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Enterobacterales use capsules, transporters, mobile genetic elements, and other evolutionary adaptations to survive antibiotics exposure in the absence of resistance genes

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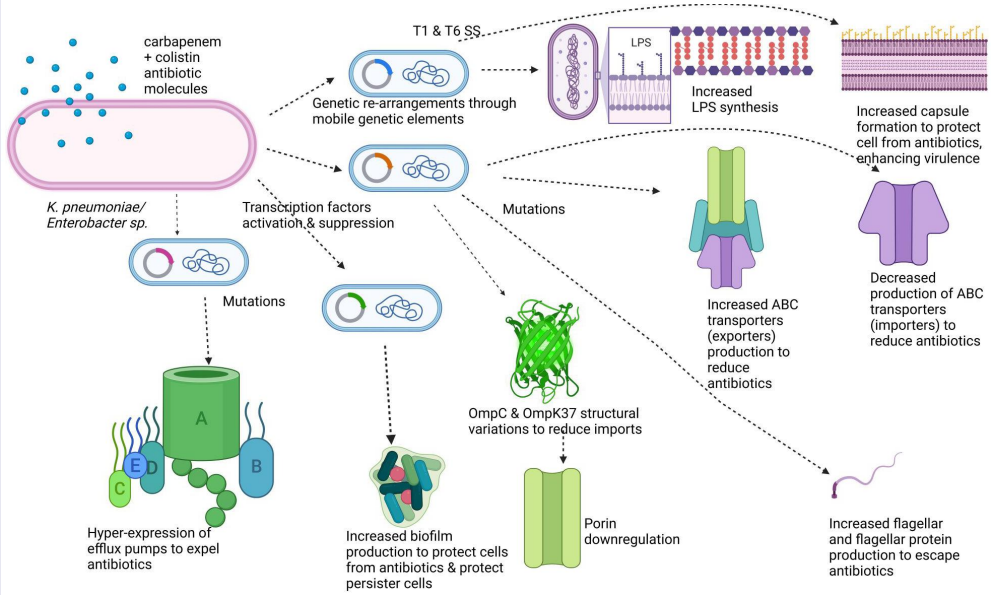
ABSTRACT

Carbapenems and colistin are last-resort antibiotics used to manage difficult-to-treat infections caused by Gram-negative bacteria. However, resistance to these two antibiotics is rising globally, and there is limited knowledge on how pathogens evolve resistance when known resistance genes are absent.

Methods: Whole-genome sequencing, transcriptomic profiling, and epigenomic analyses were performed. Phenotypic assays were used to evaluate the effects of various inhibitors on antibiotic susceptibility, while bioinformatic pipelines were used to characterize resistance determinants, virulence factors, and mobile genetic elements (MGEs).

Results: Phylogenetic analysis revealed widespread carriage of diverse resistance genes, particularly on plasmids of *K. pneumoniae*, while *Enterobacter* species possessed fewer known ARGs. Despite lacking known carbapenemase and *mcr* genes, several isolates demonstrated colistin or carbapenem resistance mediated by upregulation of efflux pumps, overproduction of capsular polysaccharides, mutations in outer membrane proteins, and potential lipopolysaccharide-modifying enzymes. Transcriptomic analysis revealed significant differential gene expression upon antibiotic exposure. Notably, genes encoding ABC transporter proteins were significantly downregulated (p-value <0.0001, fold change > 10), while genes encoding transposases were significantly upregulated (p-value <0.0001, fold change > 11). These changes underscore the critical role of transporters and MGEs in antibiotic resistance adaptation.

Conclusions: In the absence of canonical carbapenemase and *mcr* genes, *K. pneumoniae* and *Enterobacter* species can deploy a spectrum of adaptive mechanisms, including efflux pumps, mobile elements, and altered outer membrane/capsule structures, to overcome colistin and carbapenem treatments. These findings support the need for ongoing surveillance of novel or



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Tweet: "Exposure of *K. pneumoniae* and *E. cloacae* to colistin and ertapenem resulted in important genetic and transcriptional changes in transporters, transcription factors, mobile elements, and genes with unknown function: could these be new resistance mechanisms?"

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Introduction

Multidrug-resistant (MDR) *Klebsiella pneumoniae* and *Enterobacter* species have become increasingly common pathogens responsible for serious nosocomial infections, such as bloodstream, urinary tract, and respiratory tract infections [1]. Owing to the resistance of these pathogens to many first- and second-line therapies, carbapenems and colistin frequently serve as the last effective therapeutic agents. Management of such infections has resulted in the overuse of antibiotics, alongside the emergence and rapid dissemination of super bugs resistant to both carbapenems and colistin [2,3]. Unfortunately, the global surge in carbapenem and colistin resistance has severely limited treatment options.

Resistance mechanisms against colistin primarily involve modifications of the bacterial outer membrane, particularly lipid A alterations mediated by enzymes such as phosphoethanolamine transferases (encoded by mcr genes) [4–9]. Carbapenem resistance, on the other hand, frequently results from the production of carbapenemases or modifications in porins and efflux pumps, limiting drug entry or enhancing efflux [4–9]. Despite these well-characterized mechanisms, novel resistance adaptations continue to emerge, highlighting the need to explore transcriptional and genetic adaptations further.

Carbapenem-resistant *Enterobacteriaceae* (CRE) in a clinical setting is largely mediated by the acquisition of carbapenemases, which are commonly associated with mobile genetic elements (MGEs). These MGEs include plasmids, transposons, and integrons [10], which facilitate wide resistance gene dissemination between animal- and human pathogens [11]. In South Africa, there have been several reports of carbapenemase-producing *Enterobacteriaceae* outbreaks in the clinical setting [4–6,12]. Carbapenemases that have been identified in South Africa include *Klebsiella pneumoniae* carbapenemase (KPC), Verona Integron-Mediated Metallo-β-lactamase (VIM), Imipenemase (IMP), New Delhi metallo β-lactamase (NDM), and oxacillinase (OXA) [10]. Among these carbapenemases, bla_{OXA} and bla_{NDM} genes are the most common and primarily reported in South Africa [10].

Bla$_{OXA\text{-}181}$-producing *K. pneumoniae* has caused several outbreaks in several provinces in South Africa, with the ST307 being the most predominant clone [4,6,10,12,13]. Other carbapenem-resistance mechanisms include decreased membrane permeability through increased efflux activity and decreased porin expression; these are usually coupled with β- lactamase activity [14]. An observational study performed in the United States found that carbapenemase-producing Enterobacteriaceae (CPE) infections have an increased risk of fatality than non-CPE infections [15], thus highlighting the health risk imposed by these microorganisms [15]. Yet recent findings show that some carbapenem-resistant isolates lack these classic enzymes, implicating alternative pathways such as porin alterations, efflux pump overexpression, or β-lactamase gene combinations in conferring carbapenem resistance [2,3,10,11].

Colistin resistance has largely been associated with plasmid-borne *mcr* genes or mutations in genes (*mgrB, pmrAB, phoPQ*) that regulate lipopolysaccharide (LPS) modifications [7]. Colistin targets the negatively charged lipid A moiety of the bacterial outer membrane [7–9]. Resistance mechanisms typically include modifications of the lipopolysaccharide by adding positively charged moieties, thus reducing colistin binding [7–9]. These modifications are orchestrated by the two-component regulatory systems *PhoPQ* and *PmrAB*, and can be triggered by mutations in *mgrB* (a negative regulator of *PhoPQ*) [9,16,17]. The inactivation of *mgrB*, which inhibits the kinase activity of *PhoPQ*, is the most common colistin resistance mechanism in *K. pneumoniae* [16,17]. The two-component system (TCS), *PhoPQ*, are regulators of the *pbgP* operon that encodes the endogenous lipopolysaccharide modification system. This operon is also regulated by the *PmrAB* TCS. Thus, mutations within *phoP, phoQ, pmrA,* and *pmrB* results in the modification of the LPS [18], which reduces the negative net charge of the LPS [14,19,20].

Plasmid-mediated *mcr* genes add an additional layer of global concern. In both known (*mgrB, pmrAB*, and *mcr*) and emerging resistance pathways, the upregulation of efflux pumps and the overproduction of capsular polysaccharides may further fortify the outer membrane against colistin penetration [9,16–18]. However, clinical isolates increasingly exhibit colistin resistance without identifiable *mcr* or classical regulator mutations, indicating new or poorly characterized resistance pathways. Although not common in South African clinical settings, *mcr* genes are responsible for the majority of colistin resistance in *Enterobacteriaceae*, particularly in *Escherichia coli* [5,8,9].

Other colistin resistance mechanisms include the use of efflux pumps, the formation of capsules and decreasing the outer membrane proteins [14]. The prevalence of colistin- and carbapenem-resistant *Enterobacteriaceae* is increasing in South Africa and globally, necessitating surveillance studies that will monitor their epidemiology and resistance mechanisms [21]. Indeed, the high prevalence of colistin resistance in clinical CRE isolates [7] is deeply concerning as colistin is the last-resort

antibiotic that is currently being used, interchangeably, with tigecycline to manage CRE infections.

Notwithstanding, there is a growing prevalence of isolates lacking the canonical resistance determinants (i.e. carbapenemases, mcr genes). This gap in knowledge underscores the possibility of alternate genetic or transcriptional mechanisms of resistance. Our study set out to investigate MDR *K. pneumoniae* and *Enterobacter* isolates from South Africa that tested negative for these known resistance genes, yet remained clinically resistant to carbapenems or colistin. We employed an integrated approach, combining RNA-seq to capture expression profiles under antibiotic exposure, comprehensive genomic analyses to identify resistance/virulence determinants, and epigenomic methods to explore the potential roles of DNA methylation.

Our work provides fresh insights into how these pathogens adapt to high-level antibiotic exposure in the absence of well-defined resistance genes and highlights the pressing need for expanded surveillance to detect novel or evolving resistance mechanisms. These clinical isolates were part of a molecular screening that evaluated the epidemiology of carbapenemases and *mcr* genes in Pretoria, South Africa [5].

Methods

Study settings and samples collection

Nine clinical isolates (Kp_4, Kp_14, Kp_15, Kp_24, A5, G3, G5, G8, and H3) and a reference strain, Kp_13 (making 10 strains) were selected for this study. These were initially identified by MicroScan to be *K. pneumoniae* and were obtained from a collection of multi-drug resistant (MDR) Gram-negative bacteria during a molecular screening study [5]. These isolates were collected from the National Health Laboratory Service, Tshwane Academic Division (NHLS/TAD), a referral laboratory. At the time of collection, the clinical isolates were classified as carbapenem and/or colistin resistant. They were specifically selected because they tested negative for known carbapenemases and *mcr g*enes, including bla_{IMP}, bla_{KPC}, bla_{NDM}, bla_{OXA-48}, bla_{NDM}, bla_{VIM}, and *mcr 1–5* genes, as determined by multi-plex PCR screening [5]. The reference strain, Kp_13, was susceptible to both antibiotics and had none of the resistance mechanisms tested above. Ethics approval for this study was obtained from the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria (Ref. No. 581/2020).

Phenotypic testing

Minimum inhibitory concentration evaluation

The 10 clinical isolates presumed to be *K. pneumoniae* were cultured on blood agar plates and incubated at 37°C for 24 h. After incubation, the isolates underwent antimicrobial susceptibility testing and species identification using a MicroScan automated system with Combo 66 panels (Beckman Coulter). The results were interpreted according to the Clinical and Laboratory Standard Institute (CLSI) guidelines [22].

For the carbapenem- and colistin-resistant isolates, a manual broth microdilution assay was performed following ISO standard 20776-1 [23]. Ertapenem sulphate salt and colistin sulphate salt (Glentham Life Sciences, United Kingdom) were used for the assay [24]. *E. coli* ATCC 25922 was included as a quality control strain. Both antibiotics were dissolved in sterile deionized water, according to the manufacturers' instructions. The antibiotic concentrations tested were as follows: 128 µg/mL, 64 µg/mL, 32 µg/mL, 16 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL, and 0.25 µg/mL.

The assay was performed in untreated 96-well polystyrene microtiter plates, with each well containing 100 µL of antibiotic dilution and Mueller–Hinton broth (MHB) or cation-adjusted MHB for ertapenem and colistin, respectively. Subsequently, a 0.5 MacFarland suspension of bacterial strains was prepared, diluted it to 1:20 with sterile saline, and added 0.01 mL of bacterial inoculum to each well. The plates also included sensitive and negative control wells.

Following inoculation, the plates were incubated at 37°C for 16–18 h, and the minimum inhibitory concentration (MIC) was determined as the lowest antibiotic concentration without visible bacterial growth [22]. It is important to note that since the completion of this study, CLSI revised their colistin resistance breakpoint to ≥4 mg/mL, rendering the previous breakpoint of ≥2 mg/mL used in this study outdated and incorrect.

Conditional treatment with carbapenems and colistin

Conditional treatment was performed on the nine isolates before RNA extraction. The carbapenem-resistant isolates were exposed to 0.5 mg/mL of ertapenem, while the colistin-resistant isolates were exposed to 2 mg/mL of colistin. Briefly, 1 mL of a 0.5 M *K. pneumoniae* suspension was transferred to 2 mL Eppendorf tubes, and the appropriate volumes of antibiotics were added to achieve final concentrations of 0.5 mg/mL for ertapenem and 2 mg/mL for colistin. The sensitive reference isolate served as a control and was left untreated. Subsequently, all 10 isolates were incubated at 37°C for 16–18 h.

Treatment with efflux pump inhibitors and EDTA

To evaluate the change in susceptibility of ertapenem and colistin in the presence of an efflux pump inhibitor (EPIs) and EDTA, the same procedure described above in the "MIC Evaluation" section was followed. The EPIs used were carbonyl cyanide m-chlorophenylhydrazone (CCCP), reserpine (RES), verapamil (VER), and phenylalanine-arginine β-naphthylamide (PaβN). The EPIs CCCP, PaβN, and RES were diluted in dimethyl sulphoxide (DMSO), while VER was diluted in sterile distilled water.

The final concentrations of the substrates in the broth were 1.5 µg/mL for CCCP, 4 µg/mL for VER, 25 µg/mL for PAβN, 20 µg/mL for RES, and 20 mm (pH 8.0) for EDTA. Efflux pump, Metallo β-lactamase, and MCR activity were determined by observing a twofold or greater reduction in MICs of ertapenem and colistin.

Molecular investigations of resistance mechanisms

Nucleic acid extraction

For nucleic acid extractions, fresh pure colonies grown on Mueller-Hinton Agar (Diagnostic Media Products) were used. DNA and RNA were extracted using commercial kits: Quick-DNA-fungal/bacterial MiniPrep™ kit (ZymoResearch) was used for DNA and Quick-RNA-fungal/bacterial MiniPrep™ kit (Zymo Research) was used for RNA. The extraction protocols followed the manufacturers' instructions, and the concentration and purity of the DNA extracts were checked using the NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific Inc.) before sequencing. RNA samples were stored at −80°C, while the DNA samples were stored at −20°C until sequencing.

Whole-genome sequencing and RNA-sequencing

The extracted DNA samples were sent to the National Institute of Communicable Diseases (NICD) Sequencing Core Facility for whole genome sequencing using PacBio SMRT sequencing at 100× coverage. The RNA samples were sent to Inqaba Biotechnology for PacBio Isoform sequencing, which provides long and accurate HiFi reads for a diverse transcriptome.

Genomic analysis

The sequenced genomes were submitted to GenBank and assigned accession numbers under the Bioproject PRJNA861833. The Centre for Genomic Epidemiology pipeline (http://www.genomicepidemiology.org/services/) was used to analyse the sequenced DNA and retrieve information about the species identity, multi locus sequence type (MLST), antibiotic resistance genes (ARGs), and plasmids harboured by each sequenced isolate. The Kaptive-web database (https://kaptive-web.erc.monash.edu/) was used to predict the *K. pneumoniae* isolates' serotypes (K types and O types). VRprofile2 platform (https://tool2-mml.sjtu.edu.cn/VRprofile/home.php) was used to associate ARGs and virulence genes to their mobilome. PacBio's hierarchical genome-assembly process (HGAP) software was used to assemble the PacBio reads Spades was used to assemble the Illumina reads.

Epigenomic analyses

The restriction modification system (RMS), which includes DNA methylation, restriction endonucleases, and their motifs, was identified for each isolate using the Restriction Enzyme Database (REBASE), hosted by the Centre for Epidemiology. We employed PacBio MotifMaker to detect DNA methylation motifs in our isolates. Owing to financial constraints, this analysis was only conducted on three *K. pneumoniae* isolates (Kp_14, Kp_24, and H3) and two *Enterobacter* sp. isolates (A5 and G5), which were selected for PacBio SMRT sequencing.

Phylogenetics

The genetic relationships among *Enterobacter* sp. isolates, specifically focusing on *E. cloacae*, *E. bugandensis*, and *E. asburiae* were investigated. For each *Enterobacter* sp., a phylogenetic tree was generated using global whole-genome sequences of *Enterobacter* sp. Each tree included genomes of the respective species, including *E. cloacae* ($n = 33$), *E. bugandensis* ($n = 26$), and *E. asburiae* ($n = 53$).

In the case of *K. pneumoniae* isolates, a phylogenetic reconstruction was performed using 82 whole-genome sequences obtained from various settings, including South Africa ($n = 28$), other African regions ($n = 11$), and globally ($n = 43$). This analysis aimed to assess the epidemiological and evolutionary links between the clinical *K. pneumoniae* isolates examined in this study and other *K. pneumoniae* species within these three distinct geographical settings.

The 194 whole genome sequences used in the phylogenetic analysis were retrieved from the PATRIC website (https://www.bv-brc.org/), and comprehensive data on these strains are provided in Table S1. *Escherichia coli* ATCC 25,922 (GenBank accession number: CP009073) served as the reference genome. The phylogenetic analysis was conducted using the randomized axelerated maximum likelihood (RAxML) tool.

RNA-sequencing data analysis

The RNA-sequencing data analysis was conducted using the HTSeq-DeSeq2 tool for aligning, assembling, and evaluating the differential expression data from the different sample groups. Each *K. pneumoniae* isolate was compared with the carbapenem- and colistin-susceptible strain, Kp13; *K. pneumoniae* MGH64 was used as the reference genome. The differentially expressed genes (DEGs) were identified using the *K. pneumoniae* strain MGH64 genome. The function of each gene was evaluated using the genome annotations of the reference strain on the PATRIC database. The same process was followed for the *Enterobacter species* strains using the reference (wild type) genomes of *E. asburiae* 109912^{T} (ATCC 35,953), *E. bugandensis* EB-247^{T}, and *E. cloacae* ATCC 13047, respectively, for their respective species.

Porin protein's structure analysis

The structural variations observed in porin proteins as a result of mutations in the *K. pneumoniae* test strains were observed using AlphaFold [25,26] and Evo 2 [27], with Evo 2 being used for finer resolutions while AlphaFold was used for overall structural changes. The OmpK36 (OmpC) and OmpK37 porin genes' nucleotide and amino acid sequences were compared to those of the *K. pneumoniae* MGH64 reference strain to identify the mutations. The same nucleotide and amino acid sequences were, respectively, used for the proteomic structural analysis on Evo 2 and AlphaFold.

Ethical approval

This study was approved by the Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria, with reference number 581/2020. Only stored clinical samples were used and no direct interactions with patients occurred. Written informed consent is taken by the hospital and diagnostic laboratory as part of the sample collection process to store and use the samples for research. The study was conducted according to the principles and protocols of the Declaration of Helsinki. All samples were deidentified to protect the identity and demographics of the patients.

Results

Strain description

Ten putative *K. pneumoniae* isolates were selected from a collection of 302 clinical MDR Gram-negative bacteria during a molecular screening study of carbapenemases and *mcr* genes [5]. These 10 isolates, which included a carbapenem- and colistin-sensitive strain, were categorized into three groups. The first group comprised four strains that did not produce carbapenemases but were resistant to carbapenems. The second group consisted of *mcr*-negative isolates resistant to colistin. Specifically, the carbapenem-resistant isolates were Kp_4, Kp_14, Kp_15, and Kp_24, while the colistin-resistant ones were A5, G3, G5, G8, and H3. As detailed in the method section, these isolates were exposed to ertapenem and colistin for RNA-seq. The third group was the sensitive strain, Kp_13, which displayed susceptibility to colistin, imipenem, and meropenem, served as a reference genome for the subsequent RNA-seq (Table 1).

Phenotypic characterization

MIC (BMD) and MicroScan analysis

From the BMD testing, all the *K. pneumoniae* except Kp_13, were resistant to ertapenem (>16 μg/mL) and all the *Enterobacter species* were resistant to colistin (128 μg/mL) (Table 2).

The 10 isolates underwent Microscan analysis using the Neg Combo 66 panel for identification and antimicrobial susceptibility testing of 25 antibiotics, including ertapenem, imipenem, meropenem, and colistin. Table 1 reveals that seven isolates had an MIC >2 μg/mL indicating resistance to colistin, while three isolates, Kp_4, Kp_13, and Kp_15 showed susceptibility to colistin with an MIC value of ≤2. Among the non-*mcr*-producing isolates (A5, G3, G5, G8, and H3), colistin MIC values greater than 4 μg/mL were observed.

From Microscan testing (Table 1), nine of the 10 isolates demonstrated ertapenem MICs exceeding 0.5 μg/mL and were classified as ertapenem-resistant. Meanwhile, the broth microdilution (BMD) assay confirmed high-level ertapenem resistance (MIC = 128 μg/mL) in the same nine isolates, contrasting with the control *E. coli* ATCC 25,922 (MIC = 0.25 μg/mL) (Table 2). Notably, all 10 isolates remained susceptible to imipenem (MIC ≤2 μg/mL) and seven isolates were resistant to meropenem (MIC >2 μg/mL) (Table 1), suggesting that alternative mechanisms specifically target ertapenem. The reference strain, Kp_13, displayed susceptibility to colistin, imipenem, and meropenem, yet remained resistant to ertapenem at 2 μg/mL. Moreover, the non-carbapenemase-producing isolates viz., Kp_4, Kp_14, Kp_15, and Kp_24, were resistant to ertapenem (MIC >2 μg/mL) but were susceptible to imipenem (MICs ≤2 μg/mL). These findings highlight the diverse β-lactam resistance patterns within our

Table 1. MicroScan analysis providing the antimicrobial susceptibility profile and species identification of the carbapenem- and colistin-resistant isolates included in the study.

Isolate	Antimicrobial susceptibility																								Species Identification
	AMK	AMC	SAM	AMP	ATM	FEP	CTX	FOX	CAZ	CXM	CEF	CIP	CST	ETP	FOF	GEN	IMP	LVX	MEM	NIT	NOR	TZP	TGC	TOB	SXT	
A5	16	16	16	16	8	8	16	16	16	16	16	2	4	1	64	4	2	4	8	64	8	64	1	8	4	*Klebsiella pneumoniae*
G3	16	16	16	16	8	8	1	16	16	16	16	2	4	1	64	4	2	4	8	64	8	64	1	8	2	*Klebsiella pneumoniae*
G5	16	16	16	16	8	8	16	16	16	16	16	2	8	2	64	4	1	8	8	64	16	64	4	16	4	*Klebsiella pneumoniae*
G8	16	16	16	16	8	8	16	16	16	16	16	4	8	2	64	4	1	8	8	64	16	64	4	16	4	*Klebsiella pneumoniae*
H3	16	8	16	16	1	1	1	8	1	8	16	1	8	0.5	64	4	1	2	1	32	4	64	1	4	4	*Klebsiella pneumoniae*
Kp_13	32	16	16	16	8	8	16	16	16	16	16	4	2	1	64	8	1	8	2	64	8	64	1	16	4	*Klebsiella pneumoniae*
Kp_14	16	16	16	16	8	8	16	16	16	16	16	4	4	1	64	8	1	8	8	64	8	64	1	8	4	*Klebsiella pneumoniae*
Kp_15	16	16	16	16	8	8	16	16	16	16	16	4	2	1	64	8	1	8	4	64	8	64	1	8	4	*Klebsiella pneumoniae*
Kp_24	16	16	16	16	8	8	16	16	16	16	16	2	8	2	64	4	2	4	8	64	8	64	1	8	4	*Klebsiella pneumoniae*
Kp_4	16	16	16	16	8	8	16	16	16	16	16	4	2	2	64	8	1	8	2	64	8	64	2	8	4	*Klebsiella pneumoniae*

The antimicrobials abbreviations include: AMK- Amikacin, AMC- Amoxicillin/Clavulanic Acid, SAM- Ampicillin/sulbactam, AMP- Ampicillin, ATM- Aztreonam, FEP- Cefepime, CTX- Cefotaxime, FOX- Cefoxitin CAZ- Ceftazidime CXM- Cefuroxime CEF- Cephalothin, CIP- Ciprofloxacin, CST – Colistin, ERT-Ertapenem, FOF- Fosfomycin, GEN- Gentamicin, IMP- Imipenem, LVX- Levofloxacin, MEM- Meropenem, NIT- Nitrofurantoin, NOR- Norfloxacin, TZP- piperacillin-tazobactam TGC- Tigecycline, TOB- Tobramycin, SXT- Trimethoprim/sulfamethoxazole.

Table 2. Broth microdilution assay evaluating the effect of EDTA and EPIs on the MIC value (μg/mL) of Ertapenem and Colistin.

		ERT-MIC in presence of EPIs/EDTA				
Isolate	ERT-MIC	EDTA	PAβN	CCCP	RES	VER
Kp_4	16	4	16	8	16	16
Kp_13	0.5	0.5	0.5	0.5	0.5	0.5
Kp_14	16	2	16	16	8	16
Kp_15	64	32	64	32	32	32
Kp_24	128	128	128	128	128	128
E. coli ATCC 25922	0.25	0.25	0.25	0.25	0.25	0.25
P. aeruginosa ATCC 27853	4	4	4	4	4	4
		CST-MIC in presence of EPIs/EDTA				
Isolate	CST-MIC	EDTA	PAβN	CCCP	RES	VER
A5	128	128	128	128	128	128
G3	128	128	128	128	128	128
G5	128	128	128	64	128	128
G8	128	128	128	64	128	128
H3	128	128	128	64	128	128
E. coli ATCC 25922	0.25	0.25	0.25	0.25	0.25	0.25
P. aeruginosa ATCC 27853	0.25	0.25	0.25	0.25	0.25	0.25

ERT: ertapenem, CST: colistin, PaβN: phenylalanine-arginine β-naphthylamide, CCCP: carbonyl cyanide m-chlorophenylhydrazone, RES: Reserpine, VER: Verapamil, EPIs: Efflux pump inhibitors.

panel and align with the MicroScan's identification of all isolates as *K. pneumoniae* (Table 1).

Finally, all isolates, except Kp_4 (MIC of 2 μg/mL), displayed non-susceptibility to meropenem (MIC >2 μg/mL). The isolates included in the study were MDR isolates, three of which were non-susceptible to tigecycline (Table 1). Kp_13 was susceptible to colistin, imipenem, and meropenem: MICs of 2, 1, and 2 μg/mL, respectively; it was, however, resistant to ertapenem (Table 1).

Effects of EDTA and EPIs on MIC values of ertapenem and colistin

The addition of EDTA significantly impacted the ertapenem MICs of Kp_4, Kp_14, and Kp_15 isolates, while no growth inhibition was observed in Kp_24 (Table 2). Furthermore, CCCP reduced the ertapenem MIC values of Kp_4 and Kp_15 with the MIC of Kp_4 decreasing from 16 μg/ml to 8 μg/ml and the MIC of Kp_15 decreasing from 64 μg/ml to 32 μg/ml. Additionally, RES decreased the ertapenem MIC value

of Kp_15 from 16 μg/ml to 8 μg/ml. However, no growth inhibition was observed in Kp_24 with the addition of EPIs (Table 2).

In non-*mcr*-producing colistin-resistant isolates, the effects of EDTA and EPIs were evaluated (Table 2). The addition of EDTA did not inhibit the growth of the isolates in the presence of colistin. However, a decrease in MIC values was observed when CCCP was added to G5, G8 and H3, with their colistin MIC values decreasing from 128 μg/ml to 64 μg/ml. No growth inhibition was observed for the other EPIs tested.

Genomic characterization

The whole-genome sequencing analysis identified six isolates (including the reference strain) as *K. pneumoniae*, the remaining isolates were two *Enterobacter cloacae* complex strains, one *Enterobacter asburiae* and one *Enterobacter bugandensis* isolate (Table 3). Among the *K. pneumoniae* isolates, four MLST groups were identified: ST307 (Kp_4, Kp_15, and Kp_24), ST219 (Kp_14), ST25 (H3), and a novel sequence type, ST6408, for Kp_13.

K- and O-loci of K. pneumoniae *strains were clonal*

The analysis of K-loci and O-loci serotype revealed that the ST307 isolates (Kp4, Kp15, and Kp24) shared the same KL102 and O1/O2v2 results. The remaining isolates all had the same O1/O2v2 O-loci type. However, KL142, KL114, and KL2 K-loci types were found in Kp13, Kp14, and H3, respectively (Table 3).

Resistance plasmids were common in K. pneumoniae *but absent in Enterobacter*

Twelve plasmids were identified within the six *K. pneumoniae* isolates. These plasmids were associated with 10 compatibility groups, with IncFIB (K), IncFII(K), and IncR being the most common. Eight of these plasmids co-harboured multiple compatibility groups, while the remaining four were singletons (Tables 3 and S2). Among the isolates, Kp_4 hosted the highest number of plasmids ($n = 4$), followed by Kp_15 ($n = 3$). Isolates Kp_13 and Kp_25 each carried two plasmids, while both Kp_4 and H3 only hosted one plasmid.

The largest plasmid observed belonged to Kp_15, with a size of 311.9 kbp, and consisting of two incompatibility groups: IncFII(K) and IncFIB(K). The second largest plasmid belonged to H3, with a size of 216.8 kbp, and consisting of multiple replicons, including IncFIB(K), IncFII(K), and IncQ1. Notably, no plasmids were identified within the *Enterobacter* sp. Isolates (Tables 3 and 2).

Table 3. Genomic identification and characterization of the 10 presumed Klebsiella pneumoniae isolates included in the study.

					Antibiotic resistance genes	
Isolate	Species	Serotypes	MLST	Plasmids	Chromosomal	Plasmids
Kp_4	*Klebsiella pneumoniae*	K: KL102 O: O1/O2v2	ST307	IncFIA(HI1) IncFIB(K)/IncFII(K) IncL IncR	blaSHV-28, fosA6, oqxA, oqxB	blaCTX-M-15, blaTEM-1B, aac(3)-Iia, aac(6′)-Ib-cr, aadA16, aph(3″)-Ib, aph(6)-Id, ARR-3, dfrA27, qacE, qnrB6, sul1, sul2, tetD
Kp_13 (reference strain)	*Klebsiella pneumoniae*	K: KL142 O: O1/O2v1	ST6408	IncFIA(HI1)/IncR/repB(R1701) IncFIB(K)/IncFIB(K)/IncR	blaSHV-81, fosA6, oqxA, oqxB	blaCTX-M-14, blaDHA-1, blaTEM-1B, aac(3)-IId, aac(6″)-Ib-cr, aadA16, aph(3″)-Ia, aph(3″)-Ib, aph(6)-Id, armA, ARR-3, dfrA27, floR, mphA, mphE, msrE, qacE, qnrB4, sul1, sul2, tetA
Kp_14	*Klebsiella pneumoniae*	K: KL114 O: O1/O2v1	ST219	IncFIB(K) (pCAV1099–114)	blaSHV-26, fosA	blaCTX-M-15, aadA2, aph(3′)-Ia, aph(3″)-Ib, aph(6)-Id, catA2, dfrA12, mphA, qacE, qnrS1, sul1, sul2
Kp_15	*Klebsiella pneumoniae*	K: KL102 O: O1/O2v2	ST307	IncFIB(K) (pCAV1099–114) IncFIB(K)/IncFII(K) IncX3	blaSHV-28, fosA6, oqxA, oqxB	blaCTX-M-15, blaOXA-1, blaOXA-181, blaTEM-1B, aac(3)-Iia, aac(6)-Ib-cr, aadA2, aph(3′)-Ia, aph(3″)-Ib, aph(6)-Id, catA2, catB3, dfrA12, dfrA14, mphA, qacE, qnrB1, qnrS1, sul1, sul2, tetA
Kp_24	*Klebsiella pneumoniae*	K: KL102 O: O1/O2v2	ST307	IncFIB(pNDM-Mar) /IncHI1B(pNDM-MAR) IncX3	blaSHV-28, fosA6, oqxA, oqxB	blaCTX-M-15, blaOXA-1, blaOXA-181, blaTEM-1C, aac(6)-Ib-cr, aadA1, ant(3″)-Ia, catB3, dfrA15, mphA, qacE, qnrS1, sul1
H3	*Klebsiella pneumoniae*	K: KL2 O: O1/O2v1	ST25	IncFIB(K)/IncFII(K)	blaCMH-3, blaSHV-81, fosA, fosA6, oqxA, oqxB	blaTEM-1B, aph(3′)-Ia, aph(3″)-Ib, aph(6)-Id, dfrA14, mphA, sul2
A5	*Enterobacter asburiae*	N/A	ST22	None	blaACT-4, fosA, oqxA, oqxB	None
G3	*Enterobacter bugandensis*	N/A	ST632	None	blaACT-6, fosA, oqxA, oqxB	None
G5	*Enterobacter cloacae*	N/A	ST2100	None	blaCMH-3, fosA, oqxA, oqxB	None
G8	*Enterobacter cloacae*	N/A	ST2100	None	blaCMH-3, fosA, oqxA, oqxB	None

ARGs were abundant in K. pneumoniae

All the isolates harboured β-lactamase genes that influenced their phenotypic β-lactam resistance, corroborating the PCR results from the molecular screening (Table 3) [5]. The *Enterobacter* species (A5 and G3) harboured β-lactamase genes, namely $bla_{ACT\text{-}6}$ within the chromosome, while G5 and G8 harboured $bla_{\text{CMH-3}}$ genes (Table 3 and Table S2). These β-lactamase genes were not found in association with mobile genetic elements (MGEs). The *K. pneumoniae* isolates harboured multiple β-lactamase genes. Notably, bla_{SHV} variants, which are intrinsic to most *K. pneumoniae*, were found in H3, Kp_13, Kp_14, and Kp_4, along with $bla_{\text{CMH-3}}$ genes, all of which were located within the chromosome. Isolate H3 additionally harboured $bla_{\text{TEM-1B,}}$ located on an unidentified plasmid (Table S2). Kp_13 isolate harboured four additional β-lactamase genes including $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1B,}}$ which were surrounded by MGEs *IS*26 and *IS*Kpn26, respectively (Table S2).

Two other genes, $bla_{\text{DHA-1}}$ and $bla_{\text{TEM-1B,}}$ were located on the IncFIB(K) plasmid and surrounded by *IS*26 and *IS*Kpn26, respectively. Kp_14 harboured four additional $bla_{\text{CTX-M-15}}$ genes located on three contigs, along with chromosomal $bla_{\text{SHV-26.}}$ Two of the $bla_{\text{CTX-M-15}}$ genes were harboured on an IncFIB plasmid, while the other two were situated on an unidentified plasmid or transposable elements. Kp_15 harboured four additional β-lactamase, including chromosomal $bla_{\text{SHV-28}}$ and IncFIB(K)/IncFII(K) plasmid-borne $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, and $bla_{\text{TEM-1B.}}$ Additionally, $bla_{\text{OXA-181,}}$ was located on the IncX3 plasmid, also surrounded by IS26. Kp_24 harboured four additional β-lactamase genes, including $bla_{\text{SHV-28}}$; $bla_{\text{OXA-181}}$ was located on an IncX3 plasmid, also surrounded by *IS*26. The remaining genes, $bla_{\text{OXA-1}}$, $bla_{\text{CTX-M-15}}$, and $bla_{\text{TEM-1B}}$, were located on an unidentified plasmid or transposable element, and were all surrounded by *IS*26 (Tables 3 and S2).

Lastly, Kp_4 harboured five additional β-lactamase genes, including chromosomal $bla_{\text{SHV-28}}$, two $bla_{\text{CTX-M-15}}$, and three $bla_{\text{TEM-1B}}$. Two $bla_{\text{TEM-1B}}$ and one $bla_{\text{CTX-M-15}}$ were located on separate unidentified plasmids or transposable elements, while the remaining $bla_{\text{TEM-1B}}$ and $bla_{\text{CTX-M-15}}$ were located on the IncFIA (HI1) plasmid (Table S2).

ARGs were deficient in Enterobacter species

The four *Enterobacter* species (A5, G3, G5, and G8) harboured β-lactamase genes and three additional chromosomal antibiotic resistance genes (ARGs): *fosA*, *oqxA*, and *oqxB*. These ARGs were also present within the chromosomes of the *K. pneumoniae* isolates, Kp_4, Kp_13, Kp_14, Kp_24, and H3 (Table 3 and Table S3). However, the resistance genes *oqxA* and *oqxB* were not found in isolate Kp_14. The remaining ARGs listed in Table 3 were located on plasmids or extrachromosomal DNA, and included genes mediating resistance to aminoglycosides (*aac(3')-IIa, acc(6')-Ib-cr, aadA1, aadA16, aadA2, ant(3")-Ia, aph(3')-Ia, aph(3")-Ib, aph(6)-Id, armA)*, amphenicol (*catA2/B3, floR*), macrolide (*mphE, mphA, msrE*), quaternary ammonium compound (*qacE*), quinolone (*qnrB1/B4/B6/S1*), sulphonamide (*sul1, sul2*), tetracycline (*tetA, tetD*), and trimethoprim (*dfrA12/14/15/27*). The pathogen watch pipeline identified *ompK35* mutations conferring carbapenem resistance in isolates Kp_4, Kp_13, Kp_15, Kp_24, and H3 (Table S3). However, the pipeline failed to analyse the *Enterobacter* species isolates. Isolate H3 was further found to harbour *mgrB* mutations conferring resistance to colistin (Table S3).

Virulence genes analysis

Thirty virulence genes were identified on chromosomes within the 10 isolates (Table S4); they were flanked by MGEs. On average, each isolate carried 10 virulence genes, with G8 harbouring the lowest of four genes, and Kp_14 harbouring the highest number of 19 virulence genes. Certain virulence genes were found within prophage MGEs including *algU* (present in H3 and Kp_15), *hcp/tssD* (Kp_14, Kp_15, Kp_24, and Kp_4), and *rfaE* (H3). Additionally, the *hcp/tssD* gene found in Kp_4 was located near integrative conjugative elements within the chromosome (Table S4).

The different categories of virulence genes include those responsible for biofilm formation, capsular synthesis, the type VI secretion system (T6SS), and lipopolysaccharide synthesis. Biofilm formation genes were only observed in isolate Kp_14. These genes include *fimA, fimC, fimD, fimF, fimG*, and *fimI*, which are responsible for type 1 fimbriae and are involved in biofilm formation. Several capsular synthesis virulence genes were identified within *K. pneumoniae*. These include *gnd* (Kp_4, Kp_13, Kp_14, Kp_15, and Kp_24), *manB/manC* (Kp_14), *ugd* (Kp_4, Kp_13, Kp_14, and Kp_24), *wcaJ* (Kp_13), and *wza* (Kp_13, Kp_14, Kp_15, and Kp_24) (Table S4).

Multiple lipopolysaccharide synthesis genes were identified. The following genes were identified in all six *K. pneumoniae* isolates: *glf, wbbM, wbbN*, and *wzt*. The remaining genes, *wzm* (Kp_4, Kp_13, Kp_14, and Kp_24), *wbtL* (Kp_13), *wbbO* (Kp_4, Kp_14, Kp_24, and H3), and *kfoC* (Kp_4, Kp_15, Kp_24, and H3), were only found in some *K. pneumoniae* isolates.

Seven genes responsible for the T6SS were identified within both *K. pneumoniae* and *Enterobacter* species. The structural genes include *hcp/tssD* (A5, G3, H3, Kp_4, Kp_14, Kp_15, and Kp_24), *icmF/tssM* (A5, G3, G5, G8, Kp_13, and Kp_14), *sciN/tssJ* (A5 and G3), *tssF* (A5, G3, G5, G8), *tssG* (A5, G3, G5, G8), and lastly *tli1* (A5 and G3). Notably, A5 harboured all the structural genes. The last T6SS virulence gene identified as KPHS_23120, which was harboured by A5 and G3 (Table S4).

Significant upregulation was observed in virulence-associated genes including efflux pump regulators (e.g. AcrR regulator, $\log_2$ fold change = 6.87), capsular polysaccharide biosynthesis genes (*ugd, gnd, wza*; $\log_2$ fold change: 7.12–8.39), fimbrial proteins (fimA, fimC, fimD; $\log_2$ fold change = 7.85–9.07), T1 and T6 secretion systems (log2 fold change > 8; $p < 0.0005$), LysR transcriptional regulators ($\log_2$ fold change ≥ 7; $p < 0.0001$), and lipopolysaccharide biosynthesis enzymes (LPS glycosyltransferase; $\log_2$ fold change = 8.27–9.16; $p < 0.0001$), indicating their critical role in antibiotic resistance (Dataset 4).

Phylogenetic analysis

Phylogenetic analysis of the K. pneumoniae isolates

The phylogenetic analysis of the *K. pneumoniae* isolates included 81 isolates originating from five continents: Africa ($n = 39$), Asia ($n = 15$), Europe ($n = 21$), North America ($n = 6$), and South America ($n = 4$). These isolates belonged to nine sequence types (STs), with ST307 ($n = 45$), ST25 ($n = 19$), and ST219 ($n = 12$) being the most common clones. ST307 was found in eight countries, while ST25 and ST219 were found in seven countries. All *K. pneumoniae* isolates included in the phylogenetic analysis were obtained from human hosts.

The genome-based phylogeny of the South African *K. pneumoniae* isolates revealed six clades (Figure 1). Among the 28 *K. pneumoniae* isolates, 21 belonged to ST307, making up three of the six clades (Clades 4 to 6). These three clades had similar resistomes, with the highest similarities observed between Kp8, Tembi-19, Tembi-37, EC0361298, and EC03605938. In contrast, Clade 6 showed the least similarity within its isolates' resistome.

The phylogeny of the African *K. pneumoniae* isolates (Figure 2) consisted of seven clades with a high similarity within each clade concerning their resistomes. Clades 5 and 6 had similar resistome patterns. Interestingly, H2 ST501, which formed its own clade, shows its distinct resistome pattern, setting it apart from the other clades.

Figure S1 shows the genome-based phylogeny of *K. pneumoniae* from the remaining continents, revealing six clades. Kp_14 was grouped in Clade 3 alongside other *K. pneumoniae* ST219 isolates and H2 ST501 from Nigeria. Kp_13 was placed in Clade 4, along with the three Nigerian *K. pneumoniae* isolates. Lastly, Kp_4, Kp_15, and Kp_24 were assigned to Clade 5 along with *K. pneumoniae* ST307 isolates.

Phylogenetic analysis of the Enterobacter sp. isolates

For the *Enterobacter* species (*E. asburiae, E. bugandensis*, and *E. cloacae)*, three separate phylogenetic trees were constructed. The phylogeny of *E. asburiae* seen in Figure 3, included 53 isolates distributed among seven distinct clades. Interestingly, isolate A5 was placed in clade 3 alongside a South African strain (E124_11) and a Chinese strain (C210176) forming a clade with a significantly similar resistome. Clades 6 and 7 harboured a wide range of ARGs, these two clades included isolates from six to seven countries, with China being the predominant source for both. In this phylogenetic tree, the clades exhibit the presence of bla_{ACT}, fosA, and oqxB genes across most resistomes. Additionally, distinct resistome patterns are observed within each clade, indicating variations in the genes responsible for resistance mechanisms among the different groups.

The genome phylogeny of *E. bugandensis* seen in Figure 4, included 25 isolates distributed among three distinct clades. The phylogenetic tree included three isolates that carried 10 or more ARGs: IMP80 (Clade 1); C210207 and AR2787 (both in Clade 2). The remaining isolates harboured similar ARGs including bla_{ACT}, found in all isolates, and *qnrA*, found in most isolates ($n = 21$). Compared with the other phylogenetic trees, this specific tree showed a lower number of resistance genes, with bla_{ACT} and *oqxB* being the predominant ARGs among the included isolates. Only four isolates harboured more than the average three ARGs. Excluding these isolates, a consistent and similar resistance pattern is observed across the tree, suggesting a commonality in resistance mechanisms acquired by *E. bugandensis* species.

The phylogeny of *E. cloacae* (Figure 5) included 32 isolates distributed among seven distinct clades. Clade 5 had the fewest ARGs followed by Clade 6, while Clade 2 and 4 harboured the most. All the isolates from Clade 4 originated from South Africa, while Clade 6 displayed a greater diversity in terms of countries of origin.

Epigenomics

Of the four recognized RMS types (I–IV), types I–III were observed, with type II methyltransferases

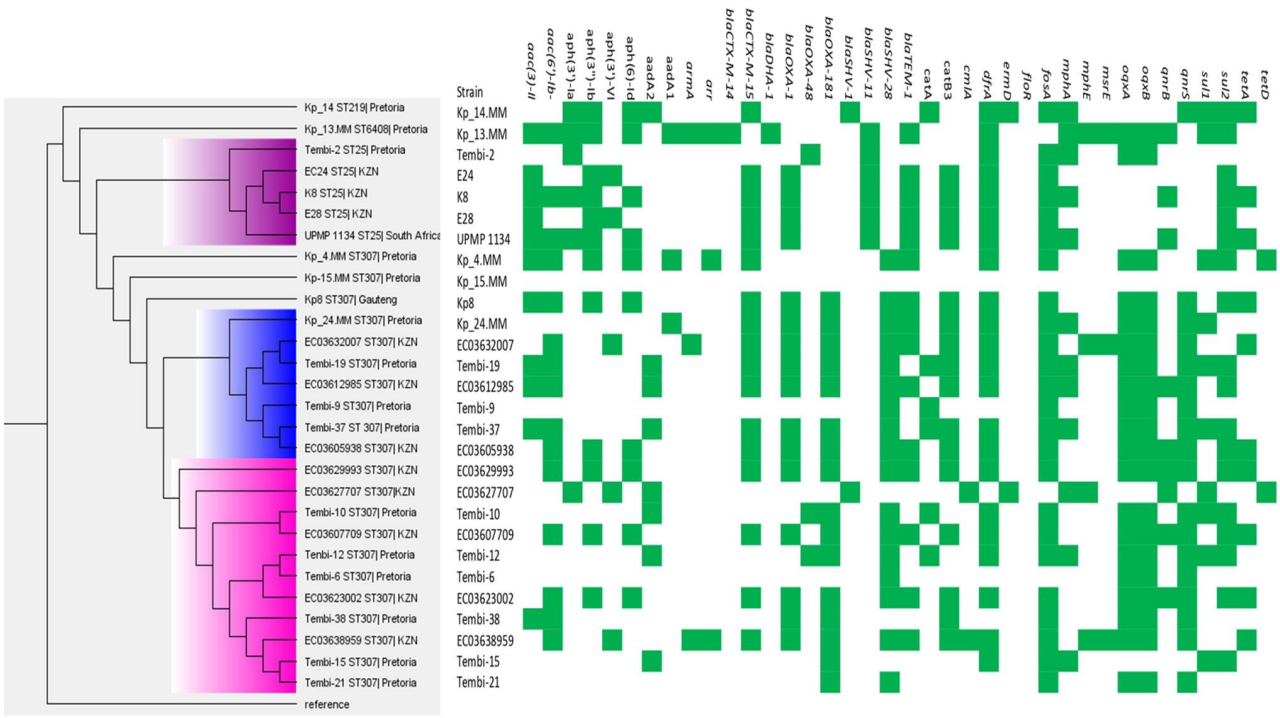

Figure 1. *Phylogenetic and resistome dynamics of K. pneumoniae isolates from South Africa collected from human samples*. Each strain is represented by its strain identifier, MLST designation, and country of origin. Strains belonging to the same clade are highlighted with the same colour on the branches. The resistome is depicted through green and white blocks, representing the presence and absence of antibiotic resistance genes, respectively.

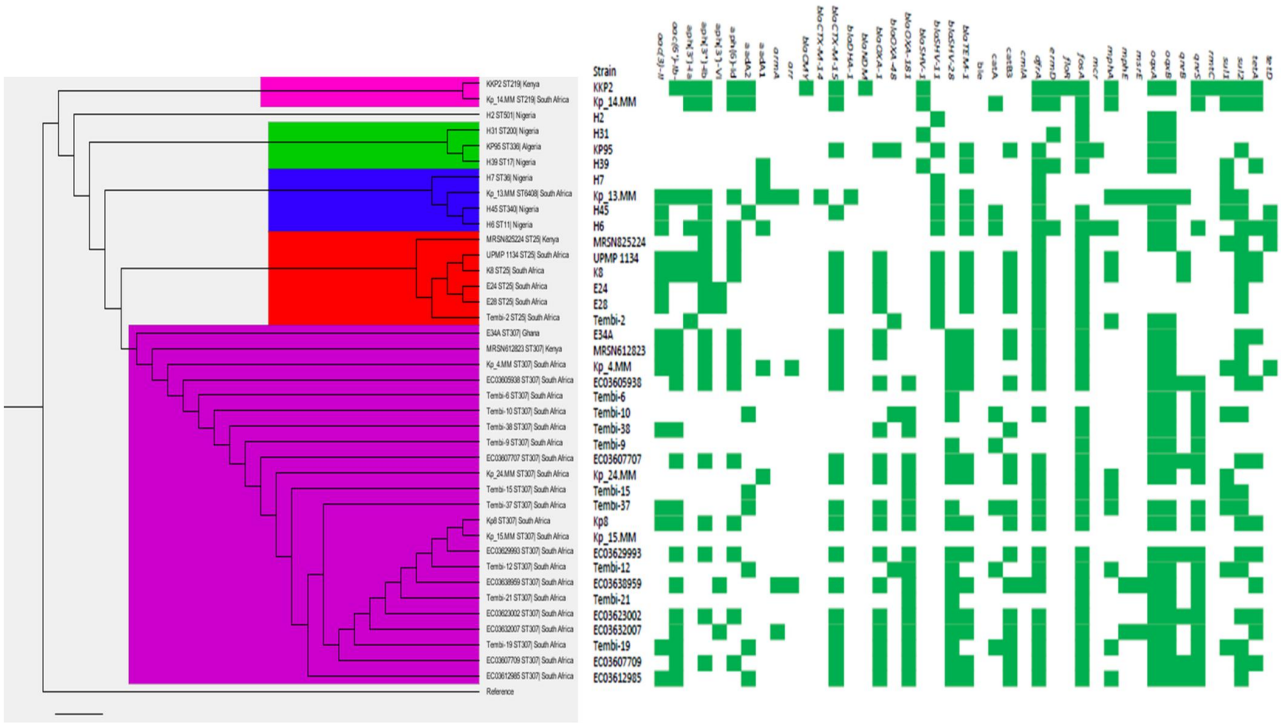

Figure 2. *Phylogenetic and resistome dynamics of K. pneumoniae isolates from Africa collected from human samples*. Each strain is represented by its strain identifier, MLST designation, and country of origin. Strains belonging to the same clade are highlighted with the same colour on the branches. The resistome is depicted through green and white blocks, representing the presence and absence of antibiotic resistance genes, respectively.

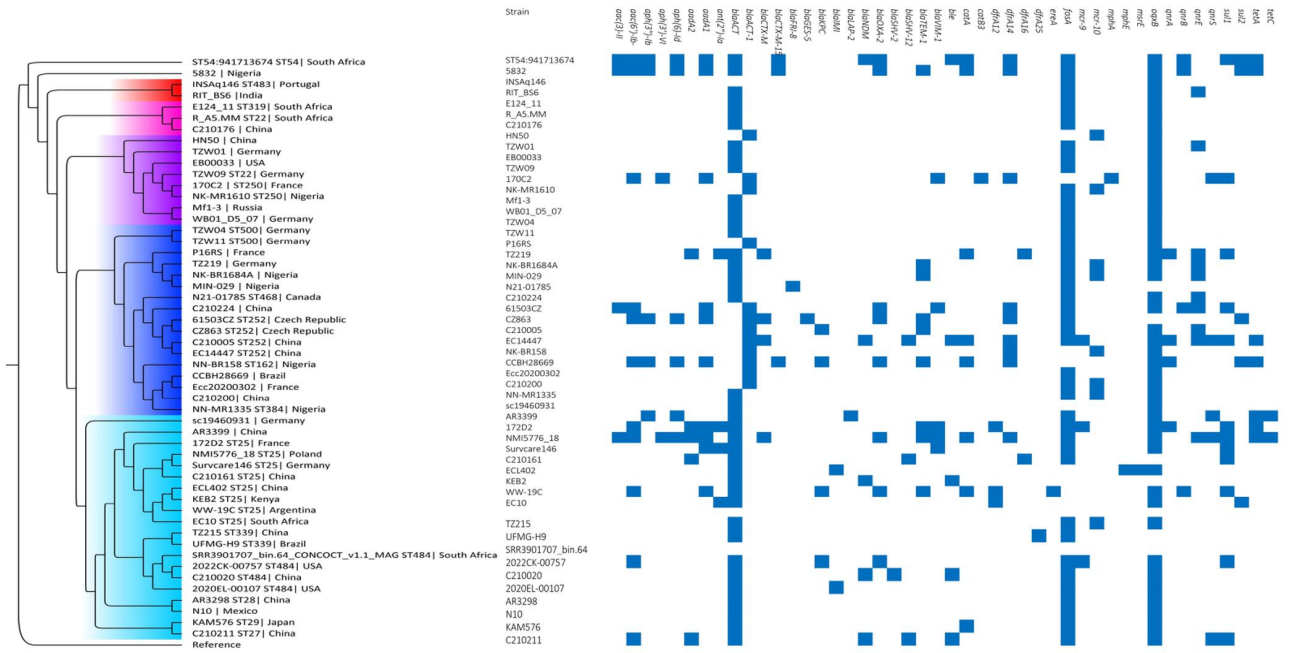

Figure 3. *Global phylogenetic and resistome dynamics of E. asburiae isolates, collected from human samples*. Each strain is represented by its strain identifier, MLST designation, and country of origin. Strains belonging to the same clade are highlighted with the same colour on the branches. The resistome is depicted through blue and white blocks, representing the presence and absence of antibiotic resistance genes, respectively.

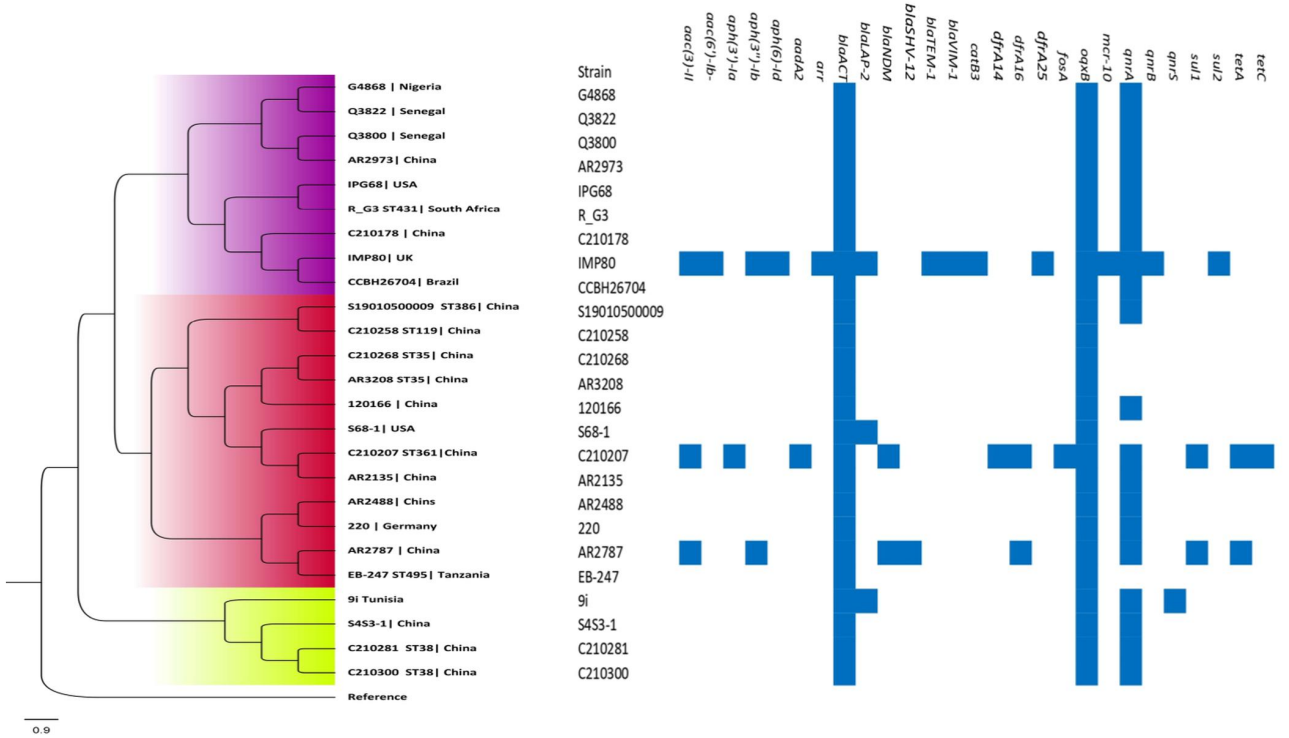

Figure 4. *Global phylogenetic and resistome dynamics of E. bugandensis isolates, collected from human samples*. Each strain is represented by its strain identifier, MLST designation, and country of origin. Strains belonging to the same clade are highlighted with the same colour on the branches. The resistome is depicted through blue and white blocks, representing the presence and absence of antibiotic resistance genes, respectively.

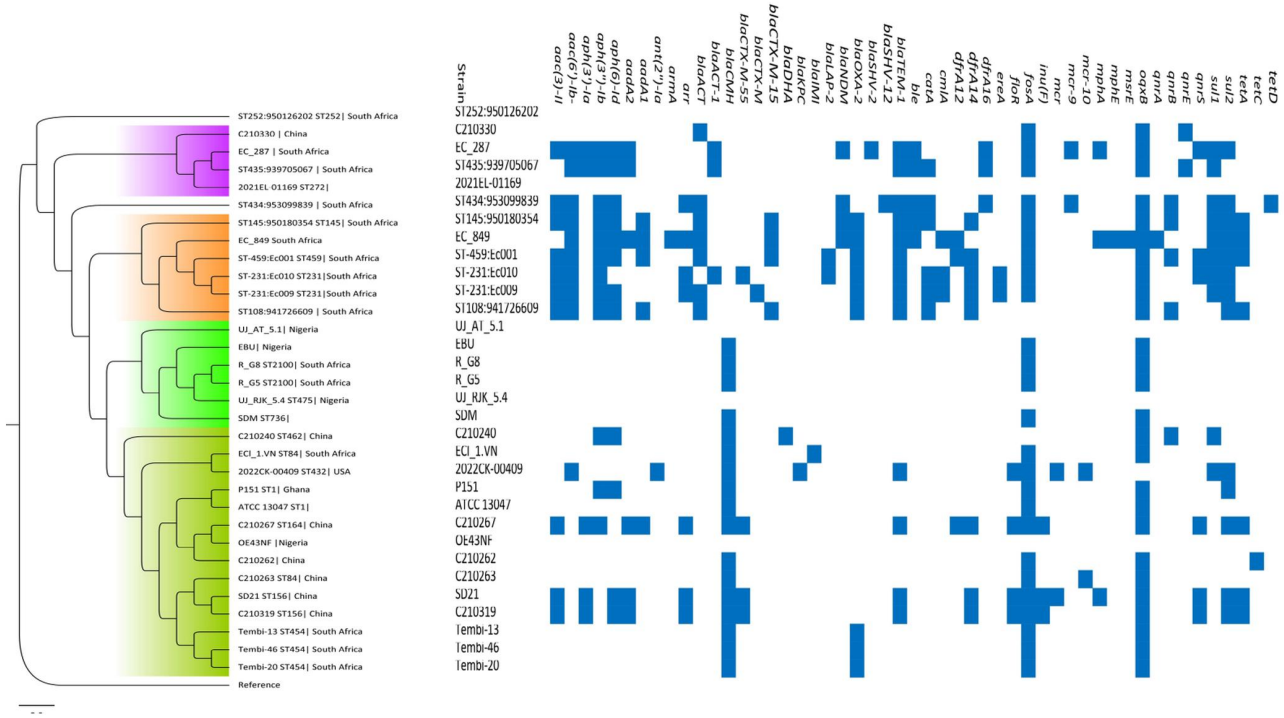

Figure 5. *Global phylogenetic and resistome dynamics of E. cloacae complex isolates, collected from human samples*. Each strain is represented by its strain identifier, MLST designation, and country of origin. Strains belonging to the same clade are highlighted with the same colour on the branches. The resistome is depicted through blue and white blocks, representing the presence and absence of antibiotic resistance genes, respectively.

predominating (followed by type I). Notably, the adenine methyltransferase (Dam) was absent, leaving cytosine methyltransferases (Dcm) as the primary epigenetic modifiers. These findings highlight the varied RMS landscape and underscore the potential for plasmid-borne epigenetic elements to influence gene regulation (Figure S2).

A single type III Mtase, *M.kpn1420I*, was located chromosomally within isolate H3, alongside a single Type I and II Mtase: *M1.Ec13497I* and *M.Kpn34618Dcm*, respectively. Each Mtase harboured by isolate H3 had its own unique recognition sequence. Lastly, isolate H3 was the only isolate that harboured three types of Mtase (Table S5).

A Type II restriction endonuclease (RE), *Eco128I*, was identified in five isolates: Kp_4, Kp_13, Kp_15, A5, and G5. Significantly, in each of these isolates, *Eco128I* was encoded on a plasmid. As well, all four Type II Restriction-Modification Systems (RMS) identified in the isolates, including the RE, shared the same recognition sequence, CCWGG. The most common of these was M.*Kpn34618Dcm*, which was present in eight of the 10 isolates. Notably, it was located chromosomally in the *K. pneumoniae* isolates Kp_4, Kp_13, Kp_14, Kp_24, and H3 while in isolates Kp_15, A5, and G5, it was plasmid encoded. This means that in isolate Kp_13, both a Type II RE and Mtase (M.*EcoRII* and *Eco128I*) were identified on a plasmid, alongside a type II Mtase (M.*Kpn34618Dcm*) within the chromosome. Notably, Type II Mtases were not identified in isolates G3 and G8 (Table S5 & Fig. S2).

The type II RMS adenine (Dam) Mtase was not found in any of the study isolates; only the type II RMS cytosine (Dcm) Mtase was present. Two Dcm Mtases were identified: M.*Kpn34618Dcm* and M.*EasL1Dcm*, with the latter only identified in isolate A5. A complete RMS consisting of REs, Mtases, and a specificity subunit was not found in any of the isolates, as no specificity subunits were identified during the analysis. Both an RE and Mtase were found in the five isolates encoding the Type II RE. These isolates, Kp_4, Kp_13, Kp_15, A5, and G5, further harboured the same type II Mtases, *EcoRII* and M.*Kpn34618Dcm*, with isolate A5 also harbouring an additional type II M.*EasL1Dcm*. The remaining five isolates only harboured MTases (Fig. S2).

Two type I Mtases were detected: *M.EcoJA03PI* and *M1.Ec134977I*. They had distinct recognition sequences, GATGNNNNNCTG and GCCNNN NNGTT, respectively, and were both located chromosomally. *M1.Ec134997I* was present in four isolates: H3, G5, G3, and G8, while *M.EcoJA03PI* was only identified in isolate Kp_4.

As described in the methods, PacBio SMRT sequencing was only performed on five isolates: Kp_14, Kp_25, H3, A5, and G5. All isolates had m6A modifications that result in N6-methyladenine (6 mA) modifications, with the GATC motif being identified in all isolates (Table S5). Moreover, the m4C modification, resulting in N4-methylcytosine (4mC), was also present in all isolates, with the VVNCYGVNYR motif identified in all cases. Due to financial constraints, SMRT sequencing was limited to five isolates (Kp_14, Kp_24, H3, A5, and G5), each exhibiting N6-methyladenine (m6A) and N4-methylcytosine (m4C) at distinct motifs.

Differential gene expression analysis

The analysis of differentially expressed genes (DEGs) was performed using HTSeq-DeSeq2, and the data was visualized using SRPlot (Figure 6–9 & S3-S19; Tables S6-S25: Datasets 1–4). The DEGs' data was further analysed on an Excel spreadsheet, wherein non-significant genes were filtered out. In the case of Kp_4, this filtering process reduced the number of DEGs from 4493 to 86, and this trend was observed across the remaining nine isolates (Dataset 4).

The patterns of DEGs were found to be similar in eight isolates (G5, G8, H3, Kp_4, Kp_14, Kp_15, and Kp_24), as seen in Table S6-S7, with capsular polysaccharide biosynthesis genes showing increased expression. This upregulation was seen in isolate Kp_14 and Kp_15. Moreover, changes were observed in the membrane area of the clinical isolates, including the downregulation of ion ABC-transporters in all *K. pneumoniae* isolates (Kp_4, Kp_14, Kp_15, and Kp_24).

Isolate G5, G8, Kp_14, and Kp_15 displayed increased expression of three ion-ABC transporters: an ATP-binding protein, permease protein, and a substrate-binding protein. Additionally, the ferric-ion transporter was upregulated in Kp_14 and Kp_15 isolates, while there was a downregulation of Iron(III) dicitrate transporter in Kp_14 and Kp_24 (Table S6).

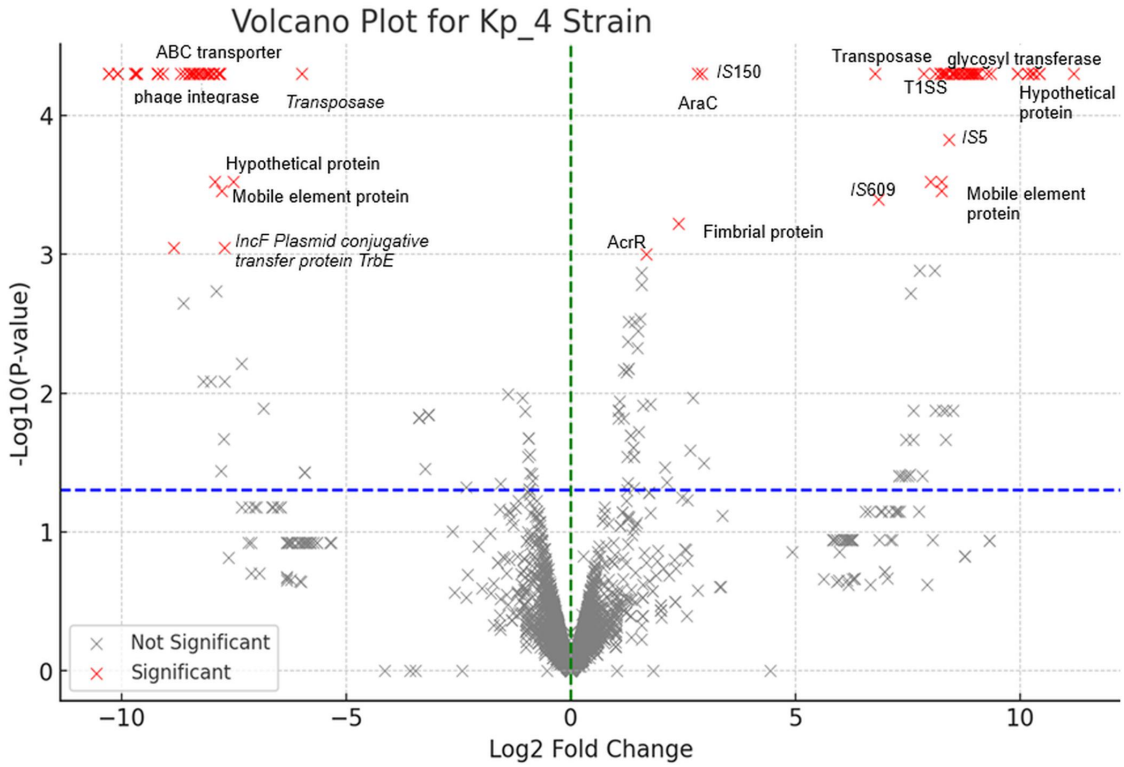

Figure 6. *A volcano plot was used to compare the Differentially expressed genes (DEGs) between the carbapenem-resistant K. pneumoniae KP_4 and the susceptible Kp_13 isolate as a reference genome.* Each data point represents a gene, and its position was determined by the Fold change (log2FC) and the statistical significance (log p-value). The x-axis shows the log2 Fold change, indicating the magnitude and direction of expression changes (left: downregulated, right: upregulated). The y-axis shows the negative log10-transformed p-values, indicating the significance of the differential expression (higher values are more significant). Red points highlight genes considered significantly differentially expressed, while grey points represent those not meeting the significance threshold. The blue dashed line represents the threshold for a p-value of 0.05, above which genes are considered significantly differentially expressed. The green dashed line marks the threshold for no fold change.

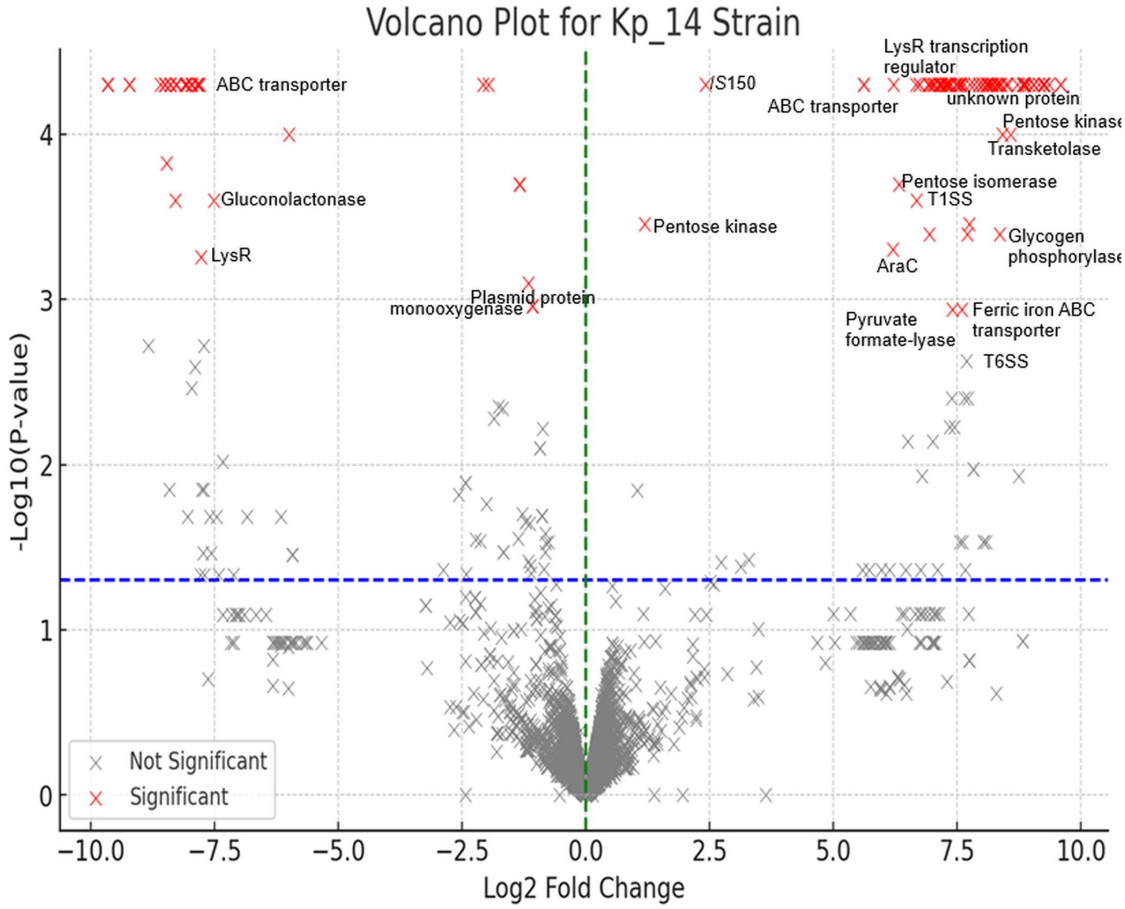

Figure 7. *A volcano plot for strain Kp_14, visually representing the differential expression analysis results*. in this plot, each point represents a gene. The x-axis shows the log2 Fold change, indicating the magnitude and direction of expression changes (left: downregulated, right: upregulated). The y-axis shows the negative log10-transformed p-values, indicating the significance of the differential expression (higher values are more significant). Red points highlight genes considered significantly differentially expressed, while grey points represent those not meeting the significance threshold. The blue dashed line represents the threshold for a p-value of 0.05, above which genes are considered significantly differentially expressed. The green dashed line marks the threshold for no fold change.

Isolate G5 had an upregulation of the ferric hydroxamate outer membrane receptor, FhuA.

The core metabolic functions also had differential expression; *sufAB*, responsible for iron-sulphur metabolism, showed increased expression in all *K. pneumoniae* isolates. On the other hand, cobalt-precorrin methyltransferase was downregulated in Kp_4, Kp_14, Kp_15, and Kp_24. The putative glycotransferase, involved in the biogenesis of natural products, was upregulated in all *K. pneumoniae* isolates; and in isolate G5, this protein was additionally upregulated along with an LPS core biosynthesis glycotransferase and an LPS core heptosyltransferase. Additionally, D-3 phosphoglycerase dehydrogenase had upregulation in Kp_4, Kp_14, Kp_15, and Kp_24. Lastly, the cellulose synthase was upregulated in Kp_14 and Kp_15 isolates, while a 3-oxoacyl-[acyl carrier protein (ACP)] synthase was upregulated in isolates G5 and G8 (Datasets 2–4).

In the *K. pneumoniae* isolates, seven transcriptional regulators were upregulated (Tables S6 and S7). Among these were a probable transcriptional regulator of MDR efflux pumps and a transcriptional regulator associated with rhamnose utilization, part of the AraC family, were upregulated in all *K. pneumoniae* isolates (Table S6). In isolate G5, four transcriptional regulators were upregulated (Table S7). One of these regulators belongs to the AcrR family, responsible for regulating the AcrAB-TolC MDR efflux system, was upregulated alongside H3. Additionally, the RND efflux pump regulator was also upregulated in isolate G5 along with isolate G8.

Components of the type 1 fimbriae were found to be upregulated in all *K. pneumoniae* isolates and in isolate G5. These components include the outer membrane usher protein, fimbrial protein *staA*, the fimbrial protein subunit precursor and the fimbrial chaperone (Datasets 2–4).

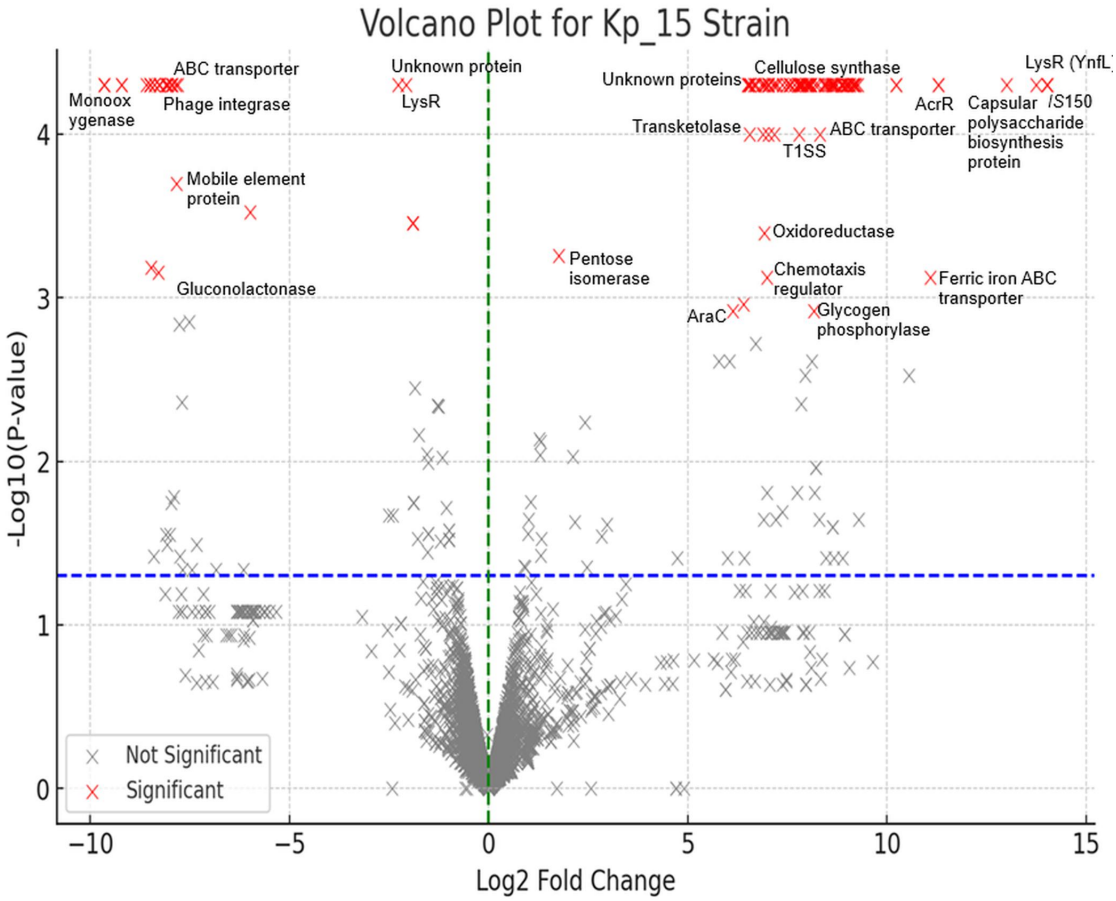

Figure 8. *A volcano plot for strain Kp_15, visually representing the differential expression analysis results*. in this plot, each point represents a gene. The x-axis shows the log2 Fold change, indicating the magnitude and direction of expression changes (left: downregulated, right: upregulated). The y-axis shows the negative log10-transformed p-values, indicating the significance of the differential expression (higher values are more significant). Red points highlight genes considered significantly differentially expressed, while grey points represent those not meeting the significance threshold. The blue dashed line represents the threshold for a p-value of 0.05, above which genes are considered significantly differentially expressed. The green dashed line marks the threshold for no fold change.

Notably, there were little to no significant expression or repression of RMS genes and significant expression of mobile genetic elements (MGEs) such as insertion sequences, transposons, phage-associated proteins, integrases, and plasmid replication genes. None of the *K. pneumoniae* strains had significant RMS DEGs while *E. asburiae* A5 (HNH endonuclease, Phage exonuclease, DNA phosphorothioation-dependent restriction protein DptH/AAA-like domain, DNA phosphorothioation-dependent restriction protein DptG, and DNA phosphorothioation-dependent restriction protein DptF) and *E. cloacae* G8 (Phage terminase, endonuclease subunit GpM) had significant RMS DEGs. Contrarily, the MGEs were highly expressed in all species and strains (Figure 7–9; Dataset 3).

Among the significant DEGs, a LysR family transcriptional regulator (XLOC_000198) was notably upregulated (log2FoldChange = 6.87–7.18, fold change < 0.01, $p \leq 0.0001$), potentially contributing to adaptive resistance regulation.

Porin protein analysis

Not all mutations in the porin proteins amino acid sequences led to structural changes. Hence, only mutations conferring structural changes were prioritized (Figure 10,11). The mutations occurring in the barrels or walls of the porins caused little to no observed structural variations while the mutations, and particularly the deletions, in the regions joining the barrel walls to each other were more likely to cause structural changes. These included differences in length, orientation, folding, and structure (Figure 10,11), showing how porin mutations can affect the permeability of the cell membrane to external molecules.

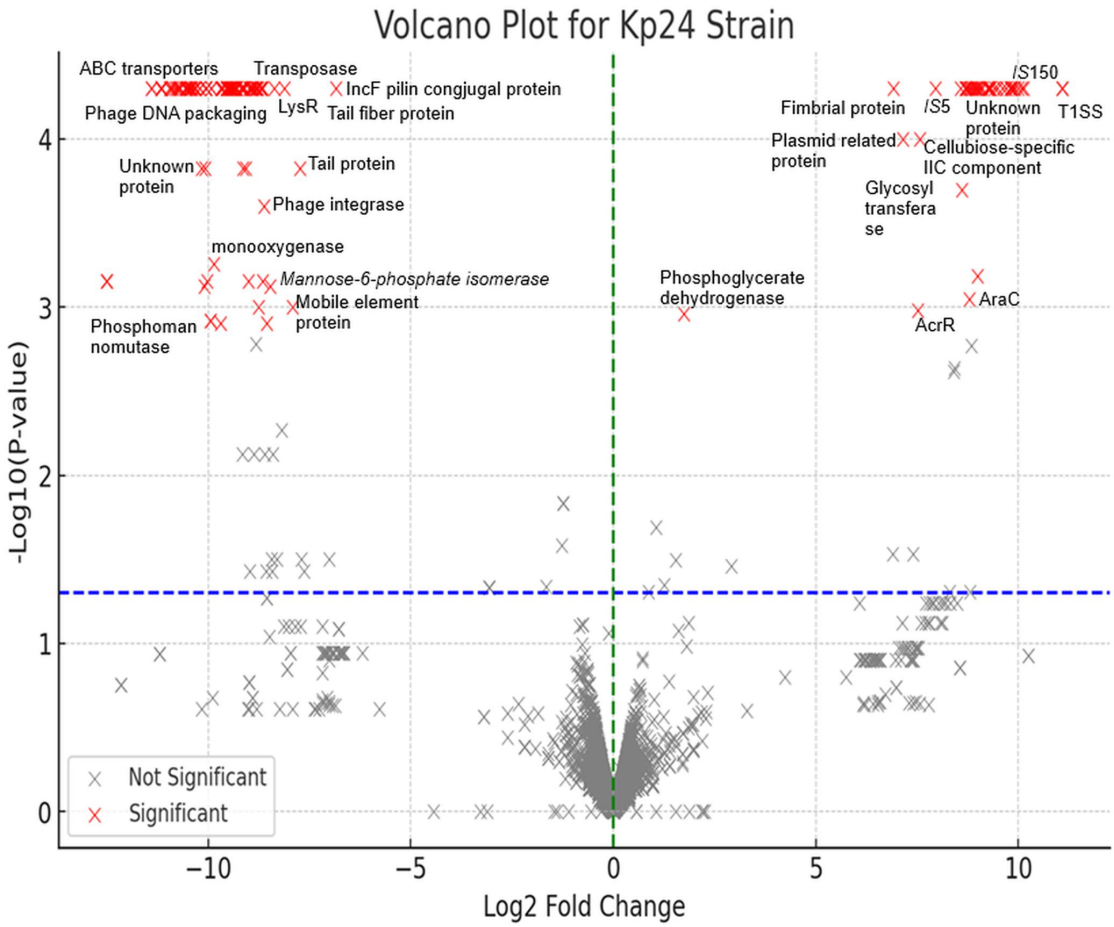

Figure 9. *A volcano plot for strain Kp_24, visually representing the differential expression analysis results*. in this plot, each point represents a gene. The x-axis shows the log2 Fold change, indicating the magnitude and direction of expression changes (left: downregulated, right: upregulated). The y-axis shows the negative log10-transformed p-values, indicating the significance of the differential expression (higher values are more significant). Red points highlight genes considered significantly differentially expressed, while grey points represent those not meeting the significance threshold. The blue dashed line represents the threshold for a p-value of 0.05, above which genes are considered significantly differentially expressed. The green dashed line marks the threshold for no fold change.

Discussion

The emergence of colistin- and carbapenem-resistant *K. pneumoniae* is a major concern owing to limited treatment options. Epidemiological data in South Africa show an increased prevalence of carbapenemase-positive Gram-negative bacteria and a low prevalence of *mcr* genes within the public health sector [8,10,28,29]. However, there are carbapenem- and colistin-resistant isolates without any known resistance mechanism. This study, therefore, aimed to characterize novel colistin and carbapenem resistance mechanisms in clinical *K. pneumoniae* isolates from South Africa.

Five non-carbapenemase producing carbapenem-resistant *K. pneumoniae* and four non-*mcr* producing colistin-resistant *Enterobacter* species were examined. Although the colistin-resistant isolates were identified by Microscan as *K. pneumoniae*, only isolate H3 was confirmed to be *K. pneumoniae*. The remaining isolates were identified as *Enterobacter* species.

The Microscan analysis showed that the *Enterobacter* species had reduced susceptibility to β- lactams, β-lactams/β-lactamase inhibitors, as well as the first- and second-generation cephalosporins. The resistance mechanisms associated with these antibiotics involve β-lactamase activity and loss of porin activity [30–32]. The *Enterobacter* species, G5, and G8 harboured bla_{CMH}, which is the most common β-lactamase gene within the *Enterobacter* genus. Additionally, bla_{ACT} which is also commonly found in this genus [33], was present within A5 and G3. The *Enterobacter* species also harboured three other resistance genes: *fosA*,

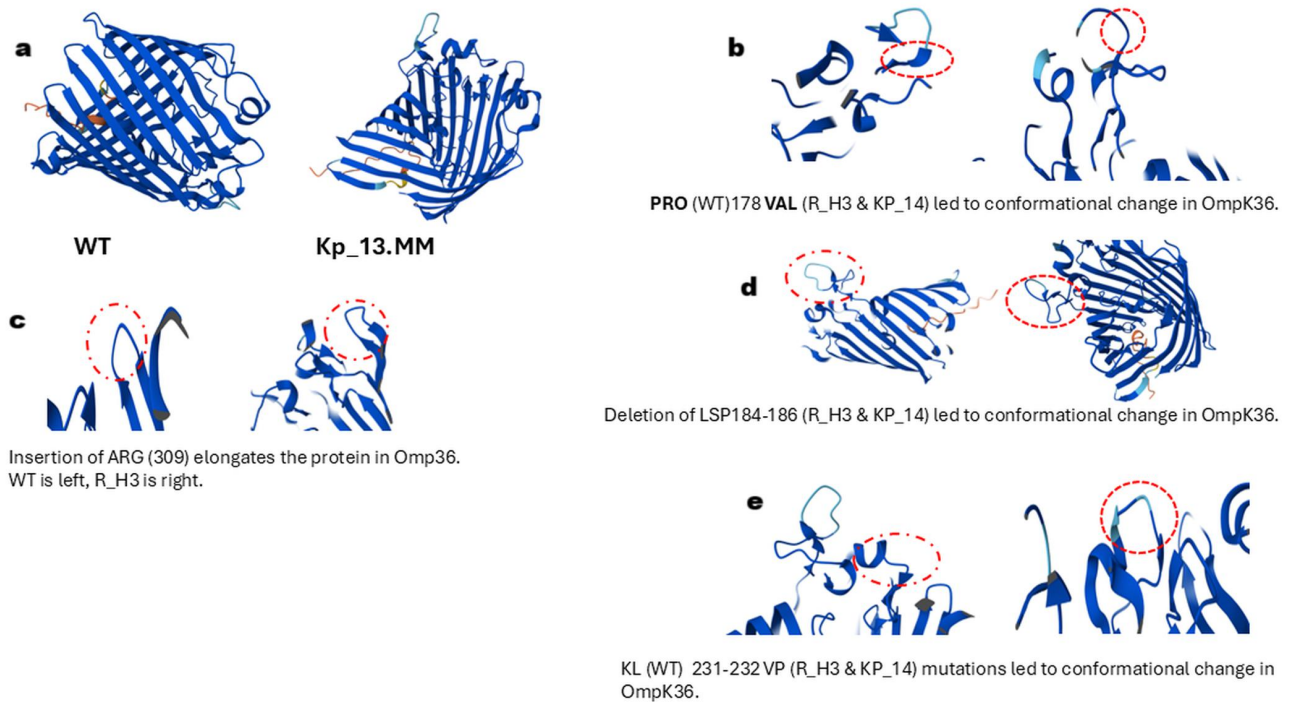

Figure 10. Structural changes in OmpK36 (OmpC) porins in *K. pneumoniae* compared to *K. pneumoniae* wild type strain. AlphaFold was used to analyse the amino acid sequences of the wild-type and carbapenem-resistance *K. pneumoniae* strains' OmpK36 porins. As shown in a-e, the mutations in the porins led to structural adjustments or conformations that likely affected the permeability of the porins to the carbapenems. Regions showing structural transformations are circled in red.

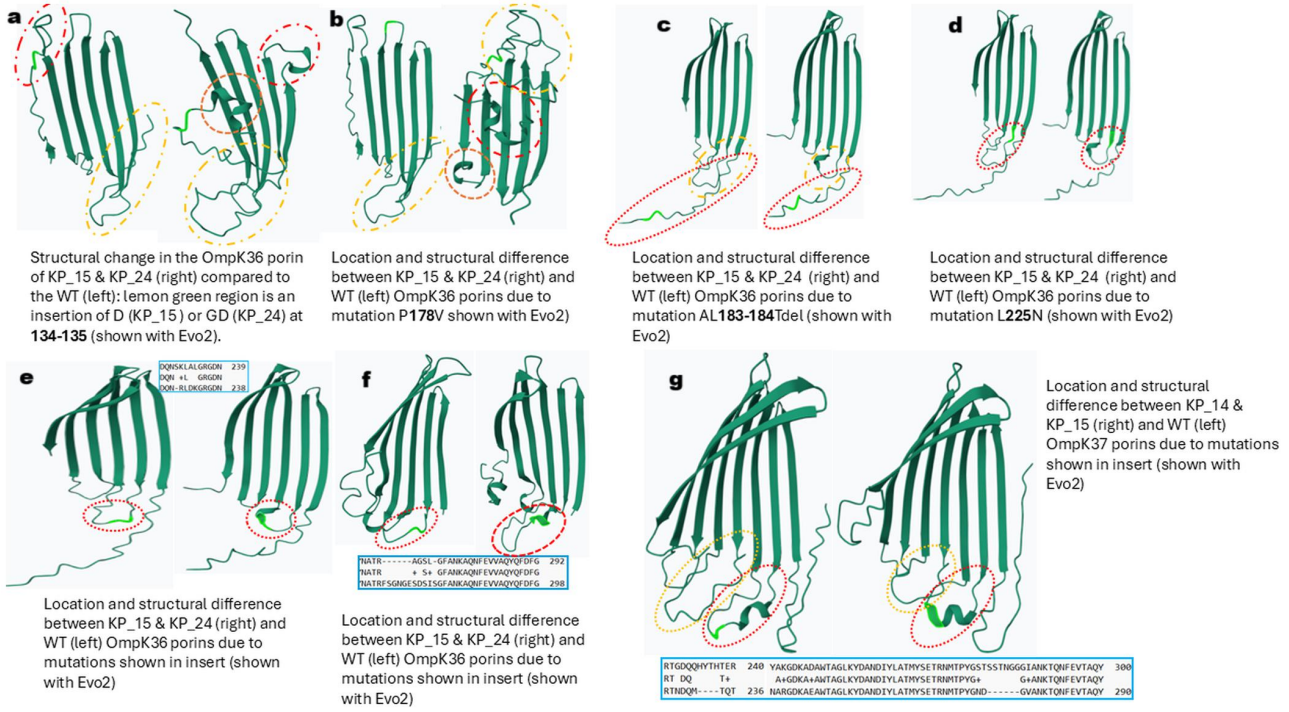

Figure 11. Structural changes in OmpK36 (OmpC) and OmpK37 porins in *K. pneumoniae* compared to *K. pneumoniae* wild type strain. Evo 2 was used to analyse the amino acid sequences of the wild-type and carbapenem-resistance *K. pneumoniae* strains' OmpK36 and OmpK37 porins. Regions showing structural transformations are circled in broken circles of red, orange, and yellow. The comparison shows how the mutations (shown as inserts in e, f, and g) affects the structure of the porins and evidently, their permeability.

conferring resistance to Fosfomycin [34], *oqxAB*, conferring resistance to quinolones, tigecycline, nitrofurantoin, several detergents, and disinfects [35]. No other resistance genes were identified. However, the phenotypic characterization of isolates revealed reduced susceptibility to ertapenem, meropenem, colistin and tobramycin. Resistance to these antibiotics can be mediated through changes in the outer membrane permeability, alteration of the lipopolysaccharide reducing porin activity and increased activity of efflux pumps [14,36].

Colistin resistance in G5, G8, and H3 was partially reversed by the efflux pump inhibitor CCCP, which reduced the BMD MIC value onefold from 128 µg/mL to 64 µg/mL, implicating active efflux in these isolates. This EPI has been shown to restore colistin susceptibility in some intrinsic colistin-resistant *Enterobacteriacae* isolates [37,38]. Such findings align with prior reports that underscore the synergy of efflux pump overexpression, capsular polysaccharide overproduction, and LPS modifications as key determinants of colistin resistance [39–42]. Moreover, the discovery of isolates that lack mcr genes yet display persistent colistin resistance reinforces the need to investigate alternative routes, such as novel regulatory mutations or understudied lipopolysaccharide-modifying enzymes. Clinically, this multi-factorial resistance pattern warrants further exploration to identify potential targets for combination therapies, including efflux pump inhibitors.

Colistin resistance has been previously linked to *mcr* activity [43], modification of the lipopolysaccharide (LPS) [44], overexpression of efflux pumps [39], and overproduction of capsular polysaccharide [40–42]. Genomic analysis reveals that the colistin-resistant isolates (A5, G3, G5, G8, and H3) were sorely negative for *mcr* gene. However, H3 harboured a *mgrB* mutation that has been demonstrated to confer colistin resistance by regulating the LPS modification system [17]. The colistin-resistant isolates, as revealed by RNA-seq analysis, showed a range of potential mechanisms for mediating resistance, including upregulation of efflux pumps, capsular polysaccharide biosynthesis, and putative glycosyltransferases. Common membrane alterations in colistin-resistant strains encompass the upregulation of MDR efflux pumps and capsular polysaccharide biosynthesis, which could potentially mediate colistin resistance. Moreover, the upregulation of the *fimH* and capsule genes, coupled with the presence of the *mrkA* virulence factor, might facilitate biofilm formation, thereby promoting antibiotic resistance [45].

The production of capsular polysaccharide was observed in isolates G5, G8, and H3. Previous studies have indicated that this activity acts as a protective barrier against cationic antimicrobial peptides like colistin [40]. As a result, this reduces the interactions between colistin and the LPS, thereby mediating resistance. Putative glycosyltransferase, notably those encoded by *crrB* gene, has been shown to mediate the LPS outer membrane modification [46]. The observed upregulation of putative glycosyltransferase in isolates G5 and H3 suggests a potential role in mediating LPS modifications. Telke *et al.* (2019) previously reported that the overexpression of the acrAB-tolC efflux pump, regulated by *soxRS* in *E. cloacae* and *E. asburiae* isolates, resulted in colistin hetero-resistance [47]. In our study, the efflux pump activity observed in G5, G8, and H3 was regulated by the *acrR*, as seen in Table S7 (Figure 6–9). However, the remaining isolates, G3 and A5 did not display significant DEGs that could confer colistin resistance, limiting the role of efflux in colistin resistance in this case. The detailed DEG tables for each isolate can be found in Table S8–25.

The *K. pneumoniae* isolates harboured a wide range of ARGs that confer resistance to various classes of antibiotics. These include aminoglycosides (*acc(3)-IId, aac(6')-Ib-cr, aadA2, aadA16, aph(3')-Ia, aph(3")-Ib, aph(6)-Id, armA, strAB)*, cephalosporins ($bla_{\text{CTX-M}}$), quinolones (*oqxA, oqxB*), fosfomycin (*fosA*), pencillins (bla_{TEM}, bla_{DHA}, bla_{OXA}, bla_{CMH}, bla_{SHV}), sulphonamides (*sul1, sul2*), tetracyclines (*tetA*), and trimethoprim (*dfrA*). The genes contributed to the observed phenotypic resistance. Isolates Kp_4, Kp_15, and Kp_24 harboured mutations in *ompK35*, which confer resistance to carbapenems [14,48].

DNA analysis revealed that all the carbapenem-resistant *K. pneumoniae* (Kp_4, Kp_14, Kp_15, and Kp_24) isolates harboured multiple β- lactamases and mutations within *ompK36* and *ompK37*, except for isolate Kp_14. The combination of porin mutations in *ompK* and β- lactamase activity contributes to carbapenem resistance. Additionally, these isolates exhibited upregulation of MDR efflux pumps [49,50].

RNA-seq analysis revealed that the carbapenem-resistant *K. pneumoniae* isolates had multiple mechanisms to confer resistance to carbapenems (Tables S6-S7; Datasets 2–4). Collectively, RNA-seq analysis revealed that carbapenem-resistant *K. pneumoniae* (Kp_4, Kp_14, Kp_15, and Kp_24) harness multiple pathways to circumvent antibiotic killing. In addition to β-lactamases and porin mutations, each isolate exhibited upregulation of efflux pump regulators (*acrR*) and capsule biosynthesis genes. Notably, Kp_14 and Kp_15 simultaneously upregulated cellulose synthase, likely fuelling biofilm formation – a synergy that can reduce antibiotic penetration [49]. Moreover, the differential

expression of ompK regulators further implicates outer membrane adaptations in enhancing carbapenem resistance. Furthermore, all the carbapenem-resistant isolates exhibited a wide variety of upregulated fimbriae products (Table S6). Taken together, these findings emphasize how *K. pneumoniae* exploits a multifaceted strategy, combining plasmid-borne β-lactamases with enhanced biofilm formation, to endure high-level antibiotic stress. It also shows how antibiotic stress can influence hypervirulence by triggering virulence genes and regulatory factors such as LysR (Figure 6–9).

In contrast, the EDTA and efflux pump inhibition analysis (Table 2) demonstrated that β- lactamase activity and efflux pumps played a role in carbapenem resistance in all isolates except Kp_24. Therefore, in this group, three distinct resistance mechanisms were observed, excluding Kp_24, where efflux pump activity did not contribute to carbapenem resistance. Further, the structural variations seen in the porins of the CRKP strains show how mutations are employed by CRKP strains to adapt to carbapenem stress by altering porin structure to reduce influx of external xenobiotics (Figure 10,11).

Isolates exhibited colistin or carbapenem resistance through significant transcriptional modulation of specific genes, including marked upregulation of efflux pump-related transporters (e.g. Copper/silver efflux RND transporter; XLOC_000016, log2FoldChange = 9.16, $p = 0.00015$) and substantial downregulation of ABC transporter proteins (e.g. permease proteins XLOC_000361–XLOC_000363, log2FoldChange = −8.27 to −8.39, fold change > 300, $p < 0.0001$). The concurrent upregulation of genes encoding transposases (XLOC_000339–XLOC_000340, log2FoldChange = 8.68–9.98, fold change < 0.002) indicates a significant role for mobile genetic elements in the adaptive resistance mechanisms observed.

The most significant transcriptomic changes were consistently associated with transporter proteins (ABC transporters, efflux pumps) and mobile genetic elements (transposases). Specifically, the extensive downregulation of ABC transporter permease and ATP-binding proteins (XLOC_000361–XLOC_000364; log2FoldChange: −8.27 to −10.06; fold change: 300–1071; $p \leq 0.0001$) suggests a strategic cellular response aimed at reducing antibiotic influx (Figure 6–9).

Table 3 shows that the majority of ARGs identified within the *K. pneumoniae* isolates were harboured on plasmids. IncFIB(K), IncFIB(K)/IncFII(K), and IncHIB harboured three or more resistance genes with the IncFIB(K)/IncFII(K) harbouring a remarkable 19 resistance genes (Table S2). These plasmid replicons, IncF and IncH, are among the most observed types of replicons in Enterobacteriaceae, and they play a significant role in facilitating the transmission of ARGs [51–53]. Studies have shown that IncFIB and IncFII replicons are capable of accommodating and stably carrying a wide variety of ARGs [54–56]. These accounts for the large number of resistance genes seen in the IncFIB(K)/IncFII(K) plasmid from this study. Furthermore, it was observed that the ARGs within these plasmids were often flanked by IS elements, particularly IS26, which is widely known to be associated with ARGs [57,58]. This underscores the potential role of IS elements in these isolates in the dissemination of these MDR ARGs, thus facilitating the wide spread of ARGs in South Africa. This may occur, through the transfer of ARGs between animal-derived and human-derived pathogens.

Indeed, the highly significant expression of MGEs in all the strains and species underscores their importance in antibiotic resistance among prokaryotes. As observed recently in *A. baumanii* [59], antibiotics exposure forces the bacterial genomes to shuttle its defences through MGEs to conserve life and adapt to the new environment. Integrases, recombinases, plasmids, integrative elements, insertion sequences, and transposons are marshalled to literally “cut and paste” resistance determinants to prevent annihilation [59].

Genomic analysis of the six *K. pneumoniae* isolates revealed that the isolates belonged to four sequence types. ST307 clone comprised three isolates, Kp4, Kp15, and Kp24, which had the same K- and O-serotypes, KL102 and O1/O2vO2. The K-antigen describes the type of capsular polysaccharide harboured by the *K. pneumoniae* isolates, and the O-antigen describes the lipopolysaccharide antigens [60]. The KL102, previously known as KN2, has been widely identified in carbapenemase-producing *K. pneumoniae* isolates in Nigeria [7], USA [61], and Switzerland [62]. These isolates were further shown to also harbour the O1/O2v2 serotype. However, in this study, despite the isolates harbouring the same sequence type and serotypes, the phylogenetic analysis of these isolates revealed an interesting pattern in their distribution and resistance profiles. The analysis included 81 *K. pneumoniae* isolates from five continents, with sequence types ST307, ST25, and ST219 being the most common. Within South Africa, the majority of *K. pneumoniae* isolates belonged to ST307, and five of the eight Clades were comprised of this sequence type. These five Clades had similar resistomes, thus highlighting the vertical and horizontal spread of this

MDR clone and ARGs within South Africa. Furthermore, in Figure 3, the study isolates (Kp_4, Kp_13, Kp_14, Kp_15, and Kp_24) clustered alongside international *K. pneumoniae* isolates, underscoring their easy transmissibility and wide distribution.

The *Enterobacter* species included isolates A5, G3, G5, and G8, which were identified as *Enterobacter asburiae*, *Enterobacter bugandensis*, and two *Enterobacter cloacae* species, respectively. The sequence types of these isolates included ST22 (A5), ST632 (G3) and a novel ST2100 for both *E. cloacae* species (G5 and G8). Fortunately, these isolates carried only a limited number of resistance genes and lacked plasmids. Moreover, they clustered with other isolates that had similar resistance patterns. Specifically, *E. asburiae* A5 clustered within clade 4 (Figure 4) and clustered with a South African strain (E124_11) and a Chinese strain (C210176). Notably, this clade displayed a distinct resistome pattern compared to the other clades; a pattern consistent with the other *Enterobacter* species analysed in this study (Figures 5 and 6). This distinction might be attributed to the presence of different plasmids that potentially encode these ARGs. A more comprehensive phylogenetic analysis, which incorporates plasmid analysis of the included isolates, could shed light on the reasons behind this clustering pattern.

The study's isolates were found to harbour a diverse array of restriction modification systems (RMS), including both restriction enzymes and methyltransferases. These RMS included Types I, II, and III RMS. Among these, the Type II *M.Kpn34618Dcm* was the most predominant and was identified in all *K. pneumoniae* isolates. Previous research, as reported by Chuckamnerd *et al.* 2022 and Ramaloko and Osei Sekyere (2022), has shown the common occurrence of this Mtase in *K. pneumoniae* [63,64]. Moreover, it is typically found alongside *M.EcoRII*, a pattern noted by Ramaloko and Osei Sekyere (2022). In this study, it was observed that four of the seven isolates harbouring the Dcm Mtase also carried a plasmid-encoded *M.EcoRII*. Interestingly, the *E. asburiae* A5 isolate displayed a similar combination of these Mtases.

In contrast, among the ST307 *K. pneumoniae* isolates ($n = 3$), *M.Kpn34618Dcm* was the sole common Mtase. Contrary to Chuckamnerd et al. (2022) findings, there was no consistent pattern observed within the RMS in this study's isolates [63]. However, it is noteworthy that all Type II Mtases, including the type II restriction endonuclease (RE), shared the same recognition sequence. This commonality facilitates the integration of plasmids encoding these Type II RMS into host bacteria, thereby enhancing the dissemination of virulence and resistance genes [4].

Only two of the three types of methylation i.e. N6-methyladenine (m6A) and N4-methylcytosine (m4C), were identified in the isolates that underwent PacBio SMRT sequencing (Kp_4, Kp_24, A5, G5, and H3). According to Militello *et al.* (2012), the methylation type N5-methylcytosine (5mC) DNA modification is not commonly found [65]. In this study, neither *K. pneumoniae* nor the *Enterobacter* species isolates encoded this type of methylation. However, m6A and m4C, representing an alternative form of cytosine methylation, were detected. It is noteworthy that only a small fraction of motif sites in the isolates remained non-methylated, as depicted in Table S5.

Contrary to what was observed in *A. baumannii* [59] and in this study's *E. asburiae* A5 and *E. cloacae* G8, no *K. pneumoniae strain* had significant expression of RMS genes, suggesting that the use of RMS to regulate resistance determinants (gene) expression is not global among all strains [59]. It could also be the case that different antibiotics elicit different genomic and epigenomic responses. As the *K. pneumoniae* were exposed to ertapenem and the *Enterobacter sp.* were exposed to colistin, the differences in RMS gene expression could stem from this. Further studies will be necessary to comprehensively delineate the role of different antibiotics on RMS transcription profile among different bacteria species.

In addition to resistance mechanisms, the *K. pneumoniae* isolates also carried various virulence genes, making them highly equipped for pathogenesis. The isolates harboured nine types of virulence genes, including adhesion, biofilm formation, efflux pumps, immune evasion, iron uptake, regulation of capsule synthesis, and secretion systems. The presence of these virulence genes further underscores the necessity for effective infection control measures to prevent the spread of these highly virulent and drug-resistant strains.

Virulence genes play a pivotal role in the pathogenesis of a pathogen, facilitating both host infection and, in this case, resistance to antibiotics [66]. The transcriptomic analysis revealed an increase in certain transporters, such as those for carbohydrates, cysteine, and ferric ions. Cain *et al.* (2018) explained that signs of stress in *K. pneumoniae* include the accumulation of compounds like cellulase, carbohydrates, and metal ions in granules at the end of active growth [67]. Thus, the upregulation of ion ABC-transporters, phosphotransferase system components, and ferric-ion transporters may indicate stress induced by antibiotic exposure in these *K. pneumoniae* isolates. Ramos *et al.* (2016) further demonstrated that intracellular regulation of iron metabolism assists bacteria in managing oxidative stress [68]. Another indicator of stress is the

upregulation of fimbriae genes, as observed by Cain *et al.* (2018) [67]. The transcriptomic data indicated upregulation of type 1 fimbriae genes, potentially mediated by *fimH* virulence gene [69].

Unfortunately, due to financial restrictions, the study was unable to employ the CRISPR-Cas system to investigate these putative resistance mechanisms in the clinical isolates. However, the combination of whole genome sequencing, epigenomics, and transcriptomics proved valuable in characterizing these resistance mechanisms.

Given the increasing prevalence of colistin and carbapenem-resistant *K. pneumoniae* in South Africa and globally, surveillance studies are essential to monitor the epidemiology and antibiotic susceptibility patterns of these MDR strains. Specifically, the emergence of carbapenem- and colistin-resistant isolates lacking canonical resistance genes poses significant clinical and public health threats as such strains may evade standard molecular diagnostics that focus solely on carbapenemase and *mcr* detection, jeopardizing timely and effective treatments. Public health agencies should therefore broaden their surveillance protocols to capture novel resistance mechanisms – particularly those involving efflux pumps, outer membrane modifications, and biofilm-associated genes. This expanded vigilance will be critical for guiding infection control strategies, informing antibiotic stewardship programs, and potentially spurring the development of targeted therapeutics such as efflux pump inhibitors or anti-biofilm agents

Conclusion

Our integrated genomic-epigenomic-transcriptomic analyses show that the three adaptive levers act in concert to undermine both colistin and carbapenem therapy in *K. pneumoniae* and *Enterobacter* isolates. In summary:

Mobile genetic elements (MGEs)

Every *K. pneumoniae* strain carried multi-replicon IncF/IncH or IncX3 plasmids densely populated with β-lactamase genes (e.g. $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-181}}$), each flanked by IS*26* or ISKpn*26*. RNA-seq revealed a > 8-$\log_2$ up-regulation of transposase and integrase transcripts (Figure 6–9; Datasets 2–3), indicating active transposition under antibiotic pressure. These MGEs therefore (i) provide an immediate enzymatic defence against carbapenems and (ii) accelerate intra- and inter-species spread of resistance cassettes, rapidly eroding treatment efficacy.

Transporters

Two opposite, yet synergistic, transporter trends were evident. (i) RND and MFS efflux components (e.g. copper/silver RND transporter, AcrAB-TolC regulators) were up-regulated 6- to 9-$\log_2$-fold, and CCCP lowered ertapenem or colistin MICs two-fold in several strains – direct evidence that active efflux diminishes intracellular drug accumulation. (ii) Conversely, core ABC import permeases were massively down-regulated (−8 to −10-$\log_2$-fold), further restricting drug entry. Together, these shifts tilt the influx – efflux balance heavily towards extrusion, blunting both colistin and carbapenem exposure at their cellular targets.

Capsule restructuring

Capsule-associated genes (*ugd, wza, gnd*, cellulose synthase) were among the most highly induced transcripts (7- to 9-$\log_2$-fold). Thickened, electro-positive capsules physically impede colistin access to lipid A and, by promoting biofilm formation, create diffusion-limited microniches that shelter the cell wall from carbapenem attack. The ST307 cluster, in particular, combined KL102 capsule up-regulation with porin-disruptive *ompK35/K36* mutations, yielding a formidable permeability barrier.

In summary, plasmid-borne MGEs supply transferable enzymatic resistance, transporter rewiring minimizes intracellular drug exposure, and capsule remodelling adds an external biophysical shield. The convergence of these three pressure-responsive systems explains how our isolates withstand colistin and carbapenem therapy despite lacking canonical *mcr* or carbapenemase genes, and underscores the urgent need to monitor non-classical resistance trajectories in routine surveillance and stewardship programs.

This study contributes significantly to our understanding of the mechanisms behind antibiotic resistance and virulence in both *K. pneumoniae* and *Enterobacter* species. It offers valuable insights into the genomic, epigenomic, and transcriptomic characterization of colistin and carbapenem resistance mechanisms in clinical *K. pneumoniae* and *Enterobacter* species. The findings underscore the importance of continuous monitoring of the epidemiology and evolution of these pathogens. Understanding the genetic basis of antibiotic resistance and virulence in *K. pneumoniae* is crucial for developing effective strategies to control and manage infections caused by these MDR bacteria. Multiple genes encoding hypothetical proteins were significantly altered upon antibiotic exposure, particularly XLOC_000339 (log2FoldChange:

8.68 to 9.99, fold change <0.002, $p < 0.0001$), suggesting potential novel roles in antibiotic resistance mechanisms that warrant further investigation.

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Author contributions

MM undertook laboratory work and manuscript drafting; NMM was a co-supervisor to the study and assisted with funding; BF was a co-supervisor to the study and assisted in reviewing of the manuscript; JOS designed and supervised the study, wrote, reviewed, and edited the manuscript, as well as assisted with analysis of the data. All authors have read and approved the final work.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement

All data used in this study are included in the supplementary files and datasets. This Whole Genome Shotgun project, epigenomic, and RNAseq data have been deposited at DDBJ/ENA/GenBank under the bioproject number PRJNA861833. All supplementary files in this article have been deposited in BioRxiv at https://www.biorxiv.org/content/10.1101/2023.12.15.571804v1.supplementary-material

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