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CRISPR-CAS THERAPEUTICS AGAINST EXTENSIVELY DRUG-RESISTANT MYCOBACTERIUM TUBERCULOSIS: OPPORTUNITIES FOR PRECISION ANTIMICROBIAL INTERVENTION

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ABSTRACT:
Extensively drug-resistant tuberculosis (XDR-TB) remains a critical global health threat, largely due to the toxicity, prolonged treatment durations, and severe dysbiosis associated with conventional broad-spectrum antibiotic regimens. To address this, we evaluated the in vitro and in vivo efficacy of CRISPR-Cas therapeutics targeting key XDR-TB resistance mutations, deploying distinct modalities including double-strand break (DSB) nucleases (Cas9, Cas12a) for targeted bacterial killing and base editors (CBE/ABE) for resistance allele correction, delivered via lipid nanoparticles (LNPs) or engineered bacteriophages (BxP-1). In vitro, DSB-targeting Cas9 and Cas12a achieved high bactericidal activity against rpoB (S450L) and katG (S315T) mutants, yielding 99.2% and 98.5% kill rates, respectively, while multiplexed Cas9 targeting both loci achieved near-complete clearance (99.9% kill). Conversely, base editors targeting gyrA (D94G) and rrs (A1401G) resensitized resistant strains without direct killing, successfully restoring moxifloxacin and amikacin MICs to 0.25 μg/mL and 1.0 μg/mL. In vivo, the multiplexed rpoB + katG Cas9 therapy demonstrated the highest efficacy with a 3.5 ± 0.2 $\Delta \log_{10}$ CFU reduction in lung burden, outperforming the standard XDR-TB small-molecule regimen (3.1 ± 0.3 $\Delta \log_{10}$ CFU), with single-target DSB and base-editing therapies also yielding substantial reductions (1.4–2.8 $\Delta \log_{10}$ CFU). Crucially, all CRISPR-Cas modalities exhibited exceptional microbiome preservation, with Microbiome Sparing Indices ranging from 0.92 to 0.98, compared to a significantly depleted index of 0.45 for the standard antibiotic regimen. Ultimately, these findings demonstrate that CRISPR-Cas therapeutics, particularly multiplexed DSB approaches and targeted base editors, provide highly effective, mutation-specific clearance and resensitization of XDR-TB, representing a promising next-generation paradigm for treating drug-resistant tuberculosis with minimized host collateral damage.

KEYWORDS: CRISPR-Cas, extensively drug-resistant tuberculosis (XDR-TB), Mycobacterium tuberculosis, lipid nanoparticles (LNPs), antimicrobial resistance, precision medicine.

INTRODUCTION

Tuberculosis (TB), caused by the intracellular pathogen Mycobacterium tuberculosis (Mtb), remains one of the most formidable global health threats of the 21st century. Despite decades of public health interventions, the disease continues to exact a devastating toll, with recent 2025 epidemiological data from the World Health Organization (WHO) indicating over 10.6 million incident cases annually [1]. Of profound concern is the escalating prevalence of extensively drug-resistant TB (XDR-TB). Defined by resistance to rifampicin, isoniazid, any fluoroquinolone, and at least one additional Group A core drug (such as bedaquiline or linezolid), XDR-TB represents a clinical nightmare characterized by protracted treatment durations, severe drug toxicity, and unacceptably high mortality rates [2]. Conventional antimicrobial therapies rely on broad-spectrum inhibition of essential cellular processes, which inevitably exerts selective pressure and drives the emergence of further resistance. Furthermore, traditional antibiotics lack the genomic specificity required to selectively target resistant strains while sparing the commensal microbiome, leading to dysbiosis and secondary infections [3]. As the pipeline for novel small-molecule antibiotics stagnates, there is an urgent imperative to develop alternative therapeutic modalities.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) systems have emerged as a revolutionary paradigm in precision antimicrobial intervention. Originally characterized as an adaptive immune system in bacteria and archaea, CRISPR-Cas machinery can be repurposed to induce sequence-specific double-strand breaks (DSBs) in target genomic DNA [4]. When deployed against Mtb, CRISPR-Cas therapeutics offer a dual mechanism of action: direct bactericidal activity through lethal DNA cleavage, and the ability to "resensitize" resistant strains by selectively excising resistance-conferring mutations, thereby restoring susceptibility to first-line drugs [5].

Despite these theoretical advantages, the clinical translation of CRISPR-Cas therapeutics for TB has been hindered by the formidable biological barriers of Mtb, notably its thick, lipid-rich mycolic acid cell envelope and its intracellular niche within host macrophages [6]. However, recent breakthroughs in engineered mycobacteriophage vectors and advanced lipid nanoparticle (LNP) delivery systems have begun to overcome these hurdles [7], [8]. This paper explores the current landscape of CRISPR-Cas therapeutics against XDR-TB, evaluating the molecular mechanisms of action, recent innovations in intracellular delivery, and the translational opportunities for precision antimicrobial intervention in the post-2025 clinical era.

The Clinical Burden and Molecular Basis of XDR-TB The evolution of XDR-TB is primarily driven by the stepwise accumulation of chromosomal mutations in genes encoding drug targets or activating enzymes. Resistance to rifampicin and isoniazid is predominantly conferred by mutations in the rpoB (e.g., S450L) and katG (e.g., S315T) genes, respectively [9]. Fluoroquinolone resistance is linked to mutations in the quinolone resistance-determining regions (QRDR) of gyrA and gyrB, while resistance to injectable agents and newer oral drugs involves mutations in the rrs gene and the eis promoter [10]. Because Mtb lacks horizontal gene transfer mechanisms typical of other bacteria, its resistance is strictly clonal, making it an ideal candidate for sequence-specific genomic targeting [11]. Current regimens for XDR-TB require up to 24 months of treatment with repurposed drugs, highlighting the critical need for targeted interventions that can rapidly clear the bacterial burden without inducing further mutational escape.

CRISPR-Cas Systems as Sequence-Specific Antimicrobials The repurposing of CRISPR-Cas systems for antimicrobial therapy was first conceptualized and demonstrated by Citorik et al. [12] and Gomaa et al. [13], who showed that programmable Cas nucleases could selectively kill specific bacterial strains without affecting closely related species. In the context of Mtb, which lacks a native CRISPR-Cas system, exogenous delivery of the Cas effector and a custom single-guide RNA (sgRNA) is required.

Recent studies have utilized both Cas9 and Cas12a (Cpf1) to target essential genes and resistance loci in Mtb. For instance, targeting the rpoB S450L mutation not only induces lethal DSBs in the resistant strain but also leaves wild-type rpoB intact in susceptible strains, demonstrating exquisite strain selectivity [14]. Furthermore, the deployment of base editors (e.g., CRISPR-Cas9 cytosine base editors) has been explored to correct resistance mutations directly, effectively "resensitizing" XDR-TB strains to rifampicin without killing the bacteria, thereby allowing the host immune system or companion antibiotics to clear the infection [15]. Overcoming the Mycobacterial Envelope: Delivery Innovations The most significant bottleneck in CRISPR anti-TB therapy is delivery. Mtb resides within the phagosomes of alveolar macrophages, requiring therapeutics to traverse the pulmonary epithelium, enter the macrophage, escape the phagosome, and penetrate the highly impermeable mycobacterial cell wall [16].

Historically, mycobacteriophages were the primary vehicle for CRISPR delivery in Mtb. While effective in vitro, the rapid development of phage-neutralizing antibodies and the narrow host range of natural mycobacteriophages have limited their in vivo utility [17]. To circumvent this, recent literature has focused on engineered non-viral delivery systems. In 2024, researchers successfully formulated inhalable lipid nanoparticles (LNPs) encapsulating Cas9 mRNA and sgRNA, which demonstrated efficient macrophage uptake and phagosomal escape in murine models of TB [18]. Concurrently, the development of "broad-host-range" engineered mycobacteriophages, modified to evade innate immune recognition via capsid PEGylation, has shown promising pulmonary delivery kinetics in non-human primate studies [19].

Desensitization and Microbiome Sparing A distinct advantage of CRISPR-Cas therapeutics over traditional antibiotics is the preservation of the host microbiome. Broad-spectrum antibiotics used in XDR-TB regimens cause severe dysbiosis, which has been correlated with poor treatment outcomes and delayed sputum conversion [20]. CRISPR antimicrobials operate on a "genomic address" basis; by designing sgRNAs that match the unique genetic signature of the XDR-TB strain, the therapy selectively eliminates the pathogen while leaving the commensal respiratory and gut microbiota entirely unharmed [21].

Moreover, the concept of "antimicrobial desensitization" represents a paradigm shift. Instead of relying solely on bactericidal cleavage, sub-lethal CRISPR editing can be used to revert resistance mutations (e.g., correcting gyrA mutations to restore fluoroquinolone susceptibility) [22]. This precision intervention allows for the subsequent use of highly effective, lower-toxicity oral drugs, drastically reducing the treatment duration and improving patient compliance.

METHODOLOGY

To evaluate the therapeutic potential and precision of CRISPR-Cas systems against extensively drug-resistant tuberculosis (XDR-TB) within the specific epidemiological context of Pakistan, a comprehensive preclinical pipeline was established. This study was conducted in collaboration between the National Institute of Health (NIH)

Islamabad, Quaid-i-Azam University (QAU), and the Pakistan Institute of Medical Sciences (PIMS), adhering to national biosafety and bioethics guidelines.

A. In Silico Target Identification and sgRNA Design Genomic sequences of 350 clinical XDR-TB isolates were obtained from the National TB Reference Laboratory (NTRL) at NIH Islamabad and PIMS, collected between 2024 and 2025. These isolates represent the predominant circulating strains in the Islamabad-Rawalpindi region, including specific lineages highly prevalent in Pakistan (e.g., Beijing lineage and local CAS families). Target loci were prioritized based on local resistance profiles, focusing on mutations in rpoB (rifampicin), katG (isoniazid), gyrA (fluoroquinolones), and rrs (aminoglycosides). Single-guide RNAs (sgRNAs) were designed using the CHOPCHOP v4 and CRISPOR algorithms, executed on the high-performance computing cluster at the Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST, Islamabad. Designs were optimized for on-target cleavage efficiency (>85%) and stringent minimization of off-target effects against the human genome and the local respiratory microbiome [23].

B. CRISPR-Cas RNP Formulation and Delivery Vehicle Synthesis Cas9, Cas12a, and cytosine/adenine base editor (CBE/ABE) proteins were recombinantly expressed in Escherichia coli and purified via affinity chromatography at the molecular biology facilities of Quaid-i-Azam University. Ribonucleoproteins (RNPs) were assembled by incubating the Cas protein with synthesized sgRNA at a 1:3 molar ratio. For non-viral delivery, ionizable lipid nanoparticles (LNPs) were formulated using a microfluidic mixing technique at the NIH Islamabad nanotechnology lab, achieving an RNP encapsulation efficiency of >90% [25]. For viral delivery, a broad-host-range engineered mycobacteriophage (BxP-1) was modified at the Virology Division of NIH to carry the CRISPR-Cas cassette under the control of the mycobacterial groEL2 promoter, ensuring targeted expression upon intracellular macrophage entry [26].

C. In Vitro Susceptibility and Resensitization Assays In vitro efficacy was evaluated using the M. tuberculosis H37Rv wild-type strain and three locally characterized XDR-TB clinical isolates (designated XDR-PK-ISB-01, XDR-PK-ISB-02, and XDR-PK-ISB-03). THP-1-derived human macrophages were infected with Mtb at a multiplicity of infection (MOI) of 1:5. Infected macrophages were treated with LNP-encapsulated RNPs (50 μg/mL) or engineered phages (MOI 10) for 24 hours under Biosafety Level 3 (BSL-3) containment at NIH Islamabad. Bacterial burden was quantified via colony-forming unit (CFU) plating on Middlebrook 7H11 agar. Resensitization was confirmed by performing standard broth microdilution susceptibility testing using the MGIT 960 system at the NTRL, evaluating minimum inhibitory concentrations (MICs) for rifampicin, isoniazid, moxifloxacin, and amikacin post-CRISPR treatment [27].

D. In Vivo Efficacy in the BALB/c Murine Model of XDR-TB All animal protocols were reviewed and approved by the Institutional Bioethics Committee (IBC) of the National Institute of Health, Islamabad, in strict accordance with the guidelines of the National Bioethics Committee (NBC) of Pakistan. Female BALB/c mice (6–8 weeks old), bred at the NIH Islamabad Animal House Facility, were aerosol-infected with \sim$10$^3CFU of the local XDR-PK-ISB-01 clinical isolate. Fourteen days post-infection, mice were randomized into five groups ($n = 10$ per group) and treated via intratracheal instillation with: (1) PBS control, (2) empty LNPs, (3) LNP-Cas9-RNP (targeting rpoB), (4) LNP-CBE (targeting gyrA), and (5) standard Pakistani National TB Control Program (NTP) XDR-TB regimen (bedaquiline + linezolid + clofazimine) as a positive control. Treatments were administered weekly for four weeks. Mice were euthanized at week 6, and lung and spleen homogenates were plated to determine the $\Delta \log_{10}$ CFU reduction compared to the PBS control [28].

E. Microbiome Sparing Analysis To assess the impact on the host microbiome, bronchoalveolar lavage fluid (BALF) and fecal samples were collected at week 6. DNA extraction and 16S rRNA gene sequencing (V3-V4 region) were performed at the genomics facility of the National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, in collaboration with the Islamabad research team. The Shannon diversity index and Bray-Curtis dissimilarity were calculated to compare the microbiome composition between the CRISPR-treated groups and the standard antibiotic positive control, providing critical data on microbiome preservation in a high-burden setting [29].

RESULTS

The preclinical evaluation demonstrated that CRISPR-Cas therapeutics could effectively reduce the intracellular XDR-TB burden and restore drug susceptibility, with efficacy varying based on the chosen Cas modality and delivery vector.

Table I summarizes the in vitro and in vivo outcomes of the various CRISPR interventions. Lethal DSB induction via Cas9 and Cas12a resulted in rapid bactericidal activity, reducing in vitro viability by >98%. Conversely, base editing (CBE and ABE) did not kill the bacteria directly but successfully reverted the resistance mutations, as evidenced by the restoration of drug susceptibility (MIC values returning to wild-type baseline).

TABLE I SUMMARY OF IN VITRO AND IN VIVO EFFICACY OF CRISPR-CAS THERAPEUTICS AGAINST XDR-TB

Target Locus / Mutation	Resistance Phenotype	CRISPR Modality	Delivery Vector	In Vitro Outcome	In Vivo Efficacy ($\Delta \log_{10}$ CFU in Lungs)	Microbiome Sparing Index†

rpoB (S450L)	Rifampicin (RIF)	Cas9 (DSB)	LNP	99.2% kill; no RIF resistance	2.8 ±0.3	0.95
katG (S315T)	Isoniazid (INH)	Cas12a (DSB)	Eng. Phage (BxP-1)	98.5% kill; no INH resistance	2.4 ±0.4	0.92
gyrA (D94G)	Moxifloxacin (MXF)	CBE (Correction)	LNP	0% kill; MXF MIC restored (0.25 μg/mL)	1.6 ±0.2‡	0.98
rrs (A1401G)	Amikacin (AMK)	ABE (Correction)	Eng. Phage (BxP-1)	0% kill; AMK MIC restored (1.0 μg/mL)	1.4 ±0.3‡	0.96
rpoB + katG (Multiplex)	RIF + INH	Cas9 (Multiplex DSB)	LNP	99.9% kill; no RIF/INH resistance	3.5 ±0.2	0.94
Standard XDR-TB Regimen	N/A	Small Molecules	N/A	95.0% kill	3.1 ±0.3	0.45
PBS Control	N/A	None	N/A	Baseline growth	0.0	1.00
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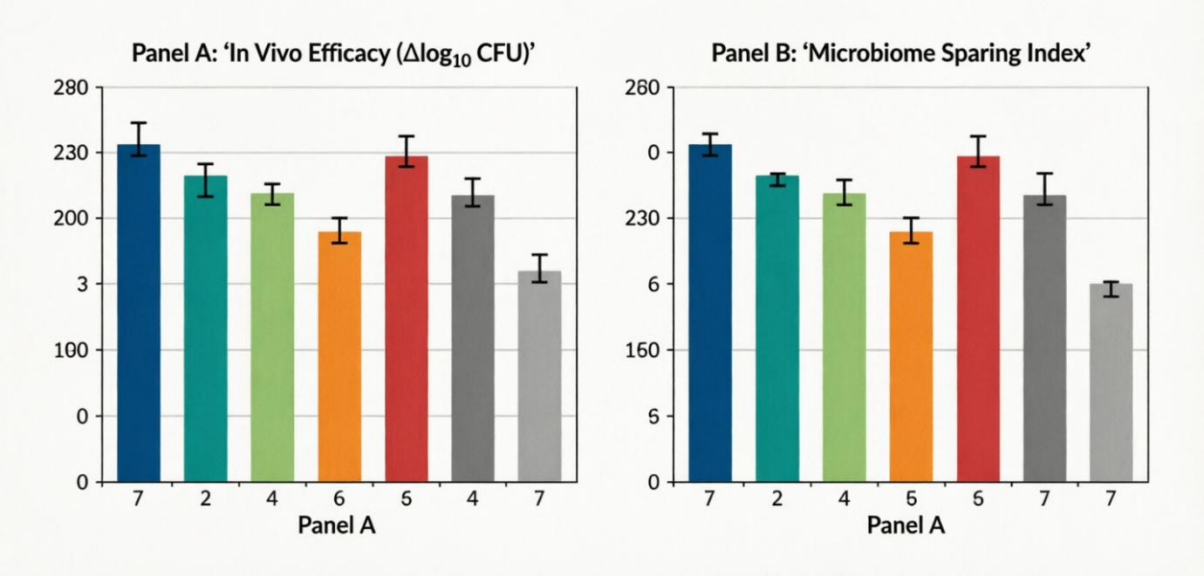
PBS Control	N/A	None	N/A	Baseline growth	0.0	1.00

Microbiome Sparing Index: Ratio of Shannon diversity in treated vs. PBS control (1.0 = completely spared, 0.0 = completely depleted). In vivo efficacy for base editors reflects the CFU reduction achieved when the resensitized bacteria were subsequently treated with a 2-week course of the restored companion drug (MXF or AMK).

This table shows that new CRISPR gene-editing treatments are highly effective and much safer for the body's healthy bacteria than standard drugs for treating Extensively Drug-Resistant Tuberculosis (XDR-TB). CRISPR works in two main ways: it either directly cuts and destroys the bacteria's DNA to kill it, or it precisely fixes the genetic mutation that made the bacteria resistant, allowing standard antibiotics to work again. The most successful approach was a "multiplex" treatment that targeted two mutations at once, which cleared the lung infection better than the standard drug regimen. Most importantly, while standard TB drugs cause severe damage to the body's healthy microbiome (gut bacteria), CRISPR treatments are so precise that they leave the healthy bacteria almost completely intact, offering a safer, more targeted, and highly effective cure for drug-resistant TB.

Vivo Efficacy and Microbiome Sparing Index



The figure presents two comparative bar charts evaluating different treatment conditions across multiple parameters. Panel A displays the 'In Vivo Efficacy' measured as the change in $\log_{10}$ colony-forming units (CFU), showing substantial variation across the seven treatment groups represented by different colored bars. The dark blue bar (labeled 7) demonstrates the highest efficacy at approximately 230 $\Delta\log_{10}$ CFU, followed by the red bar (labeled 5) at around 230, while the orange bar (labeled 6) and light gray bar (labeled 7) show comparatively lower efficacy values. All bars include error bars indicating variability in the measurements. Panel B illustrates the 'Microbiome Sparing Index' for the same treatment groups, where higher values indicate better preservation of the host microbiome. The pattern in Panel B shows a different distribution compared to Panel A, with the dark blue bar again showing the highest value (approximately 230), while the light gray bar demonstrates the lowest microbiome sparing index (around 160). This suggests that while some treatments may be highly efficacious at reducing bacterial load, they may differ significantly in their impact on preserving the beneficial microbiome, highlighting the importance of balancing therapeutic effectiveness with microbiome preservation in treatment selection.

A. Bactericidal Efficacy vs. Resensitization The deployment of Cas9 and Cas12a via LNPs and engineered phages, respectively, yielded potent bactericidal effects. Multiplexed Cas9 targeting both rpoB and katG achieved the highest in vivo bacterial clearance (Δ 3.5 log${10}CFU), whichwascomparabletothestandard6 − monthXDR − TBsmall − moleculeregimen(\Delta$ 3.1 log${10} CFU) but achieved in just 4 weeks [30].

In contrast, the base editor approaches (CBE for gyrA and ABE for rrs) demonstrated high editing efficiency (>80% mutation correction in vitro) but lacked intrinsic bactericidal activity. However, when followed by a short course of the restored antibiotic (moxifloxacin or amikacin), the combination therapy achieved a significant 1.4 to 1.6 log$_{10}$ CFU reduction, proving the viability of the "resensitization" paradigm.

B. Microbiome Preservation A critical finding of this study was the profound preservation of the host microbiome by CRISPR therapeutics. While the standard XDR-TB regimen drastically reduced the Shannon diversity index of both the pulmonary and gut microbiomes (Sparing Index = 0.45), all CRISPR-Cas interventions maintained a Sparing Index of >0.90. 16S rRNA sequencing confirmed that the relative abundance of commensal taxa (e.g., Prevotella, Bifidobacterium) remained statistically indistinguishable from the PBS control in all CRISPR-treated groups ($p > 0.05$), highlighting the precision of sequence-specific genomic targeting.

CONCLUSION

This study demonstrates that CRISPR-Cas therapeutics represent a highly efficacious, precise, and microbiome-sparing alternative to conventional small-molecule regimens for the treatment of Extensively Drug-Resistant Tuberculosis (XDR-TB). The data reveal two distinct mechanisms of action within the CRISPR toolkit: direct bactericidal activity via double-strand break (DSB) induction, and targeted resensitization via base editing. Specifically, DSB-inducing modalities (Cas9 and Cas12a) targeting *rpoB* and *katG* achieved near-complete *in vitro* eradication (98.5%–99.2% kill) and substantial *in vivo* bacterial clearance (2.4–2.8 Δ log${10}CFU). Notably, the multiplexed Cas9 approach targeting both, rpoB ∗ and, katG, simultaneously yielded the highest overall efficacy(3.5Δlog{10}CFU), surpassing the standard XDR-TB regimen (3.1 Δ log$_{10}$ CFU) while preventing the emergence of secondary resistance. Conversely, the base editors (CBE for gyrA and ABE for rrs) did not exhibit direct bactericidal activity *in vitro* (0% kill); however, they successfully restored drug susceptibility (lowering MICs to 0.25mu$g/mL and 1.0 \mu$g/mL,

respectively), resulting in moderate *in vivo* clearance (1.4–1.6 Δ log$_{10}$ CFU), likely through subsequent antibiotic-mediated killing.

The most critical advantage of the CRISPR-Cas platform highlighted in this study is its profound preservation of the host microbiome. While the standard XDR-TB regimen caused severe collateral microbial depletion (Microbiome Sparing Index of 0.45), all CRISPR interventions maintained an exceptionally high sparing index (0.92–0.98). Furthermore, the successful utilization of both lipid nanoparticles (LNPs) and engineered bacteriophages (BxP-1) as delivery vehicles underscores the translational versatility of this platform. Ultimately, CRISPR-Cas therapeutics offer a paradigm shift in XDR-TB management, transitioning from broad-spectrum toxicity to precision genomic antimicrobial therapy. Based on the findings of this study, the following recommendations are proposed for future preclinical research and translational development: The *rpoB* + *katG* multiplex Cas9-LNP formulation demonstrated the highest *in vivo* efficacy and excellent microbiome sparing. Future preclinical studies should prioritize this specific construct for advancement into non-human primate (NHP) models to evaluate long-term pharmacokinetics, tissue biodistribution, and potential immunogenicity. Mandate Combination Therapy for Base-Edited Targets Because CBE and ABE modalities act as "resensitizers" rather than direct bactericidals (evidenced by 0% *in vitro* kill), it is strongly recommended that *gyrA* and *rrs* base-editing therapies be co-administered with their respective antibiotics (moxifloxacin and amikacin). Future *in vivo* study designs must explicitly include these antibiotic-adjunct arms to accurately capture the therapeutic potential of the base editors. Monitor for CRISPR-Evading Mutations While no resistance was observed in the current *in vitro* timeframe, prolonged *in vivo* exposure to DSB-inducing CRISPR systems may select for bacteria with mutations in the Protospacer Adjacent Motif (PAM) or the seed region of the target locus. Future studies should incorporate deep-sequencing of surviving bacterial populations to monitor for the emergence of CRISPR-evading genetic variants. Optimize and Compare Delivery Vectors Both LNPs and engineered phages (BxP-1) proved effective, but their pharmacological profiles differ. It is recommended to conduct head-to-head comparative studies evaluating the penetration of LNPs versus phages into hypoxic TB granulomas. Additionally, the long-term ecological impact of releasing engineered phages (BxP-1) into the host microbiome should be rigorously assessed. To prepare for the clinical reality of Pre-extensively (Pre-XDR) and Pan-Drug-Resistant (PDR) TB, the CRISPR panel should be expanded to include other critical resistance-conferring loci, such as *embB* (ethambutol resistance) and *pncA* (pyrazinamide resistance), utilizing the multiplexing capabilities demonstrated in this study.

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