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ORIGINAL RESEARCH

Transcriptomic Insights into Adaptive Strategies of *Klebsiella pneumoniae* Co-Producing KPC-2 and NDM-5 Carbapenemases Under Meropenem Stress

Liting Dai[1,*], Huimin Zhang[1,*], Yingyi Cao[1,*], Jiacheng Ding[1], Jiaxin Tang[2], Zhirou Xiao[2], Zhemei Chen[2], Jiahui Ling[1], Mengxue Zou[1], Xiwu Cao[1], Lijuan Lin[1], Ziheng Xu[1], Wanting Liu[1], Dingqiang Chen[1], Peibo Yuan[1]

[1]Microbiome Medicine Center, Department of Laboratory Medicine, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong, 510120, People's Republic of China; [2]Department of Clinical Laboratory, National Center for Respiratory Medicine, National Clinical Research Center for Respiratory Disease, State Key Laboratory of Respiratory Disease, Guangzhou Institute of Respiratory Health, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong, 510120, People's Republic of China

*These authors contributed equally to this work

Correspondence: Peibo Yuan; Dingqiang Chen, Microbiome Medicine Center, Department of Laboratory Medicine, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong, 510120, People's Republic of China, Tel +86 15602335139; +86 15011943079, Email pbyuanpb@163.com; jyksys@126.com

Background: The emergence of *Klebsiella pneumoniae* strains that co-produce multiple carbapenemases poses significant threats to clinical management; however, the molecular adaptations driving their resilience remain poorly characterized.

Methods: This study aimed to investigate the resistance and adaptive mechanisms of the bla_{KPC-2} and bla_{NDM-5} co-producing *K. pneumoniae* KP4 strains. The antimicrobial susceptibility of KP4 was determined using Vitek, a carbapenemase inhibitor enhancement assay, and single-cell Raman spectroscopy. Transcriptomic profiling was used to identify key pathways involved in meropenem tolerance and key differentially expressed genes (DEGs). Transcriptomic analysis mapped stress-responsive pathways and DEGs, whereas qPCR validated carbapenemase gene expression and plasmid copy number variation. Biofilm dynamics were assessed under antibiotic pressure conditions.

Results: KP4 exhibited pan-drug resistance, while retaining tigecycline susceptibility. Meropenem exposure (256 mg/L) triggered 161 DEGs primarily associated with metabolic pathways, including arginine biosynthesis, amino acid metabolism, and secondary metabolite production. Notably, qPCR quantification of bacterial DNA revealed plasmid copy number amplification of bla_{NDM-5} (+2.58-fold, $p<0.05$) and bla_{KPC-2} (+1.49-fold, $p=0.156$), which may be associated with upregulation of the *repA* gene on the bla_{KPC-2}-located plasmid and the *dnaA* gene on the chromosome. Meropenem exposure enhanced biofilm formation by 42% ($p<0.01$), driven by the upregulation *trpE* (tryptophan synthesis, 4.2-fold), *wecC* (exopolysaccharide production, 2.1-fold), and *gcvA* (glycine metabolism, 2.05-fold).

Conclusion: bla_{KPC-2} and bla_{NDM-5} co-producing *K. pneumoniae* employ a two-pronged resistance strategy: bla_{NDM-5} plasmid amplification ensures enzymatic overdose, while biofilm induction creates physical barriers. These findings decode the survival strategies of pan-resistant pathogens and inform novel therapeutic approaches that target plasmid stability and biofilm disruption.

Keywords: carbapenem-resistant *Klebsiella pneumoniae*, CRKP, bla_{KPC-2}, bla_{NDM-5}, transcriptome, plasmid copy number, biofilm

Introduction

Antimicrobial resistance represents a global health emergency, with carbapenem-resistant *Klebsiella pneumoniae* (CRKP) ranking as a critical-priority pathogen by WHO.[1] The main mechanism of carbapenems resistance in *K. pneumoniae* is mediated by carbapenemases encoded by highly transmissible plasmids.[2] Among them,

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K. pneumoniae carbapenemase (KPC) and New Delhi metallo-β-lactamase (NDM) are the two main carbapenemases produced by most CRKP strains.[3–5] KPC can hydrolyze various β-lactam drugs, including carbapenem antibiotics, and can be inhibited by avibactam.[3] While NDM is capable of hydrolyzing all β-lactams except aztreonam.[6] What's more alarming is the recent emergence of *K. pneumoniae* strains that co-producing KPC and NDM.[4,5,7,8] These strains exhibit heightened resistance to carbapenems and a broader spectrum of resistance to other antibiotics, posing an even more severe threat to global public health security.[5,9–11] This surge underscores an urgent need to understand dual-enzyme dynamics under therapeutic pressure, how KPC and NDM expression/activation are temporally or genetically coordinated, and the molecular basis for plasmid coexistence under antibiotic stress. This study bridges these knowledge gaps by analyzing the $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-5}}$ co-producing CRKP strain KP4 through an integrated approach combining transcriptomics, plasmid dynamics, and biofilm assays, depicting synergistic resistance strategies of pan-resistant pathogens and offering valuable information for the development of strategies to combat antibiotic resistance.

Materials and Methods

Bacterial Strains

The strain KP4, co-producing carbapenemase genes $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-5}}$ was obtained from the sputum sample of a 72-year-old patient in September 2021 and was originally identified as *K. pneumoniae* by Vitek MALDI-TOF MS (BioMérieux, France). The strain was routinely preserved in our laboratory repository, conducted under the approved ethics protocol 2021-KY-046-01 titled "Genomic Sequencing Analysis of Clinical Bacterial Isolates and Investigation of Their Pathogenic Drug Resistance Mechanisms". No patient interventions or additional sample collections were conducted.

Antimicrobial Susceptibility Testing

The susceptibility of the KP4 strain to antimicrobial agents was initially assessed using gram-negative susceptibility cards on the Vitek system (bioMerieux, France) and broth microdilution method. The minimum inhibitory concentrations (MICs) of antimicrobial agents including ertapenem, imipenem, meropenem, amoxicillin-clavulanic acid, piperacillin/tazobactam, cefuroxime, cefuroxime axetil, cefoxitin, ceftazidime, ceftriaxone, cefoperazone/sulbactam, cefepime, amikacin, levofloxacin, and trimethoprim/sulfamethoxazole were determined using Gram-negative susceptibility cards on the Vitek system. Susceptibility testing for tigecycline, meropenem, imipenem, ertapenem, and ceftazidime/avibactam was performed using the broth microdilution method. ATCC 25922 was used as quality control. MICs results were interpreted according to the Clinical Laboratory Standards Institute (CLSI) guidelines (M100 Ed34). The breakpoint of tigecycline was based on the standards of the European Committee for Antimicrobial Susceptibility Testing (EUCAST 2024) guidelines (http://www.eucast.org/Clinical-breakpoints/).

Genome Sequencing

Total genomic DNA of the KP4 strain was extracted using the Solarbio Bacterial Genomic DNA Kit (Guangzhou Yihong Trading Co., Ltd., Guangzhou, China) and used for genome sequencing. The genome was sequenced using a combination of Pacbio/ONT+Illumina (Guangzhou Gene Denovo Co. Ltd., Guangzhou, China). Continuous long reads were attained from SMRT sequencing runs and were used for de novo assembly using Falcon (version 0.3.0).[12] Continuous long reads were attained from ONT sequencing and were used for de novo assembly using Flye (version 2.8.1-b1676).[13] Raw data from Illumina platform were filtered using FASTP (version 0.20.0).[14] Plasmid replicon typing was performed using PlasmidFinder database (https://cge.cbs.dtu.dk//services/PlasmidFinder/), and all resistance genes were detected using the Comprehensive Antibiotic Resistance Database (CARD) (http://arpcard.mcmaster.ca) and Basic Local Alignment Search Tool (BLAST).

PCR Screening of Carbapenemase Genes

KP4 was screened for the carbapenemase genes $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-5}}$ by PCR using the primers listed in Table S1. The PCR was performed for 34 cycles of 95°C for 15s, 58°C for 15s, and 72°C for 30s. The PCR products were verified using 1% agarose gel electrophoresis at 120 V for 30 min.

Carbapenemase Inhibitor Enhancement Test

The carbapenemase inhibitor enhancement test, employing combined-disk tests of imipenem alone, imipenem with either APB or EDTA, and imipenem with both APB and EDTA, was performed to detect carbapenemase production and differentiate Class A carbapenemase and metallo-β-lactamase (MBL), as previously described.[15,16] Class A carbapenemase production was considered positive if the difference in the zone of inhibition between the imipenem disk with APB and the imipenem disk alone was ≥5 mm. MBL production was considered positive when the difference in the zone of inhibition between the imipenem disk with EDTA and the imipenem disk alone is ≥5 mm. Additionally, the strain was considered negative if the difference in the zone of inhibition between the imipenem disks with APB or EDTA and the imipenem disk alone was < 5 mm. However, if the difference in the zone of inhibition between the imipenem disk with both APB and EDTA was increased by ≥5 mm compared to the imipenem disk alone, the strain was considered to produce both class A carbapenemase and MBL.[16]

Acquisition and Analysis of Single-Cell Raman Spectrum

To investigate the differences in the metabolic levels of KP4 at different meropenem concentrations, single-cell Raman spectroscopy was used to examine the C-D ratio at different concentrations. The sample preparation method was as previously described.[17] In brief, a single colony from an overnight cultured KP4 strain was picked, adjusted to a McFarland turbidity of 0.5, and then added to 2×MH broth with a gradient concentration of meropenem (0, 32, 64, 128, 256, and 512 mg/L) for 1 h. Subsequently, D_2O (99.8 atom% D, Sigma-Aldrich) was added to a final concentration of 30% and incubation was continued for another 2 h. After incubation, the mixture was centrifuged at 5000 g for three minutes, the pellet was washed with sterile deionized water, and centrifuged again at 5000 × g for another three minutes. The washing step was repeated at least thrice. The pellet was resuspended in sterile deionized water and spotted onto a CaF_2 slide to form a single layer of bacterial spots. All Raman measurements were conducted using a Raman-activated Cell Sorting and Sequencing (RACS-Seq) instrument (Qingdao Single-cell Biotechnology Co., Ltd.) with a grating of 600 g/mm and an acquisition time of 3 s, and the laser power was set to 60 mW. A minimum of 100 valid spectra was collected for each sample. The original Raman spectral data were preprocessed using the RamaAI platform (http://www.ramanai.net), including background removal, baseline correction, normalization, and smoothing. The C-D ratio, which reflects the metabolic activity of a single cell, was calculated by dividing the area of the C-D band (2050–2300 cm^{-1}) by the sum of the areas of the C-D and C-H bands (2800–3050 cm^{-1}).

Transcriptome Sequencing and Identification of Differentially Expressed Genes (DEGs)

To investigate the transcriptomic response that persisted during antibiotic treatment in the KP4 strain, RNA-seq was performed on KP4 cells after exposure to 1/2 MIC meropenem. Overnight bacterial cultures were diluted 1:100 in 20 mL of fresh LB medium and incubated until the bacterial suspension reached a McFarland turbidity of 0.5. The following day, samples from the control (no drug treatment) and treated groups (1/2 MIC=256 mg/L) were incubated at 37 °C for 24 h with shaking. Samples were recovered by centrifugation, resuspended, and rapidly frozen in liquid nitrogen. They were stored at −80°C until RNA-seq was performed. To ensure sufficient bacterial growth and to assess the changes in transcriptional expression levels associated with antibiotic treatment, we used samples treated with antibiotics for 3 h for RNA-seq analysis. This is because, under antibiotic stress, the strain can exhibit significant gene expression changes in as little as 3 h, which is largely attributed to the bacteria's rapid response mechanism and highly regulated gene expression system. RNA extraction, cDNA library construction, and paired RNA-seq (Illumina) were performed using the Gene Denovo Biotechnology (Guangzhou, China). The quality of the original sequencing data was assessed by Fastq (https://github.com/OpenGene/fastp).[14] The clean reads of the samples were mapped to the reference transcriptome using Bowtie2.[18] The transcriptional expression levels was calculated using RSEM software, FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values were calculated for gene expression quantification.[19] DEGs were identified using edgeR,[20] with thresholds of absolute |log2(fold change)| > 1 and FDR < 0.05. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using OmicShare (http://www.omicshare.com/tools/index.php/).

The Copy Number of *bla*$_{KPC-2}$ and *bla*$_{NDM-5}$

The copy numbers of *bla*$_{KPC-2}$ and *bla*$_{NDM-5}$ were quantified by qPCR.[21] Overnight bacterial cultures were diluted 1:100 in 5 mL of fresh LB medium and incubated until the bacterial suspension reached a McFarland turbidity of 0.5. Afterwards, the bacterial suspension was incubated with meropenem for 3 h and the bacterial cells were collected by centrifugation. DNA was extracted using a Solarbio Bacterial Genomic DNA Kit (Guangzhou Yihong Trading Co. Ltd). qPCR was performed using ABI 7500 (Applied Biosystems, USA) and SYBR Green Pro Taq HS qPCR kit III (Low Rox Plus) (Accurate Biotechnology (Hunan) Co., Ltd). The primers used are listed in Table S2. The copy numbers of the target genes were normalized to those of the housekeeping gene *rpoB*. The gene copy number was quantified using the ΔΔCt method, with the Ct values of each sample normalized against those of the housekeeping gene *rpoB* in *E. coli*.[22]

Biofilm Formation Assay

To measure biofilm formation ability under meropenem treatment, 96-well microtiter plates were used in a crystal violet assay. The procedure is as follows, the overnight bacterial cultures were diluted 1:100 in 10 mL of fresh LB medium and incubated until the bacterial suspension reached a McFarland turbidity of 0.5.[23] Then, incubate the bacterial culture with 1/2 MIC meropenem before transferring it to a 96-well microtiter plate and incubate at 37°C for 3 hours. Afterwards, the medium was decanted and planktonic cells were gently washed off with phosphate-buffered saline (PBS). The wells were fixed with 4% paraformaldehyde for 15 min, followed by another wash with PBS. Next, the wells were stained with 1% crystal violet for 20 min, washed three times with PBS, and allowed to dry. The bound dye was solubilized with 200 μL of 33% glacial acetic acid and quantified by measuring absorbance at 570 nm. Wells containing sterile LB without bacteria served as negative controls.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism 9 software. Inter-group differences were assessed by the Student's *t*-test. All data represent at least three independent experiments, and the results are presented as mean±standard deviation (SD). $P<0.05$ was set as the significance threshold.

Results and Discussion

Genomic Architecture and Antimicrobial Resistance Profile

The *K. pneumoniae* KP4 strain, an ST11-type *K. pneumoniae* strain isolated from a sputum sample of a 72-year-old patient in September 2021, was characterized by Vitek MALDI-TOF MS and whole-genome sequencing. Its genome comprises a 5,318,593 bp chromosome harboring *bla*$_{SHV-12}$, *qacE, aadA2*, and *fosA6* resistance determinants, along with nine plasmids (Table 1). The critical carbapenemase genes, *bla*$_{KPC-2}$ and *bla*$_{NDM-5}$ were located on distinct plasmids: *bla*$_{KPC-2}$ within a multidrug-resistant IncFII(pHN7A8)/IncR plasmid (134,874 bp) carrying *bla*$_{CTX-M-65}$, *bla*$_{TEM-1B}$, and *rmtB*, whereas *bla*$_{NDM-5}$ resided exclusively on an IncX3 plasmid (44,885 bp). Antimicrobial susceptibility testing (Table 2) revealed pan-resistance to β-lactam/β-lactamase inhibitors (amoxicillin/clavulanic acid MIC≥32 mg/L), cephalosporins (ceftazidime MIC≥64 mg/L), carbapenems (meropenem MIC=512 mg/L, imipenem MIC≥1024 mg/L, ertapenem MIC≥1024 mg/L), and non-β-lactam agents, retaining susceptibility only to tigecycline (MIC=1 mg/L). Strikingly, resistance to ceftazidime/avibactam (MIC≥1024/4 mg/L) suggests the failure of advanced β-lactamase inhibitor therapies.

Phenotypic Validation of Dual Carbapenemase Activity

Co-production of KPC-2 and NDM-5 was confirmed by PCR amplification (Figure 1A) and carbapenemase inhibition assays. Synergistic inhibition zone expansion (5 mm increase) with the EDTA/APB combination (Figure 1B) demonstrated concurrent Class A/B carbapenemase activity. The broth microdilution method determined the MIC of meropenem for the KP4 strain to be 512 mg/L (Figure 1C). Ultra-high carbapenem MICs correlated with metabolic heterogeneity observed via D_2O-Raman spectroscopy: only at 512 mg/L meropenem did significant C-D ratio divergence emerge ($p<0.001$, Figure 1D), where surviving subpopulations exhibited metabolic plasticity. This phenotypic bifurcation —complete growth inhibition versus persistent metabolic activity—suggests dual resistance mechanisms: enzymatic

Table 1 Genomic Information of The *K. pneumoniae* KP4 Strain

Genetic Context	ARG	Length	Drug Class	Plasmid Replicon
Chromosome (contig 1)	$bla_{SHV-182}$	/	Beta-lactam	/
	qacE	/	Disinfectant	
	aadA2	/	Aminoglycoside	
	fosA6	/	Fosfomycin	
pKP4-KPC-2	$bla_{CTX-M-65}$		Beta-lactam	IncFII(pHN7A8), IncR
	bla_{TEM-1B}		Beta-lactam	
	bla_{SHV-12}	134,874	Beta-lactam	
	bla_{KPC-2}		Beta-lactam	
	rmtB		Aminoglycoside	
pKP4-NDM-5	bla_{NDM-5}	44,885	Beta-lactam	IncX3
pKP4-A	/	249,775	/	IncFIB(pNDM-Mar)
pKP4-B	/	129,769	/	IncHI1B(pNDM-MAR), repB
pKP4-C	/	91,858	/	/
pKP4-D	bla_{LAP-2}		Beta-lactam	/
	qnrS1		Fluoroquinolone	
	dfrA14	84,876	Folate pathway antagonist	
	sul2		Sulfamethoxazole	
	tet(A)		Tetracycline	
pKP4-E	/	11,971	/	ColRNAI
pKP4-F	/	5,637	/	ColRNAI
pKP4-G	/	2,664	/	/

Abbreviation: ARG, antibiotic resistance gene.

Table 2 Antimicrobial Susceptibility Profiles of The *K. pneumoniae* Clinical Isolates KP4

Source	Antibiotic	Isolate (MIC mg/L)	
		KP4	ATCC 25922
Vitek	Ertapenem	≥8/R	≤0.12/S
	Imipenem	≥16/R	≤0.25/S
	Meropenem	≥16/R	≤0.25/S
	Amoxicillin/clavulanic acid	≥32/R	4/S
	Piperacillin/tazobactam	≥128/R	≤4/S
	Cefuroxime	≥64/R	4/S
	Cefuroxime axetil	≥64/R	4/S
	Cefoxitin	≥64/R	≤4/S
	Ceftazidime	≥64/R	≤0.12/S
	Ceftriaxone	≥64/R	≤0.25/S
	Cefoperazone/sulbactam	≥64/R	≤8/S
	Cefepime	≥32/R	≤0.12/S
	Amikacin	≥64/R	≤2/S
	Levofloxacin	≥8/R	≤0.12/S
	Trimethoprim/sulfamethoxazole	≥320/R	≤20/S
Broth dilution	Tigecycline	1/S	≤0.5/S
	Meropenem	512/R	0.015625/S
	Imipenem	≥1024/R	0.25/S
	Ertapenem	≥1024/R	0.015625/S
	Ceftazidime/avibactam	≥1024/4/R	/

Abbreviations: R, resistant; S, sensitive; MIC, minimum inhibitory concentration.

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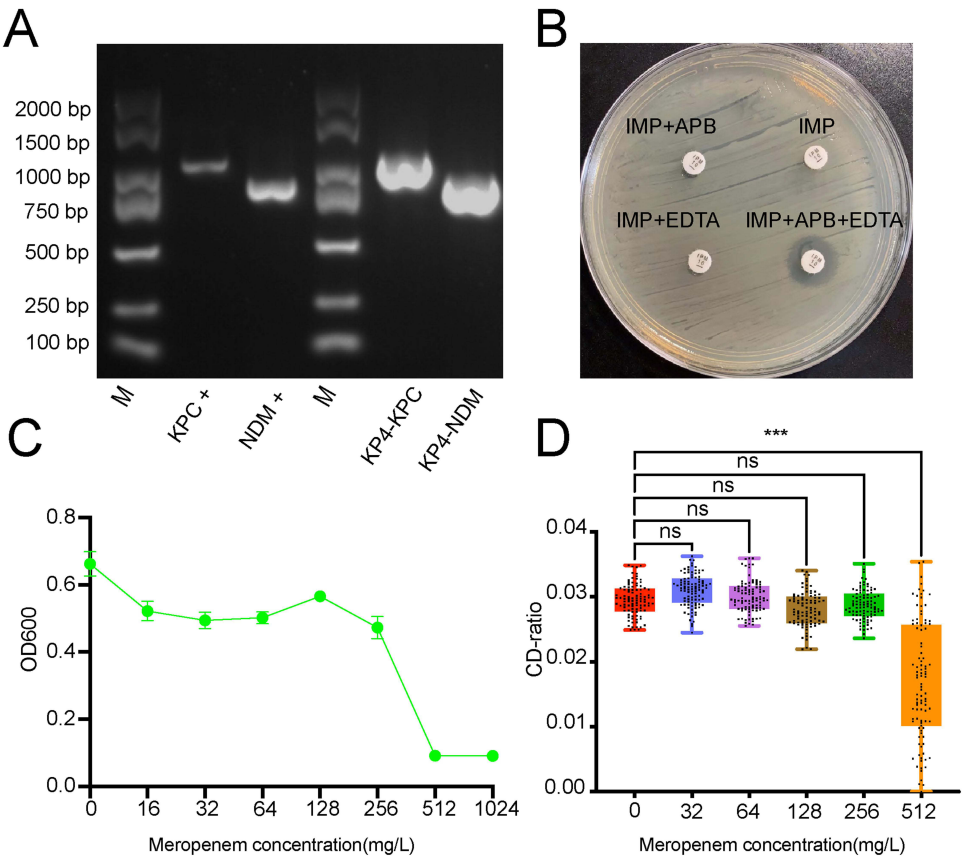

Figure 1 The phenotype experiment results of the KP4 strain. (**A**) Electrophoresis results of KPC gene and NDM gene amplification in KP4 strain. "M" represents DL2000 NDA Maker (sizes labeled: 2000, 1500, 1000, 750, 500, 250, 100 bp); "KPC+" represents positive control of the KPC gene; "NDM+" represents positive control of the NDM gene; "KP4-KPC" and "KP4-NDM" bands correspond to 893 bp (*bla*KPC-2) and 621 bp (*bla*NDM-5), respectively; (**B**) Representative results of the three combined-disc tests using discs of imipenem alone and with APB, EDTA, or APB plus EDTA for the KP4 strain; (**C**) The minimum inhibitory concentration (MIC) of meropenem for the KP4 strain using the microdilution broth method; (**D**) CD-ratio of the KP4 strain under antibiotic treatment at different meropenem concentrations (0, 32, 64, 128, 256, and 512 mg/L). Each point is a measurement of a single cell, and box plots indicate quartiles of each evolved population. ***$p<0.001$.
Abbreviation: n.s., not significant.

degradation by KPC-2/NDM-5 and epigenetic adaptation enabling subpopulation survival under lethal antibiotic pressure.

Transcriptome Analysis of KP4 Isolate Under Meropenem Stress

To investigate the transcriptomic response that persisted under meropenem pressure in KP4 strain, RNA-seq was performed on KP4 after exposure to meropenem for 3 h. Transcriptional analysis revealed 161 DEGs, comprising 100 up-regulated and 61 down-regulated genes (Table S3 and Figure 2A). KEGG pathway analysis of DEGs highlighted significant alterations in key metabolic pathways (Figure 2B), notably "arginine biosynthesis", "amino acid biosynthesis", "pyruvate metabolism", and "secondary metabolite biosynthesis". The most significantly upregulated genes were *fdhF* and *trpE*, with fold-changes of 4.50 and 4.20 respectively, which are critical for energy metabolism and aromatic amino acid synthesis. Conversely, the genes with the largest downregulation fold change were *glvA* and *glvC*, with fold changes of 6.00 and 4.52 respectively, which are involved in maltose metabolism.[24] The encoded GlvA (NAD+-dependent maltose-6'-phosphate glucosidase) and GlvC (PEP-dependent maltose transporter) coordinate carbon source utilization, suggesting resource reallocation during antibiotic challenge. GO enrichment analysis revealed predominant enrichment in catalytic activity (95 DEGs) and binding functions (89 DEGs), with cellular processes (128 DEGs) and metabolic pathways (113 DEGs) constituting the most affected biological domains (Figure 2C).

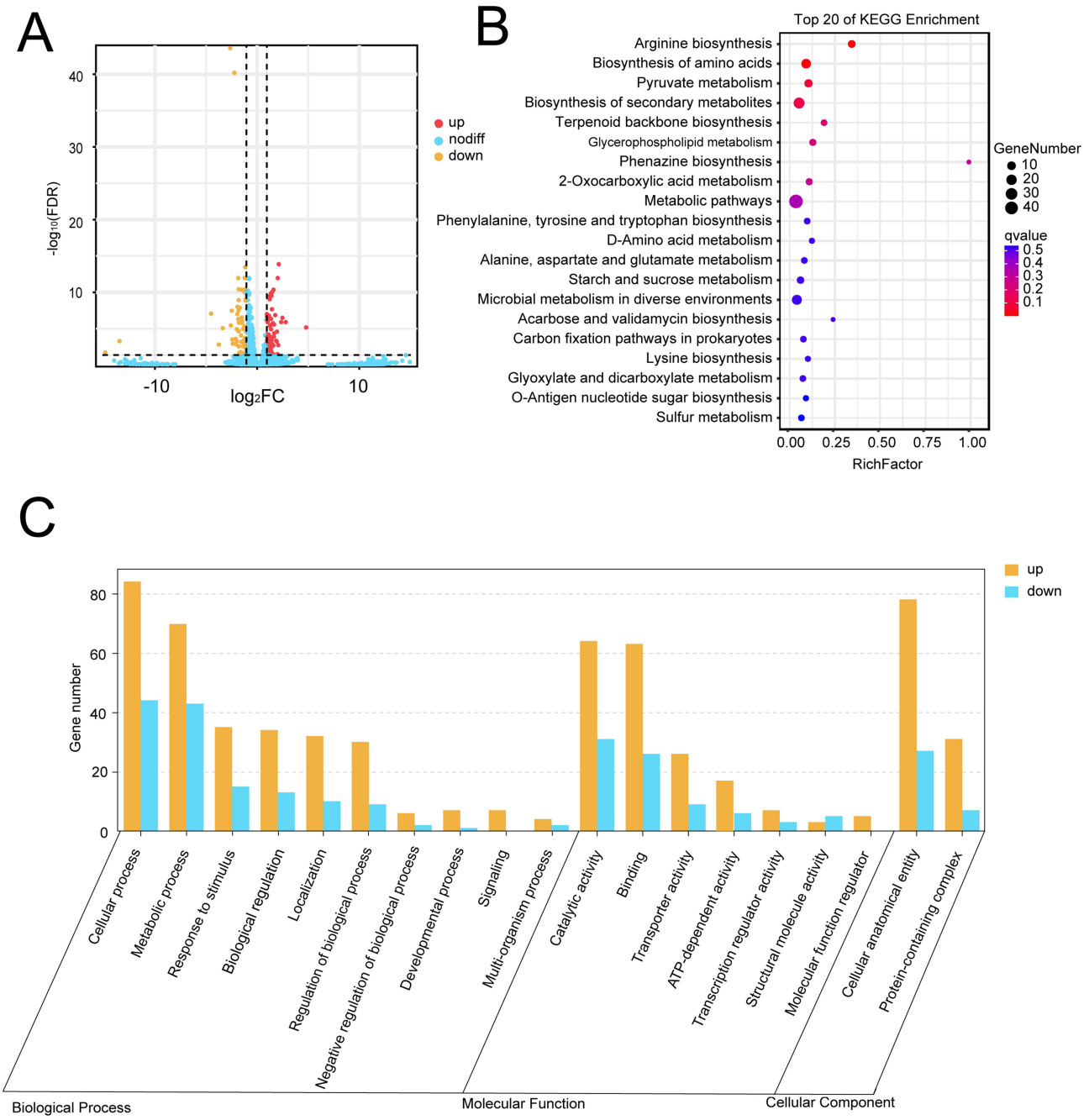

Figure 2 Transcriptome analysis of KP4 strain following exposure to meropenem for 3 h. (**A**) Volcano plot of differentially expressed genes. Each dot in the figure represents a gene. Red dots represent upregulated genes, while Orange dots represent downregulated genes; (**B**) Bubble plot of Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed genes (DEGs). The vertical axis represents the top 20 enriched pathways, and the horizontal axis represents the rich factor corresponding to the pathways. The color of the points indicates the size of the *p*-value. The smaller the *p*-value, the closer the color is to red. The size of the points represents the number of different genes contained within each pathway. The larger the points, the greater the number of genes; (**C**) Histogram of Gene Ontology (GO) enrichment analysis of DEGs. The vertical axis represents the number of genes. Orange bars represent upregulated genes, and blue bars represent downregulated genes. The horizontal axis is functional classification.

Regulation Dynamics of Carbapenemase Genes *bla*$_{KPC-2}$ and *bla*$_{NDM-5}$

Considering the critical function of carbapenemases in resistance to carbapenem antibiotics, this study focused on the modulation of the carbapenemase genes *bla*$_{KPC-2}$ and *bla*$_{NDM-5}$ in response to meropenem-induced stress.

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Notably, transcriptome analysis revealed no significant differences in the transcriptional levels of bla_{KPC-2} and bla_{NDM-5} under meropenem treatment ($p>0.05$, Figure 3A). This observation aligns with prior research, which noted only a modest 0.83-fold increase in the expression of bla_{NDM-1} under imipenem stress relative to the controls.[25]

Furthermore, qPCR quantification of bacterial DNA revealed plasmid copy number amplification of bla_{NDM-5} (2.58-fold, $p<0.05$) and bla_{KPC-2} (1.49-fold, $p=0.156$) (Figure 3B). The variation in the copy numbers of bla_{NDM-5} and bla_{KPC-2} on plasmids may represent an important resistance mechanism by which bacteria adapt to antibiotic pressure. This is consistent with previous research.[26] This research indicated that strains carrying bla_{NDM-5} exhibited a significant copy number increase when exposed to 4 mg/L of meropenem, which is closely correlated with the initiation factor *repA*. They found that the missense mutation *repA* D140Y (GAT→TAT) prevented the binding of $repA^{Mut}$ to *repAP*, thus alleviating feedback inhibition and leading to an increase in plasmid copy number. In this study, the transcriptional expression level of *repA* was significantly increased under antibiotic stress, which might contribute to the increase in bla_{NDM-5} and bla_{KPC-2} copy numbers. Moreover, transcriptomic data analysis showed an increase in the transcriptional expression level of the *dnaA* gene, which has been reported to play a vital role in maintaining plasmid copy number by enhancing the binding of DNA to the origin of replication or increasing DnaA protein expression, thereby overcoming replication inhibition caused by an excess of initiator proteins encoded by the plasmid.[27] According to the genomic annotation, *dnaA* is encoded on the chromosome of the KP4 strain, whereas *repA* is located on the KPC-type plasmid. Given that the

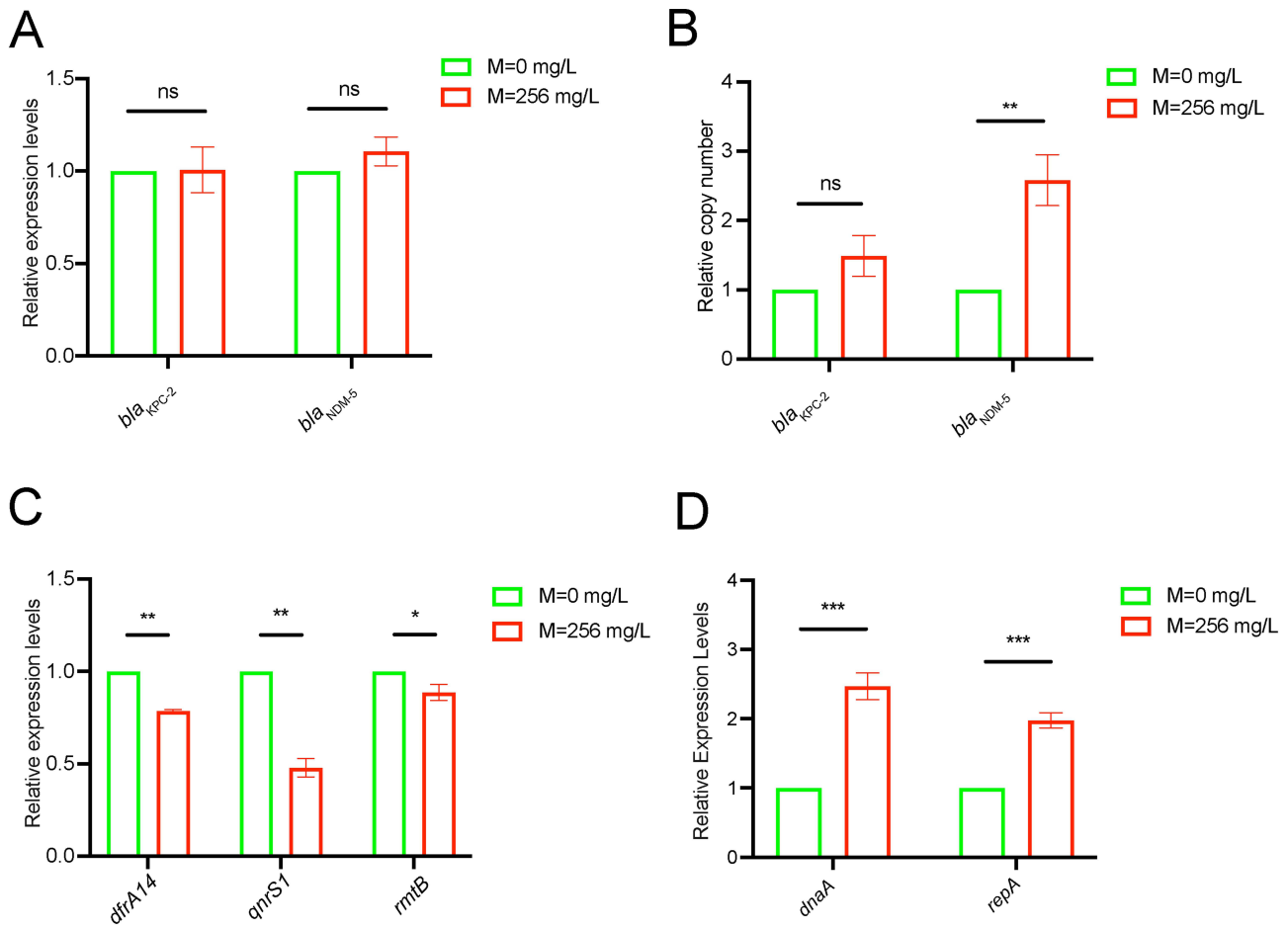

Figure 3 The regulation dynamics of antimicrobial genes in KP4 strain after 3 hours of meropenem treatment. (**A**) The transcriptional expression levels of bla_{KPC-2} and bla_{NDM-5} genes in the KP4 strain; (**B**) The relative copy number of bla_{KPC-2} and bla_{NDM-5} genes in the KP4 strain; (**C**) The transcriptional expression levels of other antibiotic resistance genes in the KP4 strain; (**D**) The transcriptional expression levels related to replication in the KP4 strain. *$p<0.05$, **$p<0.01$,***$p<0.001$. 0 mg/L (control), transcriptional expression levels (or copy number) of bla_{KPC-2} and bla_{NDM-5} without any antibiotic pressure (green bar); 256 mg/L (treatment), change in transcriptional expression levels (or copy number) of bla_{KPC-2} and bla_{NDM-5} under 1/2 MIC meropenem (red bar).
Abbreviation: n.s., not significant.

NDM-type plasmid did not encode any replication-related genes, it can be inferred that the increase in the copy number of bla_{NDM-5} on the NDM-type plasmid was closely associated with the upregulation of *repA* on the KPC-type plasmid and *dnaA* on the chromosome (Figure 3D).

In addition, analysis of transcriptome data showed that the transcriptional expression levels of other antibiotic resistance genes (*dfrA14, qnrS1, rmtB*) were significantly downregulated ($p<0.05$, Figure 3C), which aligns with the bacterial energy-conservation strategies under antibiotic pressure.[28]

Meropenem-Induced Biofilm Enhancement in KP4

To investigate potential non-carbapenemase-mediated resistance mechanisms, we quantitatively assessed the biofilm formation capacity of meropenem-exposed bacterial populations using crystal violet biomass quantification. Crystal violet assays demonstrated that meropenem exposure significantly augmented biofilm formation in KP4 cells compared to untreated controls ($p<0.05$, Figure 4A). This phenotypic adaptation aligns with clinical challenges posed by subtherapeutic antibiotic levels arising from incomplete dosing or tissue penetration,[29] which may paradoxically enhance biofilm-mediated resistance (10–1000×MIC elevation).[30] Our findings corroborate reports of biofilm induction at sub-MIC antibiotic concentrations,[31–33] suggesting such conditions promote selective enrichment of tolerant subpopulations through mutagenic pressure.[34]

The transcriptomic data further revealed that the upregulation of certain DEGs, specifically *trpE, wecC*, and *gcvA*, was correlated with biofilm formation (Figure 4B). These genes showed significant fold-changes of 4.20, 2.10, and 2.05, respectively, in comparison with bacteria not exposed to meropenem. The *trpE* gene, which is central to tryptophan biosynthesis, plays a multifaceted role in protein synthesis and generation of bioactive compounds vital for biofilm architecture. Existing literature supports the notion that *trpE* fosters biofilm formation,[35] as evidenced by the reduced adhesion observed in Δ*trpE* mutants, a defect that can be counteracted by the addition of tryptophan.[36] The *wecC* gene, encoding UDP-N-acetyl-D-mannosamine dehydrogenase, and facilitates a critical step in the synthesis of bacterial polysaccharides, which are integral to biofilm formation. This enzyme converts UDP-ManNAc to UDP-ManNAcA, a key metabolic intermediate in polysaccharide synthesis.[37] Additionally, the *gcvA* gene, a part of the LysR-type transcriptional regulators (LTTRs) family and a key transcription factor regulating the expression of the glycine cleavage system in *Escherichia coli*.[38] It has been reported that *gcvA* is upregulated as part of the stress response associated with biofilm formation.[23] LTTRs, through extensive research, have been recognized as pivotal regulators in the biofilm development process.[39] Thus, *gcvA* is crucial for the orchestration of biofilm formation and regulation by controlling the expression of pertinent genes, which in turn aids in bacterial survival and adaptation within biofilms.

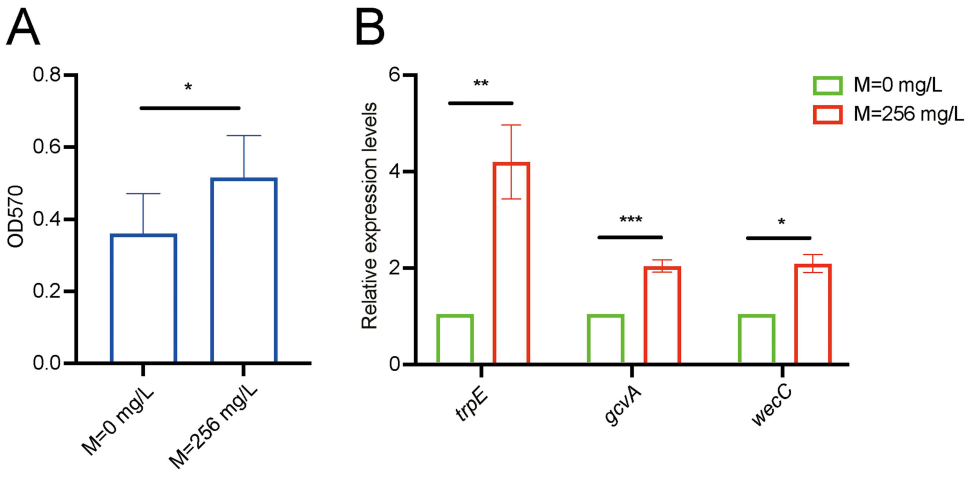

Figure 4 Biofilm formation and expression levels of biofilm-related genes in KP4 strains after 3 hours without meropenem treatment and with 1/2 MIC meropenem. (**A**) The biofilm formation capability of in the KP4 strain; (**B**) The transcriptional expression levels of genes associated with biofilm formation in the KP4 strain under conditions of meropenem-free treatment and 1/2 MIC meropenem. *$p<0.05$, **$p<0.01$, ***$p<0.001$. 0 mg/L (control), transcriptional expression levels of biofilm-related genes in the KP4 strains without any antibiotic pressure (green bar); 256 mg/L (treatment), change in transcriptional expression levels of biofilm-related genes in the KP4 strains under 1/2 MIC meropenem (red bar).
Abbreviation: n.s., not significant.

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In summary, our findings suggest that exposure to meropenem stimulates biofilm formation in the KP4 strain. This effect was mediated by the upregulation of critical genes involved in biofilm development, offering an additional explanation for the observed increase in antibiotic resistance.

Conclusion

This study comprehensively examined the antibiotic resistance and transcriptomic profiles of *K. pneumoniae* KP4 co-producing carbapenemases, KPC-2, and NDM-5. In 1/2 MIC meropenem stress induced 161 DEGs, with pathways like "arginine biosynthesis" and "amino acid biosynthesis" being notably affected. Although $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-5}}$ expression levels remained stable, $bla_{\text{NDM-5}}$ copy number increased significantly. Moreover, 1/2 MIC of meropenem also significantly enhanced biofilm formation by KP4, and *trpE* upregulation might play a crucial role. These findings shed light on KPC-2 and NDM-5 co-producing CRKP resistance and adaptation to carbapenems, underscoring the importance of understanding the bacterial responses to antibiotic stress to combat antibiotic resistance in clinical practice.

Abbreviations

CRKP, carbapenem-resistant *Klebsiella pneumoniae*; DEGs, differentially expressed genes; KPC, *Klebsiella pneumoniae* carbapenemase; NDM, new Delhi metallo-β-lactamase; MICs, minimum inhibitory concentration; ARGs, antimicrobial resistance genes; PCR, polymerase chain reaction; APB, boronic acid; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO: Gene Ontology; LTTRs: LysR-type transcriptional regulators; FDR: false discovery rate; qPCR: quantitative PCR; MBL: metallo-β-lactamase; RACS-seq: raman-activated cell sorting and sequencing; FPKM: fragments per kilobase of transcript per million mapped reads; PBS: phosphate-buffered saline.

Data Sharing Statement

The BioProject numbers under which the generated sequencing data could be found are PRJCA029936 and PRJCA035846.

Ethical Statement

Ethical clearance for the study was granted by the Ethics Committee of Zhujiang Hospital, Southern Medical University, under the reference number 2021-KY-046-01 titled "Genomic Sequencing Analysis of Clinical Bacterial Isolates and Investigation of Their Pathogenic Drug Resistance Mechanisms". The work focused exclusively on antimicrobial resistance characterization and transcriptomic investigations of a pre-existing *K. pneumoniae* strain, which falls within the scope of the approved protocol encompassing mechanistic studies on bacterial drug resistance. The strain was routinely preserved in our laboratory repository, no patient interventions or additional sample collections were conducted. Given the retrospective nature of the analysis involving anonymized, routinely collected data, the need for informed consent was dispensed with. This study adhered to the guidelines outlined in the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas, took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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