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ORIGINAL RESEARCH

The Impact of Ser165Glu Substitution Within the Ω-Loop on the Hydrolytic Activity of a Novel Ambler Class A β-Lactamase Variant LAP-3

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Introduction: Lactamase activity proteins (LAPs) are narrow-spectrum β-lactamases identified within the Enterobacteriaceae family. In this research, we uncovered a novel variant, LAP-3, while investigating quinolone and carbapenem resistance in *Klebsiella pneumoniae*. Consequently, we aimed to elucidate the hydrolytic profile and to identify and characterize the new β-lactamase, LAP-3.

Methods: Antimicrobial susceptibility was assessed using the agar dilution method. The $bla_{\text{LAP-3}}$ gene was analyzed using PCR, and genomic DNA was extracted for whole-genome sequencing and plasmid mapping. The gene was cloned to analyze the hydrolysis spectrum and biochemical characteristics of LAP-3. Protein structure was analyzed using ChimeraX.

Results: Genome sequencing and BLAST analysis revealed a substitution of glutamic acid (Glu165) with serine (Ser165) in LAP-3 compared to LAP-2 within the Ω-loop. The plasmid sequence containing $bla_{\text{LAP-3}}$ revealed that the gene was situated within the multidrug resistance unit of TnpA-tet(A)-$bla_{\text{LAP-3}}$-qnrS1-TnpR. Structural analysis revealed that Glu165 in LAP-3 formed hydrogen bonds with Glu163 and Asn167, in contrast to Ser165 in LAP-1 and LAP-2. The cloned $bla_{\text{LAP-3}}$ gene resulted in elevated MIC levels for Amoxicillin (64-fold), Piperacillin (64-fold), cefuroxime (16-fold), and Cephalothin (32-fold) and while conferring resistance to clavulanic acid and tazobactam by increasing their MICs 8- and 4-fold, respectively, thereby indicating an expanded resistance spectrum.

Conclusion: The findings reveal that the novel Ambler class A β-lactamase LAP-3 demonstrates an expanded hydrolysis spectrum against Penicillins, certain cephalosporins, and β-lactamase inhibitors. The Ser165Glu substitution within the Ω-loop may influence the resistance characteristics associated with LAP β-lactamases.

Keywords: $bla_{\text{LAP-3}}$, resistance, variant, β-lactamase, Ω-loop

Introduction

Klebsiella pneumoniae (*K. pneumoniae*) is a significant hospital-acquired pathogen, with its resistance to β-lactam antibiotics posing a considerable global threat. High Prevalence Regions like Greece, Italy, Israel, and parts of the USA report carbapenem resistance rates in invasive isolates often exceeding 25–50% in some healthcare settings, other emerging Regions like China, India, and Brazil have seen a dramatic rise in resistant *K. pneumoniae*, linked to the spread of specific plasmids carrying resistance genes. Patients infected with antibiotic-resistant strains face a grim prognosis and are susceptible to hospital outbreaks, significantly increasing the healthcare burden. A plethora of resistance genes, particularly those responsible for encoding β-lactamases, have been documented. Nonetheless, the variety of acquired narrow-spectrum penicillinases remains limited within gram-negative organisms. The most frequently cited β-lactamases belong to Ambler class A β-lactamases, such as temoniera (TEM) and sulfhydryl variable (SHV).[1] Numerous TEM- and SHV-type β-lactamases exhibit an expanded hydrolysis spectrum, encompassing that of cephalosporins.

Narrow-spectrum β-lactamases are enzymes that are effective against narrow-spectrum antibiotics, such as Penicillin antibiotics. However, the resistance of *K. pneumoniae* are predominantly attributed to extended-spectrum β-lactamases

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(ESBLs) or carbapenems rather than traditional narrow-spectrum lactamases. Therefore, *K. pneumoniae* exhibits limited expression of narrow-spectrum β-lactamases (enzymes that target Penicillin) and acquires multidrug resistance through the production of ESBLs or carbapenems.[2] According to the Ambler molecular classification system, the narrow-spectrum β-lactamases found in gram-negative bacteria, including *K. pneumoniae*, chiefly classified as A-class enzymes, exemplified by TEM, SHV, and carbenicillinases. This enzyme is characterized by its reliance on serine as the active center, predominantly hydrolyzing Penicillin antibiotics. Due to the extensive utilization of antibiotics, variants of β-lactamase have broadened their hydrolytic capabilities towards specific cephalosporins and β-lactamase inhibitors; however, they still display diminished hydrolytic activity against carbapenems.

LAP-1, a narrow-spectrum β-lactamase, was initially identified in isolates of Escherichia cloacae in 2006.[3] It exhibits a hydrolysis spectrum that includes Penicillins and several cephalosporins, such as Cephalothin and cefuroxime, while retaining susceptible to carbapenem antibiotics and the majority of cephalosporins and is inhibited by CLA. Subsequently, LAP-2—produced by clinical isolates of *Enterobacter cloacae* and *K. pneumoniae*—was recognized,[4,5] exhibiting an antibiotic spectrum akin to that of LAP-1. Although bla_{LAP} exhibits a narrow spectrum against β-lactams, it is frequently regarded as closely linked to extended-spectrum β-lactamases and carbapenemases, such as $bla_{\mathrm{CTX\text{-}M}}$, bla_{VIM}, bla_{NDM}, and bla_{KPC}, along with quinolone resistance genes *qnr*A1 and *qnr*B1.[6–9]

In this study, we detected one strain of *K. pneumoniae* harboring bla_{LAP} gene. Based on sequence analysis, we identified and characterized a novel β-lactamase, designated LAP-3, which bears resemblance to LAP-2 yet presents a distinct hydrolysis spectrum.

Materials and Methods

Bacterial Strains

In 2024, the strain of *K. pneumoniae* carrying $bla_{\mathrm{LAP\text{-}3}}$, designated as KP2CSL, was isolated from a neonatal patient in the NICU of the First People's Hospital of Yongkang, Zhejiang, China. This strain was chosen due to its resistance to quinolone and carbapenem antibiotics, as identified in a survey carried out at this facility. The study received approval from the Research Ethics Committee of The First People's Hospital of Yongkang (approval number: YKSRMYYEC2024-KT-HS-031). Species identification was conducted utilizing the Vitek-60 microorganism auto-analysis system (BioMérieux Corporate, Craponne, France), followed by biochemical identification and 16s rRNA genomic sequencing. The isolate was referred to as KP within the context of *K. pneumoniae* strains.

Antimicrobial Susceptibility Test

MICs of 12 antimicrobials (Amoxicillin, Amoxicillin+clavulanic acid, Piperacillin, Piperacillin+tazobactam, Ceftazidime, Cefepime, Cefuroxime, Cephalothin, Cefotaxime, Aztreonam, Imipenem and Levofloxacin) was determined using agar dilution with final concentration ranges of 0.0625–256 μ g/mL for Imipenem and 1–2048 μ g/mL for the other 11 antimicrobials. The strains were cultivated in a 37°C incubator for 16–24 hours. Susceptibility patterns were interpreted based on the breakpoint criteria (M100-Ed34), with *E. coli* ATCC 25922 serving as the quality control strain.

DNA Extraction and Whole-Genome Sequencing

Genomic DNA was extracted using an AxyPrep Bacterial Genomic DNA Miniprep kit (Axygen Scientific, Union City, CA, USA). A DNA library with an average insert size of 400 bp was prepared using the Next Generation Sequencing library preparation kit and sequenced using the Illumina NovaSeq platform (paired-end; 2×150 bp). Additionally, for whole-genome sequencing of a specific strain, a 10–20 kb insert library was prepared and sequenced using the Oxford Nanopore Technology sequencer (Personalbio Technology Co., Ltd., Shanghai, China).

Genome Assembly and Annotation

The complete genome was assembled using Unicycler and Flye software to obtain the contig sequences. Pilon software (https://github.com/broadinstitute/pilon) was used to correct the third-generation contig results and integrate them with

the Illumina second-generation data. A BLASTN search was performed against the assembled DNA sequences using antibiotic resistance genes from the comprehensive antibiotic resistance database (http://arpcard.mcmaster.ca) as query sequences. The search parameters included an E-value of 1e-6, amino acid sequence similarity > 45%, nucleotide identity > 70%, and alignment coverage > 80%. Basic genomic features and further analysis were performed using Proksee (https://proksee.ca/).

Cloning of the bla_{LAP-3} Gene and Purification of LAP-3 Lactamase

The bla_{LAP-3} gene along with its promoter was amplified via PCR employing forward and reverse primers that incorporated EcoRI (5' - CGGAATTCCTTTACGTGCTCCGGTGGTA-3') and HindIII (5' - CCCAAGCTGAAGGCAGTTGCCAA CAGTG-3'), respectively. The PCR product was eluted from an agarose gel, subsequently digested with EcoRI and HindIII, and then ligated into the pUCP24 vector using a T4 DNA ligase cloning kit (TaKaRa Bio, Inc., Dalian, China). The recombinant plasmids were transformed into competent *E. coli* DH5α cells using the calcium chloride method. Subsequently, the transformed DH5α colonies were cultured on agar plates supplemented with 20 μg/mL gentamicin for antimicrobial susceptibility testing. The recombinant plasmids were verified by Sanger sequencing (Personalbio Technology Co., Ltd., Shanghai, China).

Additionally, a similar procedure was used to clone the complete ORF of bla_{LAP-3} into pET-28b. The recombinant plasmid (pET-28b-bla_{LAP-3}) was transformed into competent *E. coli* BL21 cells via the calcium chloride method. The transformants were cultured in Luria-Bertani medium with 50 μg/mL kanamycin at 37 °C. The cloned fragments were confirmed using Sanger sequencing (Personalbio Technology Co., Ltd., Shanghai, China). Overnight cultures were diluted 100-fold in Luria-Bertani medium and incubated with orbital shaking for several hours. Isopropyl-β-d-thiogalactopyranoside was added to the medium at a final concentration of 1 mM when the cultures reached an OD600 of 0.6–0.8, followed by incubation for an additional 4 h. Protein LAP-3 was extracted and purified through affinity chromatography using the His-tag Protein Purification Kit.

Determination of Kinetic Parameters

Kinetic parameters for the hydrolysis of β-lactams by purified LAP-3 were assessed using ultraviolet-visible spectrophotometry at 30 °C in 10 mM phosphate buffer (pH 7.0) with a final reaction volume of 300 μL. Steady-state kinetic parameters (kcat and KM) were determined by non-linear regression of the initial reaction rates using the Michaelis–Menten equation in Prism (version 7, GraphPad Software, San Jose, CA, United States). β-Lactamase inhibition was studied using benzylpenicillin (500 μM) as the substrate. The β-lactamase inhibitors (TZB and CLA) at various concentrations were preincubated with purified LAP-3 β-lactamase for 3 min at 30 °C, followed by the addition of substrate. The inhibitor concentration required to reduce the hydrolysis of 500 μM benzylpenicillin by 50% was determined using non-linear regression with the log(inhibitor) vs response–variable slope equation in Prism software.

Analysis of Protein Structure

The protein structure PDB (Protein Data Bank) files of three β-lactamases LAP-2 (isolated from *K. pneumoniae*, UniProt: A0A8A8MW53) and SHV-1 (isolated from *K. pneumoniae*, UniProt: P0AD64) were obtained from Alphafold protein structure database (https://alphafold.com/). TEM-1 (isolated from *Escherichia coli*, PDB ID: 1ERO) was obtained from RCSB PDB (https://www.rcsb.org/).[10] Using LAP-2 as the protein template, the PDB files of LAP-1 and LAP-3 were generated using the automated protein structure homology modelling server Swiss-Model (https://swissmodel.expasy.org/). The 3D conformation of the β-lactamases was visualized using UCSF ChimeraX, which facilitated the analysis of "Atom/Bond", "Clashes", and "Contacts" to assess the structural variances among the different β-lactamases.

Nucleotide Accession Numbers

The nucleotide sequences described in this research have been deposited in the GenBank nucleotide database, assigned the accession number PRJNA1105551 for *K. pneumoniae* KP2CSL, and the number SAMN48696164 for the plasmid pKP2CSL-LAP.

Results

General Features of the K. pneumoniae Genome and Comparative Analysis of the Plasmid pKP2CSL-LAP

Whole-genome sequencing of this *K. pneumoniae* strain was previously conducted as part of the survey, and further molecular analysis of the β-lactamase gene, $bla_{\text{LAP-3}}$, is in progress. Sequencing results revealed that isolate KP2CSL carried 10 resistance genes, which included the β-lactamase gene $bla_{\text{SHV-11}}$ and aminoglycoside O-phosphotransferase gene APH(3")-Ib located on the chromosome. Additionally, five lactamase genes ($bla_{\text{SHV-66}}$, $bla_{\text{KPC-39}}$, $bla_{\text{TEM-1b}}$, and two $bla_{\text{CTX-M-65}}$) were identified on the plasmid pKP2CSL-KPC, while $bla_{\text{LAP-3}}$, tetracycline resistance transcriptional repressor TetR, as well as quinolone resistance pentapeptide gene QnrS1 were found on the plasmid pKP2CSL-LAP. Sequencing analysis of the $bla_{\text{LAP-3}}$-harboring plasmid pKP2CSL-LAP demonstrated that the plasmid was 71,414 bp in length, with 84 open reading frames (ORFs) and an average GC content of 53.74%. More than 30 sequences demonstrating the highest similarities (> 95% coverage and > 99% identity) compared to pKP2CSL-LAP were sourced from the NCBI nucleotide database. All of these were IncFII plasmid sequences from *K. pneumoniae* harboring bla_{LAP} genes, including $bla_{\text{LAP-1}}$ or $bla_{\text{LAP-2}}$; none carried $bla_{\text{LAP-3}}$. Similarly, bla_{LAP}-harbored plasmids can be categorized into two functional regions: variable and conserved (Figure 1). The conserved region includes a fragment encoding genes

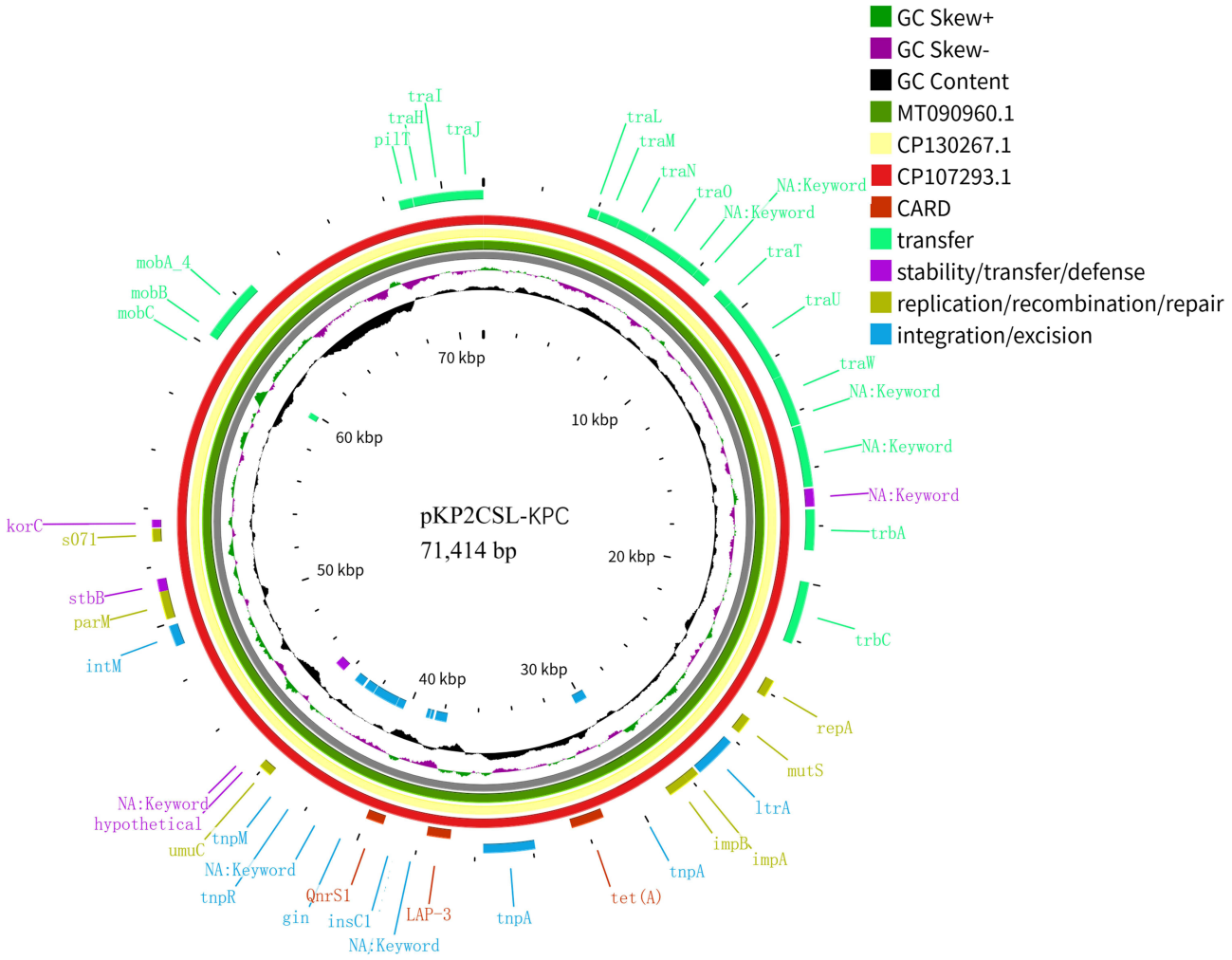

Figure 1 Circular map of plasmid pKP2CSL-LAP and comparative genomics analysis with humongous plasmids. Counting from the center toward outside: (1) the scale in bp. (2) GC skew (G-C/G+C), with a positive GC skew toward the outside and a negative GC skew toward the inside. (3) GC content, with an average of 50%, whereby a G+C content of more than 50% is shown toward the outside, otherwise, inward. (4) Backbone. The plasmid pKP2CSL-LAP (PRJNA1105551) was used as the reference sequence and was compared to the sequences of (5) pKP18-2079_tetA (MT090960.1), (6) p3-DX3-2hy (CP130267.1) and (7) pCRKP_45 (CP107293.1). Genes with different functions are shown in different colors: brown, antibiotic resistance; blue, mobile genetic elements; green, transfer conjugation; purple, plasmid stability; and dark yellow, replication.

associated with conjugative transfer region alongside another fragment dedicated to plasmid replication and stability. The variable region, referred to as the multidrug resistance (MDR) region, harbored insertion sequences (primarily transposase genes, including tnpA, tnpR, and tnpM) as well as resistance genes (*bla*$_{LAP-3,}$ TetR, and QnrS1). The sole distinction between pKP2CSL-LAP and other plasmids was the type of bla$_{LAP}$ type.

Sequencing Character of bla$_{LAP-3}$

The *bla*$_{LAP-3}$ gene has an ORF of 858 bp, encoding a preprotein of 285 amino acids. Distinct from LAP-2, which contains Ser165, LAP-3 is characterized by the presence of amino acid residueGlu165 in the same position. In comparison to LAP-1, also featuring Ser165, LAP-3 similarly exhibits Glu165. Additionally, LAP-3 and LAP-1 are differentiated by their residues at position 193, with LAP-3 possessing Glu193 and LAP-1 containing Gly193. BLASTN analysis revealed that *bla*$_{LAP-3}$ was similar to other class A ß-lactamases. The highest amino acid identity was recorded at 66.9% with TEM-1 and 58.7% with SHV-1. Amino acid sequence alignment of five β-lactamases (LAP-1, LAP-2, LAP-3, TEM-1, and SHV-1) demonstrated that LAP-3 possesses the same structural motifs (STFK, SDN, and KTG) that define the active site of Ambler class A β-lactamases. However,within a different variant of the active site (EXXLN) involved in the Ω-loop, LAP-3 has the same EPELN sequences as TEM-1, in contrast to LAP-1 and LAP-2, which exhibit EPSLN. Additionally, the SHV-1 sequences at this site reveal a distinct variation, presenting ETELN (Figure 2).

Antimicrobial Susceptibility

The antimicrobial susceptibility of five strains (KP2CSL, ATCC 25922, pUCP24-*bla*$_{LAP-3}$/DH5α, pUCP24/DH5α, and DH5α) to 12 antibiotics (Amoxicillin, Amoxicillin+clavulanic acid, Piperacillin, Piperacillin+tazobactam, Ceftazidime, Cefepime, Cefuroxime, Cephalothin, Cefotaxime, Aztreonam, Imipenem and Levofloxacin) was determined. Isolate KP2CSL displayed resistance to all antibiotics, exhibiting elevated MICs. The β-lactam MICs for the pUCP24-*bla*$_{LAP-3}$ /DH5α transformants were diminished by 2-fold or more compared to those of isolate KP2CSL. The MICs met or exceeded the resistance breakpoints according to Clinical and Laboratory Standards Institute (CLSI) M100-Ed34 for the following antibiotics—Amoxicillin (≥ 32 μg/mL), Amoxicillin+clavulanic acid (≥ 32/16 μg/mL), Piperacillin (≥ 32 μg/mL), Aztreonam (≥ 32 μg/mL), and cefuroxime (≥ 32 μg/mL). Despite the absence of interpretation criteria for Cephalothin breakpoint were available, its MIC was higher than that of cefuroxime (≥ 32 μg/mL) (Table 1).

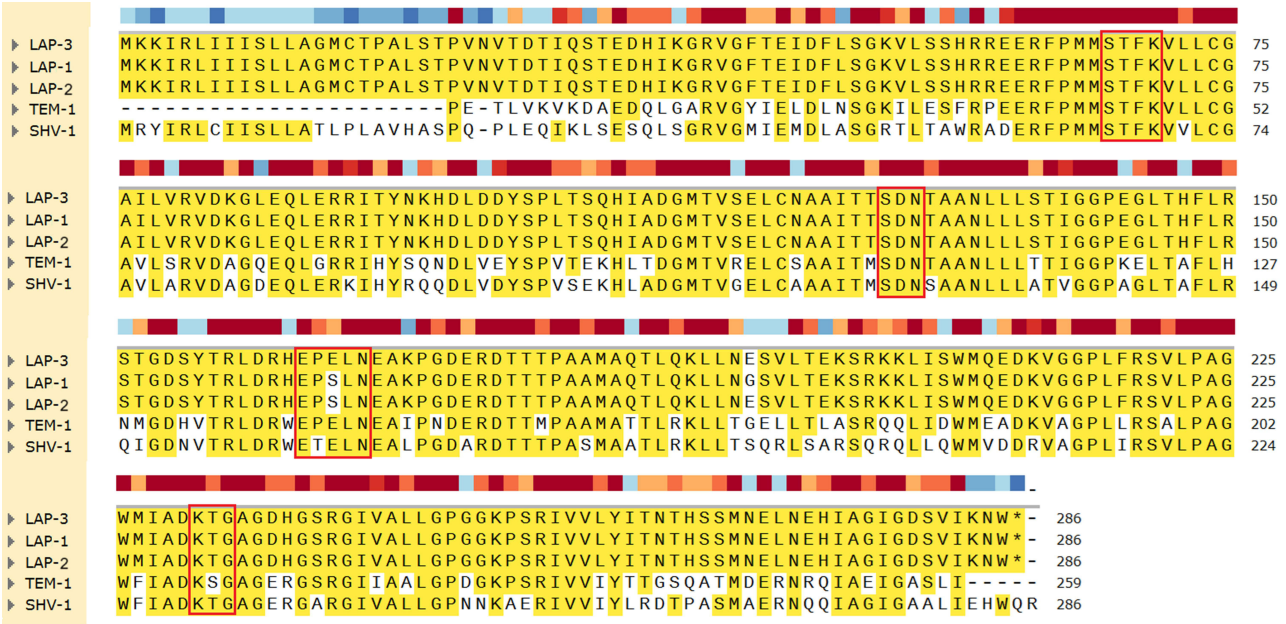

Figure 2 Amino acid alignment of LAP-3 with selected class A β-lactamases. Yellow background indicates amino acid residues identical to those of LAP-3. Active sites are enclosed within red rectangular wireframe.

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Table 1 MICs for Strains Against 12 Antibiotics (mg/L)

Antibiotic	*K. pneumoniae* KP2CSL	*E. coli* ATCC 25922	*E. coli* pUCP24-bla_{LAP-3}/DH5α	*E. coli* pUCP24/DH5α	*E. coli* DH5α
Amoxicillin	> 512	4	512	8	4
Amoxicillin+CLA	256+128	2+1	32+16	4+2	4+2
Piperacillin	256	1	64	1	1
Piperacillin+TZB	64+8	1+0.0125	4+0.5	1+0.0125	1+0.125
Ceftazidime	64	0.0625	0.125	0.0625	0.0625
Cefepime	64	0.0625	0.25	0.0625	0.0625
Cefuroxime	256	2	64	4	2
Cephalothin	> 512	4	128	4	4
Cefotaxime	64	0.125	2	0.125	0.125
Aztreonam	256	0.125	32	0.25	0.125
Imipenem	16	0.0625	0.0625	0.0625	0.0625
Levofloxacin	16	0.25	0.25	0.25	0.25

Abbreviations: CLA, clavulanic acid at a fixed concentration of 4 μg/mL; TZB, tazobactam at a fixed concentration of 4 μg/mL.

Kinetic Parameters of LAP-3

The β-lactamase LAP-3, encoded by plasmid pET-28b-bla_{LAP-3}, demonstrated significant hydrolytic activity against Amoxicillin (kcat/Km ratio, 3,367 $mM^{-1}\cdot s^{-1}$), Piperacillin (kcat/Km ratio, 2,250 $mM^{-1}\cdot s^{-1}$), cefuroxime (kcat/Km ratio, 4,000 $mM^{-1}\cdot s^{-1}$), Cephalothin (kcat/Km ratio, 5,000 $mM^{-1}\cdot s^{-1}$), and Aztreonam (kcat/Km ratio, 1,600 $mM^{-1}\cdot s^{-1}$), while displaying markedly low hydrolytic activity towards Cefotaxime (kcat/Km ratio, 0.06 $mM^{-1}\cdot s^{-1}$). Furthermore, no discernible hydrolytic activities were recorded for Cefepime, Ceftazidime, and Imipenem (Table 2). Half-maximal inhibitory concentration (IC50s) demonstrated that LAP-3 was highly susceptible to tazobactam (IC50: 0.05 ± 0.01 μM), and exhibited lesser sensitivity to clavulanic acid (IC50: 0.28 ± 0.03 μM).

Analysis of Protein Structure

The 3D structure of LAP-3 comprises a long, flexible N-terminal peptide with 26 residues, along with 13 α-helices, five β-sheets, culminating in a brief C-terminus with two residues. This is a typical 3D structure model of Ambler class A β-lactamases and resembles the structures of TEM-1 and SHV-1 (Figure 3). A careful comparison of the 3D structural map demonstrated that β-lactamase LAPs (LAP-1, LAP-2, and LAP-3) contained an Ω-loop region (residues ARG161 to ASP176) similar to other Ambler class A β-lactamases, such as TEM-1 and SHV-1. The active site located in the Ω-loop of LAP-3 contains a different sequence (EPELN; residues GLU163-ASN167) when compared to LAP-1 and LAP-2 (EPSLN) yet aligns closely with that of TEM-1 (EPELN). By utilizing the "Contacts" function within the "Structure

Table 2 Kinetic Parameters of β-Lactam Antibiotics for Purified LAP-3

Substrate	*Km* (s^{-1})	*Kcat* (μM)	*Kcat*/*Km* (mM^{-1} s^{-1})
Amoxicillin	150	700	3,367
Piperacillin	100	225	2,250
Ceftazidime	NH	NH	NH
Cefepime	NH	NH	NH
Cefuroxime	75	300	4,000
Cephalothin	80	400	5,000
Cefotaxime	60	2.86	0.06
Aztreonam	75	120	1,600
Imipenem	NH	NH	NH

Notes: Data are the means of three independent measurements. Standard deviations are within 10% of the mean values.
Abbreviation: NH, no detectable hydrolysis.

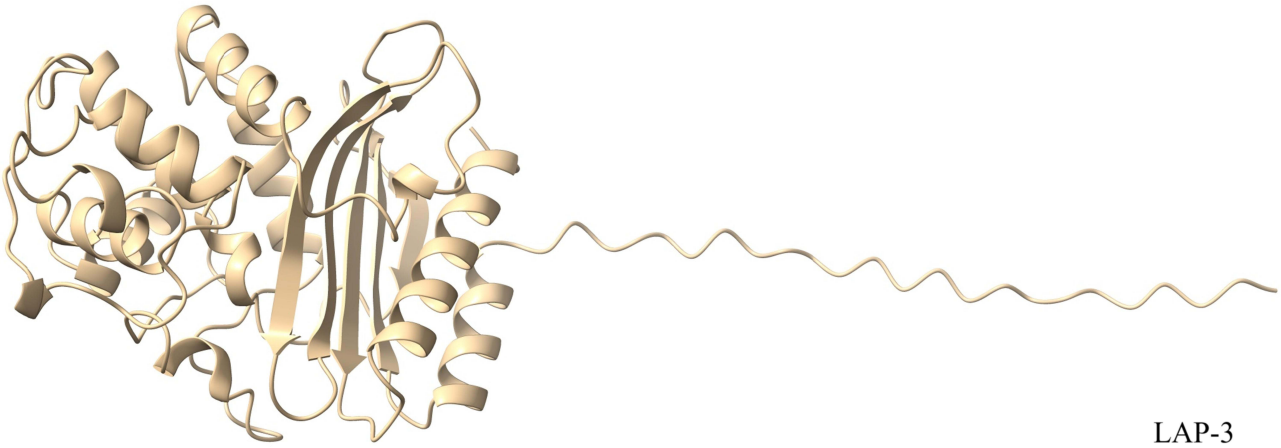

Figure 3 The general 3D structure of LAP-3 similar to TEM-1 and SHV-1.

analysis" in ChimeraX, it was discerned that the mutated residue GLU165 in LAP-3 interacts with adjacent residues, including HIS162, GLU163, ASN167, and GLU168, with interatomic distances ranging from 3.050–3.893 Å. A investigation into hydrogen bonds (H-bonds) within LAP-3 unveiled the presence of two H-bonds between GLU165 and GLU168. No H-bonds were detected between GLU165 and other residues (Figure 4). Additionally, no dashed line was noted between Ser165 and surrounding residues in LAP-1 and LAP-2.

Discussion

Antibiotic-resistant K. pneumoniae has been documented across the globe, with the widespread presence of β-lactamase genes significantly contributing to its resistance mechanisms. The predominant β-lactamases produced by K. pneumoniae belong to the TEM and SHV types of Ambler class A hydrolyzing enzymes. Nearly all Ambler class A β-lactamases exhibit an extensive hydrolysis spectrum against penicillins and cephalosporins. Furthermore, there exist a few narrow-spectrum enzymes targeting penicillinases, which, however, are inhibited by clavulanic acid. Among these is the LAP β-lactamase, which comprises two variants: LAP-1 and LAP-2. Both variants have been identified globally within the genera Klebsiella, Enterobacter, and Salmonella.

In this study, we identified a novel bla_{LAP}-type β-lactamase, designated as $bla_{\text{LAP-3}}$, while conducting a survey on resistant *K. pneumoniae*. The genome sequence of isolate KP2CSL unveiled a plasmid (pKP2CSL-LAP) that harbors a bla_{TEM}-like gene with approximately 66.5% identity to that of TEM-1. BLASTN analysis demonstrated that $bla_{\text{LAP-3}}$ shares a striking resemblance with narrow-spectrum β-lactamase genes $bla_{\text{LAP-1}}$ and $bla_{\text{LAP-2}}$, showing identity percentages of 99.3 and 99.65%, respectively. Upon comparison of the protein sequences, it was noted that two amino acids (Ser165 and Gly193) in LAP-1 are substituted by Glu165 and Glu193 in LAP-3, whereas LAP-2, only has Ser165 substituted by Glu165 in LAP-3. Subsequently, it was observed that the mutation at residue 165 is situated within the active site EPXLN, correlating to residues GLU163–ASP167 of EPSLN in LAP-1 and LAP-2, and EPELN in LAP-3. Despite the sequence EPXLN differing from LAP-1 and LAP-2, it aligns perfectly with EPELN in the TEM type. In a manner akin to other Ambler class A hydrolytic enzymes, the active site is positioned within an Ω-loop containing 16 amino acid residues. However, the amino acid sequences of the Ω-loop exhibit variability across different Ambler class A β-lactamase mutants.[11] The protein data bank (PDB) files indicate that LAPs possess possess a 16-residue Ω-loop, akin to those found in TEM-1 and SHV-1.

Plasmid-mediated determinants from Enterobacteriaceae that confer resistance to quinolones, QnrS with bla_{LAP}s, have been frequently reported.[12,13] The bla_{LAP}s and qnrS1 resistance determinant genes are often recombined and located on conjugable and untypable plasmids with various transposases.[14] The combination of $bla_{\text{LAP-1}}$ and qnrS1 is commonly observed in *Enterobacter cloacae*.[15,16] The $bla_{\text{LAP-2}}$ and qnrS1 combination is often observed in *K. pneumoniae*, residing on tet(A)-carrying plasmids that contain an MDR region with several transposases and multiple resistance genes, such as

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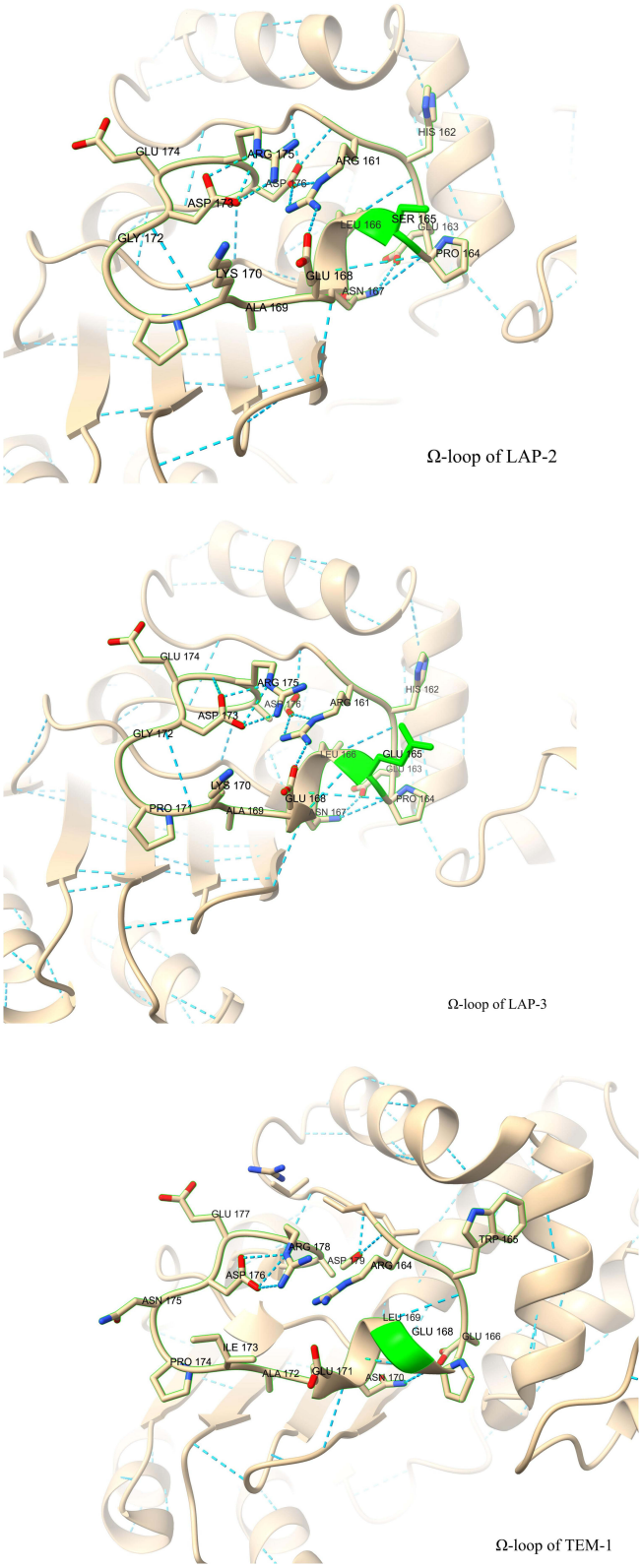

Figure 4 The Ω-loop structure of β-lactamase LAP-2 (Alphafold UniProt: A0A8A8MW53), LAP-3 (generated by Swiss-Model) and TEM-1 (isolated from Escherichia coli, PDB ID 1ERO). The blue dashed lines refer to hydrogen bonds. Residue Glu165, Ser165 and Glu168 highlighted in green.

sul2, catA2, tet(A), bla_{LAP-2}, and qnrS1.[17] Additionally, bla_{LAP-3} is harbored on a plasmid within an MDR unit of TnpA-tet(A)-bla_{LAP-3}-qnrS1-TnpR that is similar to $bla_{LAP-2.}$ Notably, except for qnrS1, no other quinolone resistance genes (QnrA, QnrB, and QnrS2) have been identified in combination with bla_{LAP-2} on any plasmid.

The spatial structures of TEM-type β-lactamases reveal that the catalytically active site of EPELN resides within a rigid conformation of the N-terminal part of the Ω-loop (residues Arg164–Asn170). Glu166 is a catalytic residue engaged in the two-step hydrolysis of antibiotics. The cis-configuration of the peptidylproline bond (Glu166–Pro167) is crucial for achieving a catalytically active orientation and forming a hydrogen-bonding network involving Glu166, Asn170, and a water molecule.[18,19] Given that most researches on the Ω - loop of TEM-1 and SHV-1 are based on Escherichia coli, the localization of amino acid residues in proteins from *K. pneumoniae* in this study may differ from which based on *Escherichia coli*. Consequently, Glu163 of LAP-3 aligns with Glu166 of TEM-1, whereas the mutated residues Glu165 and Asn167 of LAP-3 correspond to Glu168 and Asn170 of TEM-1, respectively. Although considerable research has focused on Glu166 and Asn170 of the TEM-type, other residues in the N-terminal part of the Ω-loop, including GLU168, are rarely reported. Similarly, there is currently a lack of research regarding the Ω-loop and its residues in LAPs. Using ChimeraX to analyze H-bonds revealed that Glu163 of LAP-3 interacts with Asn167 through H-bonds—paralleling the Glu166-Asn170 hydrogen bonding network in TEMs.[20] Additionally, the analysis demonstrated that Glu165 of LAP-3 forms H-bonds with Glu163 and Asn167, whereas Ser165 of LAP-1 and LAP-2 does not form such bonds with Glu163 and Asn167. This discrepancy can be attributed to the carboxyl group in the side chain of Glu, which facilitates non-covalent interactions with surrounding residues or substrates, including hydrogen and ionic bonds. However, the presence of H-bonds indicates that the N-terminal part of the Ω-loop in LAP-3 exhibits greater rigidity compared to that in LAP-1 and LAP-2.

The cloned bla_{LAP-3} gene in *E. coli* DH5α recombinant clones (pUCP24-bla_{LAP-3}/DH5α) facilitated varying elevations in MIC levels for Amoxicillin (64-fold), Piperacillin (64-fold), cefuroxime (16-fold), and Cephalothin (32-fold) when compared to the control strain (pUCP24/DH5α). Additionally, it demonstrated robust hydrolytic activity against these β-lactams (with all kcat/Km ratios $\geq$ 1600 $mM^{-1}\cdot s^{-}1$), similar to LAP-1 and LAP-2, which exhibit a narrow hydrolysis spectrum.[3,14] Although LAP-1 and LAP-2 are inhibited by β-lactamase inhibitors,[3,14] pUCP24-bla_{LAP-3}/DH5α expressed resistance to Amoxicillin-clavulanic acid and Piperacillin-tazobactam, with 8- and 4-fold higher MICs than those of pUCP24/DH5α, respectively. Moreover, although the MIC levels of cefepime, cefotaxime and aztreonam did not meet breakpoint criteria (CLSI M100-Ed34), they demonstrated 4-, 16-, and 16-fold increases in MIC levels, respectively—this expression diverging from those observed in LAP-1 and LAP-2.[3,14] Although LAP-3 did not confer resistance to alternative β-lactams, such as Ceftazidime and carbapenems, its enhanced hydrolytic ability for cephalosporins and resistance to β-lactamase inhibitors demonstrated an expanded hydrolysis spectrum. The mutation in residue Glu165, which alters the conformation of the N-terminal part of the Ω-loop, may alter its resistance spectrum, aligning its resistance characteristics with those of other Ambler A-class β-lactamases, such as TEM-1 and SHV-1.

In the sequencing era, novel alleles are frequently reported, and several *bla* genes—bla_{SHV} and bla_{TEM}—now number in the hundreds. Additionally, the reports of mutants affecting the Ω-loop are increasing. Several protein variants classified as ESBL have been reported regarding Ω-loop mutation: the substitution of Ala for Glu166 shifts SHV-1 to SHV-16, resulting in a significant loss of activity against penicillins and gain of activity against Ceftazidime when compared with those of SHV-1.[21] The substitution of Arg for Leu169 shifts SHV-1 to SHV-57, inducing a conformational alteration in the Asn170 residue. This modification affects the susceptibility pattern, resulting in resistance to Ceftazidime but susceptibility to cefazolin, and is inhibited by the β-lactamase inhibitor clavulanic acid.[22] An analysis supports that mutations at Ambler sites 238 and 179 confer ESBL activity,[23,24] whereas the majority of Ω-loop substitutions do not. The eight variants (SHV-27, SHV-38, SHV-40, SHV-41, SHV-42, SHV-65, SHV-164, and SHV-187) are devoid of ESBL-associated mutations, and phenotypic data support susceptibility to third-generation cephalosporins.[25] In TEM, the P167G and E166Y substitutions play a significant role in destabilizing the enzyme, and based on the MIC results, they contribute to the substrate specificity of the enzyme. A non-proline residue at position 167 destabilizes class A β-lactamases because of the cis peptide bond between positions 166 and 167.[26] Additionally, E-166 forms an ionic bond with K-73.[27] The W165Y single mutation has negligible effects on enzyme expression and activity. However, when the W165Y mutation is incorporated into the E166Y P167G double mutant, it enhances the hydrolytic activity of the enzyme

for ceftazidime.[28] Notably, the majority of aforementioned studies focused on residue sites 166, 167, and 170 in the N-terminal part of the Ω-loop, whereas other residues of Ambler class A β-lactamases, such as TEMs, SHVs, and SME-1,[29] are rarely reported. In our study, the substitution of Ser165Glu with Glu in LAP-3 (corresponding to residue 168 in TEM and SHV) expanded the resistance spectrum compared to that of LAP-1 and LAP-2. Structural analysis around the mutation site indicated that Ser165Glu substitution in LAP-3 may have altered the hydrolysis spectrum.

Limitations

This study has several limitations. Firstly, the characterization of LAP-3 is based on a single clinical isolate, which may limit the generalizability of the findings. Secondly, although structural modeling suggests that the Ser165Glu substitution alters hydrogen bonding within the Ω-loop, direct experimental evidence such as mutagenesis studies to confirm the functional impact of this specific residue change is lacking. Additionally, the clinical relevance of the observed resistance phenotype remains unclear, as the study did not assess the fitness cost or stability of the plasmid carrying $bla_{\text{LAP-3}}$ in bacterial populations under selective pressure. Further studies involving site-directed mutagenesis and clinical surveillance are warranted to fully elucidate the mechanistic impact of this substitution and its implications for antibiotic resistance dissemination.

Conclusion

This study identifies and characterizes a novel Ambler class A β-lactamase, LAP-3, harbored on a plasmid in a clinical isolate of *K. pneumoniae*. LAP-3 exhibits an expanded hydrolysis profile compared to its predecessors LAP-1 and LAP-2, demonstrating significant activity against penicillins (amoxicillin, piperacillin), early cephalosporins (cefuroxime, cephalothin), and notably, resistance to β-lactamase inhibitors such as clavulanic acid and tazobactam. The key genetic distinction of LAP-3 is the Ser165Glu substitution within the Ω-loop—a critical region for substrate specificity and inhibitor binding in class A β-lactamases. Structural analyses suggest that this substitution introduces new hydrogen-bonding interactions (eg, between Glu165 and Glu168), potentially increasing the rigidity of the Ω-loop and altering the enzyme's functional properties. These findings indicate the role of single residue changes in expanding the resistance spectrum of narrow-spectrum β-lactamases and underscore the ongoing evolution of plasmid-mediated resistance mechanisms in clinically important pathogens.

Data Sharing Statement

The data of whole-genome sequences have been deposited in the NCBI Sequence Read Archive (SRA) with the BioProject accession number PRJNA1105551, and SAMN48696164 for plasmid pKP2CSL-LAP.

Ethics Statement

The study involving information of strains source from human was reviewed and approved by the Research Ethics Committee of The First People's Hospital of Yongkang (approval number: YKSRMYYEC2024-KT-HS-031) and complies with the Declaration of Helsinki. Due to strain isolated from a neonatal patient, written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s) legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

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Disclosure

The authors report no conflicts of interest in this work.

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