

First Report of *bla*_{OXA-677} with Enhanced Meropenem-Hydrolyzing Ability in *Pseudomonas aeruginosa* in China

Yue Sun^{1,2}
Renru Han^{1,2}
Li Ding^{1,2}
Yang Yang^{1,2}
Yan Guo^{1,2}
Shi Wu^{1,2}
Fupin Hu^{1,2}
Dandan Yin^{1,2}

¹Institute of Antibiotics, Huashan Hospital, Fudan University, Shanghai, People's Republic of China; ²Key Laboratory of Clinical Pharmacology of Antibiotics, Ministry of Health, Shanghai, People's Republic of China

Purpose: OXA-10-type class D β-lactamases have shown their evolutionary potential of enhancing carbapenem resistance. This study aimed to elucidate the role of OXA-10 variants in clinical isolated multidrug resistant (MDR) *Pseudomonas aeruginosa* and characterize the first appearance of OXA-677 in China.

Methods: Six *bla*_{OXA-10-like}-positive strains were screened by PCR from 41 *P. aeruginosa* strains, which were resistant to both carbapenems and ceftazidime-avibactam, collected across China in 2018. The minimum inhibitory concentrations (MIC) were determined with the broth microdilution method. The resistance-associated genes and genetic environment were investigated by whole-genome sequencing (WGS). The function and mechanism of OXA-677 β-lactamase were identified by molecular cloning and protein structure modeling.

Results: All the *bla*_{OXA-10-like}-positive *Pseudomonas aeruginosa* were MDR strains. They also had outer membrane porin defects and produced β-lactam resistance gene *bla*_{PER-1}, fluoroquinolone-resistant gene *crpP*, aminoglycoside-resistance gene *aph(3')-IIb*, *aph(6)-Id*, *aacA* and *aadA*, fosfomycin-resistance gene *fosA*, sulfamethoxazole-resistance gene *sulI*, and chloramphenicol-resistance gene *catB7*. All *bla*_{OXA-10} variants were located in a Tn1403-related transposon, containing *aacA4-12-bla*_{OXA-677}-*aadA1*, *aacA4-12-bla*_{OXA-101}-*aadA5*, and *bla*_{OXA-246}-*aacA3*-*aadA13* gene cassette arrays, respectively. Notably, the *bla*_{OXA-677} producer showed a high MIC level of meropenem (MIC>64 mg/L). Compared to *bla*_{OXA-10}, *bla*_{OXA-677} was found a G-to-T transversion at position 350, leading to a phenylalanine-for-valine substitution in position 117, which is closer to leucine155 in the omega loop of the active site. MIC of meropenem for *E. coli* DH5α with the recombinant plasmid pHSG398 carrying *bla*_{OXA-677} was elevated by 8 times.

Conclusion: We speculate that the OXA-10-like enzymes and the decrease of membrane permeability confer carbapenem resistance, and the V117 substitution in OXA-677 might lead to a higher resistance level of meropenem.

Keywords: *Pseudomonas aeruginosa*, *bla*_{OXA-677}, *bla*_{OXA-10-like} gene, CHDL

Introduction

Pseudomonas aeruginosa is one of the most important and common clinical opportunistic pathogens, and its infection carries a high risk of mortality.¹ Due to various inherent and acquired resistance mechanisms, the multidrug-resistant *P. aeruginosa* has emerged globally.^{2,3} There into, carbapenem-resistant *P. aeruginosa* was included in the highest category of urgency by the World Health Organization in 2017.⁴ According to the China Antimicrobial Surveillance Network (CHINET) of 2020,

Correspondence: Fupin Hu; Dandan Yin
Tel/Fax +86 21 52888186;
+86 21 52888196
Email hufupin@fudan.edu.cn;
yindandan@fudan.edu.cn

the resistance rates of *P. aeruginosa* to imipenem and meropenem are 23.2% and 19.3%, respectively.⁵

One or more mechanisms, including alteration of outer membranes, efflux pump overexpression, and production of carbapenemases, have been found contributing to the carbapenem resistance in *P. aeruginosa* isolates.^{6–8} It is generally considered that only enzymes with broad-spectrum and significant carbapenem hydrolysis activities are the most predominant carbapenemases, such as the class B metal- β -lactamases NDM, IMP, VIM, and the class A serine- β -lactamases KPC. In recent years, special attention has been given to the class D serine- β -lactamase OXA-family.

The OXA-family counts 926 members and only several groups are considered as carbapenem-hydrolyzing class D β -lactamases (CHDLs), such as OXA-23-like, OXA-40/24-like, OXA-58-like, and OXA-48-like β -lactamases. Among these carbapenemases, only OXA-40-like genes have been identified in *P. aeruginosa*, and the rest are prevalent in *A. baumannii*. However, some studies suggest that OXA-10 and OXA-2 β -lactamases can also confer reduced susceptibility to carbapenems when they express in strains with outer membrane porin defects, just like the well-known CHDL OXA-48.⁹ Furthermore, modification of OXA-10 may enhance resistance to either carbapenem or new β -lactam/ β -lactamase inhibitor combination.^{10,11} According to the NCBI database, a total of 37 OXA-10-like enzymes have been reported around the world so far, and over 75% (28/27) of them have been detected in *P. aeruginosa*.^{12–14} OXA-14, OXA-794, OXA-795, and OXA-824, confer resistance to β -lactam/ β -lactamase inhibitor but decrease meropenem MICs.¹⁰ Kotsakis et al reported the first natural variant OXA-655 (V117L) with increased carbapenem hydrolysis produced by an environmental *E. coli* strain.¹⁵ In this study, a variant similar to OXA-655 (OXA-677, V117F) and other two variants (OXA-101 and OXA-246) were found to be produced by several clinically relevant carbapenem resistant *P. aeruginosa* isolates. Here, we tried to characterize the rare variant OXA-677 and elucidate the role of OXA-10 variants in carbapenem resistance.

Materials and Methods

Strains and Plasmid

P. aeruginosa strains named 18-W18-009, 18-W18-012, 18-W71-012, 18-W71-013, 18-W71-020, 18-W31-087 were found producing OXA-10-like enzymes during the

screen for clinical isolates, which were resistant to both carbapenems and ceftazidime-avibactam among 39 strains by the CHINET workgroup in 2018. All strains were identified using VITEK matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS, bioMérieux, Marcy l'Étoile, France). *P. aeruginosa* ATCC 27853 was used as the quality control strain for antimicrobial susceptibility testing; *P. aeruginosa* PAO1 was used as the reference strain for the whole-genome sequence blasting in the Genbank; *E. coli* DH5 α and plasmid pHSG398 were used as the recipient bacterium for transformation test and the resistance gene carrier for clone experiment, respectively.

Antimicrobial Susceptibility Testing

Minimal inhibitory concentrations (MICs) were determined with broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines of the 30th edition. Antimicrobial agents used in antimicrobial susceptibility tests were imipenem, meropenem, ceftazidime/avibactam, piperacillin/tazobactam, ceftolozane/tazobactam, cefepime, amikacin, polymyxin B, ceftazidime, aztreonam, and ciprofloxacin. MIC interpretation for most agents was performed based on the CLSI breakpoints except for polymyxin, which was interpreted using EUCAST standards.¹⁶

Whole Genome Sequencing and Bioinformatics Analyses

Total genomic DNA was extracted using the genomic DNA extraction kit (Tiangen, China). Fragmented genomic DNA (~400-bp) was obtained through the Covaris S2 (Covaris, USA) and library construction was performed by NEXTflex DNA Sequencing Kit compatible with the Biomek[®] FXp (Bio Scientific, USA). Genome-wide sequencing was performed using Illumina Solexa technology (Illumina, San Diego, CA, USA). De novo assembly was carried out with Velvet v1.2.08 and redundant contigs were removed using the Fastx toolkit.

The multilocus sequence typing (MLST) was identified by an online *Pseudomonas aeruginosa* mlst typing database (<https://pubmlst.org/>). The presence of horizontally acquired resistance mechanisms was detected with the ResFinder v4.1 server (<https://cge.cbs.dtu.dk/services/ResFinder/>) and the whole bacterial genome annotation was performed by Rapid Annotation using Subsystem Technology (RAST) (<https://rast.nmpdr.org/>). The mobile

element-related genes (MGEs) were identified by MobileElementFinder (<https://cge.cbs.dtu.dk/services/MobileElementFinder/>), ISfinder (<https://www-is.biotoul.fr/>), and Tn Number Registry (<https://transposon.lstmed.ac.uk/>). Additionally, gene alterations and disruptions of porin-encoding genes *oprD* and efflux pump repressor gene *mexR*, *nalC*, *nalD* were compared with those of *P. aeruginosa* PAO1 using SnapGene.

Molecular Cloning, Transformation Test and Protein Structure Modeling of *bla*_{OXA-677}

The gene of *bla*_{OXA-677} was amplified with Q5[®] High-Fidelity 2X Master Mix. OXA-10-F-EcoRI (5'-CGG AATTCGCCATGAAAACATTTGCCGCATATG-3') and OXA-10-R-BamHI (5' -CGCGGATCCGTTAGCCACCA ATGATGCC-3') were used as the primer pair. The cycling parameters were 98°C for 30 s, 30 cycles at 98°C for 30 s, 63°C for 30 s and 72°C for 60 s. The PCR product was ligated to the vector plasmid pHSG398 and then the recombinant plasmid was transferred into the recipient strain *E. coli* DH5 α . Mueller-Hinton agar plates containing 50 mg/L of chloramphenicol (for pHSG398) and 50 mg/L of amoxicillin (for *bla*_{OXA-677}) were used to select the potential transformants, which were confirmed finally by PCR and sanger sequencing. The three-dimensional protein structure models for OXA-677 carbapenemase were built by SWISS-MODEL Server (<https://swissmodel.expasy.org/>) and PyMOL software (<https://www.schrodinger.com/products/pymol>), using that of OXA-655 carbapenemase (PDB accession number 6SKQ; resolution, 2.10Å) as a reference.

Nucleotide Accession Numbers

The sequences of OXA-10 variants and their gene context have been deposited in the GenBank database under accession numbers OL353898 (*bla*_{OXA-101}), OL353899 (*bla*_{OXA-246}) and OL353897 (*bla*_{OXA-677}).

Results

Resistance Phenotypes and Genotypes of *bla*_{OXA-10} Variants Positive Strains

P. aeruginosa strains named 18-W18-009, 18-W18-012, 18-W71-012, 18-W71-013, 18-W71-020, 18-W31-087 expressed multidrug resistant phenotypes. They were all resistant to imipenem, meropenem, ceftazidime/avibactam, piperacillin/tazobactam, ceftolozane/tazobactam,

cefepime, amikacin, ceftazidime, aztreonam, and ciprofloxacin. *P. aeruginosa* named 18-W31-087 showed a higher MIC level of meropenem (MIC > 64 mg/L) than others (MIC = 8–32 mg/L). No carbapenemase gene could be detected except *bla*_{OXA-10-like} in these strains by the PCR (all carbapenemase genes tested in this study were shown in [Table S1](#) with primers sequence), as well as the following whole-genome sequencing (WGS). PCR and WGS data revealed that strain 18-W31-087 produced OXA-677 enzyme, strain 18-W18-009 and 18-W18-012 produced OXA-256 enzyme, strain 18-W71-012, 18-W71-013, 18-W71-020 produced OXA-101 enzyme. *bla*_{OXA-246}-positive strains belonged to ST298, and others belonged to ST244. Meanwhile, chromosomal mutations such as premature termination of the efflux pump repressor gene *mexR* and porin-encoding gene *oprD* were observed in *bla*_{OXA-246}-positive strains. And for strains producing OXA-677 or OXA-101 enzymes, premature stop codon occurred in *oprD* genes. Besides the carbapenem resistance associated genes, all the six strains produced extended spectrum β -lactamase (ESBL) gene *bla*_{PER-1}, fluoroquinolone-resistant gene *crpP*, aminoglycoside-resistance gene *aph(3')-IIb*, *aph(6)-Id*, *aacA* and *aadA*, fosfomycin-resistance gene *fosA*, sulfamethoxazole-resistance gene *sulI*, and chloramphenicol-resistance gene *catB7*. *bla*_{OXA-246}-positive strains also produced *bla*_{TEM-1B} and aminoglycoside-resistance gene *rmtB* ([Table 1](#)).

Genetic Context and Protein Structure of *bla*_{OXA-10} Variants

All three kinds of *bla*_{OXA-10} variants are located in a Tn1403-related transposon of the Tn-3 family. Each transposon contains a complex class 1 integron harboring an ISCR1-*bla*_{PER-1} unit. The difference of the three class 1 integrons was gene cassette arrays (GCA) in the variable region (VR). GCA *aacA4-12-bla*_{OXA-677}-*aadA1*, *aacA4-12-bla*_{OXA-101}-*aadA5*, and *bla*_{OXA-246}-*aacA3*-*aadA13* were detected, respectively ([Figure 1](#)). Compared to the *bla*_{OXA-10}, the sequence of *bla*_{OXA-677} was found carrying a G-to-T transversion at position 350, leading to a phenylalanine-for-valine substitution in position 117 (Val117Phe); whereas there are no base mutations for either *bla*_{OXA-101} or *bla*_{OXA-246} at the key amino acid motifs ([Figure 2](#)). Val117Phe (V117F) was located at the second catalytically important conserved motif of class D β -lactamases. Due to the substitution, the

Table 1 Demographic, Antimicrobial Susceptibility and Genotypic Data for the Clinical Isolates of *P. aeruginosa* Harboring OXA-10 Natural Variants

Strain ID	Hospital Location	Sample Type	MIC (mg/L)											MLST	β-Lactam Resistance Genes and OMP Mutation Sites	Non-β-Lactam Resistance Related Genotypes
			IPM	MEM	CZT	CZA	TZP	FEP	AMK	POL	CAZ	ATM	CIP			
18-W18-009	Liaoning	Sputum	8	16	128	64	256	64	>128	>16	>32	>128	>8	ST298	OXA-246 (K138N), PER-1, TEM-1B, OprD (237stop)*	crpP, fosA, sul1, aph(3')-IIB, aph(6)-Id, aacA3, aadA13, rmtB, catB7
18-W18-012	Liaoning	Sputum	16	8	128	64	256	64	>128	1	>32	>128	>8	ST298	OXA-246 (K138N), PER-1, TEM-1B, OprD (237stop)*	crpP, fosA, sul1, aph(3')-IIB, aph(6)-Id, aacA3, aadA13, rmtB, catB7
18-W71-012	Inner Mongolia	Urine	32	16	128	16	>256	64	128	0.5	>32	>128	>8	ST244	OXA-101 (I10T G20S S27F D55N T107S Y74F), PER-1, OprD (237stop)*	crpP, fosA, sul1, aph(3')-IIB, aph(6)-Id, aacA4-12, aadA5, catB7
18-W71-013	Inner Mongolia	Sputum	32	32	>128	32	>256	128	>128	0.5	>32	>128	>8	ST244	OXA-101 (I10T G20S S27F D55N T107S Y74F), PER-1, OprD (237stop)*	crpP, fosA, sul1, aph(3')-IIB, aph(6)-Id, aacA4-12, aadA5, catB7
18-W71-020	Inner Mongolia	Sputum	32	32	>128	32	>256	128	128	1	>32	>128	>8	ST244	OXA-101 (I10T G20S S27F D55N T107S Y74F), PER-1, OprD (237stop)*	crpP, fosA, sul1, aph(3')-IIB, aph(6)-Id, aacA4-12, aadA5, catB7
18-W31-087	Shandong	Secretion	32	>64	>128	32	>256	>128	>128	2	>32	>128	>8	ST244	OXA-677 (V117F), PER-1, OprD (237stop)*	crpP, fosA, sul1, aph(3')-IIB, aph(6)-Id, aacA4-12, aadA1, catB7

Note: *Frameshift caused by base-deletion.

Abbreviations: IPM, imipenem; MEM, meropenem; CZA, ceftazidime/avibactam; TZP, piperacillin/tazobactam; FEP, ceftepime; AMK, amikacin; POL, polymyxin B; CAZ, ceftazidime; ATM, aztreonam; CIP, ciprofloxacin; OMP, outer membrane proteins.

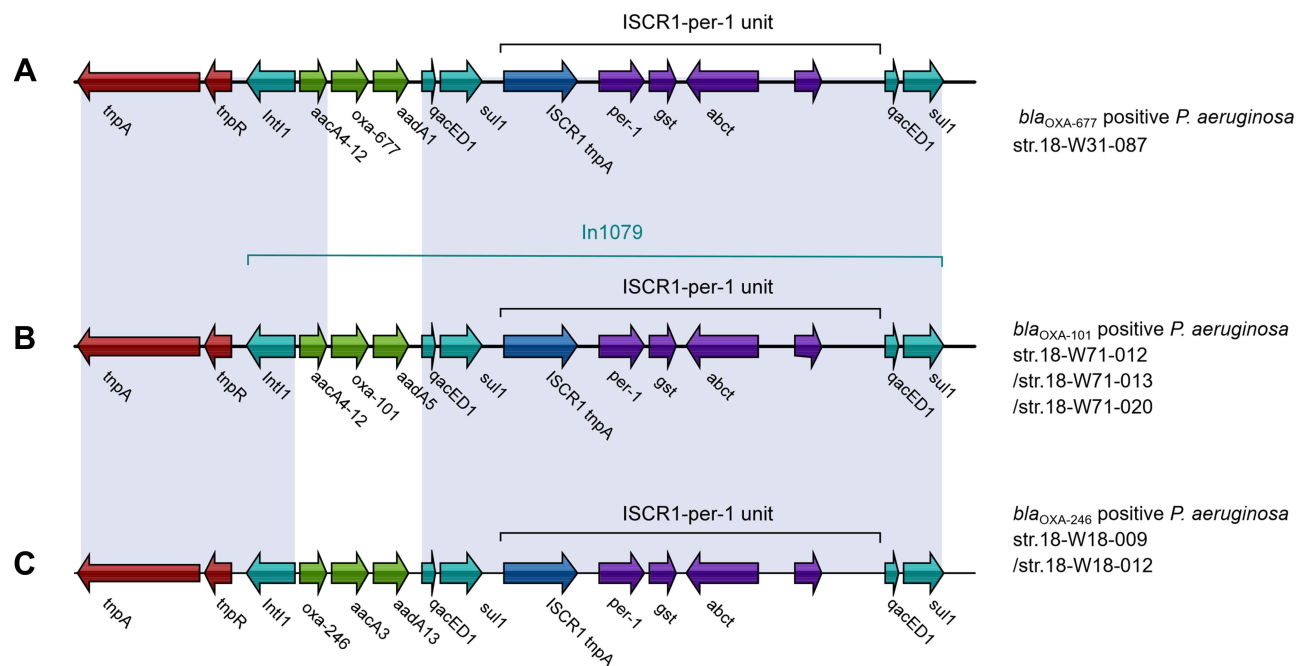


Figure 1 The genomic context of the *bla*_{OXA-677} (A), *bla*_{OXA-101} (B), *bla*_{OXA-246} (C). Genes are denoted by arrows. Genes, MGEs, and other features are colored based on their functional classification.

phenylalanine117 is closer to leucine155 in the omega loop (Figure 3).

Characteristic of Transformants Carrying *bla*_{OXA-677}

Transformants were obtained from the screening plates after verification of an 822bp PCR-amplified product of *bla*_{OXA-677}. Two sequence-confirmed transformants were randomly selected for antimicrobial susceptibility tests to ensure consistent results. Compared with the empty host, MIC of meropenem for the *E. coli* DH5 α transformant with the recombinant plasmid pHSG398 carrying *bla*_{OXA-677} was elevated by 8 times (Table 2). No significant increase was observed for imipenem, as well as ceftolozane/tazobactam, piperacillin/tazobactam, ceftazidime/avibactam, ceftazidime, aztreonam, cefepime, amikacin, polymyxin B, and ciprofloxacin (Table 2).

Discussion

OXA-10-like enzymes are widespread in *P. aeruginosa* and *A.baumannii*, and less common in *Enterobacterales* in Asia, Europe, South America, and Africa. It is the first report of the existence of *bla*_{OXA-10-like} in ST298 and

ST244 clones in China, which are two of the top 10 high-risk MDR/XDR *P. aeruginosa* clones causing nosocomial infection worldwide.¹⁷ *bla*_{OXA-101} was first reported in *Enterobacteriaceae*, *Citrobacter freundii*, *Escherichia coli*, and *Enterobacter cloacae* in Argentina in 2005 and now has been detected in *A. baumannii* and *P. aeruginosa* in China.^{18–20} *bla*_{OXA-246} was first found among ceftazidime-resistant *P. aeruginosa* clinical isolates in China in 2007.²¹ *bla*_{OXA-677} and its GCA *aacA4-12-bla*_{OXA-677}-*aadA1* were found for the first time in China in this study. Our study showed that these three variants were all embedded in class 1 integrons harboring an ISCR1-*bla*_{PER-1} unit. In public databases, the most similar integron was In1079, located on the chromosome of *P. aeruginosa*.²²

Previous studies showed that the lack of OprD protein could only raise meropenem to 2–4 mg/L.²³ Antunes et al suggested that the OXA-10 β -lactamase, which is regarded as the narrow-spectrum enzyme, is CHDL in fact.⁹ We speculate that the production of OXA-10-like enzymes and decrease of membrane permeability may cooperate kindly to confer meropenem resistance, for we found premature termination of *oprD* genes for all six *bla*_{OXA-10} variants positive strains in

OXA-10	MKTFAAYVIIACLSSTALAGSITENTSWNKEFSAEAVNGVFLCKSSSKSCATNDLARAS	60
OXA-101	-----T-----S-----F-----N--	60
OXA-246	-----	60
OXA-677	-----	60
OXA-10	KEYLPAST STFK IIPNAIIGLETGVIKNEHQVFKWDGKPRAMKQWERDLTLRGAIQV SAV PVF	120
OXA-101	-----S-----	120
OXA-246	-----	120
OXA-677	----- F ---	120
OXA-10	QQIAREVGEVRMQKYLKKFS YGN QNISGGIDK FW LEGQLRISAVNQVEFLESLYLNKLSA	180
OXA-101	-----F-----	180
OXA-246	-----N-----	180
OXA-677	-----	180
OXA-10	SKENQLIVKEALVTEAAPEYLVHS KTG FSGVGTESNPGVAWWVGWVEKETEVYFFAFNMD	240
OXA-101	-----G-----	240
OXA-246	-----	240
OXA-677	-----	240
OXA-10	IDNESKLPLRKSIPTKIMESEGIIGG	266
OXA-101	----N-----A-----	266
OXA-246	-----	266
OXA-677	-----	266

Figure 2 Amino acid alignment of OXA-10 and three OXA-10-like enzymes identified in this study. A dash indicates synonymous substitution without amino acid change. Key amino acid motifs conserved among OXA-10 are highlighted in red.

this study. Moreover, the antimicrobial susceptibility data of the clinical isolates and transformants showed that *bla*_{OXA-677} producing strain had a higher MIC level of meropenem. However, there was no difference between the resistance genes of *bla*_{OXA-677}-positive strain and *bla*_{OXA-101}-positive strains other than *bla*_{OXA-10-like} genes. Structural bases of OXA-677 may explain the high-level resistance. As previously shown, OXA-677 has a single mutation at Val117. The activity of class D β -lactamases, including OXA-10, is dependent on Lys70 carboxylation in the active site. Val117 of the S¹¹⁵A¹¹⁶V¹¹⁷ catalytic motif is the component of the hydrophobic core surrounding Lys70 and is strictly conserve.^{24,25} It was validated in OXA-655 with V117L that alterations of the interactions with carbapenems were brought by substitutions at this key site.²⁶ In both crystal structures of OXA-655 and OXA-677

proteins, their position 117 residues are closer to Leu155 in the omega loop, and their interactions seem stronger. This conformational change probably alters the accessibility of the meropenem to the active site. Further work is required to demonstrate the correlation between its structure and function.

In addition, all OXA-10 variants-producing isolates in this study harbor ISCR1-*bla*_{PER-1}. Recent studies have shown that *bla*_{PER-1} may be a source of resistance to the new class of β -lactam/ β -lactamase inhibitors ceftazidime/avibactam and ceftolozane/tazobactam.²⁷ Therefore, these β -lactamase genes combined with porin loss may confer resistance to almost all kinds of carbapenem and β -lactam/ β -lactamase inhibitors. Coupled with other gene cassettes (*aadA*, *aacA*, *sulI*) in Tn3-like transposons conferring resistance to fluoroquinolone, aminoglycosides, and sulfonamides, these

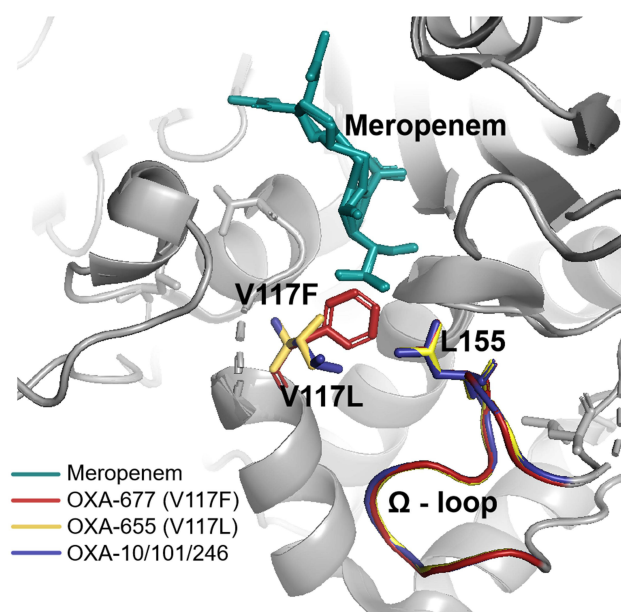


Figure 3 Superposition of OXA-10/OXA-101/ OXA-246 (blue), OXA-655 (yellow), and OXA-677 (red) with detailed view of active site. Leu155 and the omega loop are shown and labeled by the three mixed colors. Meropenem is depicted in green as a substrate. In the OXA-677 enzyme, the phenylalanine117 is closer to leucine155 in the omega loop.

clinical isolates may be more likely to develop to MDR/XDR strains. And these gene cassettes might be transmitted among different strains due to the mobile genetic element. It is unfortunate from a clinical treatment standpoint for there might be no antibiotic

available for these strains. Polymyxin B might be the only treatment option, albeit with toxicity. Therefore, screening for OXA-10 mutants during the treatment of *P. aeruginosa* infection may have some instructive significance for adjustment for medication use.

Conclusion

In summary, globally widespread OXA-10-like enzymes have shown their evolutionary potential toward enhancing carbapenem resistance. In this study, OXA-677 with increased carbapenemase activity was identified in a clinical MDR *P. aeruginosa* strain for the first time in China. It is also the first report of the existence of OXA-10 variants (OXA-677, OXA-101, and OXA-246) in ST298 and ST244 clones in China. Our findings provide evidence supporting the claim that OXA-10 should be classified as CHDL. It may help elucidate the role of OXA-10 variants in MDR *P. aeruginosa* strains. *bla*_{OXA-10-like} along with other gene cassettes in class 1 integrons and membrane permeability defects contribute to the resistance to almost all available antibiotics, which brings challenges in their treatment. Further work with a larger number of positive strains is now needed to elucidate the mechanism of OXA-677 and other OXA-10 mutants.

Table 2 β -Lactam Resistance Profile Conferred by OXA-677 in *E. coli* DH5 α Under Isogenic Conditions

Antimicrobial Agents	MIC (mg/L)				Ration of A/B
	Donor Strain	(A) DH5 α -pHSG398- <i>bla</i> _{OXA-677}	DH5 α - pHSG398	(B) Recipient Strain (DH5 α)	
Imipenem	32	0.25	0.125	0.125	2
Meropenem	>64	0.125	0.015	0.015	8
Ceftolozane/tazobactam	>128	0.25	0.5	0.25	1
Piperacillin/tazobactam	>256	8	4	4	2
Ceftazidime/avibactam	32	0.125	0.125	0.06	2
Ceftazidime	>32	0.5	0.5	≤0.5	/
Ceftriaxone	>32	≤32	≤32	≤32	/
Cefuroxime	>32	8	16	8	1
Aztreonam	>128	≤1	≤1	≤1	/
Cefepime	>128	0.125	0.125	0.06	2
Amikacin	>128	≤1	≤1	≤1	/
Polymyxin B	2	0.25	0.25	0.25	1
Ciprofloxacin	>8	≤8	≤8	≤8	/

Ethics Approval

The study protocol was approved by the Institutional Review Board of Huashan Hospital, Fudan University (No. 2020-041).

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Disclosure

The authors report no conflicts of interest in this work.

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