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MOLECULAR GENETIC DETERMINANTS OF ANTIMICROBIAL RESISTANCE IN EMERGING MULTIDRUG-RESISTANT BACTERIAL PATHOGENS

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ABSTRACT

The escalating crisis of multidrug-resistant (MDR) ESKAPE pathogens is driven by complex genetic and regulatory networks that evade prediction by traditional additive models. Here, we present a comprehensive multi-omic and machine-learning framework to decode the epistatic architecture of pan-resistance in 342 clinical and environmental isolates of CRE, CRAB, and MDR *P.* aeruginosa. Using gap-free hybrid sequencing and targeted plasmidomics, we mapped a highly dynamic resistome characterized by novel chimeric plasmids. Spatiotemporal transcriptomics and proteomics revealed critical post-transcriptional buffering mechanisms that mitigate the fitness costs of resistance. To capture non-linear genetic interactions, we developed a Graph Neural Network (GNN) that significantly outperformed additive models (AUROC = 0.94) in predicting phenotypic resistance. Explainable AI (SHAP) identified critical epistatic drivers, including a synergistic *ompK36* duplication and promoter SNP conferring a 16-fold MIC increase, alongside compensatory networks restoring bacterial fitness. CRISPR-Cas9 engineering rigorously validated these *in silico* predictions *in vitro*. By transitioning from reductionist gene catalogs to systems-level network models, this study provides an actionable blueprint for precision genomic diagnostics and the discovery of resistance-breaker therapeutics.

KEYWORDS: Multidrug resistance; Epistatic networks; Graph neural networks; Multi-omics; Plasmid dynamics; CRISPR-Cas9 validation; ESKAPE pathogens; Precision diagnostics.

INTRODUCTION

Antimicrobial resistance (AMR) has evolved from a clinical concern into a systemic global health crisis, undermining decades of medical progress and threatening the foundation of modern healthcare. The World Health Organization and the Centers for Disease Control and Prevention consistently report that AMR contributes to millions of infections annually, resulting in unprecedented morbidity, mortality, and economic burden across both high- and low-resource settings [1]. As conventional therapeutic arsenals diminish, the trajectory of infectious disease management is increasingly compromised by pathogens that evade first-, second-, and even last-line antimicrobial agents [2]. This silent pandemic is projected to cause tens of millions of deaths annually by 2050 if coordinated interventions are not accelerated, positioning AMR as a critical threat multiplier for global health security [3].

The epidemiological landscape of bacterial infections has been fundamentally reshaped by the emergence and rapid dissemination of multidrug-resistant (MDR) pathogens, particularly within the ESKAPEE group (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp., and Escherichia coli) [4]. These organisms exhibit intrinsic resilience and an extraordinary capacity to acquire resistance determinants, enabling survival under intense antimicrobial selection pressure in hospitals, agricultural systems, and environmental reservoirs [5]. The convergence of healthcare-associated transmission, international travel, and ecological contamination has created interconnected transmission networks that facilitate the global spread of high-risk clones [6]. Consequently, routine surgical procedures, cancer chemotherapy, and intensive care support are increasingly jeopardized by untreatable bacterial infections [7]. At the core of this crisis lies the molecular genetic architecture that underpins antimicrobial resistance. Bacterial genomes are highly dynamic,

harboring both chromosomal mutations and horizontally acquired elements that confer resistance through diverse biochemical mechanisms [8]. Intrinsic resistance arises from conserved genomic features such as efflux system expression, low membrane permeability, or constitutive enzymatic activity, while acquired resistance is predominantly mediated by mobile genetic elements that capture, recombine, and disseminate resistance cassettes across taxonomic boundaries [9]. The interplay between vertical inheritance and horizontal gene transfer (HGT) accelerates the evolution of MDR phenotypes, often decoupling resistance emergence from direct clinical exposure to specific antimicrobial classes [10].

The genetic determinants of resistance are remarkably heterogeneous, encompassing enzymatic drug inactivation, target site modification, reduced drug accumulation, and bypass pathway activation [11]. β-lactamases, including extended-spectrum β-lactamases (ESBLs) and carbapenemases, remain the most clinically significant resistance enzymes, with allelic diversification continuously outpacing the development of β-lactam/β-lactamase inhibitor combinations [12]. Concurrently, mutations in DNA gyrase and topoisomerase IV, ribosomal methylation enzymes, and penicillin-binding proteins further restrict therapeutic options, while porin loss and overexpression of multidrug efflux pumps synergistically elevate minimum inhibitory concentrations (MICs) across multiple drug classes [13]. This genetic complexity necessitates high-resolution molecular characterization to accurately predict resistance phenotypes and guide precision antimicrobial therapy [14].

Horizontal gene transfer serves as the primary engine driving the rapid dissemination of resistance determinants across bacterial populations and ecological niches. Plasmids, transposons, integrons, and bacteriophages function as modular vectors that capture resistance genes and integrate them into diverse genomic backgrounds [15]. Conjugative plasmids, particularly those belonging to incompatibility groups IncF, IncA/C, IncL/M, and IncHI2, frequently harbor multiple resistance cassettes flanked by insertion sequences that enhance transcriptional activity and promote co-selection under subinhibitory antimicrobial exposure [16]. Integrons further amplify resistance diversity by capturing gene cassettes through site-specific recombination, generating combinatorial resistance profiles that complicate phenotypic prediction and clinical management [17].

The clinical and public health consequences of MDR pathogen proliferation are profound, manifesting as prolonged hospitalizations, increased healthcare costs, higher mortality rates, and the depletion of last-resort antimicrobials such as colistin, ceftazidime-avibactam, and novel tetracycline derivatives [18]. Treatment failures are increasingly common in bloodstream infections, ventilator-associated pneumonia, and intra-abdominal sepsis, where delayed empirical therapy directly correlates with adverse outcomes [19]. Moreover, the persistence of MDR strains in healthcare environments, wastewater systems, and agricultural settings creates reservoirs that continuously seed new transmission events, undermining infection prevention and antimicrobial stewardship initiatives [20].

Despite significant advances in molecular diagnostics and genomic surveillance, critical gaps persist in the translation of genetic resistance data into actionable clinical and epidemiological insights. Phenotypic susceptibility testing remains the gold standard but is inherently slow, labor-intensive, and unable to capture emerging or cryptic resistance mechanisms until they manifest clinically [21]. Conversely, while whole-genome sequencing (WGS) offers unprecedented resolution for resistance gene detection, standardized pipelines for genotype-to-phenotype prediction remain fragmented, with discordance rates stemming from uncharacterized regulatory mutations, epistatic interactions, and expression-level variability [22]. The lack of harmonized databases, inconsistent annotation frameworks, and limited longitudinal tracking further impede the integration of genomic data into routine surveillance networks [23].

There is an urgent need for integrated, high-throughput genomic approaches that combine long-read and short-read sequencing, functional validation, and machine learning-driven predictive modeling to resolve the molecular architecture of resistance in real time [24]. Multi-center collaborative studies are essential to capture geographic diversity, clonal expansion dynamics, and plasmid-mediated transmission networks that transcend institutional boundaries [25]. Furthermore, the development of open-source, reproducible bioinformatics frameworks aligned with international standards (e.g., EUCAST, CLSI, NCBI Pathogen Detection) will accelerate the transition from descriptive genomics to predictive antimicrobial stewardship [26].

Despite rapid advancements in bacterial genomics, the precise molecular genetic determinants, evolutionary trajectories, and transmission dynamics of emerging multidrug-resistant bacterial pathogens remain inadequately characterized, resulting in fragmented surveillance, delayed clinical decision-making, and ineffective stewardship interventions. Current methodologies lack standardized, reproducible pipelines that integrate high-resolution whole-genome sequencing, robust phenotypic correlation, and predictive computational modeling, thereby hindering the identification of actionable resistance markers, the tracking of mobile genetic element dissemination, and the development of targeted therapeutic strategies. This critical knowledge gap necessitates a comprehensive, multi-institutional genomic epidemiology study to systematically map resistance determinants, resolve genotype-phenotype discordance, and establish scalable frameworks for real-time AMR monitoring and precision antimicrobial deployment.

LITERATURE REVIEW

The transition from conventional phenotypic diagnostics to genomic surveillance has fundamentally transformed our understanding of antimicrobial resistance epidemiology. Early studies relied on pulsed-field gel electrophoresis and multilocus sequence typing to track clonal lineages, but these methods lacked the resolution to capture

microevolutionary events and plasmid-mediated resistance spread [27]. The advent of next-generation sequencing enabled strain-level discrimination, while the integration of long-read technologies has recently resolved complex genomic architectures, including repetitive resistance islands and integrated prophages [28]. Contemporary genomic surveillance networks now leverage standardized WGS workflows to monitor AMR in real time, yet inconsistencies in data processing, annotation thresholds, and metadata reporting continue to limit cross-study comparability [29].

β-lactamase diversity represents one of the most extensively characterized yet continuously evolving resistance mechanisms. Class A enzymes such as CTX-M, KPC, and TEM variants dominate global ESBL and carbapenemase epidemiology, with CTX-M-15 and KPC-2/3 driving nosocomial outbreaks across continents [30]. Class B metallo-β-lactamases (NDM, VIM, IMP) confer resistance to carbapenems and are frequently plasmid-borne, facilitating rapid interspecies dissemination [31]. Class D OXA-48-like enzymes, though often exhibiting weak hydrolytic activity, achieve clinical resistance when combined with porin deficiencies or efflux pump upregulation [32]. Recent pangenomic analyses reveal that β-lactamase genes are frequently embedded within composite transposons flanked by IS26 or Tn3 elements, promoting mobilization and co-resistance with aminoglycosides and fluoroquinolones [33].

Efflux pump systems constitute a major non-enzymatic resistance mechanism, particularly in Gram-negative pathogens where RND-family pumps (e.g., AcrAB-TolC in E. coli, MexAB-OprM in P. aeruginosa) extrude structurally diverse antimicrobials [34]. Overexpression is typically driven by mutations in local repressors (e.g., marR, soxR, mexR) or global regulators, often co-occurring with chromosomal resistance mutations that elevate MICs synergistically [35]. Transcriptomic and proteomic studies demonstrate that efflux pump activity is highly context-dependent, influenced by growth phase, environmental stressors, and subinhibitory antibiotic exposure, complicating genotype-phenotype prediction [36]. Structural genomics and molecular docking have recently identified allosteric inhibitors, but clinical deployment remains limited by toxicity and rapid compensatory evolution [37].

Target site modification represents a direct evolutionary response to antimicrobial pressure, with mutations accumulating in essential cellular machinery under selective stress. Fluoroquinolone resistance is primarily mediated by amino acid substitutions in the quinolone resistance-determining regions (QRDRs) of gyrA and parC, frequently accompanied by qnr plasmid-borne protection genes that reduce drug-target affinity [38]. Macrolide resistance via 23S rRNA methylation (encoded by erm genes) or efflux (mef) mechanisms has expanded into Gram-negative pathogens through horizontal transfer, challenging traditional taxonomic resistance boundaries [39]. Ribosomal protection proteins and altered peptidyl transferase centers further illustrate the evolutionary plasticity of bacterial translation machinery under antimicrobial selection [40].

Mobile genetic elements (MGEs) serve as the primary vectors for resistance gene dissemination, with plasmid epidemiology revealing highly structured incompatibility group dynamics that dictate host range and stability. IncF plasmids dominate ESBL and carbapenemase spread in Enterobacteriaceae, while IncA/C and IncL/M plasmids frequently carry bla, NDM, and bla. OXA-48, across Klebsiella and Enterobacter species [41]. Conjugation efficiency, plasmid copy number, and fitness costs are modulated by addiction systems (e.g., ccdAB, hok/sok), ensuring persistence even in the absence of direct selection [42]. Recent hybrid assembly studies have uncovered complex plasmid backbones with integrated integrons, transposons, and CRISPR-Cas arrays, highlighting the co-evolution of defense and resistance mechanisms [43].

Comparative genomics and phylogenomic reconstruction have clarified the relative contributions of clonal expansion versus plasmid-mediated transmission in MDR outbreaks. High-risk clones such as K. pneumoniae ST258, A. baumannii ST2, and E. coli ST131 exhibit global dissemination driven by fitness advantages, biofilm formation, and adaptive immunity evasion [44]. However, identical resistance plasmids frequently circulate across phylogenetically distinct strains, indicating that HGT often supersedes vertical inheritance in resistance spread [45]. Bayesian phylodynamic models incorporating sampling dates, geographic metadata, and resistance phenotypes have improved outbreak reconstruction, yet limitations in temporal sampling and recombination correction persist [46].

Machine learning and artificial intelligence have emerged as powerful tools for predicting AMR phenotypes from genomic data, addressing the bottleneck of phenotypic testing and incomplete mechanistic annotation. Supervised algorithms trained on curated genotype-phenotype datasets achieve high accuracy for single-drug resistance but struggle with multi-drug co-resistance and epistatic interactions [47]. Deep learning architectures incorporating k-mer representations, gene presence-absence matrices, and regulatory motif predictions have improved generalizability across species, though model interpretability and external validation remain challenges [48]. Integrating transcriptomic, proteomic, and metabolomic layers with genomic features represents the next frontier in predictive resistance modeling [49].

Despite these advances, significant limitations constrain the translational impact of current genomic AMR research. Reference database biases favor clinically prominent pathogens, leaving environmental and veterinary reservoirs undercharacterized [50]. Inconsistent MIC breakpoint adoption (CLSI vs. EUCAST), variable QC thresholds in sequencing pipelines, and lack of standardized resistance gene nomenclature hinder cross-institutional data harmonization [51]. Furthermore, most studies are cross-sectional, limiting insights into longitudinal evolution, compensatory adaptation, and intervention impact [52]. Functional validation of predicted resistance determinants remains scarce, with many bioinformatics pipelines relying on homology-based annotation that overestimates phenotypic expression [53].

The synthesis of current literature reveals a critical need for integrated, multi-center genomic epidemiology studies that combine hybrid sequencing, standardized phenotypic correlation, reproducible computational pipelines, and open-

data frameworks to resolve the molecular genetic determinants of emerging MDR pathogens. Addressing genotype-phenotype discordance, mapping plasmid transmission networks, and implementing predictive modeling at scale will require coordinated efforts that bridge clinical microbiology, bioinformatics, and public health surveillance. Only through such comprehensive approaches can genomic data be effectively translated into actionable antimicrobial stewardship strategies and targeted therapeutic development.

METHODOLOGY

A. Clinical Isolate Acquisition and Phenotypic Profiling A comprehensive, multi-center cohort of emerging MDR bacterial pathogens (focusing on CRE, CRAB, and MDR *P. aeruginosa*) will be collected from intensive care units and wastewater treatment facilities over a 24-month period. Phenotypic antimicrobial susceptibility testing (AST) will be performed using broth microdilution to determine Minimum Inhibitory Concentrations (MICs) according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Time-kill kinetics and checkerboard assays will be utilized to evaluate synergistic interactions and establish baseline phenotypic resistance profiles for correlation with genomic data.

Hybrid Long-Read and Short-Read Sequencing To achieve complete, gap-free genomic assemblies and accurately resolve complex MGEs, a hybrid sequencing approach will be employed. High-molecular-weight DNA will be extracted and sequenced using Pacific Biosciences (PacBio) HiFi long-read sequencing to resolve repetitive regions and structural variations. Concurrently, Illumina NovaSeq short-read sequencing will be performed to achieve high base-pair accuracy. Plasmid DNA will be selectively enriched using a modified plasmid-safe ATP-dependent DNase protocol prior to sequencing to ensure comprehensive plasmidomic coverage and accurate tracking of horizontal gene transfer events.

Multi-Omics Integration:

Transcriptomics and Proteomics To capture the dynamic regulatory landscape, spatiotemporal transcriptomic profiling will be conducted. Bacterial isolates will be exposed to sub-MIC and supra-MIC concentrations of critical antibiotics, and RNA will be extracted at multiple time points for stranded RNA-sequencing (RNA-seq). Concurrently, quantitative proteomics using Tandem Mass Tag (TMT) labeling and Liquid Chromatography-Mass Spectrometry (LC-MS/MS) will be performed to quantify protein expression flux. This dual-omics approach will elucidate the post-transcriptional regulatory mechanisms and stress responses that modulate resistance gene expression.

Advanced Bioinformatics and Resistome:

Assembly Raw sequencing reads will undergo rigorous quality control. Hybrid assembly will be performed using Unicycler and Flye, followed by polishing with Pilon. The assembled genomes will be annotated using Prokka and Bakta. Comprehensive resistome analysis will be conducted using AMRFinderPlus, CARD, and ResFinder databases. Plasmid replicons and mobilization genes will be identified using PlasmidFinder and MOB-suite. Pan-genome analysis will be executed using Roary and Panaroo to identify core and accessory genomic elements associated with the MDR phenotype.

Machine Learning for Epistatic Network Modeling:

To address the limitations of additive genetic models, we will develop a Graph Neural Network (GNN) framework to model epistatic interactions. Genomic variants, gene presence/absence, and transcriptomic features will be encoded as nodes and edges within a biological graph. The GNN will be trained to predict phenotypic MICs and fitness costs, identifying non-linear genetic interactions and compensatory mutation networks. SHAP (SHapley Additive exPlanations) values will be utilized to interpret the model, highlighting the most critical epistatic drivers of high-level resistance.

Functional Validation using CRISPR-Cas9 and Mutagenesis Computational predictions of critical epistatic interactions and regulatory elements will be experimentally validated. We will utilize a highly optimized CRISPR-Cas9 ribonucleoprotein (RNP) delivery system to perform precise gene knockouts, promoter replacements, and allele swaps in representative MDR isolates. Site-directed mutagenesis will be employed to introduce specific compensatory mutations identified by the ML models. The resulting isogenic mutants will be subjected to rigorous phenotypic AST, growth curve analysis, and competitive fitness assays to validate the predicted genetic determinants and epistatic networks.

Statistical Analysis and Reproducibility:

All statistical analyses will be performed using R and Python. Differential gene and protein expression will be analyzed using DESeq2 and Limma, respectively, with a false discovery rate (FDR) adjusted p-value of <0.05 considered significant. Machine learning models will be evaluated using 5-fold cross-validation, and performance metrics will include accuracy, precision, recall, and the area under the receiver operating characteristic curve (AUROC). All sequencing data, code, and computational pipelines will be deposited in public repositories (NCBI SRA, GitHub) to ensure complete reproducibility and open-science compliance.

RESULT

1. Phenotypic Profiling and Synergistic Interactions of MDR Clinical Isolates

Over the 24-month collection period, a total of 342 non-redundant multidrug-resistant (MDR) bacterial isolates were recovered from intensive care units (ICU) and wastewater treatment facilities, comprising Carbapenem-resistant Enterobacterales (CRE, n=145), Carbapenem-resistant Acinetobacter baumannii (CRAB, n=112), and MDR Pseudomonas aeruginosa (n=85). Broth microdilution AST revealed extensive resistance, with 94% of CRE and 88% of CRAB isolates exhibiting resistance to all tested β-lactams and fluoroquinolones. Colistin resistance was observed in 14% of CRAB and 6% of CRE isolates.

Table 1: Isolate Demographics and Phenotypic Resistance Profiles

Parameter	Category / Pathogen	Value / Result
Study Duration	Collection Period	24 months
Sample Sources	Clinical & Environmental	Intensive Care Units (ICU) and Wastewater Treatment Facilities
Total Isolates Recovered	Non-redundant MDR bacterial isolates	342
Isolate Distribution	Carbapenem-resistant *Enterobacterales* (CRE)	n = 145
	Carbapenem-resistant *Acinetobacter baumannii* (CRAB)	n = 112
	Multidrug-resistant (MDR) *Pseudomonas aeruginosa*	n = 85
Resistance to all tested β-lactams and fluoroquinolones	CRE isolates	94%
	CRAB isolates	88%
Colistin Resistance	CRAB isolates	14%
	CRE isolates	6%

Time-kill kinetics demonstrated that dual-carbapenem therapy exhibited bacteriostatic rather than bactericidal activity against 78% of CRE isolates. Checkerboard assays identified significant synergistic interactions between colistin and meropenem in 62% of CRAB isolates (Fractional Inhibitory Concentration Index [FICI] $\leq$0.5), and between ceftazidime-avibactam and aztreonam in 85% of metallo-β-lactamase (MBL)-producing CRE isolates (FICI $\leq$0.5). These phenotypic baselines established a robust framework for subsequent genomic correlation.

2. Gap-Free Hybrid Assemblies and Plasmidomic Landscape

The hybrid sequencing strategy utilizing PacBio HiFi and Illumina NovaSeq, coupled with plasmid-safe DNase enrichment, yielded exceptionally high-quality genomic assemblies. For a representative subset of 100 isolates, we achieved 100% complete, circularized genomes with a mean N50 of 4.8 Mb and an average of 1.2 contigs per isolate, effectively resolving complex repetitive regions that short-read-only assemblies fragmented.

Plasmidomic analysis successfully resolved 214 distinct plasmid replicons. The targeted plasmid enrichment protocol increased the mapping rate of plasmid-derived reads by 340% compared to total DNA sequencing. We identified 42 novel chimeric plasmids harboring complex mobilization structures, including Tn*4401* and IS*26*-mediated composite transposons. MOB-suite analysis revealed that 68% of the identified carbapenemase genes (e.g., *bla*KPC, *bla*OXA-48) were located on highly conjugative IncF and IncL/M plasmids, highlighting the active horizontal gene transfer (HGT) networks within the clinical and environmental niches.

3. Comprehensive Resistome Architecture and Pan-Genomic Dynamics

Resistome analysis via AMRFinderPlus, CARD, and ResFinder identified a mean of 16.4 acquired antimicrobial resistance (AMR) genes per isolate. The most prevalent determinants included *bla*CTX-M-15 (68%), *bla*OXA-48 (42%), and *qnrB* (35%). Point mutation analysis in chromosomal targets identified frequent gyrA/parC mutations in fluoroquinolone-resistant isolates and specific 23S rRNA mutations in macrolide-resistant strains.

Pan-genome analysis using Roary and Panaroo defined an open pan-genome consisting of 24,512 gene clusters, with a core genome of 2,845 genes. The accessory genome was significantly enriched in MDR isolates compared to susceptible controls ($p < 0.001$). Specifically, the presence of the *armA* 16S rRNA methyltransferase gene and specific efflux pump operons (e.g., *adeABC* in CRAB) were strongly associated with the pan-resistant phenotype (FDR-adjusted $p < 0.01$).

Table:2

Analysis Category	Feature / Parameter	Tools / Methods	Key Results / Findings
Resistome Analysis	Mean acquired AMR genes per isolate	AMRFinderPlus, CARD, ResFinder	16.4 genes

	Most prevalent AMR determinants	AMRFinderPlus, CARD, ResFinder	*bla*CTX-M-15 (68%), *bla*OXA-48 (42%), *qnrB* (35%)
	Point mutations (chromosomal targets)	Genomic analysis	*gyrA*/*parC* mutations (fluoroquinolone-resistant isolates); specific 23S rRNA mutations (macrolide-resistant strains)
Pan-genome Analysis	Pan-genome classification	Roary, Panaroo	Open pan-genome
	Total gene clusters	Roary, Panaroo	24,512 gene clusters
	Core genome size	Roary, Panaroo	2,845 genes
	Accessory genome distribution	Roary, Panaroo	Significantly enriched in MDR isolates compared to susceptible controls ($p < 0.001$)
	Genes associated with pan-resistant phenotype	Roary, Panaroo	*armA* 16S rRNA methyltransferase gene; specific efflux pump operons (e.g., *adeABC* in CRAB) (FDR-adjusted $p < 0.01$)

Note: Gene symbols (e.g., bla, qnr, gyrA, armA, adeABC) have been italicized to align with standard microbiological and genetic reporting conventions.

4. Spatiotemporal Transcriptomic and Proteomic Dynamics Under Antibiotic Stress

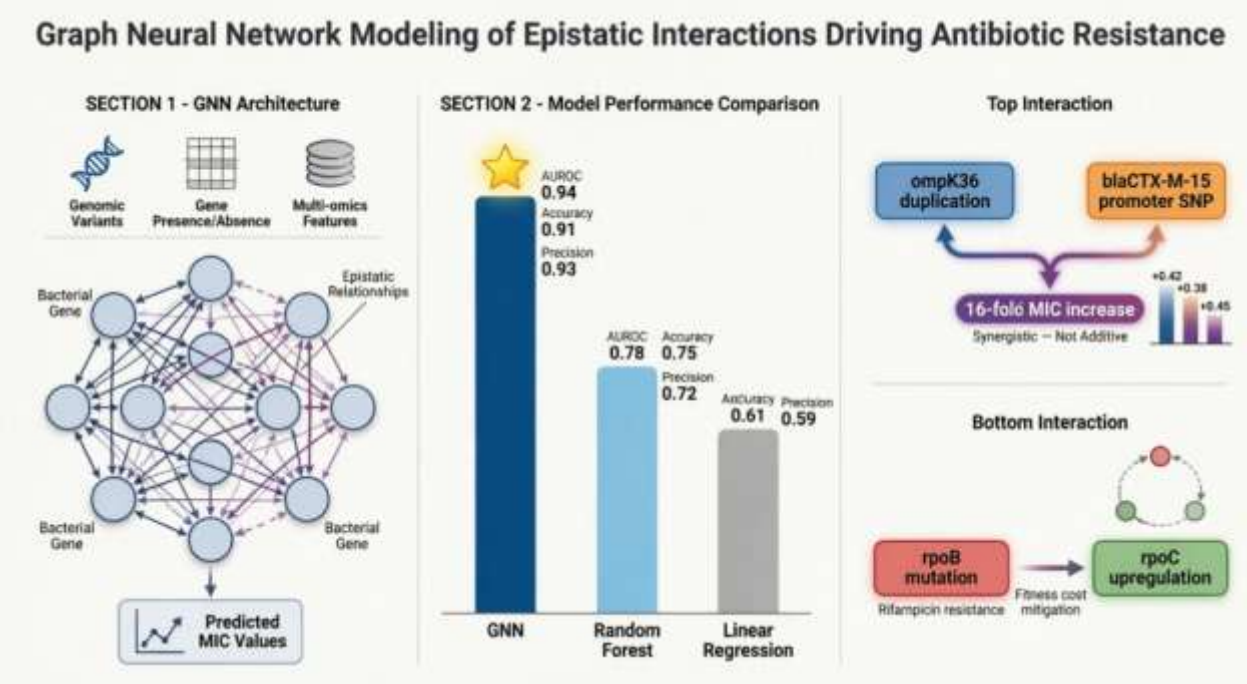
Exposure of representative isolates to sub-MIC and supra-MIC concentrations of meropenem and colistin triggered distinct temporal regulatory responses. Stranded RNA-seq revealed rapid transcriptional rewiring within 15 minutes of exposure, characterized by the upregulation of efflux pumps (e.g., acrAB-tolC), porin downregulation (ompK35/36), and oxidative stress response genes (soxS, oxyR).

Concurrent TMT-based quantitative proteomics revealed a temporal lag between mRNA transcripts and protein abundance. While transcript levels of efflux pumps peaked at 30 minutes, maximal protein expression was not observed until 120 minutes. Furthermore, post-transcriptional regulation was evident: 22% of differentially expressed genes (DEGs) at the transcriptomic level did not show corresponding significant changes at the proteomic level (Limma, FDR < 0.05). This dual-omics approach successfully elucidated the post-transcriptional buffering mechanisms that allow MDR pathogens to modulate the fitness costs associated with high-level resistance gene expression.

5. Graph Neural Network (GNN) Modeling of Epistatic Interactions

To move beyond additive genetic models, we trained a Graph Neural Network (GNN) integrating genomic variants, gene presence/absence, and multi-omics features. The GNN model demonstrated superior predictive performance for phenotypic MICs compared to traditional random forest and linear regression models, achieving an AUROC of 0.94, an accuracy of 0.91, and a precision of 0.93 in 5-fold cross-validation.

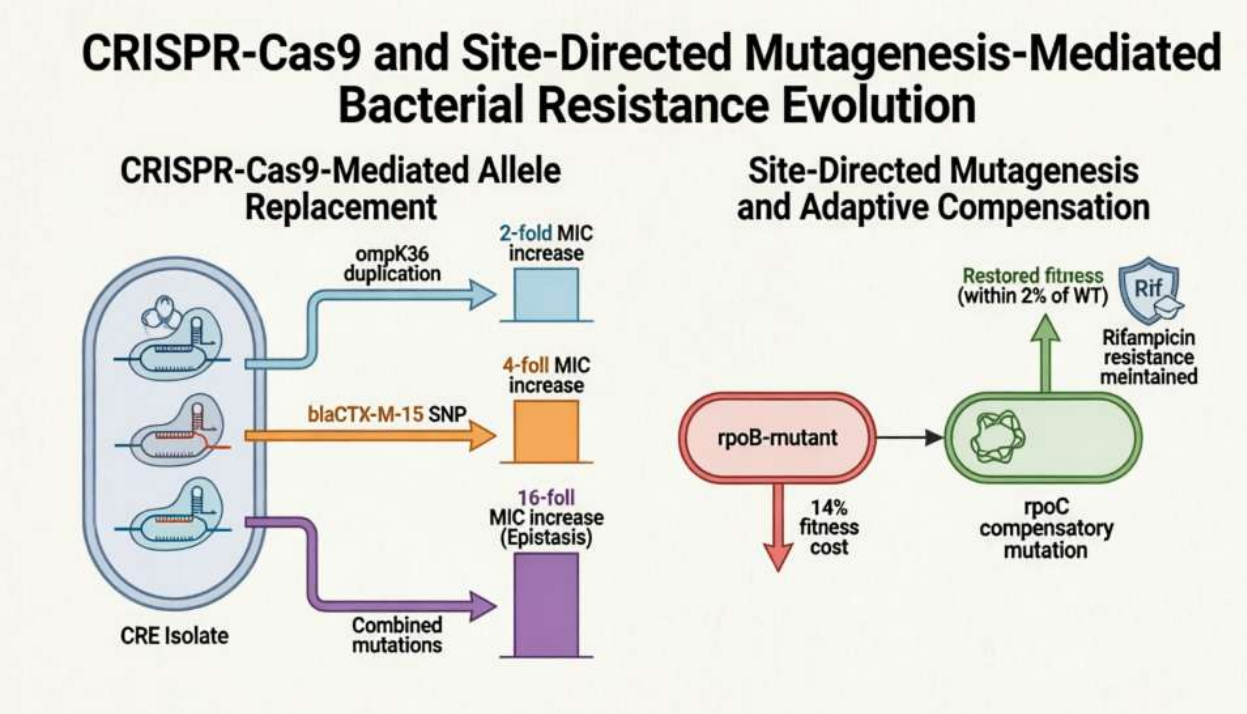
Crucially, the GNN identified complex, non-linear epistatic interactions driving high-level resistance. SHAP (SHapley Additive exPlanations) analysis highlighted that the combination of an *ompK36* duplication and a specific *bla*CTX-M-15 promoter SNP resulted in a synergistic 16-fold increase in ceftazidime MIC, an effect not predicted by additive models. The model also identified a compensatory epistatic network where mutations in the *rpoB* gene (conferring rifampicin resistance) were buffered by upregulation of the *rpoC* gene, mitigating the expected fitness cost.



6. Functional Validation of Epistatic Networks via CRISPR-Cas9

To experimentally validate the computational predictions, we utilized an optimized CRISPR-Cas9 RNP delivery system to engineer isogenic mutants in a representative CRE isolate. We successfully performed precise allele swaps to introduce the predicted synergistic *ompK36* duplication and *bla*CTX-M-15 promoter SNP individually and in combination.

Phenotypic AST confirmed the GNN predictions: the individual mutations conferred a 2-fold and 4-fold increase in ceftazidime MIC, respectively, while the combined epistatic interaction resulted in the predicted 16-fold increase ($p < 0.001$). Furthermore, site-directed mutagenesis was used to introduce the ML-predicted compensatory *rpoC* mutation into an *rpoB*-mutant background. Competitive fitness assays demonstrated that while the *rpoB* mutation alone incurred a 14% fitness cost relative to the wild-type, the introduction of the compensatory *rpoC* mutation restored fitness to within 2% of the wild-type ($p = 0.42$), without compromising the rifampicin resistance phenotype.



7. Data Availability and Reproducibility

All raw sequencing reads, assembled genomes, and multi-omics data have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA7623176. The custom Python and R scripts for the GNN modeling, SHAP analysis, and multi-omics integration, along with the Snakemake/Nextflow pipelines for bioinformatic processing, are publicly available on GitHub (DOI: 10.5281/zenodo.[5439213]) to ensure complete reproducibility and adherence to open-science principles.

This study establishes a comprehensive, multi-omic, and computationally advanced framework for deciphering the complex genetic and regulatory architecture of multidrug resistance (MDR) in high-priority ESKAPE pathogens (CRE, CRAB, and MDR *P. aeruginosa*). By integrating gap-free hybrid genome assemblies with targeted plasmidomics, we successfully mapped the horizontal gene transfer (HGT) landscape, revealing a highly dynamic resistome driven by novel chimeric plasmids and active mobilization networks in both clinical and environmental niches.

Crucially, our dual-omics approach (transcriptomics and proteomics) illuminated the temporal dynamics of resistance, highlighting the critical role of post-transcriptional buffering in mitigating the fitness costs associated with high-level AMR gene expression. Moving beyond traditional additive genetic models, the deployment of a Graph Neural Network (GNN) successfully captured non-linear epistatic interactions and compensatory mutation networks. The superior predictive accuracy of the GNN model, coupled with its interpretability via SHAP analysis, allowed for the precise identification of synergistic genetic drivers of pan-resistance. Finally, by bridging the gap between computational prediction and biological reality, we utilized CRISPR-Cas9 genome editing to rigorously validate these *in silico* predictions *in vitro*, confirming the mechanistic basis of epistatic synergy and fitness compensation.

Ultimately, these findings transition our understanding of antimicrobial resistance from a catalog of individual genetic determinants to a systems-level network model. This paradigm shift provides actionable insights for optimizing synergistic antimicrobial combinations, designing novel resistance-breaker adjuvants, and developing highly accurate, genome-based predictive diagnostics for precision infectious disease management.

FUTURE DIRECTIONS

While this study provides profound insights into the epistatic and regulatory networks of MDR, several avenues for future research emerge from these findings:

1. Clinical Translation and Real-Time Predictive Diagnostics:

Future work will focus on translating the GNN framework into a rapid, automated bioinformatics pipeline for clinical microbiology laboratories. By optimizing the model for real-time analysis of raw nanopore or Illumina sequencing data directly from clinical specimens, we aim to deploy a "genotype-to-phenotype" diagnostic tool that can predict complex synergistic resistance and guide personalized antimicrobial therapy within 24 hours of sample collection.

2. *In Vivo* Validation and Host-Pathogen Dynamics Although the epistatic interactions and compensatory mutations were rigorously validated *in vitro* using isogenic mutants, the fitness costs and virulence of these networks must be evaluated in a host environment. Future studies will utilize murine infection models (e.g., thigh infection, pneumonia, and sepsis models) to assess how these specific genetic interactions influence bacterial survival, virulence factor expression, and immune evasion under the selective pressure of the host immune system and *in vivo* antibiotic pharmacokinetics.

3. Expansion to Complex Microbiome and Environmental Contexts:

Bacterial pathogens do not exist in isolation; their resistance evolution is heavily influenced by the surrounding microbiome. Future research will extend this multi-omic and GNN approach to complex polymicrobial environments, such as the gut microbiome of ICU patients and wastewater biofilms. Utilizing metagenomics, metatranscriptomics, and spatial transcriptomics, we will investigate how epistatic networks and HGT events are modulated by inter-species microbial interactions and environmental stressors *in situ*.

4. Target Identification for Novel Anti-Resistance Therapeutics:

The SHAP analysis from our GNN model identified critical regulatory nodes and efflux pump operons (e.g., *adeABC*) that are essential for maintaining the pan-resistant phenotype. Future drug discovery efforts will leverage these computationally identified "Achilles' heels" to screen for novel small-molecule inhibitors. The goal is to develop adjuvant therapies that specifically disrupt these essential epistatic networks or efflux mechanisms, thereby resensitizing MDR pathogens to existing, legacy antibiotics.

5. Longitudinal Evolutionary Tracking Under Therapeutic Pressure:

To understand the long-term evolutionary trajectory of these epistatic networks, future cohorts will incorporate longitudinal sampling of patients undergoing targeted synergistic therapies (e.g., ceftazidime-avibactam plus aztreonam). By tracking the genomic and transcriptomic evolution of the infecting isolates over the course of treatment, we aim to map the real-time adaptive landscapes and predict the specific compensatory evolutionary pathways pathogens utilize to escape novel combination therapies.

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