

## CRISPR-CAS-BASED FUNCTIONAL GENOMICS FOR IDENTIFICATION OF GENETIC DETERMINANTS GOVERNING ANTIMICROBIAL RESISTANCE IN MULTIDRUG-RESISTANT BACTERIA

Arooj Khan<sup>\*1</sup>, Muhammad Medrar Hussain<sup>2</sup>, Saba Mubeen<sup>3</sup>,  
Rabia Siddiqi<sup>4</sup>, Sana Gul<sup>5</sup>,

<sup>\*1</sup>IMBB/CRIMM (Institute of Molecular Biology and Biotechnology/Centre for Research in Molecular Medicine),  
University of Lahore (UOL)

<sup>2</sup>Department of Health and Biological Sciences, Abasyn University Peshawar

<sup>3</sup>University of Agriculture Faisalabad

<sup>4,5</sup>Department of Biotechnology and Genetic Engineering, The University of Agriculture Peshawar

<sup>\*1</sup>arooj6098@gmail.com, <sup>2</sup>medrarbiotech@gmail.com

<sup>3</sup>sabamubeen639@gmail.com, <sup>4</sup>mas9798799@gmail.com, <sup>5</sup>sanagull887@gmail.com

DOI: <https://doi.org/10.5281/zenodo.23310754>

### Keywords

CRISPR-Cas; CRISPR  
interference (CRISPRi);  
antimicrobial resistance;  
multidrug-resistant bacteria;  
functional genomics; essential  
genes; synthetic lethality; chemical-  
genetic interactions; high-  
throughput screening

### Article History

Received: 01 August 2026

Accepted: 23 September 2026

Published: 12 October 2026

Copyright @Author

Corresponding Author: \*

Arooj Khan

### Abstract

The growing threat of antimicrobial resistance (AMR), particularly among multidrug-resistant bacteria, underscores the urgent need for innovative approaches to identify genetic vulnerabilities and potential therapeutic targets. Traditional methods, such as transposon insertion sequencing (Tn-seq), have significantly advanced our understanding of bacterial gene essentiality and the genetic determinants required for survival. They can't look at genes that are essential because changing them would kill the bacteria. CRISPR and CRISPR-associated (Cas) tools have changed the way scientists study genes by making it easier to control gene activity. CRISPR interference (CRISPRi) uses a version of Cas that doesn't cut DNA, called dCas to stop genes from working. This method works for both non-essential genes without breaking DNA. This review looks closely at CRISPR-Cas tools that help find out which genes are important for resistance. It explains how the active Cas systems work compared to the dCas ones and how CRISPRi and CRISPR activation (CRISPRa) can be used to test what happens when genes are turned off or on. The review also covers high-throughput methods for testing genes at once finding genes that work together in a bad way and looking at how genes interact with chemicals to find weak points in dangerous bacteria like *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus* and *Mycobacterium tuberculosis*. New techniques like using CRISPR tools at once testing all the genes on a plasmid and delivering CRISPR inside bacteria using modified viruses or other delivery systems are also discussed. The study ends with challenges like effects problems with gene groups, issues with how genes are turned on resistance that develops over time

and the body's immune response. Combining CRISPR tools, with research and better delivery systems could help find new ways to fight drug-resistant bacteria.

## INTRODUCTION

The fast spread of resistance (AMR) among bacterial pathogens is one of the most serious threats to modern medical care and public health worldwide. In 2019 bacterial AMR caused about 1.27 million deaths directly. Was linked to nearly 4.71 million deaths worldwide. If new treatments and new ways to find targets are not developed death rates could go above 10 million each year by 2050 (O'Neill, 2016).

Traditional discovery pipelines that rely on small-molecule screening have reached limits and cannot keep up with the evolving variety of multidrug-resistant (MDR) pathogens. The crisis worsens because resistance plasmids, transposons and integrative conjugative elements move quickly between bacterial families through horizontal gene transfer (HGT) (Johnson & Grossman 2015). Solving this treatment gap needs a system-wide

understanding of the genetic structure that controls bacterial fitness, natural resistance and conditional vulnerability. Traditional forward genetic methods, like random transposon insertion sequencing (Tn-Seq) have given insights, into how non-essential genes help bacteria tolerate antibiotics (van Opijnen & Camilli 2013).

Tn-seq cannot examine essential bacterial genes because knocking out a gene completely kills the bacteria (Todor et al., 2021). To fill this gap, CRISPR and Cas technologies, which come from immune systems have been turned into high-throughput tools for studying gene function. CRISPR interference (CRISPRi) specifically allows precise and adjustable silencing of genes and CRISPRi has broadened functional genomic screens to include essential genes and large-scale studies (Barquist et al. 2013).

## CRISPR-Cas9 Sensitization for Combating Antibiotic Resistance

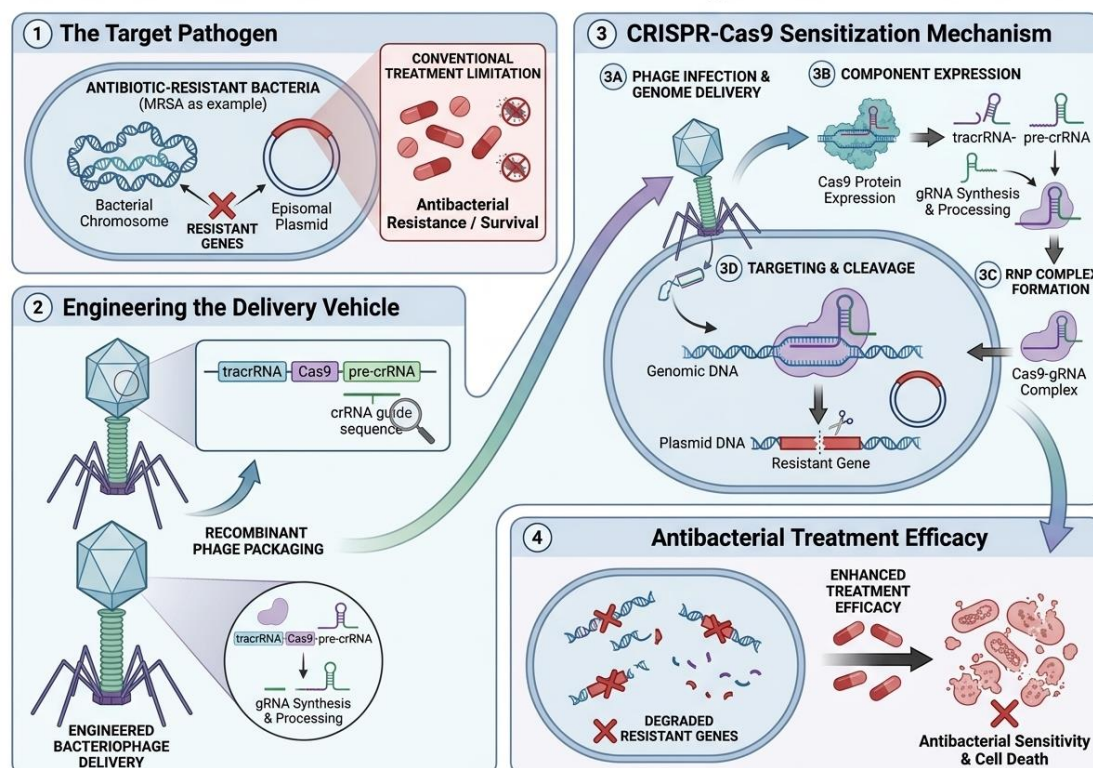


Figure 1: Overview of Diverse CRISPR-Cas Antimicrobial Strategies for Combating Antibiotic Resistance

### Mechanistic Divergence: Nuclease-Active Cas Proteins Versus Catalytically Inactive dCas Architectures

Crispr-cas adaptive immune systems are divided into Class 1 which uses many protein components and Class 2 which uses a single protein such as Cas9, Cas12a or Cas13. In cells Cas-induced double-stranded DNA breaks, known as DSBs are normally fixed by non-homologous end joining or NHEJ. This process creates insertions or deletions or indels which are perfect for knocking out genes (WHO, 2024). In contrast most bacteria do not have a working system or have repair systems that work poorly. As a result, when a wild-type Cas nuclease, for example Cas9, Cas12a or Cas3 cuts DNA in a chromosome or plasmid the damage is so severe that the cell dies of producing a mutation (Murray et al., 2022).

Wild-type Cas nucleases that cut DNA work as strong sequence-specific antimicrobials. They can

remove plasmids. Kill specific pathogens but because they kill cells, they cannot be used for large-scale genome screening. To fix these scientists change a letter in the active part of a Cas nuclease. For example, changing D10A and H840A, in *Streptococcus pyogenes* Cas9 turns it into a dead or dCas that no longer cuts DNA (Brown & Wright 2016).

### Principles of CRISPR Interference (CRISPRi) and CRISPR Activation (CRISPRa) in Bacteria

The dCas effector keeps its sequence-specific RNA-guided DNA-binding power while losing its cleavage ability. In CRISPR interference or CRISPRi a single-guide RNA or sgRNA guides the dCas protein to a target protospacer sequence that sits beside a required protospacer motif or PAM (Miethke et al. 2021). When the dCas-sgRNA pair targets the promoter region or the non-template coding strand of a gene the dCas-sgRNA complex

creates a block that stops RNA polymerase or RNAP from attaching or moving along the DNA template. This blockage turns off transcription. Unlike in eukaryotic CRISPRi systems that need repressor parts such as KRAB, the dCas9 alone can achieve strong transcriptional silencing in bacteria (Partridge et al., 2018).

On the hand CRISPR activation or CRISPRa uses dCas platforms to boost gene expression. By attaching dCas variants or sgRNA scaffolds called

scRNAs to activation modules like the N-terminal part of the RNA polymerase  $\alpha$ -subunit, RpoZ, the T4 phage protein AsiA or AraC/XylS family factors such, as SoxS, CRISPRa complexes draw host RNAP holoenzymes to weak or silent promoters. CRISPRi lets scientists knock down genes in a way while CRISPRa lets them turn genes on. Together these tools help map gene networks and uncover how resistance develops (Dorr et al., 2016).

**Table 1: Comparative Analysis of CRISPR-Cas Modalities in Bacterial Functional Genomics and AMR Research**

| System Platform /             | Effector Architecture                                     | Primary Mechanism                                           | Primary Functional Genomics / Therapeutic Application                          | Distinct Advantages & Limitations                                                                      |
|-------------------------------|-----------------------------------------------------------|-------------------------------------------------------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------|
| Nuclease-Active Cas9 / Cas12a | Single-protein Class 2 effector                           | Targeted DNA double-stranded break (DSB) cleavage           | Antimicrobial therapy, strain selection, plasmid curing                        | High killing efficiency in bacteria lacking NHEJ; limited utility for non-lethal functional screening. |
| Nuclease-Active Cas3          | Multi-protein Class 1 (Cascade) effector                  | Processive 3' → 5' single-stranded DNA degradation          | Broad chromosomal clearance, anti-plasmid therapeutics                         | Extensive genomic deletion capability; complex multi-protein expression requirements.                  |
| RNA-Targeting Cas13a          | Single-protein Class 2 effector                           | Single-stranded RNA cleavage and collateral RNA degradation | Non-DNA-altering antimicrobials, diagnostic assays                             | Avoids chromosomal DNA damage; collateral cleavage can induce non-specific dormancy or toxicity.       |
| CRISPR Interference (CRISPRi) | Catalytically dead Cas (dCas9/dCas12)                     | Steric inhibition of RNAP association or elongation         | High-throughput loss-of-function screens, essential gene vulnerability mapping | Non-lethal, tunable, reversible knockdowns; susceptibility to polar effects in polycistronic operons.  |
| CRISPR Activation (CRISPRa)   | dCas / gRNA fused to activator domains (SoxS, RpoZ, AsiA) | Recruitment of RNAP holoenzyme to promoter loci             | Gain-of-function screens, silent operon activation, efflux pump regulation     | Enables endogenous gene overexpression; strict promoter spacing and positioning constraints.           |

## **2. High-Throughput Screening Methodologies and Genomic Vulnerability Mapping**

### **2.1 Arrayed Versus Pooled CRISPRi-Seq Screening Workflows**

Bacterial CRISPRi screening strategies are carried out in either arrayed or pooled formats based on the needs of the assay including how much throughput's required and what kind of phenotyping is needed. In screens each knockdown strain is kept separate in multi-well plates. This allows for phenotypic analysis, such as measuring minimum inhibitory concentration using micro-broth dilution, high-content imaging to study bacterial cell shape and profiling of secreted proteins. However arrayed screening takes a lot of time and effort. It is hard to scale up to thousands of genes across multiple growth conditions (Barquist et al. 2013).

Pooled screening strategies solve these throughput issues by using libraries of synthetic oligonucleotides designed to produce sgRNA. In a pooled CRISPRi-seq setup a library of single-guide RNA spacers is made to target all known open reading frames, non-coding RNAs and regulatory regions. These spacers are inserted into expression vectors that can be turned on when needed. The library is then introduced into a population of the target pathogen that expresses a controlled dCas protein (Govers et al. 2017). Once induced the mutant population is grown under selective conditions—like low doses of clinical antibiotics host serum or media with limited nutrients. Genomic DNA is collected at the start and after selection. The 20-nucleotide sgRNA spacers, which act like barcodes are amplified and counted using next-generation sequencing (Todor et al., 2021). By comparing the normalized number of reads between control and treated samples researchers can spot genes whose knockdown gives a fitness advantage (seen as enrichment) or a fitness disadvantage (seen as depletion). This helps identify interactions, between drugs and genes (Shalem et al., 2014).

### **2.2 Interrogating the Essential and Conditionally Essential Genome**

Bacterial CRISPRi screening strategies can be performed in arrayed or pooled formats, chosen based on how many assays must run and what phenotypic tests are needed. Bacterial CRISPRi screening in arrayed format isolates each knockdown strain inside multi-well plates, which lets researchers do phenotypic tests such as measuring MIC by micro-broth dilution taking many images of bacterial cell shape and examining secreted proteins; however Bacterial CRISPRi screening in arrayed format is heavy work and hard to expand to thousands of genes in many growth conditions (Barquist et al. 2013). A big problem with bacterial genetics is that essential genes, which make up about ten to twenty percent of a bacterial genome cannot be studied systematically; when scientists delete or use transposon mutagenesis, they often get dead cells so the functions of essential genes in antibiotic resistance, cell envelope strength and metabolism remain poorly understood (Liu et al., 2024). CRISPRi solves the or-dead problem by making hypomorphic alleles, which are only partially inactive; by changing amounts of chemical inducers such as IPTG, aTc, L-rhamnose or L-arabinose that control dCas or sgRNA production researchers can dial repression from very low to very high (Wang, 2026). Moreover, scientists can insert one or two mismatches into the spacer, which lowers dCas binding and gives a steady intermediate knockdown without killing the cell; this approach lets researchers map vulnerabilities quantitatively showing how small drops in essential protein can lower MIC, for certain antibiotics (Qi et al., 2013).

### **2.3 Synthetic Lethality and Chemical-Genetic Interaction Landscapes**

When CRISPRi-mediated depletion of a bacterial gene works together with a low dose of a small-molecule antibiotic to kill cells the gene and the drug show a chemical-genetic interaction called synthetic lethality. Mapping these interactions over genomes creates the

chemical-genetic vulnerability landscape of a pathogen (Sullivan et al., 2026).

Chemical-genetic interaction mapping shows two types of therapeutic targets. Primary vulnerabilities are genes that encode the direct targets of an antimicrobial drug. In these cases CRISPRi-mediated suppression of gene expression reduces the amount of target protein. Lowers the amount of drug needed to stop the bacteria (Rachwalski, 2023). Secondary synergistic targets are conditionally essential parts of collateral pathways. These include DNA repair systems, cell envelope integrity pathways, two-component stress-response systems and core bioenergetics. When these parts are partially silenced the bacteria cannot use adaptations to survive drug pressure. Spotting these collateral nodes gives a plan, for antibiotic adjuvants. With adjuvants non-antibiotic small molecules block the CRISPRi-identified pathways and bring back the power of old antibiotics against resistant strains (Liu, 2023).

### **3. Uncovering Genetic Determinants of Resistance in Priority Pathogens**

#### **3.1 Gram-Negative Pathogens: *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli***

Negative ESKAPE pathogens have outer membrane barriers and active efflux networks that give them high intrinsic resistance. High-density CRISPRi screening in *Pseudomonas aeruginosa* strains PAO1 and PA14 using libraries with than 80,000 unique sgRNAs has produced high-resolution functional maps of the organism's genome. These screens have revealed metabolic nodes that control antibiotic susceptibility (Tse et al. 2025). For example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzymes have been found to be non-redundant points that control flux through glycolysis and gluconeogenesis. These enzymes help regulate persistence during chronic infections (Letizia et al. 2025).

In *P. Aeruginosa* CRISPRi screens found that ferredoxin-NADP<sup>+</sup> reductase B (fprB) is a target.

When fprB is depleted, the bacteria become more sensitive to gallium therapy, an antimicrobial that disrupts iron metabolism. Inducible CRISPRi systems in *P. Aeruginosa* using regulated rhamnose promoters (P<sub>rhaBAD</sub>) have shown that knocking down essential genes like dihydrofolate reductase (folA) or cell shape-determining proteins (mreB) greatly reduces the amount of trimethoprim needed to stop growth. This confirms that these systems are effective for validating drug targets (Tarasenko et al. 2025).

In Enterobacteriaceae, including *Escherichia coli* and *Klebsiella pneumoniae*, genome-wide CRISPRi screening showed that the homologous recombination pathway is a factor in antibiotic susceptibility. Silencing recA through CRISPRi, RecX-derived peptide fusions (RecX-20) or small-molecule inhibitors like cisplatin makes multidrug-resistant isolates more vulnerable to many different antibiotics. These include quinolones,  $\beta$ -lactams, aminoglycosides and nitrofurantoin. Blocking recA not increases the killing power of antibiotics but also stops the SOS response. This prevents stress-induced mutations. Slows the spread of resistance plasmids, through horizontal gene transfer (Sahu et al. 2026).

#### **3.2 Gram-Positive Pathogens: *Staphylococcus aureus* and *Enterococcus faecalis***

Gram-positive MDR pathogens depend largely on envelope remodeling and two-component signal transduction systems to stay strong against cell agents. In *Staphylococcus aureus* and in methicillin-resistant *S. Aureus*, MRSA scientists used genome-wide CRISPRi-seq libraries that hit both essential and core genome loci. Those studies have changed the map of which genes are vulnerable. When the bacteria faced dalbavancin stress—a lipoglycopeptide—CRISPRi screens in *S. Aureus* strain NCTC8325 found several envelope-related genes. Turning those genes off lowered MICs. Gave new ideas for drug combinations that work together (Bayer et al., 2013).

A big CRISPRi study in *S. Aureus* looked at 10 antibiotic classes. It found that essential genes play

a role in how antibiotics interact with the bacteria than non-essential ones. For example, if you lower the levels of genes that work on cell wall synthesis like tagO or on cell envelope charge like mprF or on the electron transport chain like qoxAB or on two-component systems like graRS and vraFG the bacteria become much more vulnerable to tiny doses of antibiotics (Haag & Bagnoli 2016). Another set of experiments where two genes were knocked down at once showed that lipid metabolic pathways can push the bacteria in directions. Reducing the pathway made the bacteria more prone to gentamicin while reducing fatty acid biosynthesis made them take up less aminoglycoside and become more tolerant (Worthington et al., 2013).

In *Enterococcus faecalis* researchers used CRISPRi screens to measure how much plasmid and chromosome resistance genes matter. When they turned off loci, like tetM, which gives tetracycline resistance by protecting the ribosome or ermB which gives macrolide-lincosamide-streptogramin B resistance by methylating the ribosome the bacteria became sensitive again. This shows that CRISPR loci can stop or limit the spread of resistance genes (Draper

et al., 2015).

### 3.3 Mycobacteria and Plasmid-Borne Resistance Determinants

In *Mycobacterium tuberculosis* the slow growth and thick waxy mycomembrane make traditional drug testing difficult. Large-scale CRISPRi screening in *M. Tuberculosis* has shown which essential genes are most vulnerable. When the essential two-component regulator mtrAB is partially turned down the cell envelope becomes more porous. This change makes drug-resistant strains sensitive again to treatments like rifampicin, vancomycin and linezolid (Li et al., 2022).

Beyond targeting the chromosome CRISPRi screens have also been used to study multidrug-resistant plasmids. A good example is pOXA-48, found in *Enterobacterales*. By repressing genes on the plasmid researchers can tell apart the cost of carrying the plasmid from the actual function of resistance genes like bla\_OXA-48. These studies show that turning off plasmid regulatory systems can lower the fitness burden, on the host or even remove resistance to carbapenems without removing the plasmid itself (Goff et al., 2020).

**Table 2: Key Genetic Determinants and Vulnerability Targets Identified via CRISPR Screening Across Pathogens**

| Target Organism               | Target Gene / Pathway                            | CRISPR Strategy      | Associated Antibiotic / Stress                        | Phenotypic Outcome & Mechanism                                                     |
|-------------------------------|--------------------------------------------------|----------------------|-------------------------------------------------------|------------------------------------------------------------------------------------|
| <i>Escherichia coli</i>       | recA / Homologous Recombination                  | CRISPRi (dCas9)      | Quinolones, $\beta$ -lactams, Aminoglycosides         | Broad-spectrum resensitization; suppression of SOS response and HGT.               |
| <i>Pseudomonas aeruginosa</i> | fprB (Ferredoxin-NADP+ Reductase B)              | High-density CRISPRi | Gallium inorganic therapy                             | Synergistic lethality due to collapse of iron bioenergetics and redox homeostasis. |
| <i>Pseudomonas aeruginosa</i> | GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) | Pooled CRISPRi-seq   | Carbon source transition (Glycolytic / Gluconeogenic) | Disruption of metabolic persistence during chronic pulmonary infection modes.      |

|                                   |                                                            |                               |                                           |                                                                                                     |
|-----------------------------------|------------------------------------------------------------|-------------------------------|-------------------------------------------|-----------------------------------------------------------------------------------------------------|
| <i>Staphylococcus aureus</i>      | <i>mprF</i> , <i>tagO</i> , <i>qoxAB</i> ,<br><i>graRS</i> | Genome-wide<br>CRISPRi        | Dalbavancin,<br>Gentamicin,<br>Vancomycin | Disruption of<br>envelope charge<br>balance, cell wall<br>assembly, and<br>bioenergetics.           |
| <i>Mycobacterium tuberculosis</i> | <i>mtrAB</i> (Two-<br>Component System)                    | Essential-<br>gene<br>CRISPRi | Rifampicin,<br>Vancomycin,<br>Linezolid   | Increased cell<br>envelope permeability<br>leading to multi-class<br>antibiotic<br>resensitization. |
| Enterobacteriaceae                | <i>bla</i> <sub>OXA-48</sub> / pOXA-<br>48 Backbone        | Plasmid-<br>wide<br>CRISPRi   | Carbapenems<br>(Ertapenem,<br>Meropenem)  | Elimination of<br>carbapenem<br>resistance and<br>mitigation of plasmid<br>fitness costs.           |

#### 4. Advanced CRISPR Technologies and Emerging Modalities

##### 4.1 CRISPR Activation (CRISPRa) for Gain-of-Function Profiling and Regulatory Rewiring

While CRISPRi gives us loss-of-function data knowing how bacteria resist threats also means looking at gain-of-function mechanisms. These include gene duplication, promoter activation or increased activity of efflux pumps. Using CRISPRa in bacteria comes with biochemical challenges. These are different from what we see in systems. The main problem is spatial. Activation domains must bind in the right place near promoter elements to get host RNA polymerase to work properly (Dong et al., 2018). To get past these structural issues synthetic biologists have built many different bacterial CRISPRa systems. Early designs fused dCas proteins directly to subunits of the host RNA polymerase. One example is the image subunit or even the bacteriophage T4 transcription factor AsiA. Improved versions like dCas9-AsiA have shown up to 200fold increases in gene activation across both chromosomal and plasmid locations (Fontana et al. 2020). Another way is to use scaffold RNAs or scRNAs that have special RNA sequences added. Like MS2 hairpins. To grab proteins that bring activation domains. A known system is MCP-SoxS. It turns on genes at image-dependent promoters without needing to change

the host RNA polymerase. More recent developments include effectors based on PspFDHHTH which can activate image- promoters. This opens up bacterial regulons for targeting (Ho et al., 2020).

In genomics bacterial CRISPRa helps run forward screens to find hidden or low-expression resistance genes. By turning up genes for transporters, transcription factors and metabolic enzymes these screens can reveal efflux pumps. Such as those controlled by *marA* or *soxS*. That gives resistance when they are overactive. CRISPRa has also been used in actinobacteria, like *Streptomyces*. Here it activates biosynthetic gene clusters by targeting the regulators right inside these clusters. This has led to discoveries of natural product antibiotics (Bikard et al. 2013).

##### 4.2 Multiplexed and Dual-CRISPR Repression and Activation Systems

Bacterial adaptation is often governed by genetic redundancy and complex epistatic networks, wherein single-gene knockouts yield minimal phenotypic changes due to compensatory pathways. To dissect these redundant systems, multiplexed CRISPR platforms utilize arrayed sgRNA cassettes or alternative Cas effectors (such as Cas12a, which inherently processes its own precursor crRNA array) to silence or activate

multiple genes simultaneously (Ameruoso et al., 2022).

Dual-CRISPRi libraries in *S. aureus* have mapped epistatic interactions across essential metabolic pathways, revealing synthetic lethal gene pairs within cell envelope biosynthesis pathways. Advanced platforms can also combine CRISPRi and CRISPRa within the same cell using distinct orthogonal dCas proteins (e.g., *S. pyogenes* dCas9 for repression and *Streptococcus thermophilus* dCas9 or *Lachnospiraceae* dCas12a for activation). These hybrid systems allow simultaneous down-regulation of resistance determinants and up-regulation of metabolic vulnerabilities, enabling fine-grained mapping of bacterial genetic networks (Bikard et al., 2013).

#### 4.3 Delivery Systems, In Vivo Screening, and Translational Horizons

Bringing CRISPR-based genomics into real therapies depends on delivery platforms that can work inside complex body environments. I find that Mobile-CRISPRi systems have been made using wide-host-range conjugative transposons such as Tn7 or ICE-based platforms. These systems put dCas modules directly into the chromosomes of clinical isolates that are not typical model bacteria both Gram-negative and

Gram-positive and they skip the need for strain-specific transformation methods (Todor et al. 2021).

Engineered bacteriophage vectors act as efficient species-specific carriers that deliver CRISPR constructs straight into the target bacteria. Another option is to use plasmid delivery with harmless donor bacteria for example probiotic *Escherichia coli* strains. These donors move CRISPR-encoding plasmids into the pathogens by high-frequency conjugation inside the gut or at specific mucosal sites. Non-viral methods, such as nanoparticles that carry Cas mRNA guide RNA or ready-made ribonucleoprotein complexes provide a way to deliver CRISPR to specific soft-tissue and lung infections (Zhang et al. 2017).

In animal tests Mobile-CRISPRi screens in mice with thigh, wound and lung infections have started to find virulence and fitness factors that only show up when the bacteria live inside the host. I think that these discoveries help move CRISPR approaches into clinical studies (Enright et al., 2024). Key examples are SNIPR001, which is a mix of CRISPR-guided bacteriophages aimed at killing fluoroquinolone- *E. Coli* in the human gut and is now in Phase I/II trials (NCT05277350) and LBP-EC01, a CRISPR-Cas9 engineered phage that targets urinary tract *E. Coli* and is, in Phase 2/3 trials (NCT05488444) (Prasad, 2022).

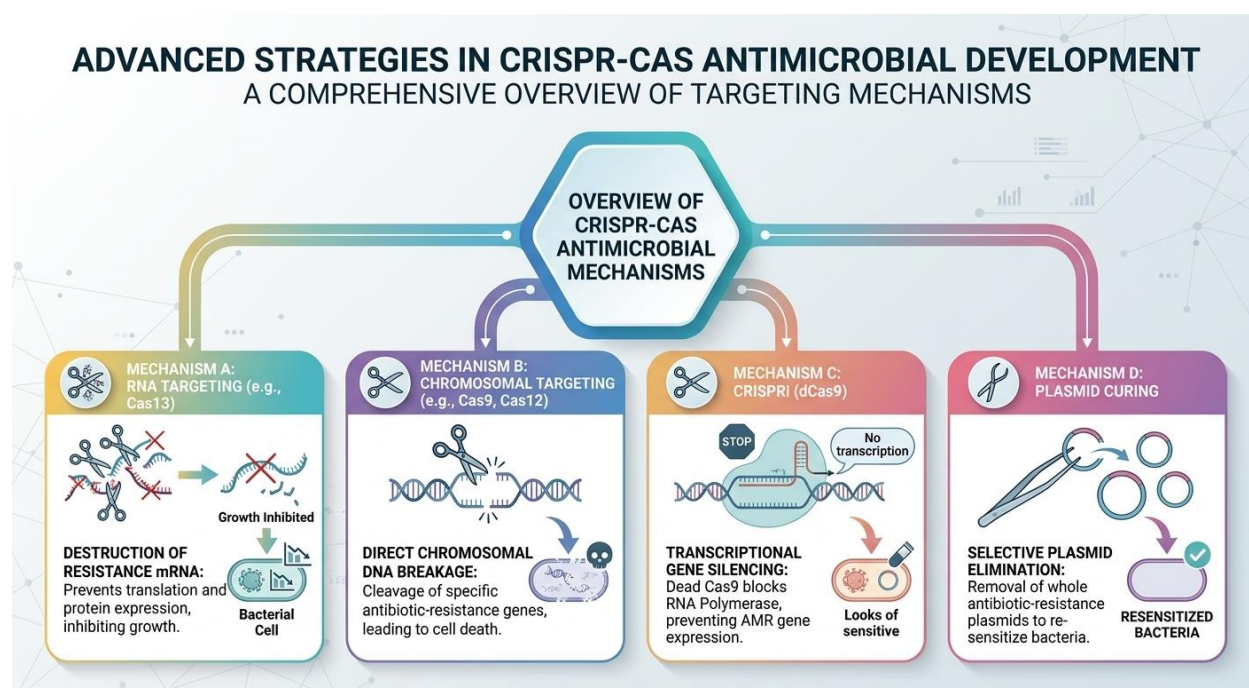


Figure 4: Schematic of Engineered Phage-Delivered CRISPR-Cas9 Re-Sensitization of Drug-Resistant Bacteria

## 5. Challenges, Limitations, and Future Perspectives

### 5.1 Off-Target Effects, Polar Effects in Operons, and Inducible Promoter Leakiness

Despite its ability to analyze genes CRISPR-based functional genomics comes with clear technical challenges that need careful planning in experiments. One major issue is off-target guide binding. This happens when the sgRNA spacer binds to the parts of the genome by mistake. Such misbinding can cause gene repression and lead to false signals about gene fitness. To reduce this problem researchers, use prediction tools to pick sgRNA spacers and work with engineered Cas proteins that are more specific to their target sequences (Kalter et al. 2025).

Another big challenge appears in operons, where multiple genes are grouped together and read as one mRNA. When dCas blocks transcription in such a setup it stops RNA polymerase from moving. This affects all the genes downstream even those not linked to the trait being studied. Because of this it's hard to know which gene is

really responsible for resistance. To solve these scientists must check results again using methods like adding back genes or placing new promoters inside the operon to test gene-specific roles.

A third issue is promoter expression. When promoters are not fully controlled, they allow low levels of dCas protein to be made even when it should not be active. This can cause loss of essential genes or harm cells even before the experiment starts. Using more tightly regulated systems, like P\_rhaBAD helps keep dCas levels low until needed reducing unwanted effects (Lotfi et al. 2025).

### 5.2 Evolutionary Counter-Resistance and Host Immune Responses

The clinical implementation of therapeutic CRISPR systems also encounters significant biological barriers. Target mutation and escape represent immediate challenges, as single-nucleotide substitutions within the target protospacer or adjacent PAM region permit bacteria to evade dCas binding. Furthermore, loss-

of-function mutations within Cas genes themselves or within vector entry receptors such as surface protein alterations conferring phage resistance allow mutant sub-populations to survive (Johnson & Grossman, 2015).

Endogenous bacterial defenses, including anti-CRISPR (Acr) proteins encoded by prophages and mobile elements, can directly bind and neutralize Cas effectors. Additionally, host immunogenicity poses a challenge for systemic delivery, as pre-existing neutralizing antibodies and adaptive T-cell responses against Cas proteins derived from *S. pyogenes* or *S. aureus* can compromise therapeutic efficacy (Partridge et al., 2018).

### Conclusion

CRISPR-Cas-based functional genomics has changed the way we study resistance. It gives researchers a flexible and precise way to explore the genes of bacterial pathogens. With the creation of catalytically Cas proteins scientists can now study genes that were once considered too important to disrupt. This has allowed them to map out spots in the genomes of many different bacteria. CRISPRi screens have helped identify drug targets and also uncovered hidden pathways that work together in ways that make bacteria more vulnerable. These screens reveal networks of gene interactions linked to drug response. When paired with CRISPRa for activating genes and multiplexed systems to study how genes interact these tools allow scientists to see the workings of antibiotic resistance with much greater clarity. These discoveries are starting to move toward treatments but there are still big challenges to overcome. Getting the CRISPR systems into the cells is difficult. Off-target effects could harm cells. Bacteria may evolve resistance again. The human immune system might react in ways that reduce effectiveness. Still progress is being made. Bacteriophage-based delivery conjugative plasmids and lipid nanoparticles have shown success in animal models. Some CRISPR-guided antimicrobials are now in trials marking a major turning point. Looking ahead combining CRISPR genomics with artificial intelligence single-cell

sequencing and multi-omics data will make target discovery faster and more accurate. As CRISPR tools become more refined and funding supports research there is real hope to renew the antibiotic discovery pipeline. This could lead to solutions, for the global antimicrobial resistance crisis.

### REFERENCES

- Ameruoso, A., Villegas Kcam, M. C., Cohen, K. P., & Chappell, J. (2022). Activating natural product synthesis using CRISPR interference and activation systems in *Streptomyces*. *Nucleic Acids Research*, 50(13), 7751-7760.  
<https://doi.org/10.1093/nar/gkac556>
- Barquist, L., Boinett, C. J., & Cain, A. K. (2013). Approaches to querying bacterial genomes with transposon-insertion sequencing. *RNA Biology*, 10(7), 1161-1169.  
<https://doi.org/10.4161/rna.24765>
- Bayer, A. S., Schneider, T., & Sahl, H. G. (2013). Mechanisms of daptomycin resistance in *Staphylococcus aureus*: role of the cell membrane and cell wall. *Annals of the New York Academy of Sciences*, 1277(1), 139-158.
- Bikard, D., Jiang, W., Samai, P., Hochschild, A., Zhang, F., & Marraffini, L. A. (2013). Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Research*, 41(15), 7429-7437.  
<https://doi.org/10.1093/nar/gkt520>
- Brown, E. D., & Wright, G. D. (2016). Antibacterial drug discovery in the resistance era. *Nature*, 529(7586), 336-343.  
<https://doi.org/10.1038/nature17042>
- Dong, C., Fontana, J., Patel, A., Carothers, J. M., & Zalatan, J. G. (2018). Synthetic CRISPR-Cas gene activators for transcriptional reprogramming in bacteria. *Nature Communications*, 9, 2489.  
<https://doi.org/10.1038/s41467-018-04901-6>

- Dorr, T., Lewis, K., & Vulić, M. (2016). Antibiotic tolerance in *Escherichia coli* and the mechanism of persistence. *Current Opinion in Microbiology*, 29, 76–83.
- Draper, L. A., Cotter, P. D., Hill, C., & Ross, R. P. (2015). Lantibiotic resistance. *Microbiology and Molecular Biology Reviews*, 79(2), 171-191.
- Enright, A. L., Heelan, W. J., Ward, R. D., & Peters, J. M. (2024). CRISPRi functional genomics in bacteria and its application to medical and industrial research. *Microbiology and Molecular Biology Reviews*, 88(2), e00170-22.
- Fontana, J., Dong, C., Kiattisewee, C., Chavali, V. P., Tickman, B. I., Carothers, J. M., & Zalatan, J. G. (2020). Effective CRISPRa-mediated control of gene expression in bacteria must overcome strict target site requirements. *Nature Communications*, 11, 1618. <https://doi.org/10.1038/s41467-020-15454-y>
- Goff, A., Cantillon, D., Muraro Wildner, L., & Waddell, S. J. (2020). Multi-omics technologies applied to tuberculosis drug discovery. *Applied Sciences*, 10(13), 4629.
- Govers, S. K., Mortier, J., Adam, A., & Aertsen, A. (2017). Intrinsic growth heterogeneity and antibiotic tolerance are both core phenotypes of bacterial populations. *Proceedings of the National Academy of Sciences*, 114(51), 13162–13167.
- Haag, A. F., & Bagnoli, F. (2016). The role of two-component signal transduction systems in *Staphylococcus aureus* virulence regulation. *Staphylococcus aureus: microbiology, pathology, immunology, therapy and prophylaxis*, 145-198.
- Ho, H.-I., Fang, J. R., Cheung, J., & Wang, H. H. (2020). Programmable CRISPR-Cas transcriptional activation in bacteria. *Molecular Systems Biology*, 16(7), e9945. <https://doi.org/10.15252/msb.20199427>
- Johnson, C. M., & Grossman, A. D. (2015). Integrative and conjugative elements (ICEs): What they do and how they work. *Annual Review of Genetics*, 49, 577–601. <https://doi.org/10.1146/annurev-genet-112414-055018>
- Kalter, N., Fuster-Garcia, C., Silva, A., Ronco-Diaz, V., Roncelli, S., Turchiano, G., ... & Hendel, A. (2025). Off-target effects in CRISPR-Cas genome editing for human therapeutics: Progress and challenges. *Molecular Therapy Nucleic Acids*.
- Letizia, M., Diggle, S. P., & Whiteley, M. (2025). *Pseudomonas aeruginosa*: ecology, evolution, pathogenesis and antimicrobial susceptibility. *Nature Reviews Microbiology*, 23(11), 701-717.
- Li, S., Poulton, N. C., Chang, J. S., Azadian, Z. A., DeJesus, M. A., Ruecker, N., ... & Rock, J. M. (2022). CRISPRi chemical genetics and comparative genomics identify genes mediating drug potency in *Mycobacterium tuberculosis*. *Nature microbiology*, 7(6), 766-779.
- Liu, H., Silvis, M. R., & Gross, C. A. (2024). CRISPRi functional genomics in bacteria and its application to medical and industrial research. *Microbiology and Molecular Biology Reviews*, 88(2), e00170-22.
- Liu, R. X. (2023). *Target-based synergy discovery using CRISPRi* (Doctoral dissertation, University of Auckland).
- Liu, Y., Wan, X., & Wang, B. (2019). Engineered CRISPRa enables programmable eukaryote-like gene activation in bacteria. *Nature Communications*, 10, 3693. <https://doi.org/10.1038/s41467-019-11479-0>
- Lotfi, M., Farshchian Yazdi, Z., Hashemi, M. R., Abbaszadegan, M. R., & Sharif, S. (2025). Guarding the genome: Strategies for off-target detection and minimization in CRISPR/Cas9 system. *Expert Review of Molecular Diagnostics*, 25(12), 883-902.

- Miethke, M., Pieroni, M., Weber, T., Brönstrup, M., Hammann, P., Halby, L., Arimondo, P. B., Glaser, P., Aigle, B., Bode, H. B., Morel, F., et al. (2021). Towards the sustainable discovery and development of new antibiotics. *Nature Reviews Chemistry*, 5, 726–749.
- Murray, C. J. L., Ikuta, K. S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S. C., Browne, A. J., Chipeta, M. G., Fell, F., Hackett, S., Haines-Woodhouse, G., Hamadani, B. H. K., Kumaran, E. A. P., McManigal, B., et al. (2022). Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis. *The Lancet*, 399(10325), 629–655. [https://doi.org/10.1016/S0140-6736\(21\)02724-0](https://doi.org/10.1016/S0140-6736(21)02724-0)
- O'Neill, J. (2016). *Tackling drug-resistant infections globally: Final report and recommendations*. Review on Antimicrobial Resistance.
- Partridge, S. R., Kwong, S. M., Firth, N., & Jensen, S. O. (2018). Mobile genetic elements associated with antimicrobial resistance. *Clinical Microbiology Reviews*, 31(4), e00088-17. <https://doi.org/10.1128/CMR.00088-17>
- Prasad, N. (2022). *A pooled CRISPRi screen to probe Pseudomonas aeruginosa gene vulnerability during murine lung infection and phage predation* (Doctoral dissertation, University of California, San Francisco).
- Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P., & Lim, W. A. (2013). Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell*, 152(5), 1173–1183. <https://doi.org/10.1016/j.cell.2013.02.022>
- Rachwalski, K. (2023). *Understanding Bacterial Stress Responses to Find New Antibiotics* (Doctoral dissertation).
- Sahu, A., Kumar, A., Vaidya, A., Mishra, J., Mishra, S., & Prajapati, S. K. (2026). Decoding the Silent Conversations: Targeting Quorum Sensing to Disarm Bacterial Pathogens in the Age of Antimicrobial Resistance. *RSC Medicinal Chemistry*.
- Shalem, O., Sanjana, N. E., Hartenian, E., Shi, X., Scott, D. A., Mikkelsen, T., Heckl, D., Ebert, B. L., Root, D. E., Doench, J. G., & Zhang, F. (2014). Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science*, 343(6166), 84–87. <https://doi.org/10.1126/science.1247005>
- Sullivan, J. R., Ferrara, K. M., Barrick, R., Romano, K. P., Warriar, T., & Hung, D. T. (2026). An inducible CRISPRi system for phenotypic analysis of essential genes in *Pseudomonas aeruginosa*. *Mbio*, 17(3), e02767-25.
- Tarasenko, A., Papudeshi, B. N., Grigson, S. R., Mallawaarachchi, V., Hutton, A. L., Warner, M. S., ... & Edwards, R. A. (2025). Reprogramming resistance: phage-antibiotic synergy targets efflux systems in ESKAPEE pathogens. *MBio*, 16(10), e01822-25.
- Todor, H., Silvis, M. R., Osadnik, H., & Gross, C. A. (2021). Bacterial CRISPR screens for gene function. *Current Opinion in Microbiology*, 59, 102–109. <https://doi.org/10.1016/j.mib.2020.11.005>
- Tse, M. W., Zhu, M., Peters, B., Hamami, E., Chen, J., Davis, K. P., ... & Blainey, P. C. (2025). Large-scale combination screens reveal small-molecule sensitization of antibiotic-resistant gram-negative ESKAPE pathogens. *Proceedings of the National Academy of Sciences*, 122(13), e2402017122.

van Opijnen, T., & Camilli, A. (2013). Transposon insertion sequencing: A new tool for systems-level analysis of microorganisms. *Nature Reviews Microbiology*, 11, 435-442. <https://doi.org/10.1038/nrmicro3033>

Wang, Y. (2026). *Engineering a Molecular Dimmer Switch to Quantify Temporal Gene Dosage Sensitivity and Essentiality*. University of California, San Francisco.

World Health Organization. (2024). *Antimicrobial resistance: Global report and key facts*. World Health Organization.

Worthington, R. J., Blackledge, M. S., & Melander, C. (2013). Small-molecule inhibition of bacterial two-component systems to combat antibiotic resistance and virulence. *Future medicinal chemistry*, 5(11), 1265-1284.

Zhang, M. M., Wong, F. T., Wang, Y., Mao, Y., Nakashima, N., & Hang, L. (2017). CRISPR-Cas9 strategy for activation of silent *Streptomyces* biosynthetic gene clusters. *Nature Chemical Biology*, 13, 607-609. <https://doi.org/10.1038/nchembio.2341>