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We use this protocol and it's working

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Abstract

The rising incidence of antibiotic resistance among pathogenic bacteria presents a significant public health challenge, particularly with regard to *Proteus vulgaris* (_P. vulgaris_). This study aimed to investigate the molecular mechanisms underlying the intrinsic resistance of *P. vulgaris* to β-lactam antibiotics, focusing specifically on the novel β-lactamase variant CumAII. Employing antimicrobial susceptibility testing, whole-genome sequencing, gene cloning, and kinetic analyses, we characterized the hydrolytic spectrum and biochemical properties of CumAII, revealing an 84.87% homology with its predecessor CumA. The isolate EC6-1 demonstrated high resistance levels, with minimum inhibitory concentrations (MICs) for Ampicillin, Cefuroxime, and Cephalothin exceeding 512 μg/mL. The introduction of CumAII into transformants decreased MICs compared to the parent strain, while co-expression with regulatory proteins CumRII and AmpD enhanced susceptibility to certain antibiotics. Molecular docking studies indicated that CumAII exhibits a varied affinity for β-lactams, reflecting changes in interacting residues. The findings elucidate the role of CumAII as a novel β-lactamase with an expanded hydrolytic spectrum against β-lactams, emphasizing the need for further research into the regulation and potential inhibition of such variants to combat the growing threat of multidrug-resistant *P. vulgaris*_.

Materials

Strains and Plasmids:
- **Pseudomonas aeruginosa (n=260):** Strains isolated in the laboratory from the First Affiliated Hospital of Wenzhou Medical University.
- **Escherichia coli DH5α:** Cloning host strain, preserved in the laboratory.
- **EC600:** Conjugation recipient strain, preserved in the laboratory.
- **H9812:** Reference strain for pulsed-field gel electrophoresis molecular weight, donated by Sir Run-Run Shaw Hospital, Zhejiang University.

Main Reagents:
- **Taq DNA Polymerase, PrimeSTAR HS DNA Polymerase, Restriction Endonucleases, T4 DNA Ligase:** Takara Bio (Dalian) Co., Ltd.
- **Loading buffer, DNA ladder:** Takara Bio (Dalian) Co., Ltd.
- **RNase A, Lysozyme:** Shanghai Generay Biotech Co., Ltd.
- **Bacterial Genomic DNA Extraction Kit:** Shanghai Generay Biotech Co., Ltd.
- **Plasmid Extraction Kit:** Shanghai Generay Biotech Co., Ltd.
- **QIAEX II Gel Extraction Kit:** Qiagen (USA)
- **PCR Purification Kit:** Shanghai Generay Biotech Co., Ltd.
- **GelRed:** Biotium (USA)
- **Various Antibiotics:** The Second Affiliated Hospital of Wenzhou Medical University
- **A Enzyme:** Genewiz (Global Gene)
- **2×GenRec Recombinase:** Genewiz (Global Gene)
- **2×PCR Mix:** Genewiz (Global Gene)
- **10mM dNTP Mix:** Shanghai Zhaowei
- **Magnetic Bead Purification Kit:** Nanjing Rebase (Rebase)
- **BDT 3.1:** ABI
- **POP-7™:** ABI
- **10×3730 Running Buffer:** ABI
- **Cofactor B:** Genewiz (Global Gene)
- **Competent Cells DH10b:** General Biosciences (General Biol)
- **Plasmid Extraction Kit:** Genewiz (Global Gene)
- **PCR Gel Recovery Kit:** Genewiz (Global Gene)
- **Restriction Enzyme HindIII and EcoRI****
- **Restriction Enzyme NcoI and XhoI****

Safety warnings

Note: Antibacterial agents must be prepared and diluted on the same day. M-H solid agar must be autoclaved and prepared into drug-containing plates on the same day.

Ethics statement

This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. The protocol was reviewed and approved by the First People's Hospital of Yongkang's Institutional Ethics Committee (approval number: YKSDYRMYIEC2024-LW-KS-005). Written informed consent was obtained from all participants prior to their enrollment in the study. Participants were informed of their right to withdraw from the study at any time without consequences. All data were anonymized to protect participant confidentiality.

Before start

Prepare a stock solution of the 12 Antibiotics at a concentration of 51.2 mg/ml. Subsequently, dilute this stock solution to final concentrations of 6.4 mg/ml and 0.8 mg/ml.

Gene Synthesis and Cloning of the Gene of CumAII, CumA, CumRII, CumR and AmpD

1 Eight gene fragments were synthesized and cloned into a plasmid following a standardized molecular cloning protocol. Specific primers were designed based on target gene sequences to facilitate PCR amplification. CumAII and CumRII were based on template CP195195 (NCBI Accession) of EC6-1, CumA and CumR were based on X80128.1, and AmpD was based on CP137920.

2 Gene Synthesis and Cloning of the transformants of pUCP24(cohesive end)-CumAII/DH10b, pUCP24(cohesive end)-CumAII-CumRII/DH10b, pUCP24(cohesive end)-CumAII-CumRII-AmpD/DH10b, pUCP24(cohesive end)-CumA/DH10b, pUCP24(cohesive end)-CumA-CumR/DH10b, pUCP24(cohesive end)-CumA-CumR-AmpD/DH10b, pET28a(cohesive end)-CumAII/DH10b, and pET28a(cohesive end)-CumA/DH10b.

Synthesis of Target Fragment

3 PCR First Round Reaction (50 µL system): Primers (each): 2 µL Template: 2 µL Polymerase Buffer: 10 µL 10 mM dNTP: 1 µL Polymerase: 1 µL ddH2O: 32 µL
Cycling Parameters (First Round): Initial Denaturation: 96°C for 5 min Cycling (23 cycles): 95°C for 25 s, 58°C for 25 s, 72°C for approx. 30 s Final Extension: 72°C for 1 min
PCR Second Round Reaction (100 µL system): Primers (each): 2 µL First Round Product: 2 µL Polymerase Buffer: 10 µL 10 mM dNTP: 1 µL Polymerase: 1 µL ddH2O: 82 µL
Cycling Parameters (Second Round): Initial Denaturation: 96°C for 5 min Cycling (23 cycles): 95°C for 25 s, 58°C for 25 s, 72°C for approx. 30 s Final Extension: 72°C for 1 min 30 s

Fragment Cloning

4 The PCR-amplified fragment was assembled into the HindIII--EcoRI sites of the pUCP24 or pET28a vector using a recombination ligation method. The ligation product was spread onto Gentamycin-resistant plates and cultured overnight at 37°C to obtain the full-length construct. The specific ligation system is shown in Table 3 below.

Reagent Name	Volume (µL)
Purified PCR Product	5
Enzyme-digested Vector	5

Table 3. Ligation Reaction System for Purified Target Fragment and Vector

5 Note: The above ligation mixture was incubated at a constant temperature of 52°C for 30 min.

Transformation Procedure

6 Absorb 10 µL of the ligation reaction product was added to approx. 100 µL of competent cells. The mixture was gently flicked to ensure contact and then placed on ice for 10 min.
Heat shock at 42°C for 90 s without shaking.
Place on ice for approx. 3 min.
Add 500 µL of pre-warmed LB medium (37°C) to each tube. Incubate at 37°C on a shaker at 200 rpm for 40 min to allow cell recovery. After recovery, centrifuge at 6000 rpm for 2 min and discard the supernatant.
Resuspend the remaining cell pellet thoroughly and spread the entire volume onto agar plates containing the appropriate antibiotic. Incubate plates overnight at 37°C.

Identification of Cloned Plasmids

7 Remove the plates the following day and observe/confirm colony growth.
Pick 4 single colonies from the plate and culture them at 37°C with shaking at 250 rpm/min, while simultaneously performing colony PCR identification.
The identification primers were prepared in-house.
Identification of Colony PCR Primer Information
Colony PCR System (30 µL): Primers (each) 1 µL, Polymerase Buffer 13 µL, ddH2O 15 µL.
Cycling Parameters: Initial Denaturation: 96°C for 3 min Cycling (23 cycles): 95°C for 30 s, 58°C for 30 s, 72°C for 50 s Final Extension: 72°C for 1 min
Perform agarose gel electrophoresis on the amplification products. Photograph the gel using a gel documentation system, analyze the image, and screen for positive clones.
Culture positive colonies at 37°C to extract plasmid DNA, and send the samples to the internal sequencing group.
Perform a double digestion on the plasmid with the correct sequencing results using HindIII and EcoRI(pUCP24), or NcoI and XhoI(pET28a).

Determination of Kinetic Parameters and Inhibition Studies for Purified CumAII and CumA β-Lactamases

8 Kinetic parameters for the hydrolysis of β-lactams by purified CumAII and CumA 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 CumAII and CumA β-lactamases 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 and molecular docking

9 The protein structure PDB (Protein Data Bank) files of CumA(isolated from *P.vulgaris_, PDB ID: 1HZO) was obtained from RCSB PDB (https://www.rcsb.org/). Using CumA as the protein templates, the PDB files of CumAII was generated using the automated protein structure homology modelling server Swiss-Model (https://swissmodel.expasy.org/).*
Small molecule ligand Sdf (Structure data file) files of Ampicillin, Piperacillin, Cefotaxime and Ceftazidime were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/),and changed to PDB files by OpenBabel software.
Before molecular docking, water molecules of protein structure were removed using Pymol software. Molecular docking performed using Autodock Vina software for each protein and ligand, and evaluated by the affinity value and RMSD between conformations. The lowest binding energy (ΔGbinding) refers to the best docking result.
Signal peptide prediction was performed with SignalP 5.0 (https://services.healthtech.dtu.dk/services/SignalP-5.0/).