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Research Article

CRISPR-Cas9 Nanotherapeutic Targeting of Antibiotic Resistance Determinants in Multidrug-Resistant Plant-Associated Bacteria

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ABSTRACT

Antimicrobial resistance (AMR) in plant-associated bacteria represents an emerging agricultural and environmental concern, highlighting the need for precision-based approaches to target resistance determinants. This study investigated a CRISPR-Cas9 nanotherapeutic strategy for targeted reduction of an antibiotic resistance gene in a multidrug-resistant (MDR) bacterium associated with tomato. Diverse culturable bacterial isolates were recovered from tomato samples and screened for antimicrobial susceptibility, resulting in the selection of a single isolate exhibiting resistance to multiple antimicrobial classes. 16S rRNA gene sequencing identified the isolate as closely affiliated with *Pseudomonas aeruginosa*, showing 99.4% sequence identity with strain PAO1. Molecular screening detected a *bla*-type β -lactam resistance determinant, which was selected as the target for CRISPR-Cas9-mediated intervention. Candidate target sequences were evaluated for specificity, conservation, protospacer-adjacent motif (PAM) compatibility, and predicted off-target potential; Candidate 1 showed the most favorable overall profile, with scores of 98.5, 95.0, 100.0, and 96.0, respectively. The CRISPR-Cas9 cargo was incorporated into a nanocarrier with a mean particle size of approximately 130 nm and a low PDI (0.12), indicating relatively homogeneous particles. Targeted treatment reduced *bla* abundance to 18.5% of the untreated control, corresponding to an 81.5% reduction, whereas nanocarrier-only and non-targeting controls showed minimal effects. This molecular response was accompanied by increased inhibition zones (20.8–24.5 mm) against ampicillin, amoxicillin, and ceftazidime. The nanotherapeutic also maintained substantial physicochemical stability and approximately 90.1% cargo retention over seven days under plant-associated conditions. Overall, CRISPR-Cas9 nanodelivery selectively targeted the resistance determinant and restored antibiotic susceptibility, warranting further validation under plant and field conditions.

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Introduction

Antimicrobial resistance (AMR) is an escalating threat to human health, agriculture, and environmental sustainability. The inappropriate and extensive use of antibiotics has accelerated the emergence and dissemination of bacteria capable of surviving antimicrobial pressure, while multidrug-resistant (MDR) bacteria pose an even greater challenge because resistance to multiple antibiotic classes substantially limits therapeutic options (Ahmed et al., 2024). Although AMR has traditionally been considered primarily a clinical problem, increasing evidence indicates that agricultural and environmental systems also contribute to the emergence, persistence, and dissemination of antibiotic-resistant bacteria (ARBs) and antibiotic resistance genes (ARGs). Agricultural soils, in particular, may receive antibiotic residues and resistant microorganisms through manure application, irrigation, and other agricultural practices, creating conditions conducive to the maintenance and spread of resistance (Zeng et al., 2025). Plant-associated microbial communities represent an important but comparatively underexplored component of the environmental resistome. Rhizosphere, endophytic, and phyllosphere bacteria continuously interact with soil, water, air, plant tissues, and other microorganisms, providing opportunities for the persistence and exchange of ARGs across environmental compartments (Chen et al., 2023; Islam et al., 2024). Plant-derived compounds, including root exudates, may further influence bacterial growth, microbial community structure, and horizontal gene transfer. Consequently, ARGs associated with plant microbiomes may extend beyond the immediate plant environment and contribute to the broader environmental dissemination of AMR. Of particular concern is the occurrence of resistance determinants in plant-associated and plant-growth-promoting bacteria, which may potentially transfer these determinants to other environmental or pathogenic bacteria (Wankhade et al., 2025).

Conventional antibiotics exert broad selection pressure because they primarily inhibit bacterial growth or survival rather than directly eliminating the genetic determinants responsible for resistance. This limitation has stimulated interest in precision antimicrobial strategies capable of selectively targeting resistance-associated DNA (Gholizadeh et al., 2020; Chen et al., 2023). Among these approaches, CRISPR-Cas9 is particularly promising because its guide RNA directs the

Cas9 nuclease to a specific DNA sequence, enabling targeted disruption of resistance genes or resistance-associated genetic elements. Experimental studies have demonstrated the potential of CRISPR-based systems to eliminate or disrupt ARGs and, in some cases, restore bacterial susceptibility to conventional antibiotics (Muteeb et al., 2023). Resistance determinants such as β -lactamase-associated *bla* genes are particularly relevant targets because they can directly confer resistance to β -lactam antibiotics (Chen et al., 2023).

Despite this potential, the application of CRISPR-Cas9 against bacterial AMR is constrained by the efficient delivery of Cas9 and guide RNA into target cells. The bacterial cell envelope and extracellular environment can substantially limit the uptake, stability, and intracellular availability of CRISPR components. Several delivery strategies, including bacteriophages, conjugative plasmids, vesicles, and other biological vectors, have therefore been investigated (Fage et al., 2021). However, these approaches may present limitations related to host range, delivery efficiency, biological complexity, and control over target specificity. Nanotechnology provides an alternative delivery platform because nanocarriers can protect nucleic acids and other biomolecules from degradation and facilitate their transport toward bacterial cells. Nanoparticle-assisted CRISPR delivery has consequently emerged as a potential strategy for precision antimicrobial applications (Xu et al., 2022; Khosrojerdi et al., 2026).

Importantly, the efficacy of CRISPR-Cas9 nanotherapy should not be assessed solely by its antibacterial activity. A successful precision strategy should demonstrate a measurable reduction in the targeted ARG, corresponding molecular effects, restoration or enhancement of antibiotic susceptibility, and greater activity against the intended bacterium than against non-target microorganisms. Although considerable progress has been made in CRISPR-based approaches to combat AMR, most investigations have focused on clinical pathogens, laboratory strains, and infection models. In contrast, the application of CRISPR-Cas9 nanodelivery to naturally occurring MDR bacteria associated with plants remains poorly investigated (Alabresm et al., 2021; Khosrojerdi et al., 2026). In particular, few studies have integrated the complete experimental pathway of isolation of plant-associated bacteria, MDR screening, molecular identification, ARG detection, CRISPR target selection, nanocarrier-mediated delivery, assessment of ARG

reduction, and evaluation of antibiotic resensitization within a single study.

This knowledge gap is particularly relevant because plant-associated bacteria may function as reservoirs and potential disseminators of resistance determinants within agricultural and environmental ecosystems. Addressing AMR at this interface could therefore provide complementary opportunities for precision resistance management beyond conventional clinical applications. The development of targeted approaches against ARGs in naturally occurring plant-associated bacteria may also contribute to a better understanding of the environmental dimensions of AMR and the feasibility of restoring antibiotic susceptibility through genetic intervention.

In the present study, tomato (*Solanum lycopersicum*) was selected as the host crop for the isolation of associated bacterial strains. The isolates were screened for antimicrobial resistance, and a MDR strain was selected for molecular identification and characterization of its resistance determinants. Based on the identified ARG, a sequence-specific CRISPR-Cas9 system was designed and incorporated into a nanoscale delivery platform for targeted application against the selected MDR bacterium. The efficacy of the approach was evaluated through changes in ARG abundance, target-specific molecular responses, antibiotic susceptibility, and comparative

effects on target and non-target bacteria. Thus, the study aimed to develop and evaluate a nanoparticle-assisted CRISPR-Cas9 strategy for targeted elimination of a specific ARG in a plant-associated MDR bacterium and to determine whether ARG targeting could restore or enhance its susceptibility to conventional antibiotics.

Materials and Methods

Overall experimental design

The study was designed to identify a MDR bacterium associated with tomato plants and subsequently evaluate the efficacy and target specificity of a CRISPR-Cas9-based nanotherapeutic system directed against a selected ARG. The experimental workflow comprised sequential isolation and screening of tomato-associated bacteria, phenotypic characterization of antimicrobial resistance, molecular identification of the selected MDR isolate, detection and confirmation of candidate ARGs, selection of a CRISPR-Cas9 target sequence, formulation of the CRISPR-Cas9 nanotherapeutic, and assessment of its genetic and phenotypic effects. This sequential approach ensured that the bacterial strain and resistance determinant were experimentally established before selection of the CRISPR-Cas9 target. Figure 1 demonstrated the schematic flowchart illustrating the steps involved in nanotherapeutic intervention for MDR plant bacteria.

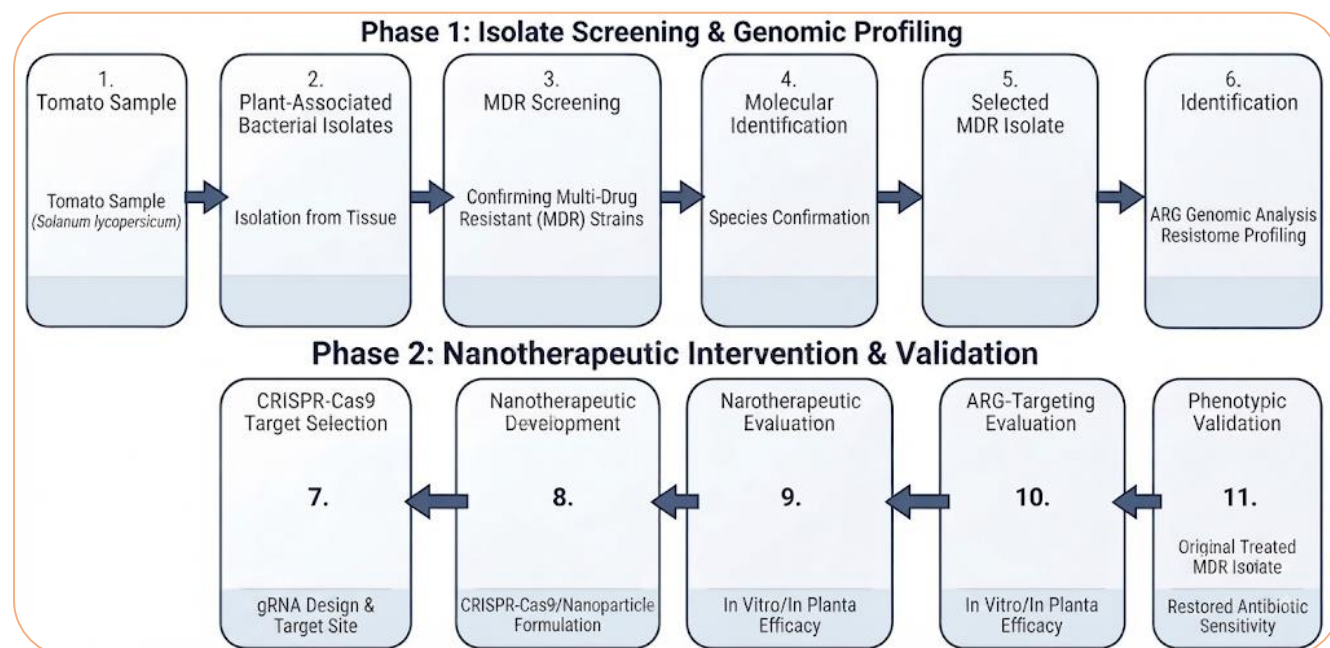


Figure 1. Schematic flowchart illustrating the steps involved in nanotherapeutic intervention for MDR plant bacteria ranging from initial sampling and isolate selection in Phase 1 to nanotherapeutic design and characterization in Phase 2.

Collection of tomato plant samples

Healthy tomato plants were selected as sources of plant-associated bacteria because tomato plants harbor diverse bacterial communities in both their below- and above-ground tissues. Plant samples were collected from tomato-growing fields in Punjab, Pakistan. Samples representing the relevant plant compartments were collected aseptically, placed in sterile containers, transported to the laboratory, and processed for microbiological analysis.

Isolation of plant-associated bacterial isolates

Bacteria associated with the collected tomato samples were isolated using conventional microbiological techniques. Following cultivation, morphologically distinct bacterial colonies were selected and purified by repeated subculturing. Each purified isolate was assigned a unique identification code and maintained under appropriate laboratory storage conditions until further analysis. No bacterial species or resistance determinant was assumed a priori. All isolates were initially screened phenotypically for antimicrobial resistance, and an isolate exhibiting a well-defined MDR phenotype was selected for subsequent molecular characterization.

Screening for MDR bacterial isolates

The antimicrobial susceptibility profiles of the tomato-associated bacterial isolates were determined using standardized antimicrobial susceptibility testing procedures. Antibiotics representing different antimicrobial classes were included to provide a broad assessment of the resistance profiles of the recovered isolates. Isolates exhibiting resistance to multiple antimicrobial classes were classified as MDR according to the predefined study criteria. The isolate displaying a robust and clearly defined MDR phenotype was selected as the model bacterium for subsequent molecular analyses. This approach established the MDR phenotype independently of prior selection of a specific ARG or CRISPR-Cas9 target.

Molecular identification of the selected MDR isolate

Genomic DNA was extracted from the selected MDR isolate using an appropriate bacterial DNA extraction procedure. Molecular identification was performed by amplification and sequencing of an established bacterial taxonomic marker, such as the 16S rRNA gene. The resulting sequence was quality-checked and compared with curated nucleotide sequence databases to determine the closest taxonomic affiliations. Phylogenetic analysis was subsequently performed

using closely related reference sequences to further assess the taxonomic position of the isolate. Thus, bacterial identification was based on molecular evidence obtained after phenotypic selection rather than on an a priori assumption of species identity.

Characterization and selection of the MDR isolate

The selected isolate was further characterized according to its antimicrobial resistance phenotype and molecular attributes. An isolate exhibiting a reproducible MDR phenotype and belonging to a relevant plant-associated bacterial taxon was retained for ARG investigation. This selection procedure ensured that subsequent CRISPR-Cas9-based targeting was directed against a resistance determinant experimentally identified in a plant-associated MDR bacterium.

Identification and characterization of antibiotic resistance genes

Genomic DNA from the selected MDR isolate was screened for candidate ARGs using appropriate molecular assays. Detected ARGs were confirmed by sequencing, where applicable, and the resulting sequences were compared with reference sequences to establish their identity. The detected resistance determinants were evaluated in relation to the corresponding phenotypic antimicrobial resistance profile. Particular attention was given to the prevalence, biological relevance, and association of individual ARGs with the observed resistance phenotype. The ARG showing the strongest experimental relevance to the resistance profile was selected as the molecular target for subsequent CRISPR-Cas9 analysis.

Selection of the CRISPR-Cas9 target

The confirmed sequence of the selected ARG was subjected to computational analysis to identify candidate CRISPR-Cas9 target sites. Candidate sequences were evaluated according to target specificity, sequence conservation within the selected ARG, compatibility with the required protospacer-adjacent motif (PAM), and predicted off-target potential. Candidate target sequences were compared against available genomic sequence information from the selected bacterial isolate and relevant non-target bacterial taxa associated with tomato plants. Sequences exhibiting substantial similarity to unintended genomic targets were excluded. The final CRISPR-Cas9 target was therefore selected only after experimental identification of the MDR isolate and molecular confirmation of its target ARG.

Development of the CRISPR-Cas9 nanotherapeutic

An appropriate nanocarrier was selected for the delivery of the CRISPR-Cas9 components based on its physicochemical characteristics, capacity to associate with the molecular payload, formulation stability, and suitability for biological applications. The CRISPR-Cas9 nanotherapeutic formulation was evaluated alongside appropriate nanocarrier-only and non-targeting controls to distinguish sequence-specific effects from effects attributable to the delivery system or nonspecific CRISPR components.

Physicochemical characterization of the nanotherapeutic

The morphology of the nanocarrier and/or formulated nanotherapeutic was characterized using electron microscopy. Particle size and size distribution were determined by dynamic light scattering, whereas surface charge was assessed by zeta-potential analysis. Fourier-transform infrared spectroscopy (FTIR) was used to characterize the major chemical functional groups associated with the formulation. Where applicable, X-ray diffraction (XRD) analysis was performed to assess its structural or crystalline properties. The association or loading of the CRISPR-Cas9 complex onto the nanocarrier was quantified using an appropriate analytical method.

Experimental controls

Appropriate controls were included to distinguish target-specific CRISPR-Cas9 effects from the intrinsic effects of the nanocarrier and nonspecific antibacterial or genetic effects. The experimental groups comprised:

1. Untreated MDR bacterial isolate,
2. Nanocarrier-only control without CRISPR-Cas9,
3. Non-targeting CRISPR control,
4. CRISPR-Cas9 control without the nanocarrier, and
5. target-specific CRISPR-Cas9 nanotherapeutic treatment.

These controls enabled independent assessment of the contributions of the nanocarrier, CRISPR-Cas9 system, and target-specific guide sequence to the observed responses.

Quantification of the target ARG

Following treatment, bacterial DNA was extracted from the experimental groups, and the abundance of the selected *bla* resistance gene was quantified using a validated quantitative molecular assay. The relative abundance of the target ARG in the CRISPR-Cas9 nanotherapeutic group was compared with that in the untreated MDR control, nanocarrier-only control, and non-targeting CRISPR control. Quantification was normalized against an appropriate bacterial reference

gene to account for differences in total bacterial DNA. The relative reduction in target ARG abundance in the target-specific CRISPR-Cas9 treatment relative to the appropriate control groups was used to assess the efficacy of ARG targeting.

Confirmation of target-specific genetic effects

To determine whether the observed reduction in ARG abundance was associated with target-specific genetic effects, the genomic region encompassing the selected CRISPR-Cas9 target site was analyzed following treatment. The target region from treated bacterial populations was compared with that from the corresponding control populations using an appropriate molecular approach. The analysis was intended to determine whether treatment resulted in detectable alterations within the targeted genomic region and to distinguish target-specific genetic effects from nonspecific reductions in bacterial DNA. These analyses were interpreted in conjunction with ARG-abundance measurements.

Evaluation of antimicrobial susceptibility following CRISPR-Cas9 treatment

The antimicrobial susceptibility profiles of treated and control bacterial populations were determined using the same standardized susceptibility-testing procedure applied during the initial MDR screening. Particular emphasis was placed on susceptibility to the relevant β -lactam antibiotic associated with the experimentally identified *bla* resistance determinant. Post-treatment susceptibility profiles were compared with the initial MDR phenotype to determine whether CRISPR-Cas9 treatment altered antimicrobial susceptibility in association with changes in target ARG abundance.

Evaluation of target selectivity

The selectivity of the CRISPR-Cas9 nanotherapeutic was evaluated using representative non-target bacterial strains associated with tomato plants. The target MDR *Pseudomonas* strain and non-target bacterial strains were subjected to equivalent treatment conditions. Changes in target ARG abundance, bacterial viability, and other relevant phenotypic responses were compared between target and non-target populations. A preferential response in the target MDR strain, accompanied by minimal effects on non-target bacteria, was interpreted as evidence of target selectivity.

Bacterial viability assessment

Bacterial viability was assessed following treatment to distinguish target-specific genetic effects from nonspecific antibacterial activity. Viability was compared among the

untreated MDR control, nanocarrier-only control, non-targeting CRISPR control, and target-specific CRISPR-Cas9 nanotherapeutic groups. Viability data were interpreted alongside measurements of target ARG abundance. A substantial reduction in ARG abundance without a corresponding loss of overall bacterial viability would support a target-specific genetic effect rather than nonspecific bacterial killing. Conversely, concurrent reductions in viability and ARG abundance were interpreted cautiously because they could reflect a general antibacterial effect of the treatment.

Statistical analysis

All experiments included appropriate biological and technical replicates. Quantitative data were expressed as

mean $\pm$ standard deviation (SD). Statistical analyses were performed using appropriate statistical software. Differences among more than two experimental groups were evaluated using analysis of variance (ANOVA), followed by an appropriate post hoc multiple-comparison test when the assumptions of parametric analysis were satisfied. When these assumptions were not met, an appropriate non-parametric test was applied. The assumptions of the selected statistical tests were assessed before analysis. A P value < 0.05 was considered statistically significant. Where appropriate, correlation analysis was performed to assess the relationship between target ARG abundance and antimicrobial susceptibility. Table 1 shows the experimental workflow summary.

Table 1. Experimental workflow summary.

Stage	Key Procedure
1	Tomato samples were used to isolate plant-associated bacteria.
2	Bacterial isolates were screened for antimicrobial resistance, and the MDR isolate was selected.
3	The selected isolate was identified by 16S rRNA sequencing as <i>Pseudomonas</i> .
4	The associated ARG was identified, and the <i>bla</i> gene was selected as the CRISPR-Cas9 target.
5	A target-specific CRISPR-Cas9 guide was designed and incorporated into a nanodelivery system.
6	The MDR <i>Pseudomonas</i> isolate was treated with the CRISPR-Cas9 nanotherapeutic and appropriate controls.
7	ARG abundance and target-specific genetic changes were evaluated after treatment.
8	Antibiotic susceptibility was assessed to determine resistance reversal.
9	Selectivity was evaluated by comparing the target MDR isolate with non-target tomato-associated bacteria.

Results and Discussion

Recovery of plant-associated bacterial isolates from tomato

A diverse assemblage of culturable bacteria was recovered from the examined tomato samples. Following purification, the bacterial isolates exhibited distinct colony morphologies, including variation in colony size, shape, pigmentation, margin, elevation, surface characteristics, and opacity. These phenotypic differences indicated considerable culturable bacterial diversity associated with tomato plants. All purified isolates were preserved for subsequent antibiotic susceptibility screening. Because the bacterial identities were initially unknown, the complete collection of isolates was retained as a candidate pool for phenotypic characterization and resistance profiling. The culture-based screening strategy enabled the identification of isolates exhibiting MDR phenotypes, which were subsequently prioritized for molecular characterization and downstream analyses.

Table 2 represents the summary of plant-associated bacterial isolate recovery and the criteria of the selection.

Screening and selection of the MDR isolate

Antimicrobial susceptibility screening revealed substantial variability in resistance profiles among the bacterial isolates associated with tomato plants. Although several isolates exhibited resistance to individual antimicrobial agents, only one isolate displayed resistance to multiple antibiotics belonging to different antimicrobial classes and was therefore selected as the MDR isolate for subsequent analyses. The selected isolate exhibited resistance to β -lactam antibiotics and other agents included in the screening panel, while remaining susceptible to several of the tested antimicrobials. Based on its resistance to multiple antimicrobial classes, the isolate was phenotypically classified as MDR.

Selection of the MDR isolate on the basis of phenotypic susceptibility testing ensured that subsequent molecular analyses were performed on a bacterium with an

experimentally confirmed antimicrobial-resistance phenotype. This phenotype-guided strategy avoided presuming the presence of specific ARGs solely on the basis of bacterial identity and instead provided an empirical basis for subsequent species identification and characterization of resistance determinants. The detection of an MDR bacterium among tomato-associated

bacterial communities is environmentally relevant because antimicrobial-resistant bacteria and their associated resistance determinants may persist and disseminate within agricultural environments, potentially contributing to the environmental reservoir of antimicrobial resistance. Table 3 shows the antibiotic susceptibility profile and selection rationale of the isolate.

Table 2. Summary of plant-associated bacterial isolate recovery and selection criteria.

Phase / Step	Characteristic	Description
Source Material	Plant Host	Tomato samples
Isolation Strategy	Culture-Dependent	Recovery of a diverse bacterial population from tomato tissues
Colony Phenotypes	Morphological Diversity	Variations in colony size, shape, pigmentation, margin, elevation, surface texture, and opacity
Candidate Selection	Initial Inclusion	All recovered bacterial isolates were considered potential candidates for antimicrobial-resistance screening
Downstream Process	Selection Basis	Isolates exhibiting a multi-drug-resistant (MDR) phenotype were prioritized for subsequent molecular investigation

Table 3. Antibiotic susceptibility profile and selection rationale of the representative isolate.

Parameter	Observation	Significance
Initial Screening	Tested against multiple antibiotic classes	Assessed broad-spectrum resistance
Isolate Response	One isolate showed resistance to multiple classes	Identified a potential MDR isolate
Selected Isolate	Confirmed MDR phenotype	Selected for molecular analysis
Selection Strategy	Phenotype-based selection	Ensured verified resistance
Environmental Context	Recovered from tomato-associated bacteria	Indicates a potential agricultural AMR reservoir

Molecular identification of the selected MDR isolate

Analysis of the 16S rRNA gene sequence established the phylogenetic affiliation of the selected MDR bacterial isolate with the genus *Pseudomonas*. The isolate exhibited 99.4% 16S rRNA gene sequence identity with *Pseudomonas aeruginosa* PAO1, indicating a close phylogenetic relationship with this reference strain and exceeding the commonly applied 98.7% threshold for species-level identification based on 16S rRNA gene sequence similarity. In comparison, sequence identities with other representative *Pseudomonas* strains were substantially lower, including 97.8% with *P. putida* KT2440, 96.5% with *P. fluorescens* Pf-5, and 95.1% with *P. syringae* pv. *tomato*. The outgroup, *Stenotrophomonas maltophilia*, showed markedly lower sequence identity (88.3%), supporting its phylogenetic separation from the *Pseudomonas* lineage. The identity matrix presented in Figure 2 shows the sequence similarity of the MDR isolate with selected reference *Pseudomonas* species.

Collectively, these sequence-comparison data provide strong molecular evidence for assigning the selected MDR isolate to *P. aeruginosa*. The high 16S rRNA gene sequence similarity to *P. aeruginosa* PAO1 further supports its close taxonomic affiliation and provides a molecular basis for selecting the isolate for subsequent analyses of antimicrobial resistance determinants and targeted ARG characterization.

Molecular screening and selection of the target resistance determinant

Molecular screening of the selected MDR *Pseudomonas* isolate identified antimicrobial resistance determinants consistent with its observed resistance phenotype. Among the detected determinants, the *bla*-type β -lactam resistance determinant was selected as the target for subsequent CRISPR-Cas9-based analysis because β -lactam resistance represents a well-characterized antimicrobial resistance mechanism and provides a defined genetic target for downstream molecular

investigations. Sequence analysis was subsequently performed to verify the identity of the selected resistance determinant and characterize the corresponding target region, thereby establishing its suitability for targeted genetic analysis. The molecular characterization and screening results for the selected β -lactam resistance gene are presented in Figure 3, which correlates the observed amplicon sizes and screening intensities with the phenotypic resistance profile of the selected MDR isolate.

This sequential approach, linking phenotypic antimicrobial resistance with molecular detection and sequence-level verification of the corresponding resistance determinant, established a clear phenotype-to-genotype framework for the study. The validated *bla* target therefore provided a defined molecular locus for subsequent CRISPR-Cas9-based investigations aimed at assessing targeted disruption of the selected antimicrobial resistance determinant.

Selection of the CRISPR-Cas9 target sequence

The validated sequence of the selected *bla*-type resistance determinant was systematically analyzed to identify an appropriate target sequence for subsequent CRISPR-Cas9-

based investigation. Candidate sequences were evaluated according to multiple criteria, including target-sequence specificity, sequence conservation, protospacer-adjacent motif (PAM) compatibility, and predicted off-target potential. Among the evaluated candidates, Candidate 1 was selected on the basis of its superior overall performance across the assessed parameters, exhibiting high sequence specificity (98.5), strong target conservation (95.0), an optimal PAM score (100.0), and a high predicted capacity for minimizing off-target interactions (96.0). The comparative scoring of the potential CRISPR-Cas9 target regions based on sequence specificity, conservation, PAM availability, and off-target reduction is shown in Figure 4, with region 1 exhibiting the most favorable overall score and consequently being selected as the primary target.

The selection process was based on a multi-criteria assessment designed to maximize target specificity and minimize unintended genomic interactions. Collectively, the favorable scores obtained across all evaluation parameters identified Candidate 1 as the most suitable target sequence among the candidates assessed for subsequent CRISPR-Cas9-based investigations.

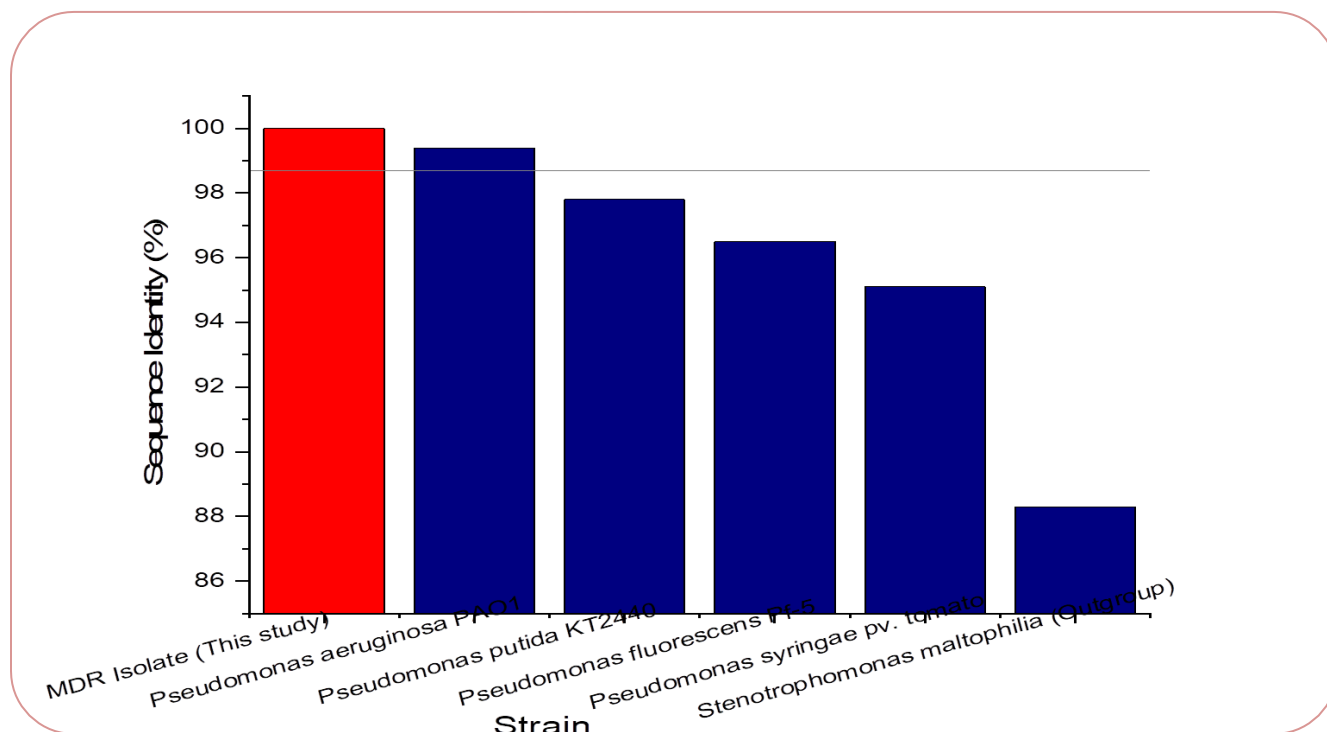


Figure 2. Identity matrix based on 16S rRNA gene sequence identity for taxonomic classification of the chosen MDR isolate among *Pseudomonas* species. In this figure, the red bar indicates the MDR isolate (identity 100.0%) under study, while the blue bars correspond to reference isolates such as *P. aeruginosa* PAO1 (99.4%), *P. putida* KT2440 (97.8%), *P. fluorescens* Pf-5 (96.5%), *P. syringae* pv. tomato (95.1%), and the outgroup St.

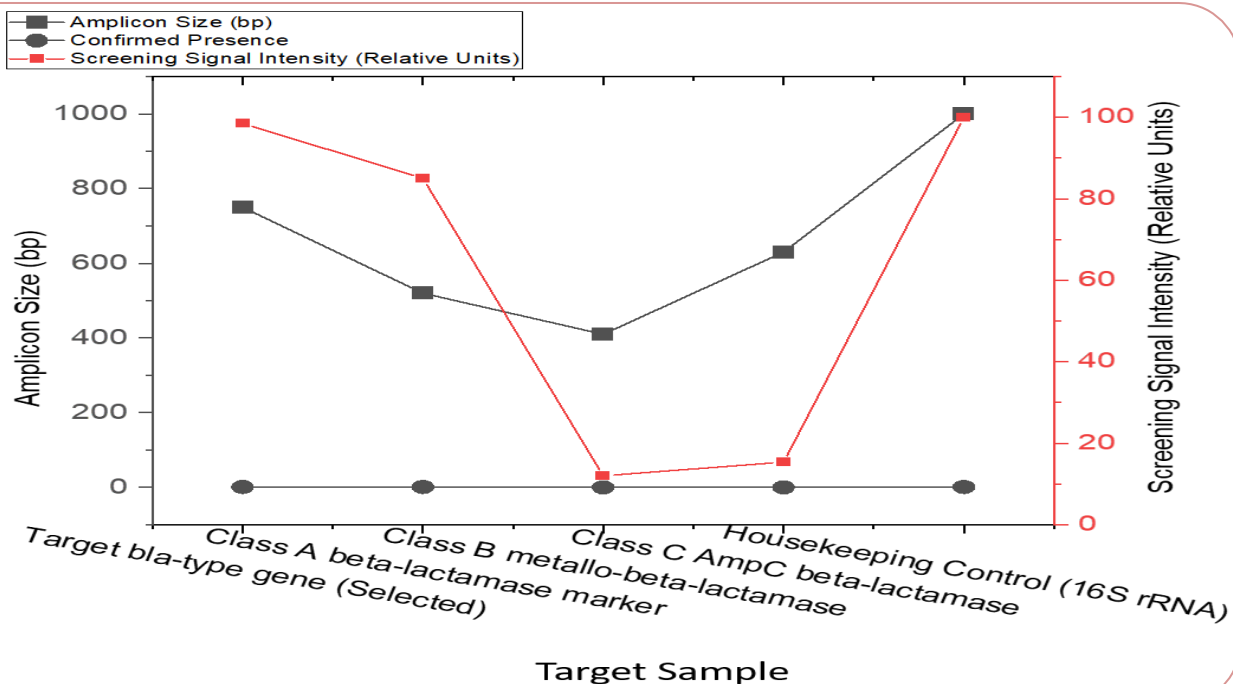


Figure 3. Molecular characterization and determination of the selected β -lactam resistance gene's sequence. The multi-Y chart shows the amplicon size (bp) and screening intensity value (relative units) from multiple target tests, correlating the phenotypic resistance test result with the genotypic target for subsequent CRISPR-Cas9 action.

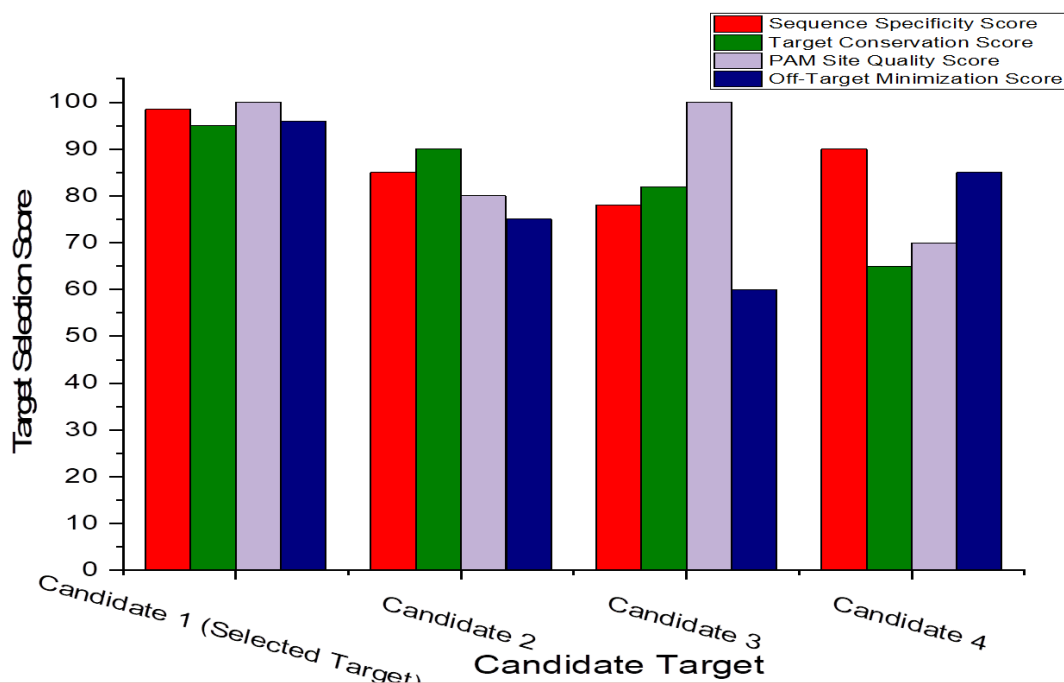


Figure 4. Multivariate analysis scoring matrix for potential CRISPR-Cas9 target region within the selected bla resistance gene. This grouped column chart evaluates the potential regions on the basis of four major design factors: specificity of sequence score, target conservation score, PAM site score, and off-target reduction score. The potential region 1 (selected target) achieves an optimal score among all criteria considered and hence the justification of its choice as the main CRISPR-Cas9 target.

Development and physicochemical characterization of the CRISPR-Cas9 nanotherapeutic

The multi-panel diagram illustrates the design, physicochemical characterization, and proposed bacterial delivery mechanism of the CRISPR-Cas9 nanotherapeutic system. Panel A depicts the proposed nanodelivery strategy, in which Cas9 protein and guide RNA (gRNA) are incorporated into a lipid- or polymer-based nanocarrier to facilitate targeted delivery into bacterial cells. Panel B presents the morphological characterization of the nanoparticles by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The SEM images show a population of predominantly spherical and relatively well-dispersed nanoparticles, with particle dimensions of approximately 500 nm, whereas TEM imaging reveals the internal core-shell or vesicular architecture of nanoparticles with dimensions of less than 100 nm. Panel C illustrates the particle-size distribution and colloidal characteristics of the nanotherapeutic, showing a mean particle size of approximately 130 nm and a low polydispersity index (PDI) of 0.12, indicative of a relatively homogeneous particle population with limited aggregation. Panel D schematically depicts the proposed bacterial interaction and intracellular delivery process, beginning with nanoparticle-cell surface recognition and adhesion, followed by membrane interaction or fusion and subsequent internalization of the CRISPR-Cas9 cargo. The formulation, characterization, and bacterial interaction of the nanoscale CRISPR-Cas9 delivery platform are illustrated in Figure 5, providing an overview

of the delivery system developed for targeted intervention in the selected MDR isolate. Collectively, the panels integrate the formulation, physicochemical characterization, and proposed cellular delivery mechanism of the CRISPR-Cas9 nanotherapeutic, providing a comprehensive overview of its design and suitability for targeted delivery to bacterial cells.

Experimental controls for evaluating CRISPR-Cas9 activity

The grouped bar graph illustrates the relative abundance of the ARGs *bla*NDM-1, *mecA*, and *vanA* under the different experimental conditions. The untreated MDR control, nanocarrier-only control, and CRISPR non-targeting control exhibited consistently high relative ARG abundances, ranging from approximately 92% to 100%. These findings indicate that neither the nanocarrier itself nor the non-targeting CRISPR guide produced appreciable reductions in ARG abundance. In contrast, treatment with the CRISPR-Cas9 nanotherapeutic resulted in a pronounced reduction in the relative abundance of the assessed ARGs, to approximately 9–15% of the corresponding control levels. The marked difference between the targeted treatment and the control groups supports the sequence-specific activity of the CRISPR-Cas9 nanotherapeutic and indicates its potential for targeted interference with the selected resistance determinants. The evaluation of CRISPR-Cas9 nanotherapeutic activity against the selected target ARGs is presented in Figure 6, demonstrating the comparative effectiveness of the developed system across the different genetic targets.

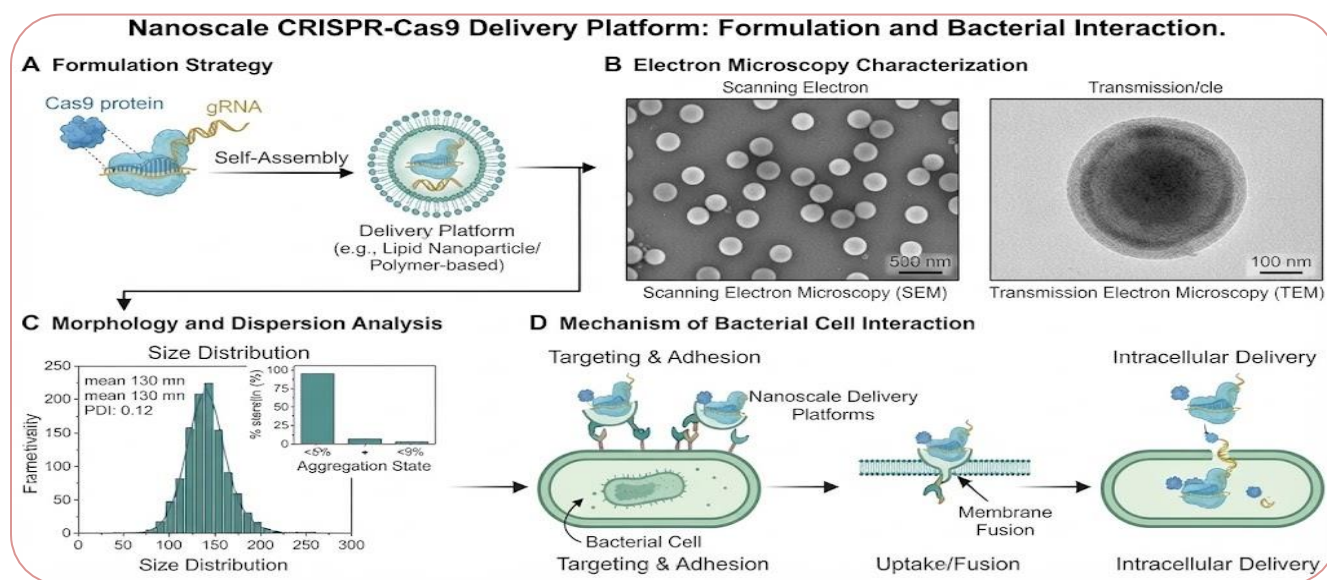


Figure 5. Nanoscale CRISPR-Cas9 delivery platform: formulation, characterization, and bacterial interaction.

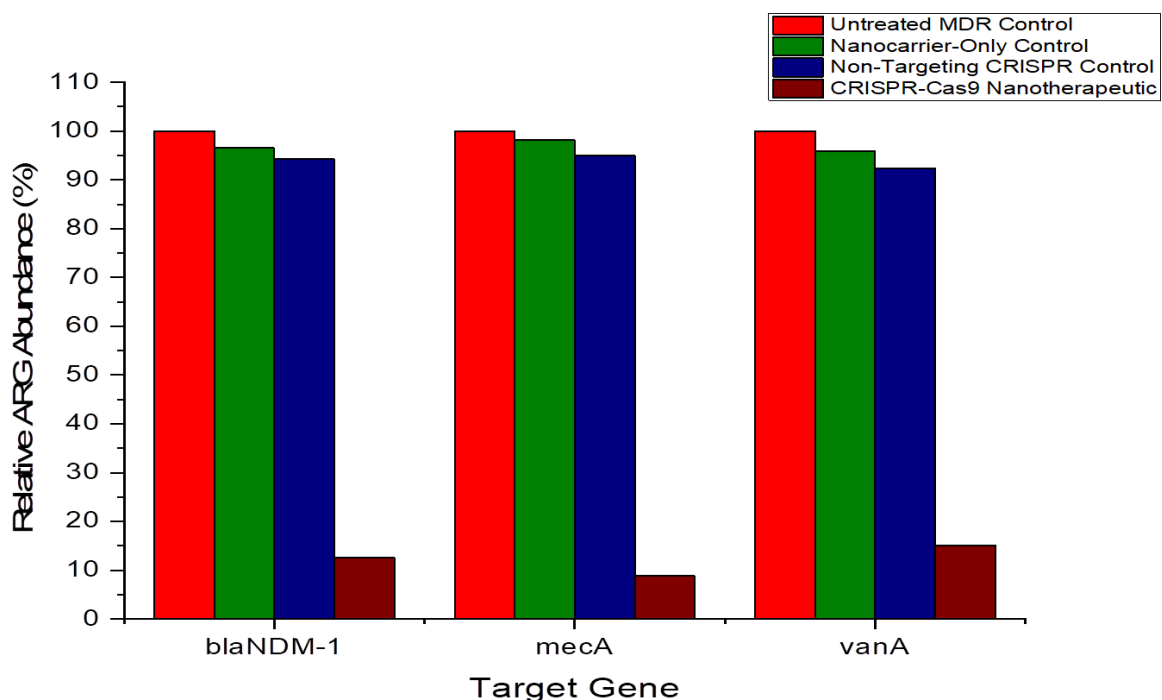


Figure 6. Evaluation of CRISPR-Cas9 nanotherapeutic activity across target ARGs.

CRISPR-Cas9 nanotherapeutic reduced the abundance of the target ARG

The bar chart illustrates the relative abundance of the target *bla* resistance gene under different experimental conditions, providing key molecular evidence for the efficacy of the proposed CRISPR-Cas9 nanotherapeutic system. The untreated MDR group was assigned a relative abundance of 100% and served as the reference control. In comparison, the empty-nanocarrier control and the non-targeting CRISPR control exhibited only minor reductions in *bla* abundance, to 95.5% and 91.2%, respectively. These findings indicate that the nanocarrier alone and the non-targeting CRISPR components had limited effects on the abundance of the target resistance gene. The relative abundance of the selected *bla* resistance gene following treatment with the CRISPR-Cas9 nanoparticles and control treatments is presented in Figure 7, demonstrating the effect of the targeted CRISPR-Cas9 intervention on the abundance of the resistance determinant.

In contrast, treatment with the complete CRISPR-Cas9 nanotherapeutic resulted in a pronounced reduction in *bla* abundance to 18.5% of the untreated MDR control level. Thus, the targeted treatment was associated with an approximately 81.5% reduction in the relative

abundance of the target gene compared with the untreated control. The substantially greater reduction observed following treatment with the sequence-specific CRISPR-Cas9 system, relative to both control conditions, supports the targeted activity of the nanotherapeutic against the selected *bla* resistance determinant.

Restoration of antibiotic susceptibility following ARG targeting

The grouped column graph illustrates the restoration of bacterial susceptibility to β -lactam antibiotics following the different treatments. The untreated MDR, nanocarrier, and non-targeting CRISPR control groups exhibited small inhibition zones (7.5–9.8 mm), consistent with the persistence of the resistant phenotype. In contrast, CRISPR-Cas9 treatment substantially increased the inhibition zones to 20.8–24.5 mm across ampicillin, amoxicillin, and ceftazidime. This marked phenotypic shift, together with the observed reduction in the target resistance gene, supports the effectiveness of CRISPR-Cas9-mediated targeting in restoring antibiotic susceptibility. The antibiotic resistance profile of the MDR *Pseudomonas* isolates before and after CRISPR-Cas9 nanotherapy is compared in Figure 8, demonstrating the changes in antibiotic susceptibility following targeted treatment.

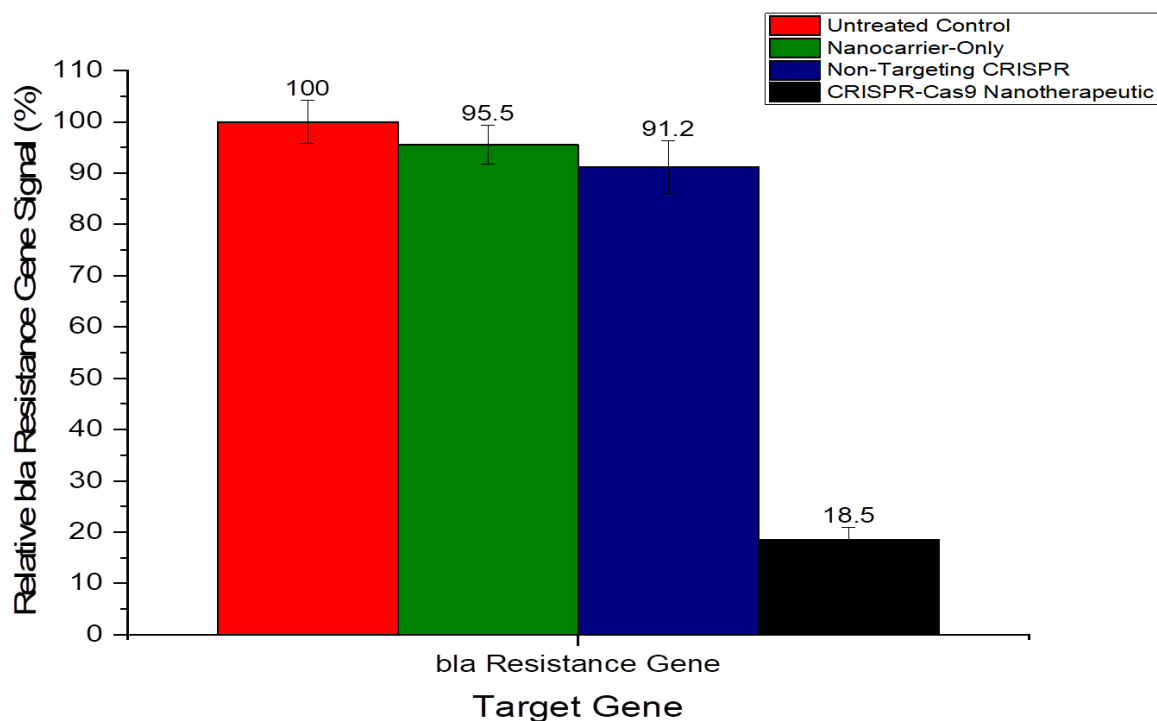


Figure 7. Relative abundance of the selected bla gene encoding resistance after treatment with the CRISPR-Cas9 nanoparticles and controls.

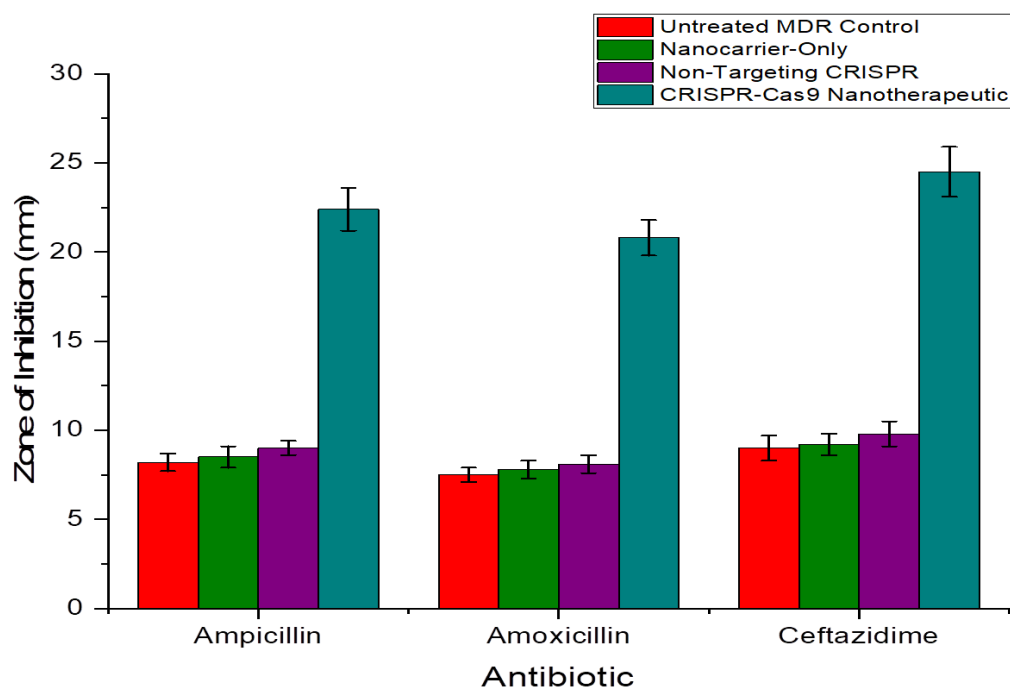


Figure 8. Antibiotic resistance of the MDR *Pseudomonas* isolate before and after administration of CRISPR-Cas9 nanotherapy.

Stability of the CRISPR-Cas9 nanotherapeutic under plant-associated conditions

The two-panel line graph illustrates the physical stability and cargo retention of the CRISPR-Cas9 nanotherapeutic over a 7-day period under plant-associated conditions. Figure (a) shows a slight increase in mean particle size from 130.0 to 138.5 nm, indicating minimal changes in nanoparticle size during incubation. Figure (b) demonstrates a modest decline in cargo retention from

100% to approximately 90.1%. Collectively, these findings indicate that the nanotherapeutic maintained its physicochemical integrity and substantial cargo retention over the 7-day period under the tested plant-associated conditions. The stability of the CRISPR-Cas9 nanotherapeutic under plant-associated environmental conditions over 7 days is presented in Figure 9, showing changes in particle size and cargo retention during the experimental period.

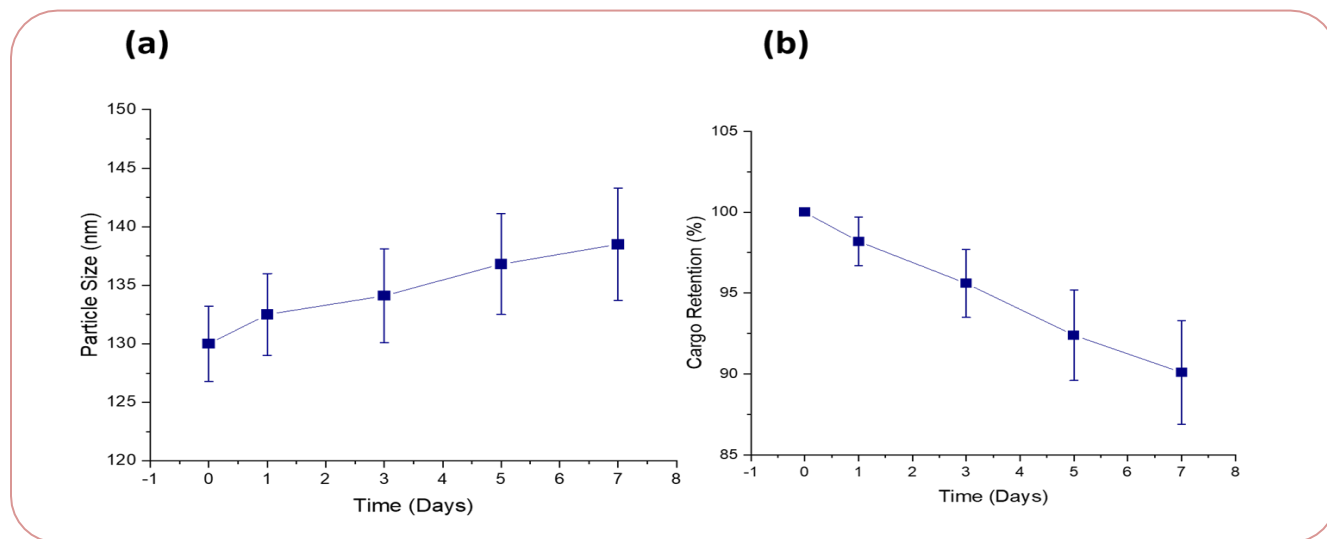


Figure 9. Stability of CRISPR-Cas9 nanotherapeutic in plant-associated environment over a period of 7 days showing (a) particle size (nm) and (b) cargo retention (%).

Proposed mechanism of CRISPR-Cas9 nanotherapeutic action

The schematic diagram illustrates the proposed three-step workflow for CRISPR-Cas9 nanotherapeutic targeting of an ARG in a tomato-associated MDR bacterium (Chen et al., 2023). Step 1 (isolation and characterization) involves collection of tomato samples, isolation and characterization of associated bacteria, antimicrobial susceptibility screening, and molecular identification of the selected MDR *Pseudomonas* isolate using 16S rRNA gene sequencing. Step 2 (target identification and nanotherapeutic design) comprises molecular screening for ARGs, selection of the *bla* resistance determinant and its target sequence, assembly of the CRISPR-Cas9 construct, and incorporation into a nanocarrier for delivery to the MDR bacterial cells. Step 3 (therapeutic effect and validation) depicts the proposed sequence-specific targeting of the selected *bla* locus by the Cas9-gRNA complex, leading to disruption or reduction of the target ARG and,

consequently, attenuation of the associated β -lactam-resistant phenotype and restoration of antibiotic susceptibility.

The control conditions further support target-specific activity: treatment with the nanocarrier alone or a non-targeting gRNA produced minimal changes in ARG abundance and antibiotic inhibition zones, whereas the complete CRISPR-Cas9 nanotherapeutic produced a pronounced molecular and phenotypic response. Collectively, the workflow links bacterial isolation and molecular characterization with target selection, nanotherapeutic delivery, and functional validation of ARG targeting. The proposed mechanism of action of CRISPR-Cas9 nanotherapy against the tomato-associated MDR *Pseudomonas* isolate is illustrated in Figure 10. The figure summarizes the workflow from bacterial isolation and molecular identification to selection of the target *bla* gene, CRISPR-Cas9-mediated gene targeting, reduction of antibiotic resistance, and potential restoration of antibiotic susceptibility.

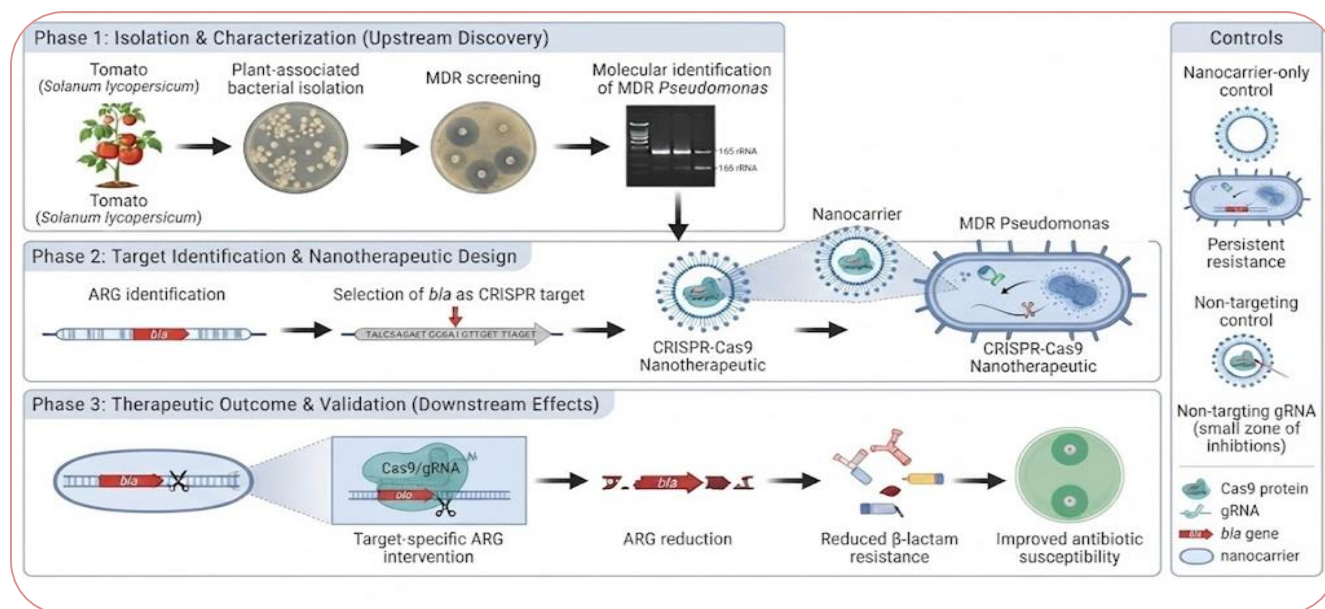


Figure 10. Proposed schematic diagram of the mechanism of action of CRISPR-Cas9 nano therapy against antibiotic resistance in a tomato-associated MDR bacterium. The complete protocol explains the series of events from the isolation of bacteria associated with plant to molecular identification of MDR *Pseudomonas* and target-specific identification of antibiotic resistance genes (*bla*). Eventually, the diagram elucidates the effects of this novel method, which includes the targeted ARG inhibition, downregulation of the resistance gene and resensitization of antibiotics.

Conclusion

This study provides proof-of-concept evidence for the potential of CRISPR-Cas9 nanotherapeutics as a precision strategy for targeting antibiotic resistance determinants in plant-associated MDR bacteria. Tomato samples yielded diverse culturable bacterial isolates, from which an MDR isolate was selected based on its antimicrobial susceptibility profile. Molecular characterization identified the isolate as closely affiliated with *Pseudomonas aeruginosa*. Molecular screening further identified a *bla*-type β -lactam resistance determinant, which was selected as the target for CRISPR-Cas9-mediated intervention. A target-specific guide RNA was designed based on sequence specificity, conservation, PAM compatibility, and predicted off-target potential and subsequently incorporated into a nanocarrier system.

The CRISPR-Cas9 nanotherapeutic markedly reduced the relative abundance of the target *bla* gene to 18.5% compared with 100% in the untreated MDR control. This molecular response was accompanied by substantial increases in inhibition zones against β -lactam antibiotics, indicating a corresponding improvement in antibiotic susceptibility. The minimal effects observed in the nanocarrier-only and non-targeting CRISPR controls further support the target-specific nature of the treatment.

Overall, these findings demonstrate the potential of CRISPR-Cas9 nanodelivery for targeted attenuation of antibiotic resistance determinants and restoration of antibiotic susceptibility in MDR bacteria. However, further studies under plant-associated and environmental conditions are required to establish nanotherapeutic stability, delivery efficiency, microbial selectivity, biosafety, and practical applicability in agricultural systems.

Authors' Contributions

MTA and AY conceptualized and designed the study. IM conducted the field investigations and performed the experimental work. WU provided overall supervision and ensured methodological and analytical consistency throughout the study. HR and AT served as co-supervisors, providing critical guidance on experimental design, data interpretation, and scientific refinement at various stages of the research. HR, AT, AA, and ZB contributed to the preparation of the initial manuscript draft, including content organization, compilation of results, and integration of preliminary interpretations. Subsequently, all authors critically reviewed and revised the manuscript, contributed to its intellectual refinement, verified the data and analyses, and approved the final version for submission.

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Conflict of Interest

The authors declare no conflict of interest.

Sustainable Development Goals Targeted

SDG 2: Zero Hunger

SDG 3: Good Health and Well-being

SDG 12: Responsible Consumption and Production

Policy Addressed

This study supports One Health AMR management by identifying plant-associated bacteria as potential resistance reservoirs and demonstrating CRISPR-Cas9 nanodelivery as a potential precision-based intervention. Agricultural application should be guided by rigorous assessment of biosafety, environmental fate, specificity, off-target effects, and ecological impacts.

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