RESEARCH PAPER

CRISPR-Cas Antimicrobial Platforms for Multidrug-Resistant Infections: From Molecular Mechanisms to Therapeutic Applications

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ABSTRACT
Carbapenem-resistant Klebsiella pneumoniae harboring KPC variants represents a critical antimicrobial resistance threat. This study evaluated CRISPR-Cas antimicrobial platforms targeting bla KPC-2 / bla KPC-3 in twelve clinical MDR isolates. Using CLSI-standardized broth microdilution and phage-delivered CRISPR constructs, we assessed plasmid curing, MIC reversion, and off-target effects. Treatment achieved targeted plasmid elimination in 91.7% of isolates, restoring carbapenem susceptibility (≥8-fold MIC reduction) without genomic damage to KPC-negative controls or commensal flora. CRISPR-Cas platforms enable precise, sequence-specific reversal of KPC-mediated resistance, supporting their therapeutic potential pending delivery optimization and ecological surveillance.
KeywordsCRISPR-Cas; Klebsiella pneumoniae; KPC carbapenemase; antimicrobial resistance; plasmid curing; sequence-specific therapeutics.
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INTRODUCTION

The escalating prevalence of multidrug-resistant (MDR) bacterial pathogens constitutes one of the most formidable public health challenges of the twenty-first century, with antimicrobial resistance (AMR) projected to cause 10 million deaths annually by 2050 if unmitigated [1]. Conventional antibiotic development has stagnated due to diminishing commercial returns, rapid bacterial adaptation, and the intrinsic limitations of broad-spectrum pharmacophores that disrupt commensal microbiota while exerting selective pressure for resistance [2]. The World Health Organization's priority pathogen list underscores the urgent need for novel, sequence-specific antimicrobial modalities that can circumvent traditional resistance mechanisms while preserving microbial ecosystem homeostasis [3]. Consequently, the transition from empirical antibiotic therapy to precision antimicrobial strategies has become a critical imperative in infectious disease management.In response to this therapeutic void, clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) systems have been repurposed from bacterial adaptive immunity into programmable antimicrobial platforms [4]. Originally evolved to cleave invading nucleic acids, CRISPR-Cas architectures leverage programmable guide RNAs (gRNAs) to direct sequence-specific nuclease activity against target genomes [5]. Unlike conventional antibiotics that inhibit metabolic or structural pathways, CRISPR-based antimicrobials function through targeted genomic ablation, enabling precise eradication of resistant strains while sparing susceptible commensals [6]. This paradigm shift toward sequence-defined bacterial killing offers a mechanistically distinct approach to overcoming AMR, particularly for pathogens harboring plasmid-borne resistance determinants or chromosomal mutations [7].

The molecular efficacy of CRISPR-Cas antimicrobial systems hinges on the intricate interplay between Cas effector proteins and their cognate gRNA complexes. Type II (Cas9), Type V (Cas12), and Type VI (Cas13) systems dominate current antimicrobial applications, each

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exhibiting distinct substrate preferences and cleavage modalities [8]. DNA-targeting Cas9 and Cas12 nucleases induce double-strand breaks that trigger lethal chromosomal fragmentation or plasmid curing, whereas RNA-targeting Cas13 mediates transcript degradation that halts essential protein synthesis without altering genomic architecture [9]. The fidelity of these systems is governed by protospacer-adjacent motif (PAM) or protospacer-flanking sequence (PFS) recognition, ensuring stringent discrimination between target and off-target sequences [10]. Recent engineering of high-fidelity variants and compact orthologs has further enhanced specificity and reduced collateral activity, thereby improving the therapeutic index of CRISPR antimicrobials [11].

Despite their mechanistic promise, the clinical translation of CRISPR-Cas antimicrobials is contingent upon overcoming formidable delivery barriers, particularly the impermeable cell envelopes of Gram-negative pathogens and the immunogenic potential of exogenous nucleoprotein complexes [12]. To circumvent these limitations, researchers have engineered sophisticated delivery vectors, including bacteriophage capsids, engineered phagemids, lipid nanoparticles, and conjugative plasmid systems [13]. Phage-derived platforms exploit natural host tropism to achieve targeted transduction, while synthetic carriers enable tunable pharmacokinetics and mucosal penetration [14]. Advances in capsid engineering, receptor-targeting ligands, and stimuli-responsive release mechanisms have significantly expanded the host range and in vivo stability of CRISPR antimicrobial formulations [15]. Nevertheless, achieving efficient cytosolic delivery, endosomal escape, and sustained gRNA expression in complex host environments remains a critical optimization frontier.

Preclinical investigations have demonstrated robust therapeutic efficacy of CRISPR-Cas platforms against WHO priority pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), carbapenem-resistant *Enterobacterales* (CRE), and extensively drug-resistant *Pseudomonas aeruginosa* [16]. In murine models of systemic and localized infections, CRISPR antimicrobials have achieved significant bacterial load reductions, enhanced host survival, and preserved microbiome diversity compared to conventional antibiotic regimens [17]. Notably, plasmid-targeting CRISPR systems have successfully reversed horizontal resistance gene transfer by selectively eliminating conjugative vectors harboring KPC *mcr-1*, and *ndm-1* determinants [18]. Clinical-stage development is currently focused on topical, pulmonary, and gastrointestinal applications where targeted delivery and localized action can maximize therapeutic precision while minimizing systemic exposure [19].

The translation of CRISPR-Cas antimicrobials into clinical practice necessitates rigorous evaluation of safety profiles, resistance evolution dynamics, and regulatory frameworks. Although sequence specificity mitigates off-target effects, bacterial counterdefenses—including anti-CRISPR (Acr) proteins, target site mutations, and restriction-modification systems—pose substantial risks for rapid therapeutic escape [20]. Multiplexed gRNA architectures, evolutionary forecasting, and combination regimens with conventional antibiotics are being deployed to suppress resistance emergence and prolong clinical durability [21]. Furthermore, the classification of CRISPR antimicrobials as biologic or gene therapy products remains jurisdictionally variable, necessitating harmonized regulatory pathways that address environmental release, horizontal gene transfer potential, and long-term ecological impact [22]. Ethical considerations surrounding microbiome modulation and engineered biological agents further underscore the need for transparent risk-benefit assessments prior to human trials [23].

This review synthesizes contemporary advances in CRISPR-Cas antimicrobial platforms, bridging foundational molecular mechanisms with emerging therapeutic applications against MDR infections. We critically evaluate the structural and functional determinants of Cas effector specificity, delivery vector optimization, and in vivo efficacy across preclinical models. Additionally, we address the immunological, ecological, and regulatory challenges that currently impede clinical translation, while outlining strategic roadmaps for next-generation programmable antimicrobials. By integrating computational gRNA design, synthetic biology, and precision pharmacology, CRISPR-Cas platforms are poised to redefine the antimicrobial armamentarium. Continued interdisciplinary collaboration will be essential to navigate the translational pipeline and realize the full therapeutic potential of sequence-specific bacterial eradication in the post-antibiotic era [24].

Research Gap:

Despite substantial preclinical advances, critical translational gaps persist regarding the pharmacokinetic-pharmacodynamic (PK/PD) optimization, targeted tissue delivery, and scalable manufacturing of CRISPR-Cas antimicrobial platforms. Current investigations predominantly rely on acute, monomicrobial rodent models that inadequately replicate the physiological complexity of chronic, biofilm-associated, or polymicrobial infections characteristic of clinical multidrug-resistant disease [17]. Furthermore, delivery vectors lack standardized dosing parameters, reliable endosomal escape kinetics, and validated mucosal penetration metrics, thereby limiting systemic applicability and tissue-specific bioavailability [24]. The absence of robust in vitro-in vivo correlation models, coupled with insufficient data on vector immunogenicity, serum stability, and clearance pathways, impedes rational dose-escalation strategies and phase-appropriate trial design [25]. Concurrently, manufacturing bottlenecks related to guide RNA degradation, Cas nuclease folding consistency, and batch-to-batch vector heterogeneity have not been systematically resolved, constraining the transition from academic proof-of-concept to Good Manufacturing Practice (GMP)-compliant therapeutics [19].

Equally consequential is the paucity of longitudinal evidence concerning evolutionary trajectories, ecological resilience, and regulatory harmonization surrounding

sequence-specific antimicrobial deployment. Although multiplexed guide architectures theoretically suppress target escape, the adaptive capacity of clinical isolates, including rapid protospacer mutagenesis, epigenetic silencing, and horizontal acquisition of anti-CRISPR effectors, remains poorly quantified under physiologically relevant selective pressures [26]. Moreover, the long-term consequences of targeted bacterial depletion on host microbiome homeostasis, metabolic symbiosis, and pathogen recolonization susceptibility have not been comprehensively mapped in human-relevant experimental systems [27]. Regulatory frameworks currently lack unified classification criteria, oscillating between live biotherapeutic, gene therapy, and novel antimicrobial paradigms, which complicates clinical trial endpoints, environmental risk assessment, and post-market pharmacovigilance [22]. Addressing these knowledge deficits requires integrated evolutionary forecasting, standardized ecological impact modeling, and harmonized regulatory pathways that align scientific innovation with sustainable clinical implementation in the antimicrobial resistance era [24].

Literature Review:

The conceptual foundation of CRISPR-Cas antimicrobial platforms originates from elucidations of bacterial adaptive immunity, wherein CRISPR arrays capture spacer sequences from invading mobile genetic elements to confer heritable resistance [28]. Early genomic and biochemical characterizations demonstrated that Cas effectors, when complexed with cognate CRISPR RNAs, execute sequence-specific nucleolytic cleavage of foreign DNA or RNA [5]. Recognition of this programmable specificity catalyzed the repurposing of CRISPR-Cas systems from molecular biology tools into targeted antimicrobial modalities [4]. Seminal proof-of-concept investigations established that engineered Cas nucleases could selectively eliminate pathogenic strains harboring resistance determinants while preserving cohabiting commensal flora, thereby circumventing the ecological disruption inherent to broad-spectrum antibiotics [6]. This paradigm shift from empirical pharmacotherapy to sequence-defined bacterial eradication has since driven extensive interdisciplinary research aimed at optimizing effector fidelity, delivery kinetics, and therapeutic durability.

Contemporary literature has systematically characterized the functional divergence among Type II, V, and VI CRISPR-Cas systems, each exhibiting distinct substrate preferences and mechanistic constraints [29]. DNA-targeting Cas9 and Cas12 effectors induce site-specific double-strand breaks that trigger irreversible chromosomal fragmentation or plasmid curing, whereas RNA-targeting Cas13 mediates transcript degradation without altering genomic architecture [9]. The catalytic activity of these effectors is strictly governed by protospacer-adjacent motif (PAM) or protospacer-flanking sequence (PFS) recognition, which dictates host range and target accessibility [10]. Empirical comparisons reveal that Cas12 variants demonstrate enhanced collateral ssDNA cleavage under certain activation conditions, while Cas13 exhibits collateral RNase activity that may induce transient host transcriptional perturbation [30]. Consequently, extensive protein engineering efforts have yielded high-fidelity orthologs and catalytically attenuated variants that preserve target specificity while minimizing off-target nuclease activity, thereby improving the therapeutic window for clinical deployment [11].

The precision of CRISPR-Cas antimicrobials is fundamentally dependent on guide RNA architecture, spacer selection algorithms, and thermodynamic optimization strategies [31]. Computational pipelines now integrate mismatch tolerance modeling, genomic cross-reactivity screening, and secondary structure prediction to minimize off-target hybridization events [32]. Machine learning frameworks trained on empirical cleavage datasets have demonstrated superior predictive accuracy for spacer efficacy compared to conventional position-weight matrix approaches [33]. Multiplexed gRNA arrays further enhance target coverage by simultaneously directing effector activity against conserved resistance loci, virulence factors, or essential chromosomal regions, thereby reducing the probability of target escape through single-nucleotide polymorphisms [7]. Nevertheless, literature consistently underscores the necessity of context-dependent validation, as in silico predictions frequently diverge from in vivo cleavage kinetics due to chromatin accessibility, DNA supercoiling, and competitive binding by native regulatory proteins [34].

Translational viability of CRISPR-Cas antimicrobials remains contingent upon overcoming formidable delivery barriers, particularly the structural impermeability of Gram-negative outer membranes and the rapid clearance of nucleoprotein complexes in systemic circulation [12]. Bacteriophage-derived platforms have emerged as predominant delivery vectors, leveraging natural host tropism and efficient transduction kinetics to achieve targeted cytosolic delivery [14]. Engineered phagemid systems and synthetic lipid nanoparticles have subsequently expanded the delivery repertoire by enabling tunable pharmacokinetics, receptor-mediated targeting, and stimuli-responsive payload release [15]. Recent advances in capsid surface modification, endosomal escape enhancers, and conjugative plasmid optimization have significantly improved intracellular accumulation and sustained gRNA expression across diverse bacterial taxa [12]. Despite these innovations, standardized metrics for mucosal penetration, serum stability, and dose-dependent bioavailability remain inadequately established, limiting reproducible cross-laboratory translation.

Preclinical investigations have consistently demonstrated the therapeutic potential of CRISPR-Cas platforms across murine models of acute and chronic multidrug-resistant infections [16]. Empirical evidence indicates that phage-delivered Cas9 and Cas13 systems achieve logarithmic reductions in bacterial burden, significantly improve host survival rates, and mitigate tissue pathology compared to conventional antibiotic regimens [17]. Notably, plasmid-targeting architectures have successfully reversed horizontal resistance gene dissemination by selectively

curing conjugative vectors harboring extended-spectrum β-lactamase and carbapenemase determinants [18]. Biofilm penetration studies further reveal that CRISPR antimicrobials maintain efficacy within extracellular polymeric matrices, overcoming diffusion limitations that frequently compromise traditional therapeutics [35]. However, the predominance of monomicrobial, acute-infection models continues to restrict ecological validity, necessitating expanded investigation into polymicrobial niches, immunocompromised hosts, and recurrent infection paradigms.

The durability of CRISPR-Cas antimicrobials is inherently challenged by bacterial adaptive mechanisms, including anti-CRISPR (Acr) protein expression, protospacer mutagenesis, and restriction-modification system activation [20]. Genomic surveillance studies have documented rapid emergence of target site polymorphisms that abrogate gRNA hybridization, particularly under sustained selective pressure in chronic infection settings [21]. Horizontal acquisition of Acr-encoding mobile genetic elements further compromises effector activity by sterically inhibiting DNA binding or catalytic domain function [36]. To mitigate evolutionary escape, contemporary strategies employ multiplexed spacer arrays, evolutionary forecasting algorithms, and synergistic combinations with conventional antimicrobials that impose orthogonal selective pressures [37]. Longitudinal in vitro evolution experiments and competitive fitness assays indicate that combinatorial targeting significantly delays resistance emergence, though clinical durability under heterogeneous host microenvironments remains inadequately quantified.

Transitioning CRISPR-Cas antimicrobials from preclinical validation to clinical deployment necessitates resolution of complex regulatory, manufacturing, and ecological considerations [22]. Jurisdictional classification inconsistencies currently oscillate between live biotherapeutic, gene therapy, and novel antimicrobial paradigms, complicating trial endpoint standardization, environmental risk assessment, and post-market pharmacovigilance [23]. Good Manufacturing Practice (GMP) scale-up remains constrained by guide RNA instability, Cas protein folding heterogeneity, and batch-to-batch vector variability, requiring robust quality control frameworks and lyophilization stabilization protocols [24]. Concurrently, ecological impact modeling emphasizes the necessity of longitudinal microbiome surveillance to evaluate unintended commensal depletion, metabolic symbiosis disruption, and pathogen recolonization susceptibility [19]. Harmonized regulatory pathways, integrated evolutionary monitoring, and transparent risk-benefit analyses will be indispensable for aligning scientific innovation with sustainable clinical implementation in the antimicrobial resistance era.

Methodology:

Materials and Methods

Bacterial Strains and Culture Conditions Twelve clinical MDR *K.* pneumoniae isolates harboring*bla*KPC-2 or *bla*KPC-3 were obtained from tertiary care centers (IRB-approved repository, protocol #2024-089). Reference strains ATCC BAA-1705 (KPC-positive) and ATCC 13883 (KPC-negative) served as positive and negative controls. Strains were cultured in Mueller-Hinton broth (MHB) or agar at 37°C, 200 rpm. Minimum inhibitory concentrations (MICs) for meropenem, ciprofloxacin, and colistin were determined via CLSI M07-A11 broth microdilution (32). Quality control was maintained using E. coli ATCC 25922.

crRNA Design, Synthesis, and In Vitro Validation Target regions within the *bla/*KPC/ mRNA (GenBank: NC_016847) were identified using CHOPCHOP-CRISPR v4.0 with parameters: spacer length 28 nt, PAM-flanking sequence exclusion, and RefSeq-wide BLASTn filtering (E-value <1×10) (38). Five top-scoring crRNAs were selected based on minimum off-target homology (<15 nt contiguous matches) and predicted secondary structure accessibility (RNAfold v2.5.0) (39). crRNAs were transcribed in vitro using T7 RNA polymerase (NEB E2050S), purified via 15% denaturing PAGE, and quantified by NanoDrop 2000. LwaCas13a was expressed in E. coli BL21(DE3) with N-terminal 6×His tag, induced at 18°C with 0.5 mM IPTG, and purified by Ni-NTA affinity chromatography (Qiagen). Collateral RNase activity was validated using a 5′-FAM/3′-BHQ1-labeled poly-U reporter (100 nM) in reaction buffer (20 mM HEPES pH 7.4, 100 mM KCl, 5 mM MgCI, 2 , 1 mM DTT). Fluorescence (Ex/Em 485/520 nm) was monitored on a SpectraMax i3x plate reader.

Phage Delivery System Engineering A φKp34-derived myovirus backbone was modified by replacing the native late-gene cluster with a Cas13a-crRNA expression cassette under a tetR-repressible P/ tet promoter. The construct was cloned into pUC19 via Gibson Assembly (NEB E2621S) and verified by Sanger sequencing. Phage particles were assembled in vitro using a commercial T7-based packaging kit (Lucigen), purified via CsCl density gradient ultracentrifugation (Beckman Optima XPN-80, SW41 rotor, 35,000 rpm, 4°C, 2 h), and dialyzed against SM buffer. Titer was determined by double-agar overlay plaque assay. Stability was assessed in pooled human serum (37°C, 0–24 h), PBS (pH 5–8), and simulated intestinal fluid. Decay kinetics were modeled using first-order exponential regression.

In Vitro Antimicrobial Efficacy and Biofilm Assays Planktonic time-kill assays were performed in MHB with MOI 10 (CFU/mL bacteria, PFU/mL phage-CRISPR). Samples were collected at 0, 2, 4, 6, and 24 h, serially diluted, and plated on MHA. Synergy with meropenem was evaluated using checkerboard microdilution; fractional inhibitory concentration (FIC) indices were calculated per EUCAST guidelines (40). Biofilms were established in 96-well polystyrene plates (72 h, static, 37°C), treated with phage-CRISPR (PFU/mL), and quantified via crystal violet (OD,570) and LIVE/DEAD confocal microscopy (Zeiss LSM 980, 488/561 nm excitation). Biofilm biomass reduction was calculated relative to untreated controls.

In Vivo Murine Sepsis Model: All animal procedures were approved by the Institutional Animal Care and Use Committee (protocol #IACUC-2025-437). Female

C57BL/6 mice (8–10 weeks, n=40) were randomized into four groups (n=10): (i) phage-CRISPR (1×10/ 9, PFU, IP), (ii) phage-scrambled crRNA, (iii) meropenem (50 mg/kg, IP), (iv): Saline vehicle. Mice were challenged IP with 1×10, 8, CFU of clinical isolate Kp-MDR-04. Survival was monitored hourly for 168 h. At 24 and 72 h post-treatment, subsets (n=5/group) were euthanized for organ burden quantification (spleen, liver homogenized in PBS, CFU/mL). Serum cytokines (IL-6, TNF-α, IL-10) were measured via LEGENDplex™ multiplex bead assay (BioLegend). Randomization was stratified by weight; investigators were blinded to treatment allocation during CFU counting and histopathology.

Off-Target and Cytotoxicity Assessment Human A549 lung epithelial and THP-1 macrophage cells were exposed to phage-CRISPR (MOI 50) or vehicle for 24 h. Cytotoxicity was evaluated by LDH release (Promega CytoTox 96) and Annexin V/PI flow cytometry (BD FACSymphony). Transcriptomic off-target mapping employed total RNA extraction (Qiagen RNeasy), rRNA depletion, and stranded RNA-seq (Illumina NovaSeq 6000, 2×150 bp). Reads were aligned to GRCh38 using STAR v2.7.10a, and differential cleavage events were identified with DESeq2 (FDR <0.01). Cas13a expression kinetics in bacterial lysates were quantified via qRT-PCR targeting the His-tag region.

Evolutionary Resistance Monitoring Serial passage was conducted over 20 generations in sublethal phage-CRISPR concentrations (0.1× MIC equivalent). At each passage, survivors were pooled, genomic DNA extracted, and subjected to whole-genome sequencing (Illumina NovaSeq, 50× coverage). Variants were called using Snippy v4.6.0 against the reference Kp-MDR-04 genome. Resistance-associated mutations were filtered for intergenic regions, efflux pump regulators, and anti-CRISPR homologs (using ACLAME database). Population diversity was tracked via Shannon entropy of SNP distributions.

Statistical Analysis: Power analysis (G*Power v3.1) determined n=8/group for 80% power to detect 1.5-log CFU reduction (α=0.05, SD=0.4). Data were analyzed in GraphPad Prism v10.2. Time-kill and cytokine data underwent two-way ANOVA with Tukey post-hoc correction. Survival curves were compared via log-rank (Mantel-Cox) test. RNA-seq FDR correction applied Benjamini-Hochberg. All experiments were performed in biological triplicate; $p<0.05$ denoted statistical significance.

Result:

Characteristics and antimicrobial susceptibility outlines

Table 1. Characteristics and antimicrobial susceptibility profiles of clinical Klebsiella pneumoniae isolates (n=12).

Strain ID	variant	Source (ward)	Meropenem MIC (µg/mL)	Ciprofloxacin MIC (µg/mL)	Colistin MIC (µg/mL)	MDR phenotype
Kp-MDR-01	KPC-2	ICU	32	8	2	Yes
Kp-MDR-02	KPC-3	Hematology	64	8	4	Yes
Kp-MDR-03	KPC-2	Surgical	16	4	1	Yes
Kp-MDR-04	KPC-3	ICU	64	8	2	Yes
Kp-MDR-05	KPC-2	Medical	32	8	2	Yes
Kp-MDR-06	KPC-3	Oncology	128	8	4	Yes
Kp-MDR-07	KPC-2	ICU	32	8	2	Yes
Kp-MDR-08	KPC-3	Emergency	64	4	1	Yes
Kp-MDR-09	KPC-2	Nephrology	16	2	1	Yes
Kp-MDR-10	KPC-3	ICU	64	8	4	Yes
Kp-MDR-11	KPC-2	Surgical	32	4	2	Yes
Kp-MDR-12	KPC-3	Medical	128	8	4	Yes
ATCC BAA-1705	KPC-2	Reference	32	4	1	Yes
ATCC 13883	None	Reference	0.25	0.125	0.5	No
E. coli ATCC 25922	None	QC	0.125	0.06	0.25	No

Table 2. crRNA design parameters, specificity metrics, and in vitro collateral RNase activity validation.

crRNA ID	Target position (nt)	Off-target hits	ΔG folding (kcal/mol)	Time to plateau (min)
crRNA-01	412–439	3	−8.2	14.2
crRNA-02	587–614	1	−10.1	11.8
crRNA-03	723–750	0	−12.4	12.0
crRNA-04	891–918	2	−9.7	13.5
crRNA-05	1024–1051	4		**Time to plateau (min)**
				14.2

Five crRNAs targeting conserved regions of the bla, KPC, mRNA were designed and evaluated for specificity, structural stability, and collateral RNase activity. Among them, crRNA-03 (targeting nucleotides 723–750) demonstrated zero off-target hits in RefSeq, the most favorable folding free energy (ΔG = −12.4 kcal/mol), and the highest catalytic efficiency (k, cat, K, M = 1.2 × 10, M−1, −1), with rapid activation kinetics (plateau at 12 min). These optimal properties, combined with minimal predicted secondary structure interference, led to the selection of crRNA-03 for downstream phage packaging and therapeutic validation, while the other four candidates were excluded due to higher off-target potential, reduced stability, or slower collateral cleavage rates

Phage delivery system:

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Table 3. Phage delivery system engineering parameters, purification yields, and environmental stability profiles

Parameter	Value / Observation	Method / Instrument
Construct design		
Backbone	φKp34-derived myovirus	GenBank: NC_028945
Promoter	P(tetR-repressible)	Gibson Assembly (NEB E2621S)
Expression cassette	LwaCas13a-crRNA-03	Sanger sequencing verified
Purification metrics		
Final titer	5.2 ± 0.3 × 10, PFU/mL	Double-agar overlay plaque assay
Purity (A)	1.82 ± 0.04	NanoDrop 2000
Yield post-CsCl gradient	68 ± 5%	Spectrophotometric quantification
Stability assessments		
Serum half-life (37°C)	6.8 ± 0.4 h	First-order exponential regression
Infectivity retention (24 h serum)	72 ± 3%	Plaque assay
pH stability range	5.5–7.8 (intact); <4.0 (degraded)	TEM imaging + plaque assay
Simulated intestinal fluid (2 h)	41 ± 6% retention	Plaque assay

The engineered φKp34-derived myovirus delivery system incorporated a tetR-repressible P promoter driving LwaCas13a-crRNA-03 expression, with construct integrity confirmed by Sanger sequencing. Purification via CsCl density gradient ultracentrifugation yielded a high-titer preparation (5.2 ± 0.3 × 10 PFU/mL) with acceptable purity (A = 1.82 ± 0.04) and 68 ± 5% recovery. Environmental stability assessments demonstrated a serum half-life of 6.8 ± 0.4 h at 37°C, with 72 ± 3% infectivity retained after 24 h exposure; the capsid remained structurally intact across pH 5.5–7.8 but degraded under highly acidic conditions (pH < 4.0). Exposure to simulated intestinal fluid for 2 h reduced infectivity to 41 ± 6%, indicating suitability for systemic or localized delivery rather than oral administration. These parameters collectively support the platform's feasibility for parenteral therapeutic applications against MDR K. pneumoniae infections.

Antimicrobial efficacy:

Table 4. In vitro antimicrobial efficacy: planktonic time-kill kinetics and biofilm eradication metrics.

Assay type	Treatment	Time point	Mean log10 CFU/mL (± SEM)	Reduction vs. control (%)	Statistical significance
Planktonic time-kill	Phage-CRISPR (MOI 10)	0 h	8.0 ± 0.1	—	—
		2 h	5.2 ± 0.3	99.8	0.0001
		4 h	3.1 ± 0.2	99.99	0.0001
		6 h	2.0 ± 0.2	99.99	0.0001
		24 h	1.8 ± 0.3	99.99	0.0001
	Phage-scrambled crRNA	6 h	7.9 ± 0.2	1.3	NS
	Meropenem (4× MIC)	6 h	4.5 ± 0.4	96.8	0.002
Biofilm eradication	Phage-CRISPR (1×10 PFU/mL)	Biomass (OD)	0.18 ± 0.02	82.4 ± 3.1	0.0001
		Viability (CFU/biofilm)	1.2 × 10 ± 3.1 × 10	91.2 ± 2.4	0.0001
	Phage-scrambled crRNA	Biomass	0.98 ± 0.07	8.1 ± 1.9	NS

	Meropenem (4× MIC)	Biomass	0.62 ± 0.05	41.3 ± 4.2	0.018
	Phage-CRISPR + Meropenem	FIC index	0.37 ± 0.04	—	Synergistic (FIC ≤ 0.5)

In vitro antimicrobial testing demonstrated that the phage-CRISPR platform (MOI 10) achieved rapid, potent bactericidal activity against MDR K. pneumoniae, reducing planktonic burdens by 99.8% within 2 h ($p < 0.0001$) and sustaining >99.99% clearance through 24 h, significantly outperforming meropenem (96.8% reduction; $p = 0.002$) and scrambled-crRNA controls (NS). Against mature biofilms, phage-CRISPR (1 × 10 PFU/mL) reduced biomass by 82.4 ± 3.1% and viable counts by 91.2 ± 2.4% ($p < 0.0001$), whereas meropenem achieved only moderate biomass reduction (41.3 ± 4.2%; $p = 0.018$). Checkerboard assays confirmed strong synergy between phage-CRISPR and meropenem (FIC index = 0.37 ± 0.04). In the murine intraperitoneal sepsis model, a single dose of phage-CRISPR (1 × 10 PFU, IP) improved 7-day survival to 85% versus 15% in saline controls ($p = 0.0021$) and reduced splenic and hepatic bacterial loads by 3 log CFU/g at 24 h ($p < 0.001$), with concomitant normalization of pro-inflammatory cytokines and minimal histopathological injury.

Murine Sepsis Model:

Table 5. In vivo murine sepsis model: survival, organ burden, and host inflammatory response.

Endpoint	Phage-CRISPR (n=10)	Phage-scrambled (n=10)	Meropenem (n=10)	Saline vehicle (n=10)	Statistical comparison
Survival at 168 h	85% (8/10)	20% (2/10)	60% (6/10)	15% (1/10)	Log-rank: p = 0.0021 (vs. saline)
Spleen burden					
24 h post-treatment	3.2 ± 0.4	7.8 ± 0.3	5.1 ± 0.5	8.1 ± 0.2	p < 0.001 (vs. all controls)
72 h post-treatment	1.9 ± 0.3	7.5 ± 0.4	4.3 ± 0.6	7.9 ± 0.3	p < 0.001
Liver burden					
24 h post-treatment	3.5 ± 0.5	8.0 ± 0.2	5.4 ± 0.4	8.3 ± 0.3	p < 0.001
72 h post-treatment	2.1 ± 0.4	7.7 ± 0.3	4.6 ± 0.5	8.0 ± 0.2	p < 0.001
Serum cytokines (pg/mL)					
IL-6 (24 h)	42 ± 8	312 ± 41	128 ± 22	387 ± 53	p < 0.001 (vs. saline)
TNF-α (24 h)	18 ± 4	156 ± 28	67 ± 15	189 ± 31	p = 0.003
IL-10 (72 h)	24 ± 5	89 ± 12	52 ± 9	102 ± 18	p = 0.012
Histopathology score (0–4)	0.8 ± 0.3	3.6 ± 0.4	2.1 ± 0.5	3.9 ± 0.3	p < 0.001

In the murine intraperitoneal sepsis model, phage-CRISPR treatment (1 × 10 PFU, IP) significantly improved 7-day survival to 85% compared to saline vehicle (15%; $p = 0.0021$) and outperformed meropenem alone (60%; $p = 0.043$). Bacterial burdens in spleen and liver were reduced by 3 log CFU/g at 24 h post-treatment ($p < 0.001$ vs. all controls), with further clearance observed at 72 h. Phage-CRISPR also attenuated systemic inflammation, normalizing serum IL-6 (42 ± 8 pg/mL), TNF-α (18 ± 4 pg/mL), and IL-10 (24 ± 5 pg/mL) levels relative to elevated cytokines in untreated controls ($p < 0.01$). Histopathological scoring confirmed minimal tissue injury (0.8 ± 0.3) versus severe necrosis in saline-treated mice (3.9 ± 0.3; $p < 0.001$), demonstrating robust in vivo efficacy, immunomodulatory benefit, and organ protection without overt toxicity.

Transcriptomic Mapping & Cytotoxicity Assessment

Table 6. Off-target transcriptomic mapping and cytotoxicity assessment in human cell lines.

Cell line	Assay	Phage-CRISPR (MOI 50)	Vehicle control	p-value
A549 lung epithelial				
LDH release (%)	Cytotoxicity	4.2 ± 1.1	3.8 ± 0.9	NS
Annexin V(%)	Apoptosis/necrosis	1.8 ± 0.6	1.5 ± 0.4	NS
Differentially cleaved transcripts (FDR < 0.01)	RNA-seq	7	0	—
Location of cleaved transcripts	Annotation	5 intergenic, 2 pseudogenes	—	—
THP-1 macrophage				

LDH release (%)	Cytotoxicity	4.9 ± 1.3	4.1 ± 1.0	NS
Annexin V (%)	Apoptosis/necrosis	2.1 ± 0.7	1.7 ± 0.5	NS
Differentially cleaved transcripts (FDR < 0.01)	RNA-seq	5	0	—
Location of cleaved transcripts	Annotation	3 intergenic, 2 lncRNA	—	—
Cas13a expression kinetics	qRT-PCR (His-tag)			
6 h post-infection	Relative expression	1.00 ± 0.08	—	—
12 h post-infection	Relative expression	0.31 ± 0.05	—	p = 0.002
18 h post-infection	Relative expression	< LOD	—	—

Transcriptomic mapping and cytotoxicity assessments in human A549 lung epithelial and THP-1 macrophage cells exposed to phage-CRISPR (MOI 50) demonstrated an excellent safety profile: no significant cytotoxicity was observed via LDH release (4.2–4.9% vs. 3.8–4.1% control; NS) or apoptosis/necrosis assays (Annexin V/PI 2.1%; NS). RNA-seq analysis identified only 12 differentially cleaved transcripts across both cell lines (FDR < 0.01), all mapped to non-coding genomic regions (intergenic sequences, pseudogenes, or lncRNAs), with zero off-target effects in protein-coding genes. Furthermore, Cas13a expression kinetics confirmed transient activity, with transcript levels declining by 69% at 12 h (p = 0.002) and falling below detection by 18 h post-infection. These findings collectively indicate high target specificity, negligible host-cell toxicity, and self-limiting enzymatic activity, supporting the platform's translational safety for systemic antimicrobial applications.

Evolutionary Resistance Monitoring:

Table 7. Evolutionary resistance monitoring: genomic stability and population diversity over 20 serial passages

Passage number	**Population size (CFU)**	**Fixed mutations in *bl/*KPC/locus**	**Anti-CRISPR homologs detected**	**Shannon entropy (SNP distribution)**	**Growth rate vs. ancestor**
0 (ancestor)	1.0 × 10	—	—	2.14	1.00 ± 0.03
5	8.2 × 10	0	0	2.18	0.98 ± 0.04
10	7.9 × 10	0	0	2.21	0.97 ± 0.05
15	8.5 × 10	0	0	2.19	0.99 ± 0.03
20	8.1 × 10	0	0	2.22	1.01 ± 0.04
Control: Meropenem sub-MIC					
20	9.3 × 10	3 (promoter, signal peptide)	N/A	1.87	1.12 ± 0.06

Serial passage experiments over 20 generations demonstrated that the phage-CRISPR platform effectively suppresses resistance evolution, with zero fixed mutations detected in the (bla) KPC locus and no emergence of anti-CRISPR homologs. Population genetic diversity remained stable (Shannon entropy: 2.14 → 2.22), and bacterial growth rates were unchanged relative to the ancestral strain, indicating an absence of selective sweep or fitness compensation. In stark contrast, sub-MIC meropenem exposure induced three fixed mutations in promoter and signal peptide regions, reduced genomic diversity (entropy: 1.87), and conferred a significant growth advantage (1.12 ± 0.06 vs. ancestor; p < 0.01). These findings confirm that transient, RNA-targeted Cas13a activity imposes a high evolutionary barrier to resistance, circumventing the adaptive selection and fitness gains typically driven by conventional antibiotic pressure.

Conclusion & Recommendation:

This study establishes a precision CRISPR-Cas13a antimicrobial platform capable of sequence-specific elimination of multidrug-resistant “Klebsiella pneumonia” through targeted transcript depletion and collateral RNase activation. Delivered via an engineered, serum-stable φKp34 myovirus vector, the system demonstrated rapid bactericidal kinetics, profound biofilm penetration, and significant survival benefits in a murine sepsis model, while preserving host cell viability and exhibiting negligible off-target cleavage in human transcriptomes. Critically, longitudinal evolutionary monitoring revealed a high genetic barrier to resistance, with no fixed mutations in the “bla” locus or emergence of anti-CRISPR determinants over 20 serial passages, contrasting sharply with conventional antibiotic-driven selective sweeps. By circumventing chromosomal DNA damage and SOS-mediated mutagenesis, this RNA-targeted modality offers a mechanism-agnostic strategy that synergizes with existing β-lactams and inherently restricts horizontal resistance transfer. Collectively, these findings validate a

reproducible, clinically aligned pipeline that bridges molecular specificity with robust in vivo pharmacodynamics, positioning CRISPR-Cas platforms as a viable therapeutic class for recalcitrant Gram-negative infections.

This study establishes a methodologically rigorous, translationally aligned CRISPR-Cas13a antimicrobial platform that addresses critical gaps in precision infection control. By targeting the bla KPC transcript rather than genomic DNA, the system circumvents SOS-mediated mutagenesis and horizontal resistance transfer, limitations inherent to Cas9/Cas12a platforms (41,12). The engineered φKp34 capsid provides species-specific tropism, serum stability, and biofilm penetration, resolving delivery bottlenecks that have historically constrained CRISPR antimicrobials (7).

The in vivo data demonstrate robust pharmacokinetics, with rapid bacterial clearance and survival benefits comparable to first-line carbapenems, yet without inducing cross-resistance. Transcriptomic off-target mapping confirms high fidelity, a prerequisite for regulatory approval (42). Notably, the absence of resistance emergence over 20 passages suggests that Cas13a-mediated RNA depletion imposes a fitness cost that suppresses adaptive evolution, contrasting with conventional antibiotics where sub-MIC exposure routinely selects resistant mutants (43).

Limitations include the immunogenic potential of repeated phage administration, which may necessitate capsid PEGylation or alternating serotype cycling in chronic regimens (44). Additionally, phage packaging capacity restricts multiplexing to ≤3 crRNAs per construct, though cocktail formulations can expand target breadth. Scalability of in vitro phage assembly remains cost-intensive, though recent advances in continuous-flow bioreactor production may mitigate this (45).

Clinical translation will require IND-enabling toxicology, pharmacokinetic modeling in humanized models, and phase I dose-escalation trials in localized infections (e.g., diabetic foot ulcers, ventilator-associated pneumonia) before systemic deployment. Integration with rapid diagnostic sequencing will enable patient-specific crRNA matching, realizing precision antimicrobial stewardship.

Translation of this platform to clinical practice requires coordinated advancement across delivery engineering, manufacturing, diagnostics, and regulatory science. First, the immunogenic potential of repeated phage administration must be mitigated through capsid surface modification (e.g., PEGylation, glycan shielding) or rational serotype-rotation protocols to prevent neutralizing antibody formation. Second, production workflows should transition from bench-scale ultracentrifugation to GMP-compliant continuous-flow bioreactor systems, coupled with standardized potency, endotoxin, and sterility release assays to ensure batch consistency. Third, clinical deployment will be maximally effective when integrated with rapid diagnostic sequencing; point-of-care metagenomic or targeted qPCR panels will enable patient-specific crRNA matching, real-time resistance tracking, and adaptive treatment regimens. Fourth, IND-enabling toxicology, humanized infection pharmacokinetic modeling, and phase I/II dose-escalation trials in localized indications (e.g., diabetic foot ulcers, ventilator-associated pneumonia, catheter-associated infections) should precede systemic administration. Finally, next-generation cassette optimization should explore multiplexed crRNA arrays, orthogonal Cas effectors with reduced collateral activity, and synthetic promoter systems for tunable expression kinetics. With rigorous preclinical validation, scalable manufacturing, and precision diagnostic integration, CRISPR-Cas antimicrobial platforms are poised to establish a new paradigm in targeted, resistance-suppressive infection control..

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