a water molecule — positioned within the active site — attacks the acyl–enzyme carbonyl, regenerating the free enzyme and releasing the inactivated antibiotic. This water approaches from the β -face of the carbonyl, which is a distinctive feature of class C enzymes compared to class A. Proton transfers between Lys67, Tyr150, and the deacylating water coordinate the hydrolysis, with Tyr150 remaining largely protonated throughout the cycle. Depending on the substrate, alternative routes may also contribute, but the central mechanism involves Lys67-mediated activation of serine for acylation and orchestrated proton transfers during deacylation. Class C β -lactamases are generally not efficient carbapenemases, and their contribution to carbapenem resistance typically arises when overexpressed enzymes are combined with reduced outer-membrane permeability, allowing carbapenems to be sequestered before reaching PBPs. Normally, the orientation of carbapenems within the active site of class C enzymes is unfavorable for rapid deacylation, which limits turnover. However, certain class C variants, such as CMY-10 and ADC-68, have acquired the ability to hydrolyze carbapenems in addition to extended-spectrum cephalosporins. Structural modifications, including deletions or substitutions in key loops of the active site, expand the substrate-binding cavity and reduce steric hindrance, facilitating accommodation of the bulky carbapenem side chains. These changes may also enhance deacylation by allowing the catalytic water molecule better access to the acyl–enzyme intermediate, although the exact mechanism remains under investigation^{49,50,51}.

2.2.1.1 ADC β -lactamases

The intrinsic β -lactamase repertoire of *A. baumannii* includes multiple enzyme families that contribute to its remarkable resistance to β -lactam antibiotics, among which chromosomally encoded *Acinetobacter-derived cephalosporinases (ADC)*, belonging to Ambler class C β -lactamases, play a central role. These enzymes hydrolyze the β -lactam ring through a serine-based catalytic mechanism, leading to inactivation of penicillins and cephalosporins and

clinical treatment failure when expressed at sufficient levels⁵². Large-scale genomic analyses have revealed extensive diversity within the ADC family; in a dataset of over 28,000 *A. baumannii* isolates, ADC genes accounted for more than 28,900 β -lactamase sequences, forming one of the dominant β -lactamase families alongside intrinsic OXA variants, with 203 assigned alleles and hundreds of additional unassigned variants, highlighting an underappreciated reservoir of genetic diversity. Among the assigned alleles, *bla*_{ADC-73} and *bla*_{ADC-30} were most prevalent globally, accounting for approximately 28 % and 25 % of isolates, respectively, while *bla*_{ADC-222} and *bla*_{ADC-33} appeared at lower but significant frequencies, reflecting dynamic geographic and temporal distributions influenced by clonal expansion and selective pressures⁵³. ADC genes are typically chromosomal, distinguishing them from plasmid-borne β -lactamases, and their expression is generally low; however, insertion sequences such as ISAbal upstream of ADC genes can enhance transcription, resulting in increased enzyme production and elevated β -lactam MICs⁵⁴. In outbreak settings, this mechanism often co-occurs with other resistance determinants, reflecting the genomic plasticity that underpins multidrug resistance⁵³. Among ADC variants, ADC-25 has emerged as a prevalent and clinically relevant allele in multiple geographic regions. **Whole-genome sequencing** (WGS) of clinical isolates from China showed universal presence of *bla*_{ADC-25}, which resided on chromosomal contigs and was associated with resistance to monobactams such as aztreonam⁵⁵. Similarly, Egyptian surveillance studies reported 100 % prevalence of *bla*_{ADC-25} in clinical isolates, frequently co-existing with OXA-23, OXA-66, and class A β -lactamases⁵⁶. Environmental and nosocomial studies from the Middle East further confirm the widespread detection of ADC-25 in both clinical and environmental reservoirs, often alongside other resistance determinants⁵⁷. ADC enzymes exhibit a characteristic spectrum of hydrolytic activity that includes penicillins such as Ampicillin (AMP) and piperacillin, narrow-spectrum cephalosporins like cephaloridine and cephalothin, and third-generation cephalosporins such as ceftazidime and cefotaxime, with variable activity against monobactams like aztreonam depending on the allele, while extended-spectrum cephalosporins such as cefepime are generally poorly hydrolyzed except in extended-spectrum variants⁵⁸. Among the most frequent ADC variants, ADC-30 contributes to third-generation cephalosporin resistance and can reduce susceptibility to Sulbactam when overexpressed, whereas ADC-33 and ADC-56 demonstrate broader hydrolytic activity including cefepime and aztreonam due to structural modifications in the Ω -loop and active site⁵⁹. ADC-25, although less extensively biochemically characterized, is expected to efficiently hydrolyze penicillins, early cephalosporins, and third-generation

cephalosporins but likely exhibits limited intrinsic activity against fourth-generation cephalosporins or monobactams beyond baseline ADC activity, with potential modulation if overexpressed or in the presence of regulatory elements such as *ISAbal1*. In summary, ADC β -lactamases constitute a diverse and globally distributed intrinsic resistance mechanism in *A. baumannii*, with allelic variants such as ADC-73 and ADC-30 dominating surveillance datasets, while ADC-25 consistently appears across multiple regions, often as part of multidrug resistance profiles, highlighting the importance of integrating biochemical characterization with genomic epidemiology to predict β -lactam susceptibility and guide clinical therapy^{55,56,60}.

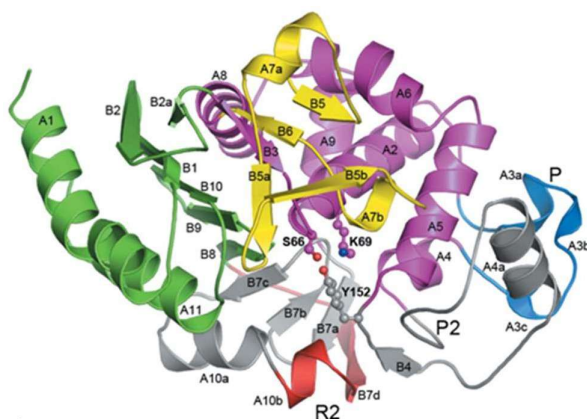


Figure 5. Crystal structure of ADC-1 β -lactamase showing the enzyme fold and active site residues involved in β -lactam hydrolysis⁶¹.

2.2.2 Class D β -lactamases

Class D β -lactamases rely on a **carboxylated lysine residue** in the active site, which acts as the **general base** for both **acylation** and **deacylation**. This lysine undergoes reversible carboxylation by atmospheric CO₂, and its carboxylate form is essential to activate the catalytic serine. During **acylation**, the carboxylated lysine deprotonates the nucleophilic Ser-OH, enabling nucleophilic attack on the β -lactam carbonyl and formation of the acyl-enzyme intermediate. In the **deacylation** step, the same lysine carboxylate activates the deacylating water molecule, promoting hydrolysis of the acyl-enzyme bond and release of the inactivated antibiotic. The relatively hydrophobic environment of the OXA active site lowers the pK_a of the conserved lysine, favoring its carboxylation and thereby sustaining catalytic turnover. Although mutation of this lysine severely impairs catalysis, the modest residual acylation observed in some variants suggests that additional active-site features also contribute to the activation of the nucleophilic serine⁴⁴. Recent research has increasingly focused on the

interactions between OXA enzymes and carbapenems, an area that continues to evolve rapidly. Within the broader OXA family, five distinct clades are generally recognized as capable of hydrolyzing carbapenems. However, some authors have proposed—largely based on the ability of these enzymes to raise carbapenem MICs in organisms with reduced permeability, such as *Acinetobacter*—that all OXA enzymes could be considered carbapenemases. Among the five established OXA carbapenemase groups, four (**OXA-23**, **OXA-24/40**, **OXA-51**, and **OXA-58**) are predominantly associated with *A. baumannii*. The **OXA-51** group consists mainly of intrinsic, chromosomally encoded enzymes that can confer carbapenem resistance when overexpressed, for instance due to promoter insertions, while the remaining groups are more commonly plasmid borne. In contrast, **OXA-48-like enzymes** are primarily found on plasmids within Enterobacteriaceae, where their rising prevalence, contribution to carbapenem treatment failures, and notoriously challenging detection have made them an increasingly significant concern for clinical microbiology⁶².

2.2.2.1 OXA β -lactamases

The **OXA β -lactamases** were among the earliest β -lactamase families to be identified. Initially, class D β -lactamases were relatively uncommon, strictly plasmid-encoded, and displayed a narrow substrate profile largely restricted to penicillins, although some variants gradually acquired the capacity to degrade cephalosporins. Beginning in the 1980s, CRAB isolates emerged, carrying plasmid-borne β -lactamases—later designated OXA-23, OXA-40, and OXA-58—classified as OXA enzymes based on their sequence relatedness to earlier oxacillinases. It soon became evident that all *A. baumannii* strains possess a chromosomally encoded OXA-type enzyme (OXA-51-like). While most of these intrinsic enzymes do not inherently confer carbapenem resistance, certain genetic contexts—such as insertion elements activating their promoters—can drive expression to clinically significant levels. Closely related *Acinetobacter* species also harbor chromosomal OXA β -lactamases, some of which can be mobilized into *A. baumannii* and contribute to transferable carbapenem resistance. In some instances, carbapenem-hydrolyzing OXA enzymes, particularly OXA-48, have disseminated into Enterobacteriaceae, becoming an increasingly important cause of carbapenem resistance. Overall, the rise of OXA enzymes capable of degrading carbapenems—especially within *A. baumannii*—has transformed this class from a relatively minor concern into a major clinical challenge that threatens the therapeutic utility of carbapenems⁶². Hedges and colleagues first showed that plasmid-encoded β -lactamases in Gram-negative bacteria could be divided into

two major categories: the widespread TEM enzymes and a smaller group distinguished by their ability to hydrolyze oxacillin. Unlike TEM enzymes, this second group exhibited heterogeneous substrate profiles and was associated with a more limited range of plasmid backbones⁶³. These enzymes resembled a β -lactamase originally identified on plasmid R1818 (later renamed R46) by Datta and Kontomichalou. Compared with TEM enzymes, this group displayed lower specific activity toward penicillin but markedly higher activity against oxacillin and methicillin. Their emergence likely paralleled the clinical adoption of flucloxacillin and methicillin to treat staphylococcal infections. They were less efficient against first-generation cephalosporins and showed poor inhibition by classic class A β -lactamase inhibitors such as clavulanic acid⁶⁴. Initially, related β -lactamases were differentiated by their isoelectric points. Sykes and Matthew identified three oxacillinases — OXA-1, OXA-2, and OXA-3⁶⁵. The first two exhibited a characteristic feature seen in most members of this class: inhibition by chloride ions⁶⁶. The first sequenced gene from this group corresponded to *bla*_{OXA-2} carried on plasmid R46^{67,68}. The *bla*_{OXA-1} gene, later sequenced from a Tn21-derived transposon on plasmid RGN238, shared only ~48% sequence identity with *bla*_{OXA-2} but exhibited conserved motifs around the catalytic serine and a leucine at position 179—two hallmark residues now recognized as defining motifs of OXA enzymes. Notably, both *bla*_{OXA-1} and *bla*_{OXA-2} were found integrated into the same region of the Tn21 transposon, suggesting strong evolutionary pressure not only to maintain these genes but to preserve their association with this specific mobile element. Subsequent OXA variants were assigned numerical designations based on chronological discovery, later confirmed by unique amino acid sequences curated by the Lahey Clinic, although numbering does not reflect phylogenetic relatedness⁶⁹. A new set of plasmid-borne β -lactamases was later identified in *Pseudomonas aeruginosa*. These enzymes were initially termed PSE (Pseudomonas-specific enzymes) because they were thought to be restricted to this species. Four main variants—PSE-1 through PSE-4—were described, and their genes could be transferred to *Escherichia coli* both experimentally and in clinical isolates⁷⁰. The assumption of species specificity was ultimately incorrect, and sequencing revealed that the PSE enzymes were not a homogeneous family. All were active against the anti-pseudomonal penicillin carbenicillin; however, one enzyme, PSE-2, also hydrolyzed oxacillin. Sequence comparison showed that PSE-2 was closely related to the OXA β -lactamases—sharing only ~36% identity with OXA-2 but exhibiting stronger similarity to OXA-5. The pronounced diversity among these enzymes suggested ancient divergence from a common ancestral gene. Due to its strong structural alignment with the OXA family, PSE-2 was later redesignated OXA-10⁷¹.

2.2.2.2 OXA Extended-Spectrum β -Lactamases (ESBLs)

Historically, class D OXA enzymes played a secondary role compared with the more prevalent class A β -lactamases, especially in mediating penicillin resistance. Some OXA variants had initially been described in *P. aeruginosa*. During the 1980s, this organism remained susceptible to the third-generation cephalosporin ceftazidime, until a multidrug-resistant isolate from a Turkish patient in 1991 demonstrated transferable ceftazidime resistance⁷². Sequencing showed that this enzyme carried two mutations relative to OXA-10—Arg143→Ser and Gly157→Asp. While OXA-10 exhibited weak hydrolysis of cefotaxime, ceftriaxone, and aztreonam, these mutations significantly increased ceftazidime binding, and the resulting variant—OXA-11—became the first example of an OXA enzyme that had evolved into an extended-spectrum β -lactamase (ESBL)⁷³. Several additional ESBLs have since emerged from the OXA-10 lineage, including OXA-13, OXA-14, OXA-16, OXA-17, OXA-19, and OXA-28—almost all identified in *P. aeruginosa*. These enzymes carry up to nine amino acid substitutions relative to OXA-10, though the functional consequences of these mutations remain largely uncharacterized. Notably, the Gly157→Asp substitution first seen in OXA-11 also appears in four other OXA-10-derived ESBLs. OXA-14 contains this single mutation, strongly implying that it is the critical determinant of high-level ceftazidime resistance. Although the mechanistic basis is unclear, the mutation affects kinetic behavior and appears to influence enzyme dimerization rather than directly altering the active site, which may enhance cephalosporin binding^{74,75,76,77,78,79}. Other OXA-10-derived ESBLs harbor additional mutations—such as Asn143→Ser in OXA-11 or Ala127→Thr in OXA-16—yet these changes are absent from more recent ESBL variants, suggesting they are not essential for cephalosporin resistance. OXA-19, with nine substitutions relative to OXA-10, appears to have spawned several related ESBLs (OXA-28, OXA-35, OXA-145, OXA-147), indicating that this cluster has been particularly successful evolutionarily. In contrast, OXA-17 carries a unique substitution (Asn73→Ser) near the active site, conferring resistance to cefotaxime and ceftriaxone but little activity against ceftazidime⁷⁶. The OXA-2-like ESBLs are more heterogeneous. Six have been described, each defined by a single amino acid substitution relative to OXA-2. In two cases—OXA-15 and OXA-36—the mutation affects the same position (Asp149), though the substituted residues differ (Gly in OXA-15, Tyr in OXA-36). This pattern suggests that the loss of Asp149, rather than the identity of the replacing residue, is the key factor enabling cephalosporin hydrolysis⁸⁰. Despite their functional relevance in *P. aeruginosa*, OXA-derived ESBLs have rarely been found in other species and do not appear to

be widely disseminated. One exception, OXA-21, was identified in *A. baumannii*, marking the first report of an OXA β -lactamase in this organism. Overall, the clinical impact of OXA-type ESBLs remains uncertain due to limited epidemiological data, and their relevance is modest when compared with globally dominant CTX-M ESBLs⁷³.

2.2.2.3 OXA-23 and OXA-23-LIKE

The **OXA-23** group represents the earliest cluster of carbapenem-resistant OXA-type β -lactamases identified in *A. baumannii*. The prototype enzyme, OXA-23, was first detected in an *A. baumannii* isolate recovered in Edinburgh, UK, in 1985—the same year imipenem (IPM) entered clinical use. This isolate exhibited IPM MICs of 16 mg L⁻¹, and the resistance phenotype was transferable, indicating that the gene resided on a plasmid. The sequence of *bla*_{OXA-23} was later published in 2000, and since then an additional 18 allelic variants have been reported (Table 1)⁸¹. Genes belonging to this group are frequently plasmid-encoded and have been identified not only in multiple *Acinetobacter* species but also among members of the Enterobacteriaceae. The detection of several *bla*_{OXA-23-like} genes (*bla*_{OXA-23}, *bla*_{OXA-102}, *bla*_{OXA-103}, *bla*_{OXA-105}, *bla*_{OXA-133}, and *bla*_{OXA-134}) on the chromosome of *Acinetobacter radioresistens* strongly suggests that this species represents the natural reservoir of the OXA-23 lineage⁸². **Kinetic analyses** have so far been conducted for three members of this family—OXA-23, OXA-27, and OXA-146. Kinetic parameters vary widely, even for the same enzyme, complicating direct comparisons across studies. Nevertheless, these enzymes consistently display hydrolytic activity not only toward carbapenems but also against oxyiminocephalosporins, aminopenicillins, piperacillin, oxacillin, and aztreonam. Within the carbapenem class, OXA-23 shows a markedly higher turnover rate for IPM than for meropenem (MEM), ertapenem, or doripenem⁸³. Despite the relatively modest catalytic efficiencies reported for carbapenem hydrolysis, these enzymes appear to compensate through the presence of a “hydrophobic bridge” spanning the top of the active site, formed by Phe110 and Met221. A similar structural feature in OXA-40 has been shown to promote tight carbapenem binding. More recently, OXA-146 was shown to possess an expanded substrate profile that includes ceftazidime—an activity absent in other class D OXA-type carbapenemases—while retaining carbapenem hydrolysis. This finding marks the first example of an OXA carbapenemase exhibiting ESBL-like properties, raising concerns for future clinical impact. The broadened spectrum of OXA-146 results from the duplication of an alanine residue at position 220 within the β 5– β 6 loop. This duplication shifts Met221 away from its position

in the hydrophobic bridge, eliminating steric hindrance with ceftazidime and aztreonam⁸⁴. Expression of OXA-23 in an *A. baumannii* strain is sufficient to confer carbapenem resistance. Some studies reported that introducing a cloned *bla*_{OXA-23} gene on a low-copy plasmid into the susceptible type strain CIP 70.10 raises carbapenem MICs to 16 µg mL⁻¹, meeting the resistance breakpoint. When *bla*_{OXA-23} is expressed in a background that also harbors the AdeABC efflux pump, MICs further increase to ≥32 µg mL⁻¹. This demonstrates that, unlike some other OXA carbapenemases, OXA-23 is capable of conferring resistance, although additional mechanisms can amplify resistance levels substantially⁸⁵.

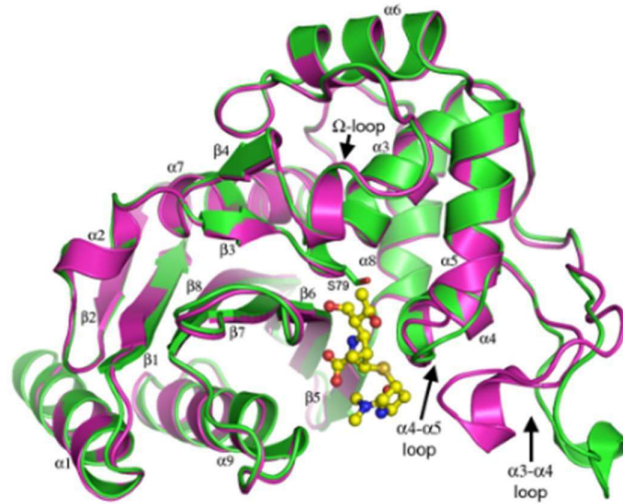


Fig.6. Crystal structure of the OXA-23 β-lactamase in complex with meropenem, showing the enzyme fold, the active site, and key residues

Enzyme group	Enzyme(s)	No. of enzymes in group	Location(s)	Host species
OXA-23-like	OXA-23, OXA-27, OXA-49, OXA-73, OXA-102, OXA-103, OXA-105, OXA-133, OXA-134, OXA-146, OXA-165–OXA-171, OXA-225, OXA-239	19	C and P	<i>A. baumannii</i> , <i>A. junii</i> , <i>A. radioresistens</i> , <i>A. pittii</i> , <i>Proteus mirabilis</i> , <i>Acinetobacter phenon</i> 5, <i>Acinetobacter phenon</i> 6/ct 13TU, <i>A. nosocomialis</i> , <i>Acinetobacter</i> genomic species 10/11, <i>A. lwoffii</i> , <i>Klebsiella pneumoniae</i> , <i>A. baylyi</i>
OXA-40-like	OXA-40, OXA-25, OXA-26, OXA-72, OXA-139, OXA-160, OXA-207	7	C and P	<i>A. baumannii</i> , <i>A. haemolyticus</i> , <i>A. pittii</i> , <i>A. baylyi</i> , <i>Pseudomonas aeruginosa</i> , <i>A. calcoaceticus</i> , <i>K. pneumoniae</i>
OXA-51-like	OXA-51, OXA-64–OXA-71, OXA-75–OXA-80, OXA-82–OXA-84, OXA-86–OXA-95, OXA-98–OXA-100, OXA-104, OXA-106–OXA-113, OXA-115–OXA-117, OXA-120–OXA-128, OXA-130–OXA-132, OXA-138, OXA-144, OXA-148–OXA-150, OXA-172–OXA-180, OXA-194–OXA-197, OXA-200–OXA-203, OXA-206, OXA-208, OXA-216, OXA-217, OXA-219, OXA-223, OXA-241, OXA-242, OXA-248–OXA-250, OXA-254	95	C and P	<i>A. baumannii</i> , <i>A. nosocomialis</i> , <i>Enterobacter cloacae</i> , <i>Escherichia coli</i> , <i>K. pneumoniae</i>
OXA-58-like	OXA-58, OXA-96, OXA-97, OXA-164	4	C and P	<i>A. baumannii</i> , <i>A. pittii</i> , <i>A. nosocomialis</i> , <i>Acinetobacter phenon</i> 6/ct 13TU, <i>A. junii</i> , <i>Acinetobacter</i> genomic species 9, <i>A. bereziniae</i> , <i>A. calcoaceticus</i> , <i>A. radioresistens</i> , <i>E. cloacae</i> , <i>Comamonas testisteroni</i> , <i>E. coli</i> , <i>K. pneumoniae</i> , <i>Delftia acidovorans</i>
OXA-134a-like	OXA-134a, OXA-186–OXA-191	7	C	<i>A. lwoffii</i>
OXA-143-like	OXA-143, OXA-182, OXA-231, OXA-253, OXA-255	5	P	<i>A. baumannii</i> , <i>A. pittii</i>
OXA-213	OXA-213	17	C	<i>A. calcoaceticus</i>
OXA-214-like	OXA-214, OXA-215	5	C	<i>A. haemolyticus</i>
OXA-211-like	OXA-211, OXA-212, OXA-309	6	C	<i>A. johnsonii</i>
OXA-229-like	OXA-228–OXA-230, OXA-257	8	C	<i>A. bereziniae</i>
OXA-235-like	OXA-235–OXA-237, OXA-278	7	C	<i>A. schindleri</i>
OXA-48-like	OXA-48, OXA-48b, OXA-162, OXA-163, OXA-181, OXA-199, OXA-204, OXA-232, OXA-244, OXA-245, OXA-247	11	C and P	<i>E. cloacae</i> , <i>K. pneumoniae</i> , <i>E. coli</i> , <i>Shewanella xiamenensis</i> , <i>Citrobacter freundii</i> , <i>Serratia marcescens</i> , <i>Providencia rettgeri</i> , <i>Klebsiella oxytoca</i> , <i>Enterobacter sakazakii</i> , <i>A. baumannii</i>

Table 1. Carbapenem-hydrolyzing OXA-type carbapenemases⁸⁷

2.2.2.4 OXA-51 and OXA-51 like

The *OXA-51-like β -lactamases* represent the largest and most diverse collection of OXA enzymes described so far. The prototype enzyme, OXA-51, was first identified in *A. baumannii* isolates recovered in Argentina in 1996. These enzymes are intrinsic to *A. baumannii*, encoded on its chromosome, and this intrinsic origin has likely driven the remarkable diversification of the group, which now includes 95 known variants. The sheer number of alleles strongly suggests that these chromosomal enzymes have been exposed to substantial antibiotic-driven selective pressure and that they actively participate in resistance rather than being harmless background elements. Among this large family, only OXA-51 and OXA-69 have been investigated kinetically⁸⁸. Although they display different affinities for IPM and MEM, both exhibit only weak catalytic activity toward carbapenems in vitro. Despite this modest hydrolysis, in vivo expression of OXA-51-like enzymes can markedly elevate carbapenem MICs. As demonstrated experimentally, expression of these enzymes in carbapenem-susceptible *A. baumannii* or *Acinetobacter nosocomialis* strains can raise MICs to 32 mg/L—comparable to the resistance levels produced by acquired OXA carbapenemases. Whether all members of the OXA-51-like family can confer carbapenem resistance remains to be clarified, but the possibility that every *A. baumannii* isolate carries the genetic potential for carbapenem resistance remains a major clinical concern. Although no crystal structure is currently available for an OXA-51-like enzyme, structural modeling and sequence-based approaches have helped pinpoint features relevant to carbapenem interaction. Docking simulations suggest that IPM interacts least strongly with modeled OXA-51 structures, whereas ceftazidime appears to bind most favorably—though this clashes with enzymatic assays showing no detectable ceftazidime hydrolysis. Additional modeling work indicates that hydrolysis of oxacillin is energetically more favorable than hydrolysis of IPM and that IPM induces a larger conformational rearrangement in the enzyme's tertiary structure, consistent with earlier kinetic observations. Comparative modeling further predicts that OXA-51-like enzymes lack the hydrophobic bridge spanning the active-site cleft that characterizes OXA-23, OXA-40, and OXA-58⁸⁴. Amino acid substitutions predicted to lie within the active-site cleft may alter its geometry and, consequently, substrate binding and hydrolysis profiles, implying functional variability across the OXA-51-like family^{88,89}. Sequence analysis of *bla*_{OXA-51-like} alleles provides striking evidence of positive selection acting on specific regions of the gene. Elevated N/S ratio (*indicative of selective pressure favoring amino acid-altering mutation*) are observed in three major regions:

- residues 64–97, surrounding the active-site serine
- residues 144–160, corresponding to the start of the Ω -loop,
- residues 180–200, overlapping regions implicated in dimer formation in OXA-13.

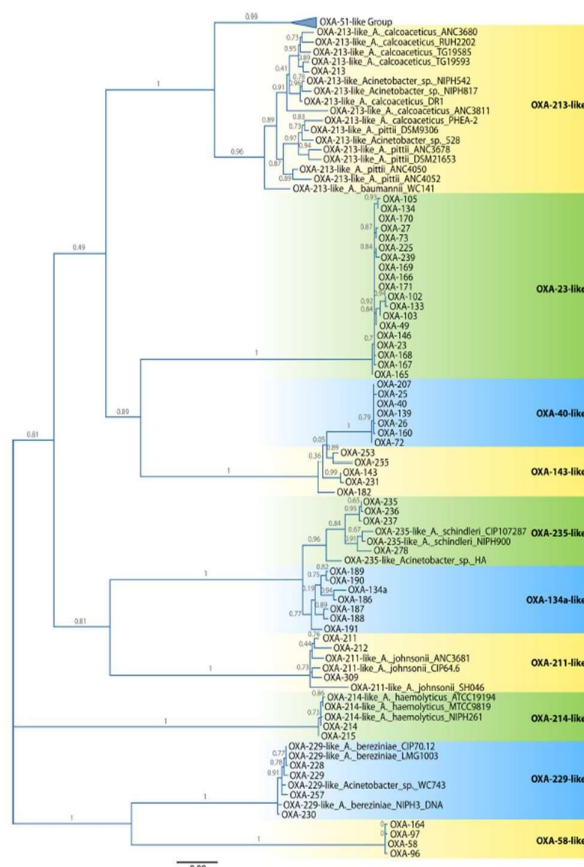


Table 2. Maximum likelihood amino acid tree of the OXA-type β -lactamases found in *Acinetobacter* species⁸⁷.

Additional segments of the gene contain exclusively nonsynonymous polymorphisms, reinforcing the idea that these positions are evolving under selective pressure. These include nearly the entire Ω -loop (positions 147–167) and residues 214–237, which encompass amino acids homologous to functionally critical residues in OXA-40—among them position 222, associated with carbapenem specificity and forming half of the hydrophobic bridge in OXA-40⁹⁰. Phylogenetic analysis of amino acid sequences further supports the presence of selection. Six amino acid positions appear as polymorphic in multiple phylogenetic clades, suggesting repeated, independent mutations at positions likely to influence enzyme behavior. Five of these lie in functionally relevant regions (Ω -loop residues 146 and 167; position 194 in the putative

dimer interface; positions 222 and 225 in the carbapenem-interaction region). The remaining polymorphic position, residue 36, has no known functional role in other OXA enzymes but appears within a genomic region displaying high N/S ratios, warranting further investigation. Together, these findings indicate that OXA-51-like enzymes are not functionally uniform. Instead, significant diversity likely exists in their catalytic profiles, and deeper biochemical characterization of individual variants is essential to understand their contributions to β -lactam and carbapenem resistance⁹¹.

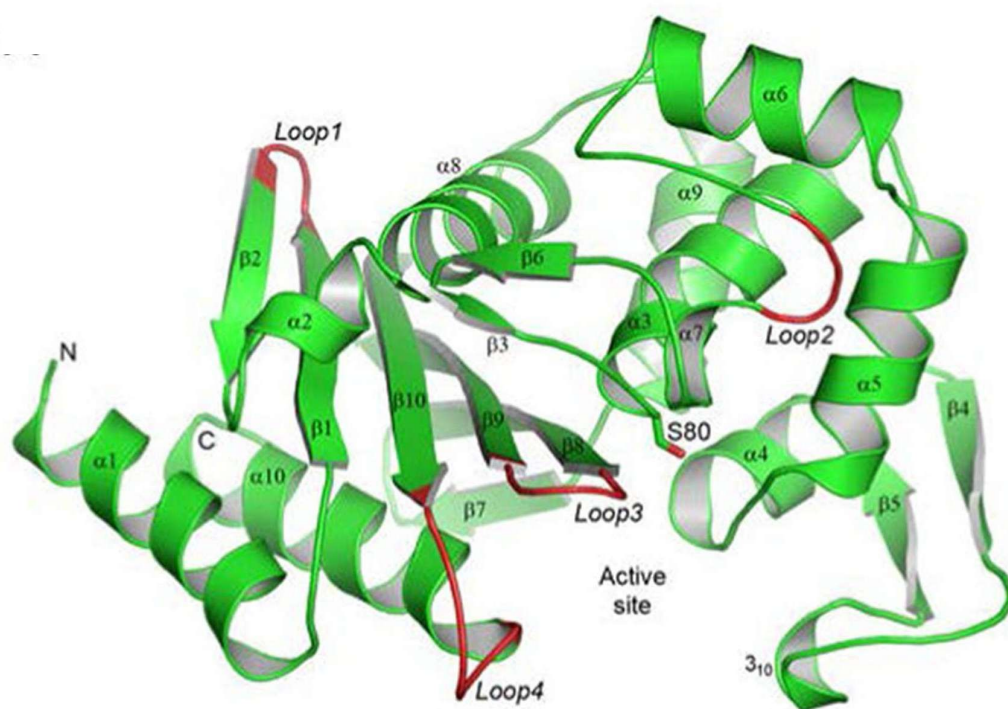


Fig.7. Three-dimensional structure of the OXA-51 β -lactamase, highlighting the overall protein fold and the active site residues involved in carbapenem hydrolysis⁹²

2.2.3 Genetic Location, Mobilization, and Regulation

- **ISAbal**

Insertion sequences (ISs) play a central role in both the mobilization and the regulation of OXA-type β -lactamase genes, particularly within *Acinetobacter* spp., where IS elements are frequently found adjacent to these resistance determinants. Among them, **ISAbal** is by far the most widespread. Although sequences related to ISAbal had been detected earlier, the element was formally described in *A. baumannii* in 2001⁹³. Its initial discovery upstream of *bla_{ADC}* revealed that ISAbal can act as a potent promoter, driving overexpression of *bla_{ADC}* and conferring resistance to cephalosporins. The transposase encoded by ISAbal is unusual, it is generated from two overlapping ORFs, requiring a programmed ribosomal frameshift for the production of the functional protein. Transposition frequencies measured in *E. coli*—around 2×10^{-7} transconjugants per recipient, increasing to $\sim 10^{-4}$ when a frameshift mutation was introduced—suggest that conditions favoring frameshift events greatly enhance ISAbal mobility. Indeed, exposure to the frameshift-inducing agent acriflavine has been shown to stimulate ISAbal mobilization⁹⁴. ISAbal has been found adjacent to several OXA-type β -lactamase genes—including *bla_{OXA-23-like}*, *bla_{OXA-51-like}*, *bla_{OXA-58-like}*, and *bla_{OXA-235-like}*^{95,96}. When positioned upstream of these genes, ISAbal typically increases their expression to levels sufficient to confer carbapenem resistance. For example, ISAbal located 25 bp upstream of *bla_{OXA-23}* provides a strong promoter that enhances transcription. Similarly, an ISAbal insertion 7 bp upstream of *bla_{OXA-51-like}* can elevate expression by approximately 50-fold, resulting in carbapenem MICs comparable to those mediated by acquired OXA carbapenemases. These events frequently appear to be selected during carbapenem therapy⁹⁷.

Beyond transcriptional activation, ISAbal also contributes directly to gene mobilization, forming part of several transposons that carry *bla_{OXA}* genes. One notable example is Tn2006, composed of two oppositely oriented ISAbal copies flanking *bla_{OXA-23}*. The presence of a 9-bp target site duplication at both ends indicates that the entire unit transposes as a single structure. ISAbal-containing transposons carrying *bla_{OXA}* and other resistance genes are widespread across *Acinetobacter*, highlighting the critical role of this element in the genomic plasticity of the genus^{98,99}.

- ***ISAb*a3**

The insertion sequence ***ISAb*a3** is commonly associated with *bla*_{OXA-58-like} genes, often flanking the gene on both sides to generate a composite transposon. *ISAb*a3 contains a single ORF encoding its transposase and was first identified in *A. baumannii* in 2003¹⁰⁰. When positioned upstream of a *bla*_{OXA} gene in the opposite orientation, *ISAb*a3 can provide a promoter that drives expression in a manner analogous to *ISAb*a1. However, *ISAb*a3-driven mobility differs from *ISAb*a1: the absence of target-site duplications surrounding *ISAb*a3 suggests that it spreads *bla*_{OXA} genes primarily through homologous recombination rather than classical transposition. *ISAb*a3 is frequently found disrupted by other insertion sequences—such as *ISAb*a2, *IS*1008, *IS*18, or *ISAb*a825—forming chimeric IS elements with hybrid promoter regions. These composite promoters often enhance *bla*_{OXA-58-like} expression to varying degrees, likely reflecting selective pressure imposed by carbapenem treatment⁹⁶.

- **Other Insertion Sequences in *Acinetobacter***

Although *ISAb*a1 and *ISAb*a3 are the most common partners of *bla*_{OXA} genes, several additional IS elements contribute to their dissemination and regulation.

- *ISAb*a125 has been detected in association with *bla*_{OXA-58} and appears to mediate gene duplication. In an *Acinetobacter pittii* isolate, strains containing one, two, or three copies of *bla*_{OXA-58} exhibited progressively higher IPM MICs (16, 32, and 128 mg/L), indicating a clear dosage effect. The duplications were flanked by IS26 elements, suggesting an IS26-mediated replication of the *ISAb*a2/*ISAb*a3–*bla*_{OXA-58}–*ISAb*a3 region¹⁰¹.
- *ISAb*a4 has been identified 25 bp upstream of *bla*_{OXA-23}, providing a promoter. Along with a downstream fragment of its right inverted repeat, this forms the basis of Tn2007, a transposon mobilized via one-ended transposition⁹⁸.
- *ISAb*a9 and *ISAb*a10 have independently inserted into *ISAb*a1 elements upstream of *bla*_{OXA} genes¹⁰².

- A hybrid *ISAba9/ISAba1* promoter upstream of *bla_{OXA-51}* increased expression eightfold relative to *ISAba1* alone¹⁰².
- A *ISAba10/ISAba1* element upstream of *bla_{OXA-23}* increased expression two- to fivefold, with corresponding rises in carbapenem MICs¹⁰³.

These findings underscore that IS-mediated promoter evolution is a powerful driver of carbapenem resistance. In addition to enhancing expression, some IS elements can inactivate *bla_{OXA}* genes by inserting directly into their coding sequences. Although rare, *ISAba15* and *ISAba19* have been found disrupting *bla_{OXA-66}* and *bla_{OXA-78}*, respectively. The low frequency of such events suggests that maintaining a functional OXA enzyme generally offers a stronger selective advantage.

2.3 Alternative Mechanisms of Antibiotic Resistance in *A. baumannii*

In addition to β -lactamase-mediated resistance, *A. baumannii* employs multiple alternative strategies to withstand antibiotic treatment. These include aminoglycoside resistance, target site modifications, reduced membrane permeability, biofilm formation, efflux pump overexpression, and quorum sensing-mediated regulation. Understanding the interplay of these mechanisms is essential for developing effective therapeutic strategies against this multidrug-resistant pathogen.

Resistance to aminoglycosides is predominantly mediated by aminoglycoside-modifying enzymes (AMEs), which inactivate the drug through phosphorylation, adenylation, or acetylation, preventing binding to the 30S ribosomal subunit. These enzymes are often plasmid-borne, facilitating horizontal dissemination. High-level resistance can also arise from the activity of **16S rRNA methyltransferases**, such as ArmA, which methylate the aminoglycoside binding site on the ribosome, thereby blocking drug activity. While aminoglycoside resistance is mainly based on enzymatic inactivation or ribosomal protection, *A. baumannii* also employs strategies that directly alter the antibiotic targets themselves, thereby reducing drug affinity¹⁰⁴. **Target modification** further contributes to resistance without compromising essential bacterial functions. In the case of β -lactams, mutations or the acquisition of altered alleles of **PBPs** reduces drug affinity¹⁰⁵. Resistance to colistin is frequently linked to modifications of lipid A—such as the addition of phosphoethanolamine—

mediated by the **pmrCAB operon**, or to complete/partial loss of lipopolysaccharide (LPS) caused by mutations in *lpxA*, *lpxC*, or *lpxD*. Target modification, however, is only one facet of the bacterium's defense; alterations in outer membrane permeability further restrict antibiotic entry and potentiate resistance¹⁰⁶. **Reduced permeability** of the outer membrane also plays a key role, especially against hydrophilic antibiotics. Alterations or loss of porins such as **OmpA** and **CarO** significantly reduce drug influx, while changes in LPS charge and composition further limit permeability. In addition, a thick polysaccharide capsule can impair aminoglycoside uptake and enhance survival under antibiotic stress. Reduced permeability not only limits drug access to the periplasm but also synergizes with another survival strategy: the formation of biofilms, which provide a protective niche against antimicrobial activity¹⁰⁷. **Biofilm formation** represents another important mechanism of antimicrobial tolerance. Within biofilms, the extracellular polymeric matrix—composed of polysaccharides, proteins, lipids, and extracellular DNA—impedes antibiotic penetration and fosters persistence in hostile environments. Key determinants involved in biofilm formation include *bap*, *ompA*, *csuE*, and *pgaB*, which mediate initial attachment, biofilm maturation, and structural stability. Yet even within biofilms, antibiotics that manage to penetrate the extracellular matrix may be expelled through active efflux systems, further lowering intracellular drug concentrations^{110,111}. **Active efflux** is mediated primarily by resistance-nodulation-division (RND) family transporters. The **AdeABC system** is one of the most important efflux pumps in *A. baumannii*. Its expression is regulated by the two-component system **AdeRS**, and mutations in this regulatory system can cause overexpression, leading to multidrug resistance. Other efflux systems, including AdeIJK and AdeFGH, further contribute to the extrusion of diverse antibiotic classes. Finally, the regulation of these multifaceted mechanisms is strongly influenced by quorum sensing, which coordinates biofilm development and virulence in a population density-dependent manner¹⁰⁸. **Quorum sensing (QS)** provides an additional regulatory layer that coordinates resistance and virulence. In *A. baumannii*, QS is primarily mediated by the **AbaI/AbaR system**, which synthesizes and detects N-acyl homoserine lactones (AHLs) such as OHC12-HSL. Importantly, disrupting QS through quorum sensing inhibitors (QSIs), quorum quenching (QQ) enzymes, or certain fatty acids has been proposed as a promising strategy to reduce biofilm formation and pathogenicity^{112,113}.

In conclusion, antibiotic resistance in *A. baumannii* is multifaceted, involving intrinsic and acquired β -lactamases, aminoglycoside-modifying enzymes, target modification, porin loss, efflux pumps, and biofilm-associated tolerance, all of which can be further modulated by

quorum sensing. This complex interplay of mechanisms underscores the remarkable adaptability of *A. baumannii* and highlights the urgent need for novel therapeutic strategies to combat its multidrug-resistant and extensively drug-resistant phenotypes.

2.4 Current treatments

Currently, the therapeutic landscape against *A. baumannii* relies on both established regimens and innovative strategies, reflecting the urgent need to address infections caused by multidrug-resistant and carbapenem-resistant strains. Among the conventional agents, Sulbactam-based therapies represent a cornerstone, while tetracycline derivatives such as Tigecycline and Eravacycline have expanded the armamentarium. More recently, novel β -lactam/ β -lactamase inhibitor combinations and entirely new approaches, such as phage therapy and targeted inhibitors of bacterial processes, are being explored, highlighting the dynamic and evolving nature of treatment options for this pathogen.

Sulbactam (SUL), a first-generation narrow-spectrum β -lactamase inhibitor commonly combined with **AMP**, plays a key role due to its intrinsic bactericidal activity. Unlike other inhibitors, SUL not only blocks β -lactamase enzymes but also directly targets PBPs, notably PBP1 and PBP3, which are essential for cell wall synthesis. Clinical studies have shown that AMP/SUL achieves outcomes comparable to those of colistin or IPM, with favorable efficacy and safety profiles. Standard dosing regimens are effective in mild to moderate infections, but severe carbapenem-resistant infections require escalation to high-dose or continuous infusion strategies. Importantly, combination regimens pairing high-dose SUL with a second active agent, such as Tigecycline or **Cefiderocol (FDC)**, have been associated with reduced mortality and lower nephrotoxicity compared with **colistin-based therapies**. Even in settings with extensively drug-resistant isolates, optimizing SUL exposure through prolonged infusion may allow clinicians to overcome resistance mechanisms^{114,115}. In clinical practice, Tigecycline has emerged as an alternative option, particularly valuable where therapeutic choices are limited. Its broad-spectrum activity extends to many multidrug-resistant and extensively drug-resistant *A. baumannii* strains, although clinical results have been mixed. Standard regimens employ a moderate loading and maintenance dose, while more severe infections require higher dosing to improve outcomes, especially in ventilator-associated pneumonia. Although Tigecycline is not consistently superior in terms of mortality reduction, higher doses have been linked to better success in critically ill patients. However, the risk of resistance development during therapy remains a significant concern. The primary mechanism involves overexpression of the

AdeABC efflux pump, but also mutations and insertion sequences that enhance efflux activity can rapidly reduce Tigecycline efficacy, particularly under sub-inhibitory drug pressure. This highlights the importance of careful dosing, monitoring, and often combining Tigecycline with other agents to mitigate resistance emergence^{116,117}. To expand beyond Tigecycline, Eravacycline, a synthetic Fluorocycline structurally related to Tigecycline but chemically optimized, has recently been introduced. Approved for complicated intra-abdominal infections, it demonstrates strong in vitro activity against Gram-positive and Gram-negative organisms, including CRAB, although it lacks activity against *P. aeruginosa*. Clinical trials have confirmed its non-inferiority to carbapenems in intra-abdominal infections, with high cure rates and a favorable safety profile. Notably, Eravacycline offers the advantage of both intravenous and oral formulations, making it versatile in clinical practice. Retrospective real-world data further support its effectiveness against *A. baumannii*, including carbapenem-resistant strains, although more robust evidence is needed to define its role fully. Its safety profile and the potential for use in patients with β -lactam allergies increase its attractiveness as a therapeutic option^{118,119}. While these agents provide valuable tools, resistance has progressively limited their effectiveness, prompting the development of innovative therapies. One of the most promising is Sulbactam/Durlobactam, a novel β -lactam/ β -lactamase inhibitor combination specifically designed to overcome the resistance mechanisms of CRAB. **Durlobactam (DURL)**, a diazabicyclooctane inhibitor, protects SUL from enzymatic degradation by OXA-type carbapenemases and other prevalent β -lactamases, thereby restoring and enhancing SUL activity. In vitro and in vivo studies have shown potent activity, with significant improvements in bacterial clearance and survival compared to colistin-based regimens. Clinical trials have confirmed its efficacy and safety, culminating in regulatory approval for the treatment of hospital- and ventilator-associated pneumonia caused by carbapenem-resistant strains. Importantly, SUL/DURL not only improves survival outcomes but also significantly reduces nephrotoxicity compared to older alternatives, making it a critical addition to current treatment options^{120,121}. Parallel to this development, Cefepime/Zidebactam has emerged as another promising β -lactam/ β -lactamase inhibitor combination. Cefepime, a fourth-generation cephalosporin, gains enhanced activity against resistant Gram-negative bacteria when paired with Zidebactam, a non- β -lactam inhibitor that also targets PBPs. Early studies indicate that this combination may effectively counteract resistance in MDR *A. baumannii*, with ongoing clinical trials evaluating its safety and efficacy in serious infections^{122,123,124}. Similarly, the combination of Imipenem/Cilastatin/Funobactam represents another innovative approach. Funobactam, a novel diazabicyclooctane inhibitor, neutralizes both serine and metallo- β -

lactamases, restoring IPM's antibacterial potential against resistant pathogens, including *A. baumannii*. Early-phase trials have demonstrated favorable pharmacokinetics and safety, and large phase 3 trials are underway to establish its clinical utility^{125,126}. Another agent under development is Xeruborbactam, a benzoxaborole-based β -lactamase inhibitor with a unique mechanism of action. Unlike traditional inhibitors, Xeruborbactam effectively blocks both serine and metallo- β -lactamases, thereby extending the activity of partner β -lactam antibiotics against highly resistant organisms. Although it displays modest intrinsic antibacterial activity, its synergy with β -lactams positions it as a strong candidate for treating carbapenem-resistant infections. Early clinical studies suggest it is safe and well tolerated, with both intravenous and oral formulations under investigation, highlighting its potential for widespread clinical use. Beyond β -lactam combinations, entirely novel approaches are being explored. Phage therapy, employing lytic bacteriophages to selectively target *A. baumannii*, has shown encouraging results in animal models of bacteremia and pneumonia, with improved survival and reduced inflammation. Importantly, phages isolated from hospital environments have demonstrated potent lytic activity against MDR and carbapenem-resistant strains, offering a highly specific and potentially resistance-proof strategy. Although still experimental, phage therapy represents a promising adjunct to conventional antibiotics, particularly when combined with standard regimens to enhance efficacy and prevent relapse^{127,128}. Other innovative strategies focus on disrupting bacterial physiology at unique targets. LPS transport inhibitors, such as Zosurabalpin, act by blocking the LptB2FGC complex, essential for transporting lipopolysaccharides to the bacterial outer membrane. By disrupting this process, Zosurabalpin effectively compromises membrane integrity, leading to bacterial death. Preclinical studies have shown remarkable activity specifically against *A. baumannii*, with minimal impact on commensal microbiota, reducing the risk of dysbiosis. Its specificity and novel mechanism also limit the likelihood of cross-resistance with existing antibiotics. Early clinical trials are ongoing to assess its safety and potential for human use¹²⁹. In parallel, new protein synthesis inhibitors, such as apramycin and zifanocycline, are being evaluated. Apramycin, an aminoglycoside derivative, and zifanocycline, a third-generation tetracycline, both act on the 30S ribosomal subunit but evade traditional resistance mechanisms. Preclinical studies have shown promising efficacy in murine infection models, with potent bactericidal activity and efficient tissue penetration¹³⁰. Novel polymyxin derivatives, including MRX-8, QPX9003, and SPR206, are also in early-phase clinical development. These agents retain the traditional mechanism of binding to lipopolysaccharides and disrupting the bacterial outer membrane but exhibit reduced toxicity compared with older polymyxins such as colistin¹³¹. Finally, ***bacterial cell division***

inhibitors have attracted attention as another potential class of antimicrobials. Targeting FtsZ, a highly conserved cytoskeletal protein essential for bacterial cytokinesis, these agents prevent the formation of the Z-ring, thereby halting cell division and leading to bacterial death. Natural compounds such as cinnamaldehyde and synthetic molecules like PC190723 have demonstrated significant activity in preclinical models, underscoring the potential of this approach as a novel therapeutic avenue. Overall, the treatment of *A. baumannii* is rapidly evolving from reliance on traditional antibiotics to a more diversified arsenal that includes chemically optimized derivatives, innovative inhibitor combinations, and entirely new therapeutic paradigms. While SUL remains a cornerstone and tetracycline derivatives provide valuable alternatives, the future of therapy increasingly depends on novel molecules and strategies designed to bypass or suppress resistance mechanisms. This multifaceted approach offers hope for improving patient outcomes in infections caused by one of the most formidable pathogens in modern medicine¹³².

CHAPTER 3

“Penicillin-Binding Proteins: Structure and Function”

3.1 Introduction

Bacterial peptidoglycan provides both mechanical strength to withstand the high intracellular pressure present in the cell and a well-defined shape that is faithfully maintained across generations. It is composed of glycan chains formed by alternating *N-acetylglucosamine* and *N-acetylmuramic acid* residues, which are cross-linked through short stem peptides attached to the N-acetylmuramic acid. PBPs are responsible for catalyzing the polymerization of glycan strands through *transglycosylation* and forming peptide cross-links between glycan chains via *transpeptidation*. Certain PBPs also exhibit *DD-carboxypeptidase* activity, removing the terminal D-alanine of stem pentapeptides, or endopeptidase activity, cleaving the peptide bonds connecting two glycan strands, with *endopeptidation* functioning as the reverse of transpeptidation. Due to the structural similarity between the *D-Ala-D-Ala* moiety of the stem peptide precursors and penicillin, these enzymes involved in late-stage peptidoglycan synthesis are highly susceptible to β -lactam antibiotics, which form stable acyl-enzyme complexes that inhibit their ability to cross-link peptidoglycan. Bacteria contain a variable number of PBPs, a number that has been precisely determined thanks to extensive genome sequencing efforts⁴³. PBPs are generally classified into two main groups based on molecular weight and domain organization: **High Molecular Mass (HMM) PBPs** and **Low Molecular Mass (LMM) PBPs**. HMM PBPs are multimodular enzymes that mediate peptidoglycan polymerization and its insertion into the existing cell wall, and their topology typically includes a cytoplasmic tail, a transmembrane anchor, and two domains connected by a β -rich linker exposed on the outer surface of the cytoplasmic membrane where cell wall synthesis occurs¹³². Depending on the structure and catalytic function of their N-terminal domain, *HMM PBPs* are further divided into *class A* and *class B*. The C-terminal **Penicillin-Binding (PB) domain** in both classes functions as a transpeptidase, catalyzing cross-linking between adjacent glycan chains. In class A PBPs, the N-terminal domain exhibits glycosyltransferase activity and is responsible for elongating uncross-linked glycan chains, whereas in class B PBPs, the N-terminal domain is thought to contribute to cell morphogenesis through interactions with other proteins involved in the cell cycle¹³³. Some bacteria also possess **Monofunctional Glycosyltransferases** (MGTs) that resemble the N-terminal domain of class A PBPs, although their precise physiological role

remains unclear. LMM PBPs, frequently referred to collectively as class C PBPs, are sometimes subdivided into C1, C2, and C3 subgroups and are primarily involved in the remodeling and maturation of peptidoglycan rather than its polymerization. PBPs share a common ***DD-peptidase activity***, which can manifest as ***DD-transpeptidase***, ***DD-carboxypeptidase***, or ***DD-endopeptidase*** functions¹³⁴. The carboxypeptidation and transpeptidation reactions follow a three-step mechanism. First, a rapid and reversible formation of a noncovalent Henri–Michaelis complex occurs between the enzyme and the peptidoglycan stem pentapeptide, known as the donor strand. This is followed by nucleophilic attack of the active site serine on the carbonyl carbon of the C-terminal D-Ala-D-Ala peptide bond, resulting in the formation of an acyl-enzyme intermediate and the release of the terminal D-alanine (acylation). The final step, deacylation, involves either hydrolysis, releasing the shortened peptide in carboxypeptidation, or cross-link formation with an acceptor stem peptide in transpeptidation. The DD-endopeptidase activity corresponds to hydrolysis of the peptide cross-bridge formed during transpeptidation. This DD-peptidase activity is catalyzed by a conserved PB domain, which also serves as the target for β -lactam antibiotics. These antibiotics exploit the structural similarity between penicillin and the D-Ala-D-Ala dipeptide of the natural substrate, forming a stable acyl-enzyme complex that inhibits cross-linking activity⁴³. Structurally, the PB domain consists of two subdomains: one composed of a five-stranded β -sheet covered by three α -helices, and an all-helical domain. The active site is located at the interface between these subdomains, and flexibility between them may influence ligand binding. Nine residues within the active site are broadly conserved. The nucleophilic serine at the beginning of helix α 2 forms the ***SXXK motif***, followed by the SxN motif located in a loop between α 4 and α 5 helices, and the KTG(T/S) motif with a conserved glycine positioned at the rear of the active site⁴³.

These conserved residues play specific roles in substrate recognition and catalysis. The penultimate D-alanine of the donor stem peptide fits precisely within the active site, with its amide group interacting with the asparagine of the second motif and the β 3 strand backbone. The carbonyl oxygen of this D-alanine occupies the oxyanion hole, while its methyl group is accommodated in a hydrophobic pocket, highlighting the importance of the conserved glycine. The carboxylate group of the leaving D-alanine interacts with the hydroxyl groups of the third motif, ensuring proper orientation. Collectively, these conserved residues facilitate accurate positioning of the substrate, prevent steric hindrance, and contribute to the specificity of PBPs toward the penultimate D-alanine of the stem peptide. The acylation of PBPs involves the

nucleophilic attack of the active serine on the carbonyl carbon of the penultimate D-alanine in the donor stem peptide, accompanied by the release of the terminal D-alanine. This process requires the abstraction of a proton from the serine residue, a step facilitated by the lysine of the first conserved motif, which is assumed to be deprotonated. The proton can then be transferred to the leaving amine group of the D-alanine via the serine of the second motif. Alternatively, a concerted one-step mechanism may occur, in which the proton is directly transferred from the active serine to the serine of the second motif and simultaneously to the amine group of the leaving D-alanine. During the deacylation step, a proton from the acceptor group, either an amino group in transpeptidation or a water molecule in carboxypeptidation, must be abstracted to activate it for nucleophilic attack on the carbonyl carbon of the acyl-enzyme intermediate. This proton can subsequently be returned to the active serine. The lysine of the third motif, in its deprotonated form, may facilitate the abstraction of the proton from the acceptor, while the serine of the second motif can assist in this process. The active site of PBPs can thus be considered as a dual lysine–serine system, one dyad promoting acylation and the other promoting deacylation, although these two systems are not entirely independent. Proton transfer in these dyads is likely delocalized between the hydroxyl oxygen of serine and the nitrogen of lysine, and the precise orientation of the electron lone pairs is critical for the catalytic mechanism. Understanding the dynamics of these lysine–serine interactions at a quantum mechanical level may provide further insight into the mechanisms of acylation and deacylation in PBPs^{135,136}.

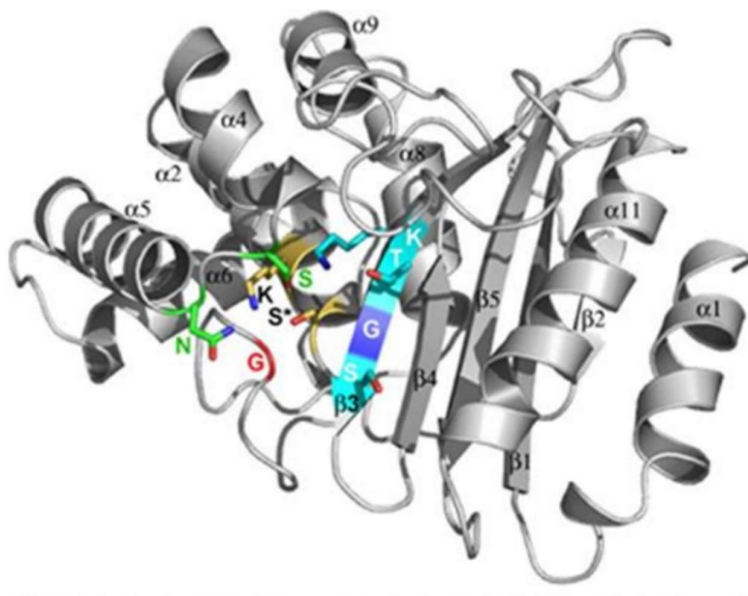


Figure 8. PB domain and conserved motifs of the active site (PBP4a)

Class A PBPs are *bifunctional* enzymes that integrate two catalytic modules within a single polypeptide. The **N-terminal module** belongs to the glycosyltransferase family GT51 and is responsible for polymerizing the glycan strands of peptidoglycan by incorporating Lipid II precursors into growing glycan chains. This glycosyltransferase domain is defined by a series of conserved sequence motifs and uses lipid-linked intermediates as substrates. The **C-terminal module** is a transpeptidase domain — the canonical PB domain — that catalyzes peptide cross-linking between stem peptides attached to adjacent glycan chains. Structurally, these two modules are connected by an interdomain linker composed of a short β -sheet and an α -helix, positioning the catalytic domains to coordinate polymerization and cross-linking functions in a coordinated manner¹³⁷. Some class A PBPs also contain additional accessory domains, such as **PASTA (Penicillin-binding protein And Serine/Threonine kinase Associated)** domains, **Fn3 (fibronectin type III)** domains, or **FHA (forkhead-associated)** domains, which are indicative of further regulatory or interaction roles and suggest that these proteins may participate in broader protein networks or cell surface interactions. The precise biological roles of these accessory domains remain an area of ongoing investigation, but they are thought to contribute to localization, complex formation, and engagement with other cell wall biosynthetic factors¹³⁸. In addition to these bifunctional enzymes, many bacteria encode **monofunctional glycosyltransferases (MGTs)** that resemble only the glycosyltransferase domain of class A PBPs. These enzymes also belong to the GT51 glycosyltransferase family and can polymerize uncross-linked glycan chains, although their exact physiological roles have not yet been definitively established. The presence of MGTs across diverse bacterial genomes indicates that monofunctional activities can complement or substitute for the glycosyltransferase activity of bifunctional PBPs under certain conditions¹³⁹.

Class B PBPs are *monofunctional transpeptidases*. Unlike class A PBPs, they lack an intrinsic glycosyltransferase domain and instead comprise a complex **N-terminal non-catalytic module** coupled to the C-terminal PB domain. The N-terminal region does not itself catalyze glycan polymerization but is believed to mediate essential interactions with other components of the cell envelope synthesis machinery. This module positions the transpeptidase domain within defined cellular complexes that guide cell elongation, division, or morphogenesis^{140,141}. The transpeptidase domain itself contains the canonical active site motifs that define PBPs — the conserved **SXXK**, **(S/Y)xN** and **(K/H)(S/T)G** motifs — and catalyzes the cross-linking of peptide stems to form the structural meshwork of peptidoglycan¹⁴¹. The distinction between class A and class B transpeptidase domains is reflected not only in domain architecture but also

in subtle sequence variations of these motifs, which correlate with their specialized cellular functions and interactions.

Class C PBPs represent the LMM family of PBPs and are predominantly *hydrolases* that modulate peptidoglycan architecture rather than directly synthesizing it. These include enzymes with DD-carboxypeptidase and DD-endopeptidase activities, which trim terminal D-alanines from stem peptides or cleave peptide cross-bridges, respectively, contributing to peptidoglycan maturation, remodeling, and recycling¹⁴². The structural organization of class C PBPs varies among subclasses, but all retain the central PB domain that coordinates DD-peptidase activity. Some class C PBPs contain additional domains either inserted within the PB fold or appended at terminal positions, such as β -rich modules or amphipathic helices that facilitate membrane association or substrate interaction; these structural features are consistent with specialized roles in spatially regulated peptidoglycan modification¹³⁵.

Overall, across all classes, the **PB domain** itself shares a conserved fold composed of two subdomains — a five-stranded β -sheet covered by α -helices and an all-helical domain — with the active site located at their interface. This domain harbors the conserved catalytic motifs central to DD-peptidase activity. The diversity in domain composition and organization among PBPs reflects the multiplicity of roles these enzymes play in bacterial cell physiology: class A PBPs catalyze the core polymerization and cross-linking reactions of peptidoglycan assembly, class B PBPs integrate transpeptidation with cell cycle and morphogenetic processes through protein interactions, and class C PBPs remodel and mature the cell wall structure. Collectively, this modular architecture and functional specialization enable PBPs to orchestrate both the construction and dynamic adaptation of the peptidoglycan network essential for bacterial growth and survival¹⁴³.

3.2 Specific Characteristics of Penicillin-Binding Proteins in *A. baumannii*

Genomic analyses have shown that *A. baumannii* encodes a defined and conserved set of PBPs involved in peptidoglycan synthesis and remodeling. Specifically, seven PBP-encoding genes and one gene encoding a MtgA are consistently present across diverse clinical isolates. The PBP repertoire includes the HMM PBPs - PBP1a and PBP1b - which are **bifunctional enzymes** possessing both transglycosylase and transpeptidase activities, as well as the class B PBPs PBP2 and PBP3, which function as monofunctional transpeptidases involved in cell elongation and septal peptidoglycan synthesis, respectively. In addition, *A. baumannii* encodes several

LMM PBPs, including PBP5/6, PBP6b, and PBP7/8, which primarily exhibit DD-carboxypeptidase and/or DD-endopeptidase activities and are thought to fine-tune peptidoglycan maturation¹⁴⁴.

Genetic inactivation studies have demonstrated that **PBP3** plays a unique and essential role in *A. baumannii*. Attempts to delete the gene encoding PBP3 consistently result in nonviable cells, indicating that this enzyme is indispensable for bacterial survival. Although deletion events can transiently occur at the chromosomal level, cells lacking PBP3 fail to sustain growth, leading to rapid loss of viability. This finding identifies PBP3 as the only strictly essential PBP in *A. baumannii* and highlights its central role in septal peptidoglycan synthesis and cell division. In contrast, PBP1a, PBP1b, and PBP2 are individually nonessential, although they contribute differently to optimal growth and cell wall integrity. Selective inactivation of the transpeptidase activity of PBP1a results in a measurable reduction in growth rate and cell viability, indicating that this enzyme plays a prominent role in supporting normal peptidoglycan synthesis during cell elongation. The TP activities of PBP1b and PBP2 appear to be more functionally redundant, as their individual inactivation has little impact on growth under laboratory conditions. Combinatorial inactivation of PBPs reveals a hierarchy of functional importance and partial redundancy among these enzymes. Simultaneous inactivation of the TP domains of PBP1a and PBP1b, or of PBP1a and PBP2, leads to progressively more severe growth defects, increased cell lysis, and reduced viability. These observations indicate that while individual PBPs can partially compensate for one another, the TP activity of PBP1a represents a major contributor to cell wall stability. Loss of PBP1a TP activity is the primary driver of cell lysis, an effect that is exacerbated when combined with the inactivation of additional PBPs. Remarkably, *A. baumannii* remains viable even when the TP activities of PBP1a, PBP1b, and PBP2 are simultaneously inactivated, provided that PBP3 remains functional. Although such mutants display severely impaired growth and increased cell lysis, their survival underscores the dominant physiological role of PBP3 in maintaining essential peptidoglycan cross-linking during cell division. These findings demonstrate that PBP3 alone is sufficient to sustain minimal cell wall synthesis compatible with viability, whereas the remaining PBPs primarily contribute to growth efficiency, robustness, and resistance to cell wall stress¹⁴⁵.

Overall, the physiological roles of PBPs in *A. baumannii* are characterized by a combination of essentiality, redundancy, and specialization. PBP3 emerges as the central and indispensable enzyme required for survival, while PBP1a, PBP1b, and PBP2 collectively support cell

elongation, cell wall integrity, and protection against lytic events. This functional organization highlights the critical importance of PBP3 as a core component of the divisome and suggests that simultaneous interference with PBP3 and selected elongation-associated PBPs may represent an effective strategy for compromising cell wall synthesis in this pathogen¹⁴⁵.

3.3 PBPs as targets of selective pressure and contributors to β -lactam resistance in *A. baumannii*

In *A. baumannii*, PBP3 (*FtsI*) represents a primary target of several β -lactam antibiotics, particularly cephalosporins and carbapenems. Over time, sustained exposure to β -lactam antibiotics has likely exerted selective pressure on PBP3, leading to the accumulation of subtle amino acid substitutions that only minimally affect the overall protein structure but strategically reduce antibiotic binding affinity. These structural changes, although often minimal, can significantly reduce antibiotic affinity, thereby contributing to decreased susceptibility and the emergence of resistance phenotypes. Detailed analyses of PBPs in *A. baumannii* have identified specific amino acid substitutions that accumulate in clinical isolates under β -lactam selective pressure. Although most PBP genes are highly conserved, several studies have reported point mutations affecting both HMM and LMM PBPs. In a large comparative study of carbapenem-susceptible and -resistant clinical isolates, Cayô et al. identified multiple point mutations in genes encoding PBPs, including *ponA* (PBP1a), *mrda* (PBP2), *ftsI* (PBP3), and *dacC/dacD* (PBP5/6 and PBP6b)¹⁴⁴. Notably, a **G523V substitution in PBP3 (*FtsI*)** was detected in carbapenem-resistant isolates belonging to specific clonal lineages. This mutation is located outside the conserved catalytic motifs of the transpeptidase domain, suggesting that it does not abolish enzymatic activity but may subtly alter local conformation and antibiotic binding properties. The same study reported additional substitutions in other PBPs, such as **V509I** and **E110Q** in **PBP2**, and multiple substitutions in PBP1a (including **L147I**, **A244T**, and **S382N**)¹⁴⁴. Importantly, these mutations were observed in both susceptible and resistant isolates, indicating that individual substitutions are not sufficient on their own to confer resistance but may contribute cumulatively to reduced β -lactam affinity in combination with other mechanisms. Phenotypic studies focusing on carbapenem-resistant isolates further support the functional relevance of such subtle alterations. Vashist et al. reported altered PBP binding profiles in CRAB, characterized by reduced affinity of HMM PBPs for β -lactams, including carbapenems. Although this study did not systematically map individual amino acid substitutions, the observed changes in binding

behavior are consistent with the presence of minor structural variations in PBPs such as those described by Cayô et al.¹⁴⁶. In another study, WGS performed on several strains during a prolonged infection revealed that *A. baumannii* can accumulate mutations over time under sustained carbapenem exposure. Hawkey et al. showed that resistance evolution is driven by stepwise genetic changes affecting multiple cellular functions, including cell wall synthesis pathways. While carbapenem resistance in this context was multifactorial, the study provides a clear evolutionary framework in which **incremental mutations—including those in essential proteins such as PBPs—can be selected without compromising bacterial viability**. Earlier biochemical analyses also highlighted that carbapenem resistance correlates with changes in PBP profiles rather than with complete loss or inactivation of these enzymes¹⁴⁷. Fernández-Cuenca et al. reported reduced expression and altered binding of HMM PBPs, particularly PBP2, in carbapenem-resistant isolates. In several cases, no mutations were detected within PBP coding sequences, indicating that **structural mutations and regulatory changes affecting PBP expression or accessibility may act synergistically**¹⁴⁴.

Overall, the available evidence indicates that specific point mutations in PBPs—such as the G523V substitution in PBP3—represent adaptive changes that preserve enzymatic function while subtly reducing β -lactam affinity. These mutations, when combined with β -lactamase production, porin loss, and regulatory adaptations, contribute to clinically relevant resistance phenotype in *A. baumannii*. The progressive accumulation of subtle structural changes in essential PBPs, particularly PBP3, highlights the limitations of conventional β -lactams whose activity relies on high-affinity binding to these enzymes. In this context, the development of novel β -lactam antibiotics capable of overcoming PBP-mediated resistance mechanisms has become a major therapeutic priority, leading to the introduction of siderophore-conjugated agents such as FDC.

CHAPTER 4

“CEFIDEROCOL: a novel therapeutic strategy against *A. baumannii*”

4.1 Introduction

Cefiderocol is a novel siderophore cephalosporin structurally related to ceftazidime and cefepime, with specific modifications that enhance its activity against Gram-negative bacteria. Its 7-position side chain features an amino-acyl group with an aminothiazole ring and a carboxypropyl-oxyimino chain, which provides stability against β -lactamase hydrolysis and improved activity against Gram-negative bacilli. The key distinction of FDC lies in its 3-position catechol moiety, which chelates ferric iron and enables active transport into the periplasmic space via bacterial iron uptake systems (Fig. 9).

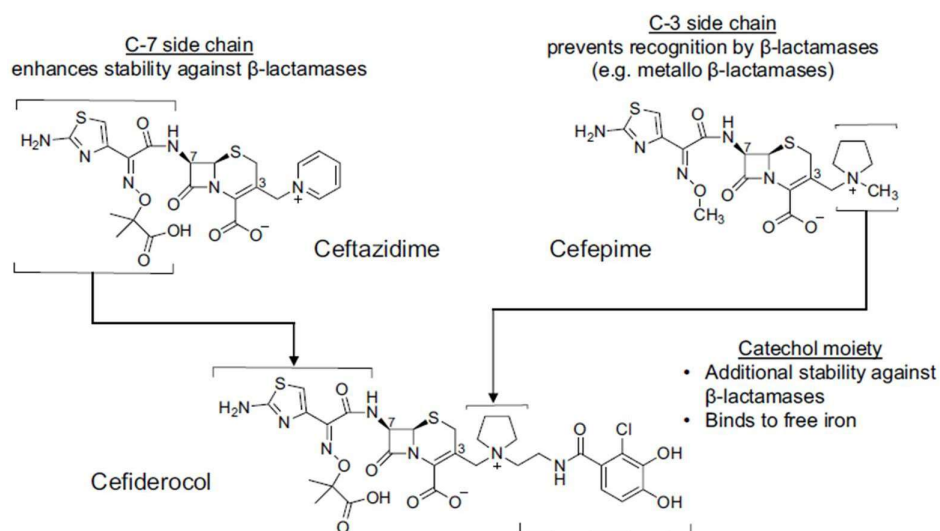


Figure 9. Chemical structure of Cefiderocol, highlighting the catechol moiety responsible for iron chelation

In *A. baumannii*, FDC exploits *TonB-dependent* siderophore receptors, such as **PirA**, to actively enter the periplasmic space by mimicking ferric-siderophore complexes, enabling efficient delivery to PBPs and bypassing the limitations of passive diffusion through porins. Once inside the periplasm, FDC dissociates from iron and binds to PBPs, primarily PBP3, inhibiting peptidoglycan synthesis and leading to bacterial cell death. This “Trojan horse” strategy, combined with broad stability against serine- and metallo- β -lactamases, allows FDC to maintain activity even against strains of *A. baumannii* that have accumulated subtle PBP3 mutations reducing the affinity of conventional β -lactams. In addition to PBP3, FDC also

inhibits PBP1a, PBP1b, and PBP2, contributing to its potent antibacterial profile (Fig. 10)^{148,149,150}.

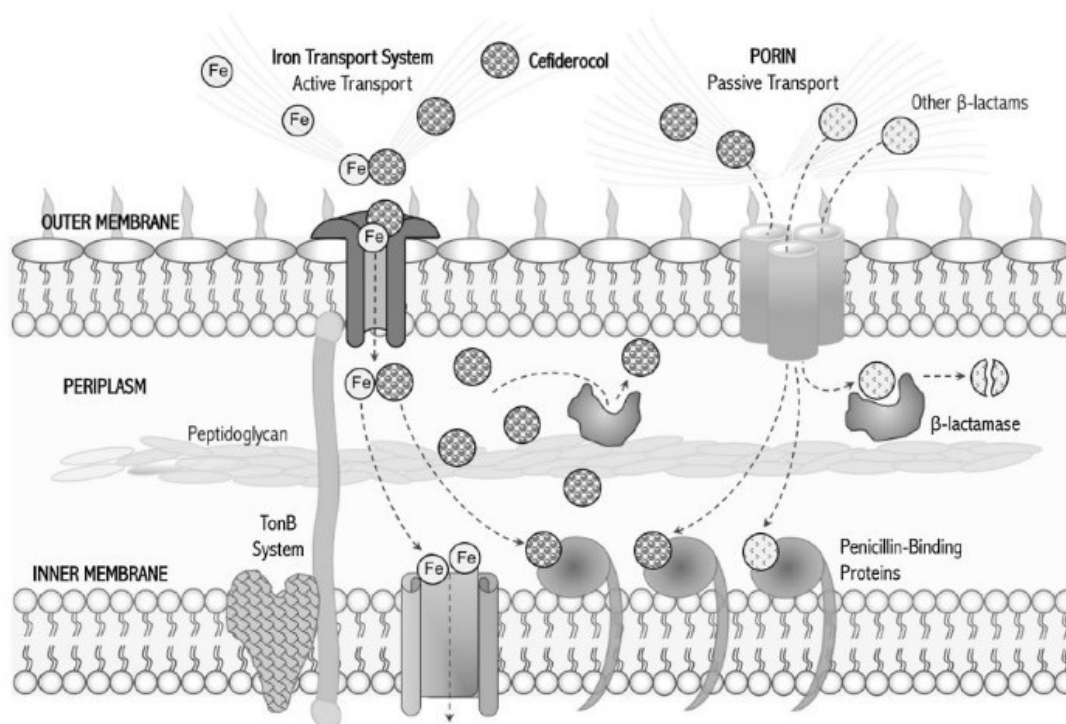


Figure 10. Mechanism of action of Cefiderocol against Gram-negative bacilli

4.2 Cefiderocol Resistance Mechanisms

FDC is a siderophore-cephalosporin specifically designed to overcome traditional β -lactam resistance mechanisms in Gram-negative bacteria. Its catechol moiety chelates ferric iron and facilitates active uptake through siderophore receptors, such as *PirA*, *PiuA*, and homologous *TonB-dependent transporters*, enabling high periplasmic concentrations that distinguish it from conventional β -lactams. Once inside the periplasm, FDC binds primarily to PBP3, inhibiting peptidoglycan cross-linking and compromising cell wall integrity. Despite these innovations, resistance to FDC can emerge under selective pressure via multiple, often synergistic mechanisms. **Reduced drug influx** is a major pathway, with in vitro selection experiments revealing loss-of-function mutations in genes involved in iron uptake, including *envZ* (a regulator of porins and iron transport genes), *tonB*, *cirA*, and *piuC*. These mutations, often frameshifts, premature stop codons, or critical amino acid substitutions, impair siderophore-mediated entry and reduce periplasmic drug accumulation^{151,152}. **Efflux pump**

modulation also contributes, with mutations in regulatory loci such as *baeS*, *czcS*, and *nalC* enhancing the expression of multidrug pumps like *AdeABC* in *A. baumannii*, thereby lowering intracellular FDC concentrations¹⁵³. A central mechanism involves **target-site modifications**, particularly in PBP3. While most clinical isolates maintain a wild-type sequence, point mutations in the transpeptidase domain can subtly alter the enzyme's structure, reducing drug binding without abolishing physiological function. Malik et al. identified two key substitutions, **Ile236Asn** and **His370Tyr**, in a FDC-resistant ST2 isolate of *A. baumannii*. The *Ile236Asn* mutation replaces a hydrophobic isoleucine with a polar asparagine near the catalytic site, disrupting local hydrophobic interactions, modifying hydrogen-bond networks, and subtly reorienting active-site residues. *His370Tyr* introduces a bulkier aromatic side chain that likely affects the positioning of the catalytic serine and the geometry of the transpeptidase groove^{153,154}. Although these changes minimally affect overall protein folding, they are strategically positioned to diminish FDC affinity. Similarly, in vitro studies by Kriz et al. identified mutations in other PBP family members, including an R363C substitution in PBP1b (*mrcB*), illustrating that alterations across multiple PBPs can modulate susceptibility under selective pressure. Importantly, these **target-site mutations alone usually confer modest MIC increases**, but in combination with reduced iron transport, efflux upregulation, or β -lactamase activity, they can produce clinically significant resistance. In vitro resistance development models have shown MIC elevations up to 512-fold, accompanied by complex mutational patterns across regulatory, metabolic, and structural genes. This multifactorial nature underscores that FDC resistance rarely arises from a single alteration; rather, it reflects the cumulative effect of impaired uptake, enzymatic activity, efflux, and finely tuned target-site changes in PBP3 and other PBPs¹⁵⁵. Collectively, these observations highlight the sophisticated interplay of mechanisms underlying FDC resistance in *A. baumannii*, emphasizing the need for vigilant molecular surveillance, stewardship strategies, and consideration of both structural and regulatory PBP alterations when predicting therapeutic outcomes (Fig.11).

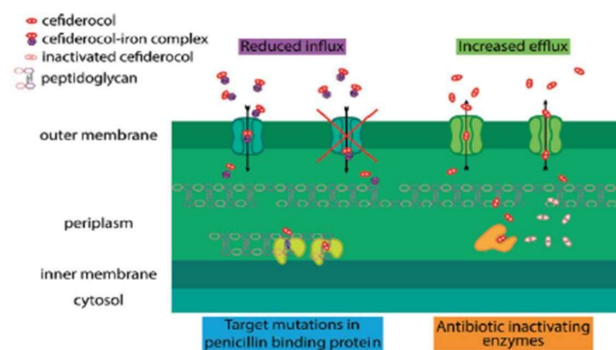


Figure 11. Graphical representation of resistance mechanisms associated with Cefiderocol resistance¹⁵

PROJECT'S AIM

The aim of this project is to achieve a comprehensive genomic and functional characterization of five clinical *A. baumannii* strains, with a particular focus on carbapenem and emerging β -lactam resistance, through the integration of advanced WGS and detailed biochemical analyses. WGS is employed to reconstruct the complete genomic architecture of each strain, enabling the identification insertions and deletions, resistance genes, plasmid content, mobile genetic elements, and virulence factors, thereby providing a thorough understanding of both the pathogenic potential and antibiotic resistance profiles of these clinical isolates. This approach highlights the critical role of WGS in the characterization of clinical strains, allowing precise mapping of resistance determinants and offering a robust framework for mechanistic and epidemiological studies. In parallel, the project investigates the expression, structural features, and activity of PBPs, using kinetic assays with selected β -lactam antibiotics—including carbapenems, cephalosporins, and the novel siderophore cephalosporin, FDC—to evaluate how specific amino acid substitutions affect substrate binding and catalytic efficiency. Particular attention is given to the emergence of FDC resistance, primarily linked to modifications in PBPs, which represents a mechanism of resistance that is difficult to detect without targeted assays and is increasingly reported in clinical isolates. By integrating genomic, bioinformatic, and functional data, the study aims to correlate genetic mutations with phenotypic outcomes, constructing a detailed profile of each strain that elucidates both established and emerging resistance mechanisms. Ultimately, this work addresses the urgent clinical threat posed by CRAB, reinforces the importance of WGS in clinical microbiology, and emphasizes the critical need to study in depth antibiotic target modifications, which, together with other resistance mechanisms, contribute to antibiotic treatment failure and can complicate already compromised clinical scenarios, sometimes resulting in fatal outcomes, thereby guiding more informed therapeutic strategies and enhancing our comprehension of this critical pathogen at both the genetic and functional levels.

CHAPTER 5

“Genotypic and Phenotypic Characterization of Carbapenem-Resistant *A. baumannii* (CRAB)”

5.1 Specific aim

The aim of this chapter is to provide an in-depth characterization of five clinical *A. baumannii* strains, integrating genomic and phenotypic data to better understand their diversity, behavior, and clinical potential. Chapter 5 is structured in two sections:

- 1) in the first section, the genome of five *A. baumannii* isolates was analyzed using NGS sequencing;
- 2) in the second section, the antimicrobial susceptibility was evaluated by determining MIC values and performing time-kill assay using selected β -lactams;

5.2 Clinical cases

The CRAB isolates analyzed in this study were obtained from four male patients admitted to Cotugno Hospital (Naples, Italy) for severe respiratory failure requiring prolonged hospitalization in the ICU or SICU. Three patients were affected by coronavirus disease 2019 pneumonia, while one patient presented with bilateral H1N1 pneumonia. All patients had received broad-spectrum antimicrobial therapy before the onset of *A. baumannii* infection.

The first case concerned a 68-year-old man admitted to the SICU for COVID-19 pneumonia and managed with non-invasive ventilation for an extended period. After 10 days of hospitalization and prior treatment with MEM and linezolid, he developed fever, increased inflammatory markers, and radiological worsening. *A. baumannii* was isolated from urine, bronchoalveolar lavage, and rectal swab. Treatment with FDC (2 g every 8 h, continuous infusion) combined with AMP/SUL (4/2 g every 6 h, continuous infusion) was administered for 14 days, resulting in clinical resolution and complete microbiological eradication (designated as Ab. 1). The second case involved a 77-year-old patient admitted to the ICU for acute respiratory deterioration requiring intubation and subsequent tracheostomy. Approximately 20 days after admission, he developed fever and elevated inflammatory markers, and *A. baumannii* bacteremia was documented (Ab. 2). Colistin therapy was initiated but discontinued after the onset of acute kidney injury requiring continuous venous

hemodiafiltration. Following transient microbiological clearance, bloodstream infection relapsed, prompting initiation of FDC in combination with colistin. Due to persistent bacteremia and clinical deterioration, colistin was replaced with AMP/SUL in combination with FDC. This regimen led to clinical improvement and microbiological clearance from blood, urine, and rectal swab. The third case described a 49-year-old man with severe bilateral H1N1 pneumonia who required intubation and prolonged extracorporeal membrane oxygenation support. During the ECMO period, *A. baumannii* was isolated from bronchoalveolar lavage, urine, and central venous catheter (Ab. 3). Initial therapy with FDC plus colistin was associated with microbiological failure, leading to a therapeutic switch to FDC combined with AMP/SUL for 14 days. Blood and respiratory cultures subsequently turned negative, although rectal colonization by MDR *A. baumannii* persisted. Despite microbiological clearance, the patient died from refractory septic shock. The absence of microbiological evidence of ongoing infection and the presence of unresponsive vasoplegic shock supported the hypothesis of immune paralysis as the main contributor to the fatal outcome. The fourth case involved a 70-year-old man with COVID-19 pneumonia initially admitted to the SICU for non-invasive ventilation and later transferred to the ICU for invasive mechanical ventilation and tracheostomy. During his ICU stay, he received MEM and linezolid and subsequently developed *Clostridioides difficile* infection treated with fidaxomicin. Clinical deterioration prompted further investigation, and *A. baumannii* was isolated from bronchoalveolar lavage and rectal swab (Ab. 4 and Ab.5). Based on susceptibility testing, therapy was modified to FDC combined with AMP/SUL, leading to clinical improvement and negative follow-up cultures after 14 days.

<i>Clinical cases/strains</i>	<i>Clinical history</i>
Case 1/ Ab.1	COVID-19 pneumonia requiring prolonged non-invasive ventilation; developed <i>A. baumannii</i> infection after prior broad-spectrum therapy. Combined FDC and AMP/SUL treatment led to clinical resolution and microbiological eradication.
Case 2/Ab.2	ICU patient with severe respiratory failure who developed <i>A. baumannii</i> bacteremia. Initial colistin therapy was discontinued due to nephrotoxicity, and infection relapsed. Persistent bacteremia during FDC plus colistin resolved after switching to FDC combined with AMP/SUL, leading to clinical and microbiological recovery.
Case 3/ Ab.3	Severe H1N1 pneumonia requiring ECMO support; developed multi-site <i>A. baumannii</i> infection. Initial FDC plus colistin therapy failed, while FDC combined with AMP/SUL achieved microbiological clearance. The patient died due to refractory septic shock, likely related to immune dysfunction rather than persistent infection.
Case 4/Ab. 4 -Ab.5	COVID-19 pneumonia with progression from non-invasive to invasive ventilation; developed <i>A. baumannii</i> infection following broad-spectrum antibiotic exposure and <i>C. difficile</i> infection. Targeted therapy with FDC plus AMP/SUL resulted in clinical improvement and microbiological clearance.

Table 3. Summary of clinical cases, including patient characteristics and corresponding *A. baumannii* isolates obtained from each patient.

5.3 Methods

5.3.1 Whole-genome sequencing, quality control and bioinformatic analysis

Short-read sequencing libraries were prepared with an Illumina DNA Prep Kit (Illumina Inc. San Diego, CA) and sequenced on an Illumina MiSeq instrument with a 2×300 bp paired-end protocol, as previously described [21]. Quality control and sequence filtering were performed using DRAGEN FastQC + MultiQC version 3.6.3 (<https://basespace.illumina.com/apps/10,562,553/DRAGEN-FastQC-MultiQC> , access date = 20 March 2024). Adapter sequences and low-quality bases were removed using Trimmomatic version 0.39 (https://usegalaxy.eu/?tool_id=toolshed.g2.bx.psu.edu%2Frepos%2Fpjbbriggs%2Ftrimmomatic

%2Ftrimmomatic%2F0.39%2Bgalaxy2&version=latest , access date = 20 March 2024) with standard filtering parameters. Post-trimming quality control was conducted with FastQC to ensure high-quality input for downstream analysis.

ResFinder version 4.4.2 was used to detect acquired ARGs and chromosomal mutations ([https://www.genomicepidemiology.org/ services/](https://www.genomicepidemiology.org/services/) , access date = 22 March 2024). Mobile genetic elements were identified by MobileElementFinder version 1.0.3 (<https://cge.food.dtu.dk/services/MobileElementFinder/> , access date = 25 March 2024). The genome was also assigned to ST using MLST 2.0.9 (<https://cge.food.dtu.dk/services/MLST/> , access date = 25 March 2024). The Virulence Factor Database was used to detect vir- ulence factors (<http://www.mgc.ac.cn/VFs/> , access date = 27 March 2024).

Paired-end reads were assembled with SPAdes Genome Assem- bler version 3.9.0 ([https://usegalaxy.eu/?tool_id=toolshed.g2.bx.psu.](https://usegalaxy.eu/?tool_id=toolshed.g2.bx.psu.edu%2Frepos%2Fnml%2Fspades%2Fspades%2F4.1.0%2Bgalaxy0&version=latest)

[edu%2Frepos%2Fnml%2Fspades%2Fspades%2F4.1.0%2Bgalaxy0&version=latest](https://usegalaxy.eu/?tool_id=toolshed.g2.bx.psu.edu%2Frepos%2Fcrs4%2Fprokka%2Fprokka%2F1.14.6%2Bgalaxy1&version=latest) , access date = 20 March 2024) and the result- ing genomes were annotated using Prokka version 1.14.6 (https://usegalaxy.eu/?tool_id=toolshed.g2.bx.psu.edu%2Frepos%2Fcrs4%2Fprokka%2Fprokka%2F1.14.6%2Bgalaxy1&version=latest , access date = 21 March 2024). Whole-genome sequences of the f ive *A. baumannii* isolates analysed in this study have been de- posited in the GenBank database under the BioProject accession number PRJNA1277157.

5.3.2 Antimicrobial resistance test (MIC)

The phenotype profile has characterized by microdilution method using a bacterial inoculum of 5×10^5 CFU/ml according to clinical and laboratory Standards Institute (CLSI) performance standards. The β -lactams used were FDC, AMP, SUL and FDC/SUL and AMP/SUL combinations. MIC experiments were performed in triplicate. Antimicrobial susceptibility testing was performed using iron-depleted cation-adjusted Mueller–Hinton broth (ID-CAMHB), strictly following EUCAST recommendations to avoid potential bias related to iron availability.

5.3.3 Time kill assay

Time–kill assays were performed to evaluate the bactericidal activity of the tested compounds over a 24-h period. Bacterial strains were grown overnight in Mueller–Hinton broth (MHB) at 37 °C with shaking. The overnight cultures were diluted in fresh MHB to obtain an initial inoculum of approximately 1×10^6 CFU/mL.

Aliquots of the bacterial suspension were exposed to the tested antibiotics at the desired concentrations ($2\times$ MIC), while growth control without antibiotics was included in each experiment. Cultures were incubated at 37 °C with agitation.

At predefined time points (0, 4, 8, 12, and 24 h), samples were collected, serially diluted in sterile saline solution, and plated onto Mueller–Hinton agar plates. Plates were incubated at 37 °C for 18–24 h, after which colonies were counted to determine viable bacterial counts.

5.4 Results and Discussion

5.4.1 Molecular analysis of *A. baumannii* clinical isolates

Given the complexity of the clinical courses and the MDR phenotype observed, WGS was performed to achieve an in-depth genomic characterization of the *A. baumannii* isolates. An additional *A. baumannii* strain was identified, harboring the same resistance genes as the other strains. MLST analysis revealed that all isolates belonged to the same sequence type, namely ST2, which represents the most widely disseminated clone at the international level and is particularly prevalent in Italy. With regard to antimicrobial resistance determinants, WGS identified the presence of multiple resistance genes. Specifically, genes conferring resistance to aminoglycosides were detected, including *aadA*, *aac(6')-Ib3*, *aph(6)-Id*, and *aph(3'')-Ib*, as well as genes associated with resistance to tetracycline and chloramphenicol, namely *tetB* and *catB8*. Resistance to β -lactam antibiotics was mediated by the presence of *ADC-25*, a class C β -lactamase, and *OXA-23* and *OXA-66*, a class D β -lactamases. The *ADC-25* β -lactamase confers resistance to cephalosporins, whereas OXA enzymes are *oxacillinases*. Notably, *OXA-23* and *OXA-66* function as carbapenemases, enzymes capable of hydrolyzing carbapenems and rendering them ineffective. The bioinformatic analysis indicated that no plasmids were found in all strains sequenced. *OXA-66* is an *OXA-51*-like enzyme which differs from *OXA-51* by a single amino acid substitution (L167V); however, this minimal variation is sufficient to confer carbapenemase activity, highlighting the strong impact that subtle sequence changes may have on enzyme function. In contrast, *OXA-23* and *OXA-66* differ by 90 amino acid substitutions and belong to distinct OXA subfamilies, which is consistent with their markedly different carbapenem-hydrolyzing efficiencies. While *OXA-23* is a highly efficient and clinically relevant carbapenemase, *OXA-66* displays a weaker intrinsic activity. Nevertheless,

the expression of OXA-66 represents a potential risk, as its widespread presence in *A. baumannii* and its ability to contribute to reduced carbapenem susceptibility—particularly when overexpressed or combined with additional resistance mechanisms—may facilitate the emergence and amplification of carbapenem resistance (Fig. 12).

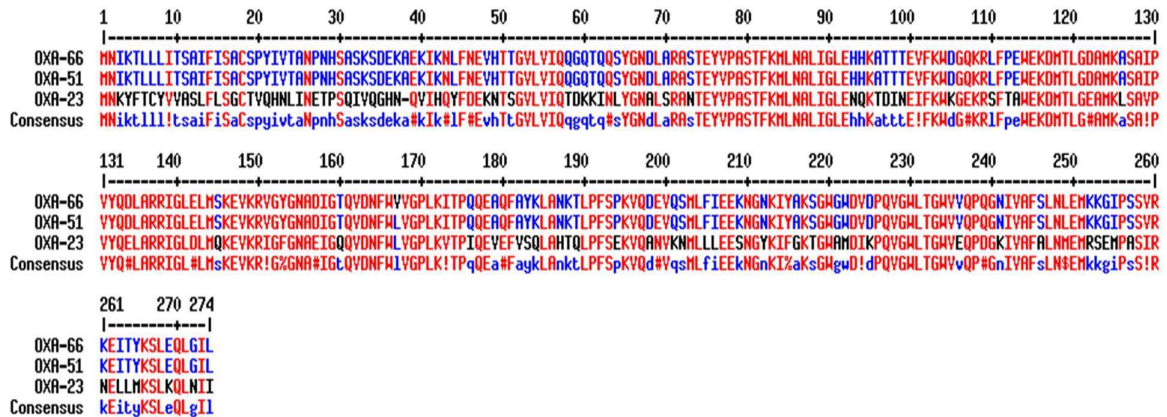


Figure 12. Multiple sequence alignment of OXA-51, OXA-66 and OXA-23 β-lactamases. OXA-66 differs from OXA-51 by a single amino acid substitution (L167V), which is sufficient to confer carbapenemase activity. In contrast, OXA-66 and OXA-23 differ by 90 amino acid substitutions, reflecting their belonging to distinct OXA subfamilies and their different carbapenem-hydrolyzing efficiencies.

In the absence of plasmid-mediated resistance, the dissemination and maintenance of antimicrobial resistance determinants in these *A. baumannii* isolates appear to be primarily driven by chromosomally encoded mobile genetic elements. WGS identified several insertion sequences, including *ISVsa3* (*IS91* family), *ISAb24* (*IS66* family), and *ISAb25* (*IS3* family), highlighting a genomic background characterized by high plasticity. *ISVsa3*, belonging to the *IS91* family, is associated with a rolling-circle-like transposition mechanism and has been implicated in chromosomal rearrangements and mobilization of adjacent genetic regions.

ISAb24, a member of the *IS66* family, is frequently detected in MDR *A. baumannii* and is thought to contribute to genome instability and structural reorganization of resistance-associated loci. *ISAb25*, belonging to the *IS3* family, is widely distributed among high-risk clones and may facilitate intrachromosomal mobility and, in some cases, modulate the expression of nearby resistance genes. Although these insertion sequences do not mediate classical horizontal gene transfer, their combined presence may enhance the stability, expression, and evolutionary persistence of resistance determinants within the chromosome, thereby supporting the multidrug-resistant phenotype observed in ST2 *A. baumannii* isolates (Table 4).

Clinical Isolates	ARGs	B-lactamases	Mobile Genetic Elements (MGEs)
<i>A. baumannii</i> (Ab.1 to Ab.5)	<i>aadA1</i>	<i>blaOXA66</i>	<i>ISVsa3</i> (IS91 family)
MLST ST 2	<i>armA</i>	<i>blaOXA 23</i>	<i>ISAbA24</i> (IS66 family)
	<i>aac(6')-Ib3</i>	<i>blaADC 25</i>	<i>ISAbA25</i> (IS30 family)
	<i>aph(6)-Id</i>		
	<i>aph(3'')-Ib</i>		
	<i>mrsE</i>		
	<i>mphE</i>		
	<i>catB8</i>		
	<i>sulI</i>		
	<i>tetB</i>		
	<i>qacE</i>		

Table 4. MLST, ARGs, and chromosomally encoded MGEs identified by WGS in *A. baumannii* isolates.

The presence of several virulence factors was identified in all five strains analyzed (Table 4). In particular, the genes *adeF*, *adeG*, *adeH*, *bap*, *csuA*, *csuB*, *csuC*, *csuD*, *csuE*, *pgaA*, *pgaB*, *pgaC*, and *pgaD* are involved in biofilm formation, which reduces antibiotic efficacy and mediates adhesion to eukaryotic cells. The presence of genes encoding OMP, such as *OmpA*, was also observed; mutations in these proteins may impair drug entry, as many antibiotics use these channels as their primary route of access into the bacterial cell. Additional virulence factors contributing to bacterial survival within the human host were identified, including the acinetobactin-mediated iron acquisition system (*barA*, *barB*, *basA*, *basB*, *basC*, *basD*, *basF*, *basG*, *basH*, *basI*, *basJ*, *bauA*, *bauB*, *bauC*, *bauD*, *bauE*, *bauF*, *entE*), which plays a crucial role in iron uptake, an essential process for cellular viability. Other virulence determinants involved in immune evasion and adaptation to stress conditions, such as oxidative stress (*katA*), were also detected and are reported in Table 5.

Category	Virulence factors	Target genes
Adherence	Outer membrane protein	<i>ompA</i>
Biofilm formation	AdeFGH efflux pump	<i>adeF</i> ; <i>adeG</i> ; <i>adeH</i> ;
	Biofilm-associated protein	<i>bap</i>
	Csu fimbriae	<i>csuA</i> ; <i>csuB</i> ; <i>csuC</i> ; <i>csuD</i> ; <i>csuE</i> ;
	Polysaccharide poly-N-acetylglucosamine	<i>pgaA</i> ; <i>pgaB</i> ; <i>pgaC</i> ; <i>pgaD</i> ;
Enzyme	Phospholipase C	<i>plc</i>
	Phospholipase D	<i>plcD</i>
Immune evasion	LPS	<i>lpsB</i> ; <i>lpxA</i> ; <i>lpxB</i> ; <i>lpxD</i> ; <i>lpxL</i> ; <i>lpxM</i> ;
Iron uptake	Acinetobactin	<i>barA</i> ; <i>barB</i> ; <i>basA</i> ; <i>basB</i> ; <i>basC</i> ; <i>basD</i> ; <i>basF</i> ; <i>basG</i> ; <i>basH</i> ; <i>basI</i> ; <i>basJ</i> ; <i>bauA</i> ; <i>bauB</i> ; <i>bauC</i> ; <i>bauD</i> ; <i>bauE</i> ; <i>bauF</i> ; <i>entE</i> ;
	Heme utilization	<i>hemO</i>
Regulation	Quorum sensing	<i>abaI</i> ; <i>abaR</i> ;
	Two-component system (BfmRS)	<i>bfmR</i> ; <i>bfmS</i> ;
Serum resistance	PbpG (Penicillin-binding protein)	<i>pbpG</i>
Stress adaption	Catalase	<i>katA</i>

Table 5. Virulence factors identified by whole genome sequencing in *A. baumannii* isolates.

5.4.2 Antimicrobial susceptibility test

A phenotypic analysis of antimicrobial resistance was performed by determining the minimum inhibitory concentrations (MICs) for all five isolates. Susceptibility testing was carried out against FDC, SUL, and AMP, both alone and in combination with SUL. The Ab.2, Ab.3, and Ab.4 isolates were resistant to FDC, AMP, and AMP/SUL. The addition of SUL resulted in a decrease in MIC values exclusively for FDC, lowering them from 16/32 mg/L to 8 mg/L. In contrast, isolates Ab.1 and Ab.5 remained susceptible to FDC (Table 6). It is important to note that FDC activity can be influenced by iron availability in the testing medium due to its siderophore-mediated uptake mechanism; however, testing was performed under standard CAMHB conditions to ensure consistency across all antimicrobial agents and comparability between isolates.

The determination of MIC values allows the selection of optimal conditions for performing time kill experiments.

Clinical isolates	FDC	FDC/SUL	SUL	AMP	AMP/SUL
Ab.1	0,5	0,25	8	>128	32
Ab.2	32	4	8	128	32
Ab.3	16	4	8	>128	32
Ab.4	32	8	8	>128	16
Ab.5	0,25	0,25	32	>128	64

Table 6. Antimicrobial susceptibility test of the five CRAB against selected β -lactams

5.4.3 Time-kill assay

The time-kill assay is a dynamic, quantitative method used to evaluate the bactericidal activity of antimicrobial agents over time. Unlike static measurements such as MICs, which provide a single endpoint of growth inhibition, time-kill studies capture the kinetics of bacterial killing and regrowth, offering a more comprehensive understanding of antibiotic efficacy. In this assay, bacterial cultures are exposed to one or more concentrations of antibiotic, and viable cell counts are determined at multiple time points to assess the rate and extent of killing.

Time-kill experiments are particularly informative for MDR pathogens, such as CRAB, where subtle differences in bactericidal activity may have clinical relevance. This method allows the evaluation of both single-agent activity and potential synergistic effects in combination therapies. In this study, time-kill assays were performed to assess the bactericidal effect of selected β -lactams, including novel agents such as FDC, against clinical *A. baumannii* ST2 isolates. The results were intended to complement genomic analyses, including WGS and resistome profiling, thereby linking genotypic determinants of resistance with their phenotypic consequences on bacterial survival and antibiotic susceptibility. Results were expressed as $\log_{10}$ CFU/mL and plotted as a function of time. Bactericidal activity was defined as a $\geq 3 \log_{10}$ CFU/mL reduction in bacterial count relative to the initial inoculum at each time point. A bacteriostatic effect was defined as a $< 3 \log_{10}$ CFU/mL reduction. Synergy in time-kill experiments was defined as a $\geq 2 \log_{10}$ CFU/mL decrease in bacterial burden for the combination compared with the most active single agent at the same time point.

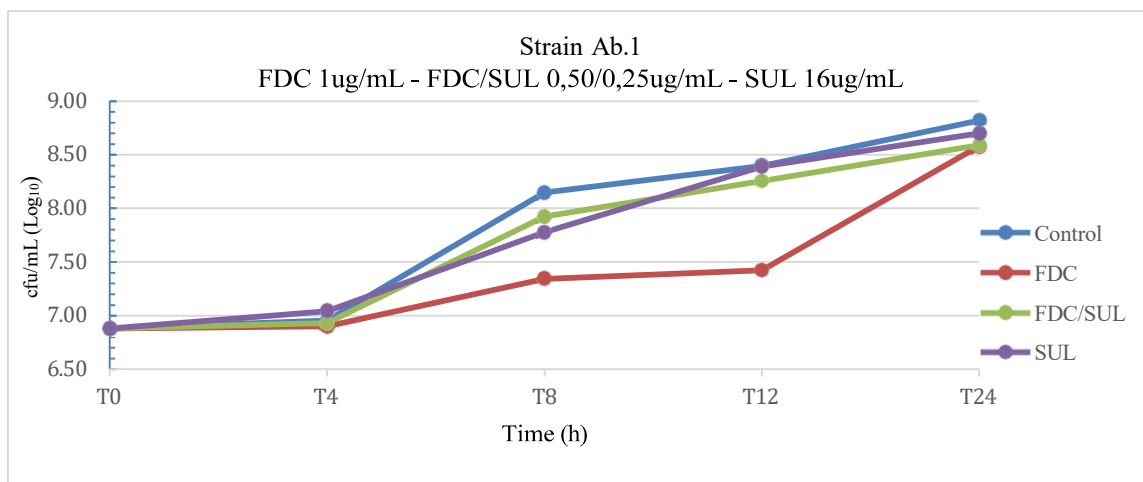


Figure 13. Time-kill curve of *A. baumannii* strain 1 over 24 hours, showing bacterial viability ($\log_{10}$ CFU/mL) in the presence of Cefiderocol (FDC), Cefiderocol–Sulbactam combination (FDC–SUL), and Sulbactam(SUL)

Time-kill experiments performed on isolate Ab.1 (Fig. 13) showed that FDC alone resulted in a reduction of bacterial counts consistent with a bacteriostatic effect, as no $\geq 3 \log_{10}$ CFU/mL decrease from the initial inoculum was observed over the tested time course. SUL alone displayed minimal antibacterial activity. The combination of SUL and FDC did not result in enhanced activity compared with FDC alone, as no $\geq 2 \log_{10}$ CFU/mL difference between the combination and the most active single agent was observed at any time point, indicating absence of synergistic interaction according to the defined criteria.

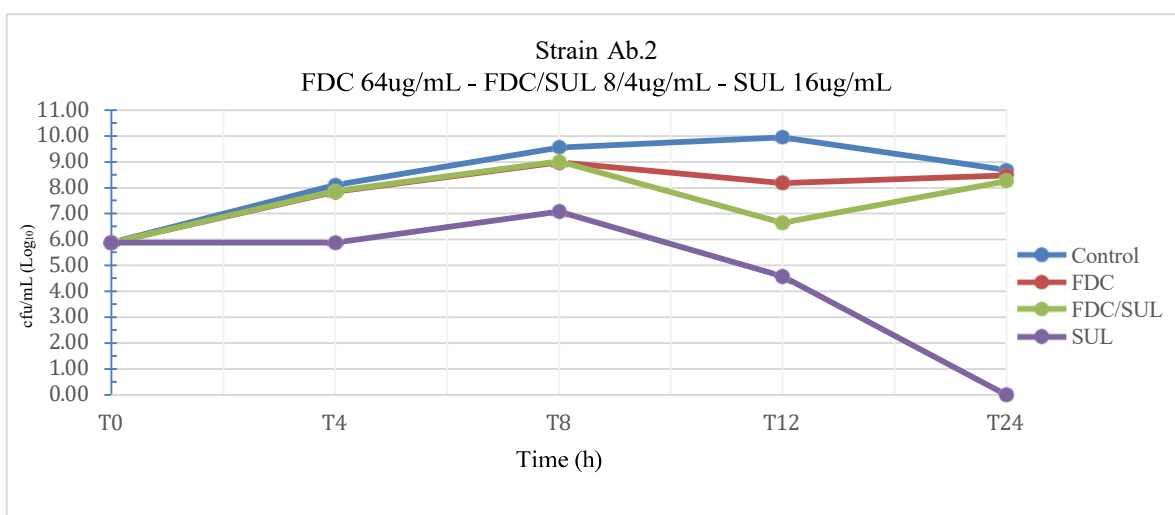


Figure 14. Time-kill curve of *A. baumannii* strain 2 over 24 hours, showing bacterial viability ($\log_{10}$ CFU/mL) in the presence of FDC, FDC-SUL, and SUL.

For isolate Ab.2, FDC alone did not exhibit significant antibacterial activity. The addition of SUL to FDC led to a more pronounced reduction in bacterial counts compared to single-agent treatment with FDC, particularly at intermediate time points. However, regrowth was observed at 24 hours, and bactericidal thresholds were not reached. The SUL showed a better bactericidal capacity. (Fig.14).

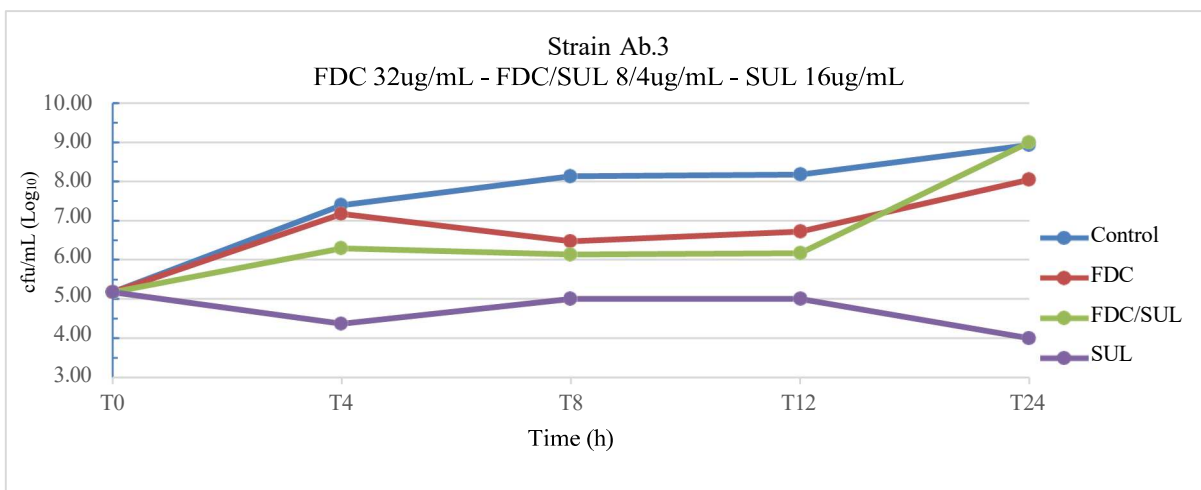


Figure 15. Time-kill curve of *A. baumannii* strain 3 over 24 hours, showing bacterial viability ($\log_{10}$ CFU/mL) in the presence of FDC, FDC-SUL, and SUL.

In isolate Ab.3, SUL alone showed the greatest antibacterial activity over time, resulting in a reduction of bacterial counts but not reaching the $\geq 3 \log_{10}$ CFU/mL threshold required to define bactericidal activity. The combination of FDC and SUL did not display synergistic effects compared with SUL alone, as no $\geq 2 \log_{10}$ CFU/mL additional reduction was observed at any time point. FDC alone showed no appreciable antibacterial activity (Fig. 15).

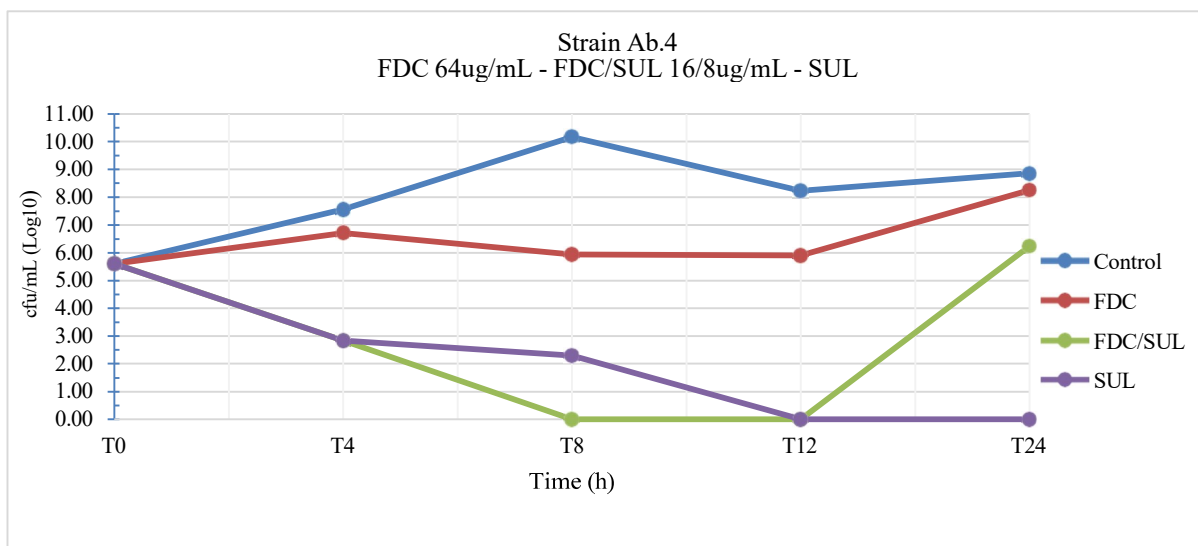


Figure 16. Time-kill curve of *A. baumannii* strain 4 over 24 hours, showing bacterial viability ($\log_{10}$ CFU/mL) in the presence of FDC, FDC-SUL, and SUL.

For isolate Ab.4, the FDC-SUL combination produced a reduction in bacterial burden compared to FDC alone, particularly during the early phases of the assay. SUL showed strong and sustained antibacterial activity over time. Overall, combination of two antimicrobial agents improved activity than FDC alone (Fig.16).

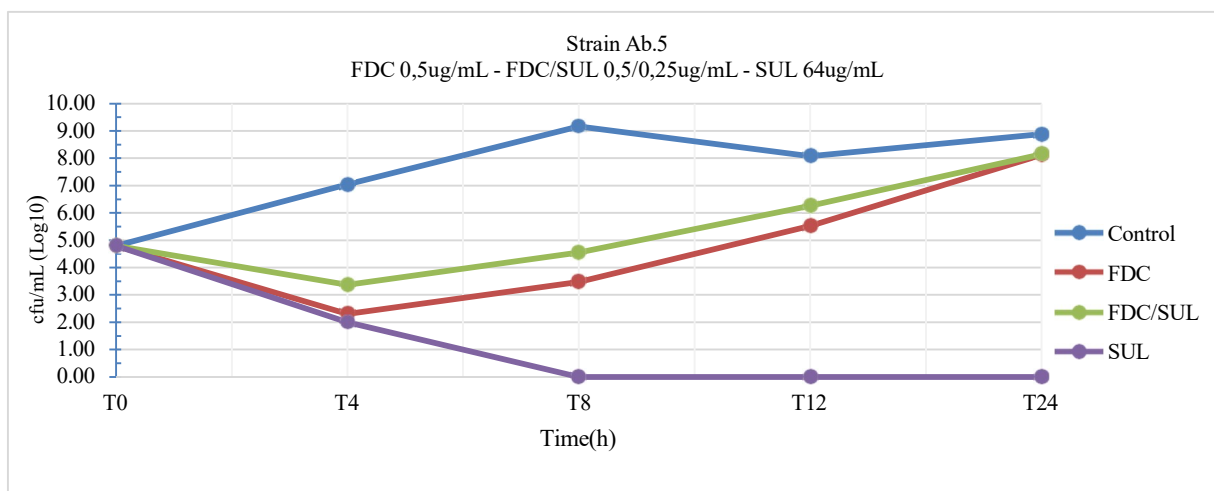


Figure 17. Time-kill curve of *A. baumannii* strain 5 over 24 hours, showing bacterial viability ($\log_{10}$ CFU/mL) in the presence of FDC, FDC-SUL, and SUL.

For Ab.5 isolate, SUL alone resulted in a reduction of bacterial counts over the course of the experiment. FDC did not demonstrate sustained antibacterial activity. The addition of SUL to FDC did not enhance or prolong the inhibitory effect compared with SUL, with no evidence of

synergistic activity according to the predefined criteria.

The time-kill experiments revealed a marked strain-dependent variability in the activity of FDC, SUL, and their combination. Overall, these data highlight the limited and inconsistent efficacy of FDC alone, contrasted with the more robust and sustained antibacterial activity of SUL, either alone or, in selected cases, in combination.

Across the tested isolates, FDC alone generally failed to achieve bactericidal activity, exerting at best a bacteriostatic or transient inhibitory effect. In several isolates (Ab.2, Ab.3, and Ab.5), the FDC alone did not demonstrate significant or sustained antibacterial activity, with bacterial regrowth observed at later time points. These findings suggest that, in this strain collection, FDC activity may be compromised by isolate-specific resistance mechanisms, limiting its effectiveness as a single agent.

In contrast, SUL displayed a more consistent antibacterial profile, showing effective and sustained bacterial killing in multiple isolates (Ab.3, Ab.4, and Ab.5), and outperforming FDC in terms of bactericidal capacity. The combination of FDC-SUL did not consistently result in synergistic effects. While in Ab.2 and Ab.4 isolates the addition of SUL to FDC led to an enhanced early reduction in bacterial counts compared to FDC alone, this effect was often transient, with regrowth occurring at 24 hours and bactericidal thresholds not being achieved. In other isolates (Ab.1, Ab.3, and Ab.5), the combination failed to improve activity relative to SUL alone, indicating a lack of additive or synergistic interaction. Taking together, these results suggest that the therapeutic benefit of combining FDC with SUL is isolate-dependent and largely driven by the activity of SUL rather than true pharmacodynamic synergy. The absence of sustained bactericidal effects with the combination, particularly in comparison to SUL, underscores the need for cautious interpretation of combination strategies involving FDC against *A. baumannii*. Further investigations integrating molecular resistance profiling would be required to better define the conditions under which this combination may provide clinical benefit.

CHAPTER 6

“Molecular Characterization of PBP3”

6.1 Specific Aim

The aim of this chapter is to molecularly and functionally characterize the **PBP3** gene in the five clinical *A. baumannii* strains through Sanger sequencing and subsequent analysis of PBP activity. The decision to investigate PBP3 was driven by the resistance profiles observed in the strains, where resistance to FDC and other β -lactams could potentially be mediated by mutations or alterations in this PBP. The presence of mutations K235N/H370Y in the analyzed PBP3 prompted a detailed investigation to understand the impact of these mutations on protein binding, with *PBP activity* characterized using *bocillin*, evaluating protein binding at different concentrations of the antibiotic of interest, and *binding kinetics* of PBP with bocillin.

6.2 Methods

6.2.1 PCR amplification of PBP3

The PBP3 genes were amplified by PCR from Ab.1, Ab.3, and Ab.5 strains. The reaction mix (total volume 50 μ L) contained 10 ng of genomic DNA, forward and reverse primers at a final concentration of 0.4 μ M each, 10 μ L 5 $\times$ Reaction Buffer (Wonder Start), 0.5 μ L Wonder Taq Hot Start (5 U/ μ L), and nuclease-free water. PCR amplification was performed under the following cycling conditions: initial denaturation at 95°C for 1 min, followed by 30 cycles of 95°C for 40 sec, annealing at primer-specific temperatures determined for each primer pair on their melting temperatures (T_m), set 1–2°C below the lowest T_m of each pair to ensure optimal specificity (Table 7), and 72°C for 1 min, with a final extension at 72°C for 7 min, followed by storage at 4°C. PCR products were verified by agarose gel electrophoresis (1% agarose) and purified using the Gel & PCR DNA Purification Kit (Euroclone).

Primers	Target	Sequenza (5'-3')	T _m (°C)
FOR	PBP3	ATGTGGCGGTTTTATCTGCT	57.0
REV	PBP3	TTACCTGCGAATAGGATTTTCTG	55.25
1R	PBP3	TCTTCAAACGTGGGTTCTGC	57.3
2F	PBP3	TCGGCAARGTTTCTTTCG	51.4
3R	PBP3	TTATTACCCRCAACCTCAGC	55.24
4F	PBP3	TCAAACCAATAATTGGGCATT	57.7

Table 7. Primers used for PCR amplification and sequencing of PBP3 gene

6.2.2 Sanger sequencing of PBP3

Purified PCR products were sequenced using the Sanger method. The sequencing reaction contained 3 μ L purified PCR product, 4 μ L 5 $\times$ sequencing buffer, 4 μ L primer (0.8 pmol/ μ L), 2 μ L BigDye terminator, and 7 μ L nuclease-free water. The sequencing cycling protocol consisted of an initial denaturation at 94°C for 10 min, followed by 25 cycles of 96°C for 10 sec, 55°C for 5 sec, and 60°C for 4 min, with a final hold at 10°C. The sequencing products were purified by ethanol/NaAc precipitation: 20 μ L DNA, 2 μ L NaAc, and 50 μ L 100% ethanol were incubated at room temperature for 10–15 min, centrifuged at 14,000 rpm and 4°C for 35 min, washed with 100 μ L 70% ethanol, and air-dried at 37°C for 30 min. The pellet was resuspended in 20 μ L formamide and diluted 1:10, followed by denaturation at 100°C for 10 min. Capillary electrophoresis was performed using the 3500 Genetic Analyzer (Applied Biosystems), and sequence data were analyzed accordingly.

6.2.3 Cloning and subcloning of PBP3 variants

The amplified PBP3 genes from strains Ab.1, Ab.3, and Ab.5 were initially cloned into the pCR-Blunt-II-TOPO vector (Figure 19) and subsequently subcloned into the pET-28a(+) expression vector (Figure 19) to introduce an N-terminal 6 $\times$ His tag. Recombinant plasmids were inserted by transformation into *E. coli* BL21(DE3) competent cells. Cultures were grown

in superoptimal broth (SOB) supplemented with M9 salts at 37°C with shaking until reaching an optical density at 600 nm of 0.6. Protein expression was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), and cultures were incubated at 16°C with shaking for 18 h.

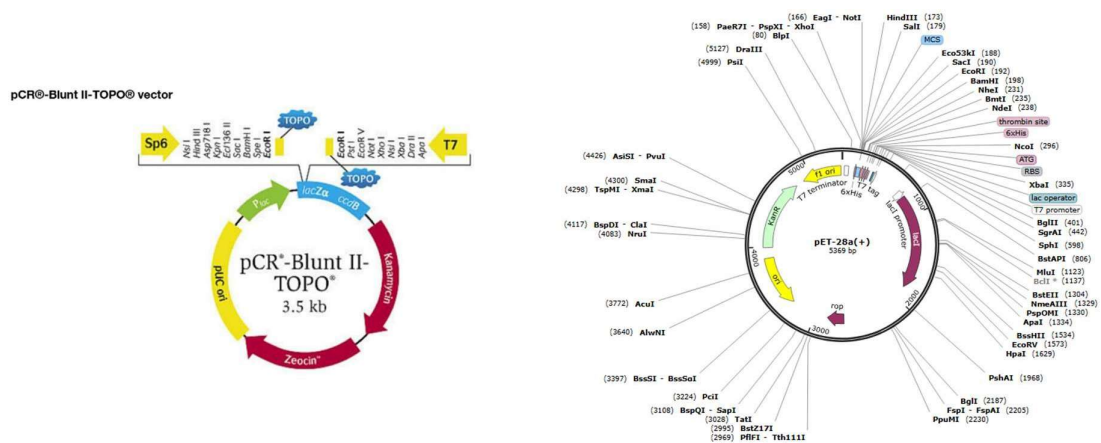


Figure 19. Schematic representation of the two cloning vectors used in this study: pCR™-Blunt II-TOPO®, employed for initial PCR product cloning, and pET-28a(+), used for expression of recombinant proteins.

6.2.4 Purification of His-tagged PBP3

Cells were harvested by centrifugation, and His-tagged PBP3 proteins were purified using Ni²⁺-nitrilotriacetic acid affinity chromatography (Qiagen), following the manufacturer's instructions. The purity of the eluted fractions was assessed by SDS-PAGE.

6.2.5 Bocillin assay protocol

The binding affinity between *PBPs* and selected antibiotics, as well as the impact of mutations on inhibitor interaction, was evaluated using a *bocillin*-based competition assay. Fluorescence was detected on 12.5% SDS-PAGE gels using a ChemiDoc imaging system. Fluorescence intensity was inversely correlated with the affinity of the *PBP* for the tested antibiotic. Increasing concentrations of antibiotics were incubated with *PBP3* (5 μ g) at 37 °C for 20 minutes. A fixed concentration of 100 μ M of *bocillin* was added, followed by an additional incubation at 37 °C for 20 minutes. Prior to antibiotic exposure, inhibition of β -lactamase activity was required to prevent antibiotic degradation. Therefore, SUL was included at a constant concentration of 1200 μ M and incubated with the protein for 30 min at 37 °C (Figure 20). Specifically, PBP3 and its variants were pre-incubated with SUL prior to the assay in order to minimize hydrolysis of the β -lactam substrate during the experimental readout,

resulting from the intrinsic low-level β -lactam hydrolytic activity of PBP3. Although SUL is known to interact with PBPs, including PBP3, it was used at a fixed concentration across all experimental conditions. Therefore, any potential contribution of SUL to PBP binding is expected to be constant and not to affect the relative comparison of IC₅₀ values among different β -lactams. Moreover, its inclusion allowed preservation of substrate stability, ensuring more reliable binding measurements.

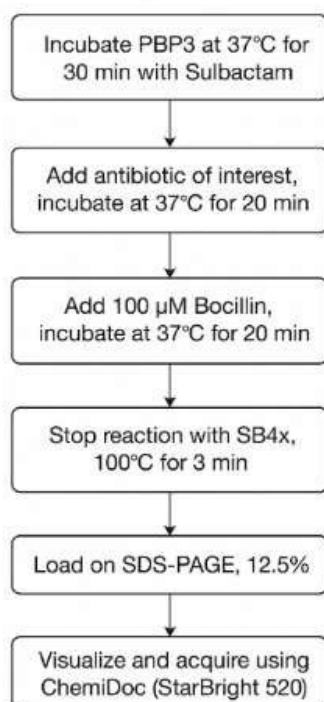


Figure 20: Overview of the experimental workflow for the assay

6.2.6 Determination of kinetic parameters for the *Bocillin*–PBP3 interaction

To characterize the kinetics of the interaction between *bocillin* and PBP3, the initial reaction velocity (v) was analyzed as a function of substrate concentration ($[S]$). The experimental data were interpreted according to the **Michaelis–Menten** model:

$$v = \frac{V_{\max}[S]}{K_m + [S]}$$

where $V_{\max}$ represents the maximum reaction velocity of the enzymatic system, and K_m is the substrate concentration required to reach half of $V_{\max}$.

In our assays, the fluorescence intensity (FI) of *bocillin* bound to PBP3 was used as a direct readout of enzyme-substrate interaction. The observed FI was integrated into the Michaelis–Menten equation as follows¹⁵⁶:

$$FI_{\text{observed}} = FI_{\max} \cdot \frac{[Bocillin]}{K_m + [Bocillin]}$$

where $FI_{\max}$ represents the maximum fluorescence at substrate saturation, and K_m corresponds to the Michaelis constant of *bocillin* for PBP3. This approach allows direct quantification of the enzyme kinetics while accounting for the fluorescent readout of substrate binding. The Michaelis–Menten model was applied as an empirical approach to describe the saturation behavior of fluorescence binding data. It should be noted that this model was used as a phenomenological fit and does not reflect catalytic turnover, but rather provides an approximation of binding kinetics under the experimental conditions.

6.3 Results and Discussion

6.3.1 PBPs molecular analysis

The PBP3 gene was amplified using genomic DNA of the five clinical *A. baumannii* strains as template. The sequencing of corresponding amplicons revealed distinct mutational profiles (Figure 21 and Table 7). Amino acid substitutions were annotated using the *A. baumannii* PBP3 (*FtsI*) reference sequence (NCBI Protein accession: SUU29918), and all positions refer to the mature protein after signal peptide cleavage.

Strain Ab.1 presented K235N amino acid substitution in PBP3, the Ab.2, Ab.3, and Ab.4 strains harbored both K235N and H370Y mutations. Ab.5 strain did not show any mutations in PBP3. The genes encoding for PBP3, PBP3^{K235N}, PBP3^{K235N/H370Y} were cloned in overexpression system and the corresponding proteins were purified. The purification was performed using one chromatographic step, the affinity chromatography by His-tag system. At the end of protocol purification, we obtained the following quantity of proteins: 0.5 mg/ml for PBP3, 0.4 mg/ml for PBP3^{K235N}, 0.5 mg/ml for PBP3^{K235N/H370Y}. Despite using the pET-28 cloning system, no marked overexpression of the proteins was observed in the employed biological system.

Clinical isolates	PBP3_variant
Ab.1	K235N
Ab.2	K235N, H370Y
Ab.3	K235N, H370Y
Ab.4	K235N, H370Y
Ab.5	No mutation

Table 7. Mutations identified in PBP3 genes from *A. baumannii* strains



Figure 21. Amino acid sequence comparison of the three PBP3 proteins from clinical *A. baumannii* isolates relative to the WT reference. Amino acid substitutions identified in each PBP are highlighted.

PBP3 enzymes are essential in clinical settings, as they represent the most common and ideal targets of β -lactam antibiotics. The constant selective pressure at the clinical level can drive the emergence of mutations such as those observed, which may contribute to antibiotic resistance and help explain the complex MDR profiles of these isolates, as previously discussed. Among the identified substitutions, **H370Y** appears to be the one most likely involved in carbapenem resistance. Observing the clinical case, the resistance profile, and the presence of PBP3 mutations, it was deemed particularly interesting to characterize each PBP3 variant at the kinetic level.

6.3.2 Characterization of PBP Activity in Response to β -Lactam Antibiotics

The K_m values for bocillin of PBP3, PBP3^{K235N} and PBP3^{K235N/H370Y} were determined using a gel-based activity assay and were $23 \pm 1.4 \mu\text{M}$, $38 \pm 2.7 \mu\text{M}$ and $80 \pm 4.1 \mu\text{M}$, respectively. The presence of mutations in PBP3 reduced the affinity for bocillin, as indicated by the increased K_m values. The ability of β -lactams to interact with PBP3 and its variants was assessed using a bocillin-based competition assay in SDS-PAGE. IC₅₀ values were determined as the β -lactam concentration causing a 50% reduction in bocillin fluorescence intensity (FI). The β -lactams tested included AMP, FDC, MEM and IPM. As shown in Figure 22 and 23, increased concentration of AMP and FDC (10 -1200 μM) did not result in the reduction of bocillin fluorescence in PBP3, PBP3^{K235N} and PBP3^{K235N/H370Y}. However, no differences were observed in the affinity of AMP and FDC across all PBPs.

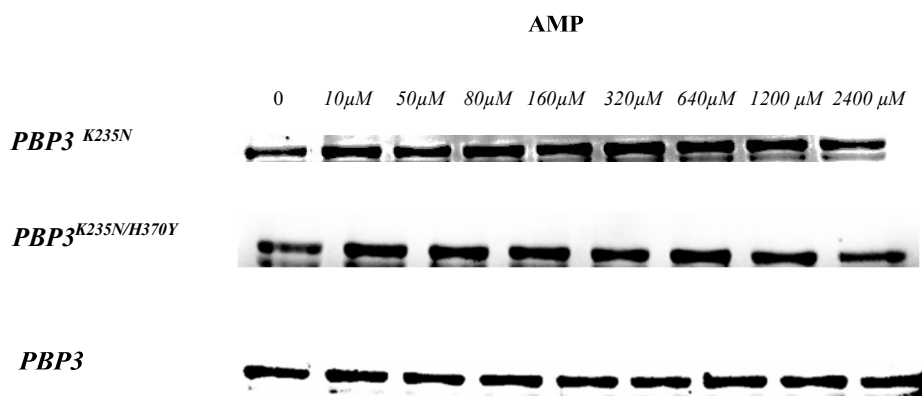


Figure 22. Binding pattern of AMP in PBP3 variants. The indicated β -lactam concentrations were used during the 20 minute binding reaction before bocillin FL was added.

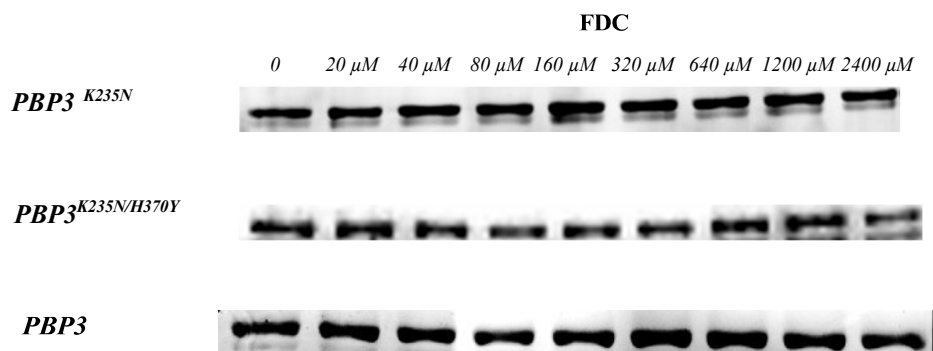


Figure 23. Binding pattern of FDC in PBP3 variants. The indicated β -lactam concentrations were used during the 20 minute binding reaction before bocillin FL was added.

Markedly different behavior was observed for the carbapenems IPM and MEM. In the case of **PBP3**, gel images showed a progressive decrease in fluorescence intensity with increasing carbapenem concentrations (Fig.24, 25). Under these conditions, it was possible to calculate IC₅₀ values for both carbapenems, which were estimated at $110 \pm 3.1 \mu\text{M}$ for **IPM** and $80 \pm 1.3 \mu\text{M}$ for **MEM**. In contrast, neither IPM nor MEM displayed significant inhibitory activity toward the mutants, since no decrease in band fluorescence intensity was observed for **PBP3**^{K235N} and **PBP3**^{K235N/H370Y}, suggesting a substantial reduction or loss of carbapenem binding. These findings indicate that the introduced mutations may directly affect PBP–carbapenem interactions, thereby reducing inhibitory efficacy relative to **PBP3**. Consistent with these observations, previous studies have implicated the *H370Y* mutation in carbapenem resistance mechanisms. Although the precise contribution of this mutation remains to be fully elucidated, the results obtained in this study appear to be in agreement with existing literature.

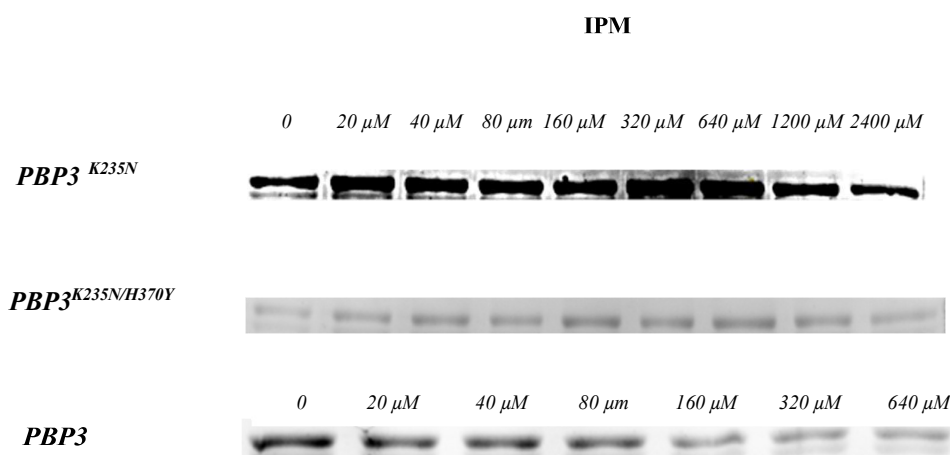


Figure 24. Binding pattern of IPM in PBP3 variants. The indicated β -lactam concentrations were used during the 20 minute binding reaction before bocillin FL was added.

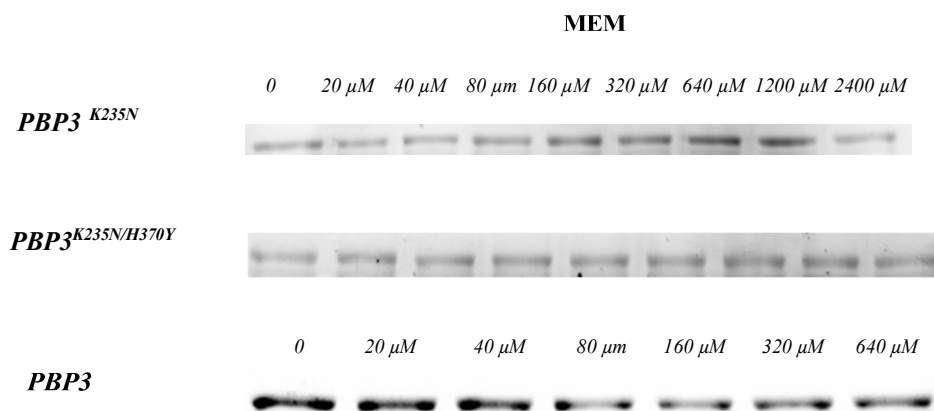


Figure 25. Binding pattern of MEM in PBP3 variants. The indicated β -lactam concentrations were used during the 20 minute binding reaction before bocillin FL was added.

The selection of β -lactam compounds used in the bocillin competition assays was influenced by both experimental and clinical considerations. Initial assays were affected by rapid substrate degradation, consistent with the intrinsic low-level β -lactam hydrolytic activity associated with PBP3 and its variants. To address this limitation, assay conditions were optimized through SUL pre-incubation to ensure reliable assessment of PBP3– β -lactam interactions. In addition, the panel of compounds was expanded to include the clinically relevant carbapenems IPM and MEM, in order to provide a broader assessment of PBP3 binding spectrum and to incorporate antibiotics of high therapeutic importance in Gram-negative infections. This choice was guided by both previously published literature and the need to strengthen the clinical relevance of the biochemical findings. However, MIC determinations for IPM and MEM were not performed on the clinical isolates within the timeframe of the study, and therefore phenotypic susceptibility data are not available for direct comparison with the biochemical binding results. Future studies will focus on determining the MIC values of IPM and MEM in the clinical isolates in order to complement the biochemical binding data and enable a more comprehensive comparison between target affinity and phenotypic susceptibility.

CONCLUSIONS

The production of carbapenem-hydrolysing β -lactamases represents one of the most widespread mechanisms responsible for CRAB. Several carbapenemases belonging to class D and class C serine β -lactamases have been described; however, resistance in this pathogen cannot be attributed exclusively to enzymatic hydrolysis. Additional mechanisms, including efflux pump overexpression, porin alteration or loss, and modifications affecting PBPs, contribute substantially to the resistant phenotype observed in clinical isolates.

Therapeutic options for CRAB remain extremely limited. Clinicians treating critically ill patients frequently rely on β -lactam–SUL combinations or on agents with suboptimal efficacy or toxicity concerns, such as polymyxins and tigecycline. In this context, FDC has emerged as a promising option, although variability in clinical and microbiological responses has been increasingly reported.

In the present study, five *A. baumannii* clinical isolates (Ab.1–Ab.5), recovered from critically ill patients treated with FDC plus AMP/SUL therapy, were investigated through an integrated genomic, phenotypic, and biochemical approach combining WGS, MIC determination, time-kill assays, and functional characterization of PBP3 variants.

WGS analysis highlighted the importance of comprehensive genomic characterization for understanding antimicrobial resistance. Multiple antimicrobial resistance genes, virulence determinants, and mutations located in functionally relevant genomic regions were identified across the isolates, supporting the concept that resistance in MDR *A. baumannii* results from the combined action of several mechanisms rather than from a single determinant.

MIC analyses demonstrated isolate-dependent susceptibility patterns. Isolates Ab.2, Ab.3, and Ab.4 exhibited reduced susceptibility to FDC, whereas Ab.1 and Ab.5 remained susceptible. The addition of SUL reduced FDC MIC values in Ab.2–Ab.4, suggesting a partial restoration of activity likely linked to inhibition of β -lactamase-mediated effects. However, SUL did not universally restore susceptibility, indicating that β -lactamase inhibition alone is insufficient to overcome resistance and that additional mechanisms, including target modification and strain-specific genomic context, contribute to antimicrobial response.

The efficacy of the combination therapy was further evaluated through time-kill assays. For most analyzed strains, particularly Ab.2–Ab.4, the combination of SUL with FDC enhanced antibacterial activity during the first 12 hours of incubation, restoring early bactericidal effects. Nevertheless, this activity was not maintained at 24 hours.

This time-dependent reduction in activity may suggest a decrease in FDC effectiveness during prolonged incubation, potentially related to antibiotic instability or adaptive bacterial responses; however, no stability measurements were performed in this study, and this interpretation remains hypothetical.

Overall, these results indicate that the benefit of combination therapy is strain-dependent rather than universally demonstrable, emphasizing the importance of dynamic pharmacodynamic evaluation beyond static MIC measurements.

Sequencing analysis revealed distinct PBP3 mutational profiles. The Ab.1 isolate carried the K235N substitution, Ab.2–Ab.4 harbored combined K235N/H370Y mutations, and Ab.5 showed no detectable PBP3 alterations. Fluorescence-based kinetic analyses using bocillin as a reporter provided a detailed characterization of PBP3–antibiotic interactions. Under the experimental conditions employed, PBP3 exhibited limited apparent affinity for AMP and FDC, whereas measurable binding was observed for IPM and MEM, allowing determination of IC₅₀ values. Mutant PBP3 variants, particularly K235N and K235N/H370Y, showed marked reduction or complete loss of carbapenem interaction, indicating that these substitutions significantly alter the antibiotic binding site. None of the additional tested β -lactams demonstrated substantial inhibition of mutant PBP3, suggesting a severely restricted therapeutic window for isolates carrying these variants. Importantly, the limited apparent affinity detected for FDC does not contradict its recognized role as a PBP3-targeting antibiotic. Rather, it suggests that the interaction may not be fully captured by the *in vitro* fluorescence assay and may depend on structural or dynamic properties not reproduced under experimental conditions. The observed phenotypes align with previously reported roles of the H370Y substitution in carbapenem resistance and increased β -lactam resistance.

Integration of phenotypic data with biochemical analyses indicates that PBP3 mutations contribute to resistance phenotypes but do not independently determine antimicrobial susceptibility. Isolates carrying K235N/H370Y mutations (Ab.2–Ab.4) exhibited reduced FDC susceptibility and derived the greatest early benefit from SUL combination therapy, yet variability in MIC values and killing kinetics demonstrates that antibiotic response results from the interaction between target modification, β -lactamase activity, and broader genomic background.

Collectively, these findings support a multifactorial model of resistance in which enzymatic degradation, altered target affinity, and strain-specific determinants act simultaneously to modulate antimicrobial efficacy.

This work also highlights the utility of bocillin-based kinetic assays as tools for probing PBP function and inhibitor susceptibility. Although the kinetic framework applied represents an empirical model for binding behavior rather than catalytic turnover, it enabled comparative evaluation of antibiotic–PBP interactions and provided mechanistic insights into resistance-associated mutations.

Several limitations should be acknowledged. The study involved a limited number of clinical isolates, and experiments were conducted under controlled in vitro conditions that may not fully reflect in vivo pharmacokinetics or host environments. Furthermore, the potential contribution of antibiotic stability over extended incubation periods was not experimentally assessed.

Future studies involving larger isolate collections, structural analyses, and in vivo validation will be necessary to further clarify the clinical relevance of these observations.

From a clinical perspective, the present findings suggest that FDC-based combination therapies may provide benefit primarily in selected isolates with reduced baseline susceptibility rather than representing a universally effective strategy against MDR *A. baumannii*. The pronounced isolate-dependent variability observed throughout this study supports precision antimicrobial stewardship approaches integrating genomic, phenotypic, and functional data to guide therapeutic decisions.

Final remarks

Overall, this thesis provides an integrated characterization of resistance mechanisms in MDR *A. baumannii*, demonstrating how β -lactamase activity, PBP3 mutations, and genomic background collectively shape antibiotic response. Importantly, this work underscores the critical role of target-site mutations in shaping antibiotic resistance. Alterations affecting key therapeutic targets, such as PBP3, represent a frequently underestimated variable in clinical resistance assessment. As demonstrated in this study, mutations within the antibiotic target can significantly impair drug binding and contribute to a multilayer resistance phenotype, further restricting therapeutic options. Awareness of this target-mediated component is essential from a clinical perspective, as failure to recognize its contribution may lead to underestimation of resistance complexity and compromise effective management of MDR *A. baumannii* infections. By combining WGS, phenotypic susceptibility testing, time-dependent pharmacodynamic analyses, and functional protein characterization, this work contributes mechanistic insights into β -lactam resistance and offers a framework for future strategies aimed at overcoming therapeutic limitations in this clinically challenging pathogen.

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