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ORIGINAL ARTICLE

In-Silico Systems Biology Analysis of the *Klebsiella spallanzanii* Defensome Against Bacteriophages

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ABSTRACT

Introduction: A common source of nosocomial infections, *Klebsiella pneumoniae* has a high morbidity and fatality rate, particularly in immunocompromised patients and infants. Public health is seriously threatened by its multidrug-resistant (MDR) strains. Despite therapy, the death rate from hospital-acquired pneumonia caused by *K. pneumoniae* is higher than 50%. The newly discovered species *Klebsiella spallanzanii* (Ko3 phylogroup) and *K. oxytoca* have recently become clinical concerns. Bacteriophage therapy is a promising alternative in light of the growing antibiotic resistance, however its effectiveness may be hampered by bacterial defence mechanisms. In order to improve phage therapy tactics, this work attempts to describe the defence mechanisms of *K. spallanzanii*. **Methods:** The three webservers PADLOC, CRISPRCasFinder, and DefenseFinder were used to analyse the defence mechanisms of *Klebsiella spallanzanii*, which was isolated from a Chinese patient suffering from urinary tract infections. DefenseFinder checks for a variety of defence mechanisms, CRISPRCasFinder finds CRISPR-Cas systems, and PADLOC finds prokaryotic antiviral defence systems. These methods were used to map and categorise the strain's defence mechanisms using genomic data. **Results:** *Klebsiella spallanzanii* has a strong anti-phage arsenal, as evidenced by the discovery of a CRISPR-Cas system and seven other defence mechanisms, including as restriction-modification, abortive infection, and toxin-antitoxin systems. **Interpretation & conclusions:** The discovery of seven additional defence systems, including CRISPR-Cas, indicates that *K. spallanzanii* has a variety of defence mechanisms against phage invasion, which may limit the effectiveness of bacteriophage therapy. These results demonstrate how intricate bacterial defences are, requiring specialised phage designs to avoid or interfere with these systems.

Keywords: *Klebsiella pneumoniae*, *K. oxytoca*, *Klebsiella spallanzanii*, Immunocompromised individuals, PADLOC webserver, CRISPRCasFinder

INTRODUCTION

The birth of Next Generation Sequencing (NGS) techniques allowed extensive sequencing of microbial genomes. The comparative genomics studies of these organisms revealed the dynamic nature of prokaryotic genome. Horizontal gene transfer (HGT), which restructures and expands the gene pool for selection, is the cause of this plasticity in microbial evolution. Genetic material can spread by transformation, transduction, and conjugation, and the main mobile genetic elements (MGEs) that support horizontal gene transfer (HGT)

include viruses and bacteriophages, plasmids, transposons, and integrative conjugative elements (ICEs). These elements provide their hosts adaptive features like either virulence or antibiotic resistance, many MGEs increase their fitness[1].

The interactions between bacterial genomes and MGEs have multiple facets. Among these interactions, parasitic interaction was studied for a long time. For instance, parasitic virulent phages lyse their hosts after infections, which releases new virions for further infections. However, temperate phages and plasmids, for instance, can live steadily inside bacteria and confer mutualistic features, they come with costs associated with conjugation, proliferation, and self-serving maintenance through post-segregational death. Finally, by using phage "superinfection exclusion" and other incompatibility or competition mechanisms, MGEs can restrict the acquisition of additional MGEs with potentially advantageous features[2].

Prokaryotes have a diverse range of defense mechanisms that function at various phases of MGE invasion in order to control MGEs. Although surface receptor mutation or alteration is a first line of defense, it usually comes at a fitness cost since it interferes with biological functions of the host cells. Additionally, prokaryotes can also conceal receptors by generating alginate, exopolysaccharides, or capsules, or they can control receptor expression in response to particular environmental cues. As an alternative, several bacteria generate outer membrane vesicles with receptors that bind phages and decrease productive infections, or proteins that bind receptors and stop phage attachment[3].

Moreover, prokaryotes have developed specialised defense mechanisms that are collectively referred to as the "***prokaryotic immune system***.". Over the brief evolutionary periods, bacterial cells often encode several defense mechanisms that are capable of aborting infections from phages. The majority of genomes (78%) encode at least two known defense systems, with an average of six. The distribution of these systems varies among species, with some having dozens and others having none[4].

Defense systems are often found co-located in genomic regions termed “defense islands,” although the reasons for their clustering remain unclear. Their frequent association with phages, phage satellites, ICEs, plasmids, and transposons suggests that genomic clustering may facilitate horizontal dissemination of defense systems. Furthermore, defense islands may emerge due to synergy between defense systems and/or a need for coordinated regulation. However, certain defense systems may exhibit antagonistic relationships that prevent their stable co-existence but incompatibility can be overcome by mechanisms such as epigenetic silencing[5].

Klebsiella spallanzanii is a gram-negative, non-motile, and encapsulated bacterium and it is a member of the *Klebsiella oxytoca* species complex. It was first identified as a novel species (phylogroup Ko3) in 2019 and was given the name Lazzaro Spallanzanii in honour of the Italian researcher. This organism is frequently found in environmental sources such soil, water, and plants. It also functions as a commensal in both human and animal gastrointestinal tracts. Human urine, faecal samples, and farm environments—including cow feces—have all yielded strains[6].

K. spallanzanii is an opportunistic pathogen that affects human health, especially in medical environments. It frequently affects immunocompromised people or those with underlying diseases like diabetes or long-term antibiotic use. It also leads to bacteraemia, urinary tract infections, and antibiotic-associated hemorrhagic colitis. It is linked to nosocomial (hospital-acquired) infections and has the potential to develop antibiotic resistance, which could make treatment more difficult and fuel outbreaks, just like other *Klebsiella* species[7].

In this work, we downloaded *Klebsiella spallanzanii* reference genome sequences from the GenBank for the existence of the defensome. We then further studied the CRISPR-Cas systems and their spacers that were found.

METHODOLOGY

To find and describe CRISPR-Cas systems in the *Klebsiella spallanzanii* genome, this work used a computational method. Identifying the CRISPR-Cas system and acquiring genomic data were the two stages of the methodology.

Genomic Data Acquisition

The National Centre for Biotechnology Information (NCBI) database provided the full genomic sequence of *Klebsiella spallanzanii* strain SB6411. GCA_902158555.1 (genomic assembly) and GCF_902158555.1 (RefSeq assembly) were the specific accession numbers that were utilised. To guarantee that the entire sequence information was accessible for further analysis, the data was downloaded in FASTA format (Fig. 1). The availability of this specific strain and the thorough annotation that NCBI gave led to its selection.

CRISPR-Cas System Identification

Potential CRISPR-Cas loci and the genes linked to them were predicted and annotated using a variety of bioinformatics tools and online servers on the obtained genomic sequence. By cross-referencing the results, three

different web platforms were employed to guarantee the findings' accuracy and robustness

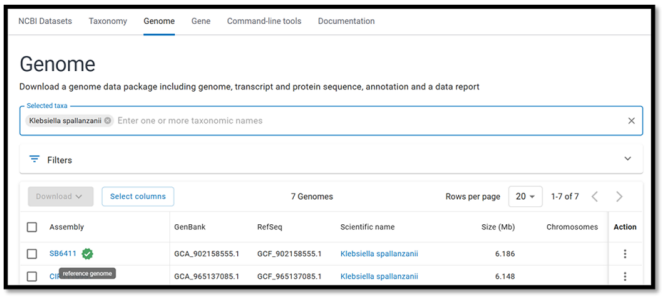

Fig. 1: NCBI genome with *Klebsiella spallanzanii* datasets

PADLOC webserver

The bacterial genome's defense system genes, including those connected to CRISPR-Cas, were found using the online service PADLOC (Prediction and Analysis of Defense Loci- https://padloc.otago.ac.nz). PADLOC offers a thorough summary of prospective defense systems and searches for known defense-related protein families[7].

CRISPRCasFinder Webserver

This program (https://crisprcas.i2bc.paris-saclay.fr.) is made especially to find Cas genes and CRISPR arrays in a given sequence. The CRISPR-Cas system type can be classified by identifying the spacer sequences, distinctive repeating sequences (CRISPR repeats), and neighbouring cas genes[8].

Defense Finder Webserver

Defense Finder (https://defensefinder.mdmlab.fr/), like PADLOC, was used to check for a variety of bacterial defense mechanisms, such as CRISPR-Cas. It offers a thorough report on the existence and location of these systems using a carefully curated database of known defense genes[9-11].

To confirm the existence and variety of CRISPR genes throughout the genome, each tool was used using its default settings, and the outcomes were compared. By using these three independent platforms, we were able to comprehensively screen the *Klebsiella spallanzanii* genome for CRISPR-Cas systems and validate the presence of any identified loci.

RESULTS

Potential CRISPR-Cas defense mechanisms were identified and described by analysing the entire genomic sequence of *Klebsiella spallanzanii* strain SB6411 (accession numbers GCA_902158555.1 and GCF_902158555.1). To guarantee reliable identification and result confirmation, a multi-tool strategy was used.

Defense System Identification Using PADLOC

Analysis of the *Klebsiella spallanzanii* genome using the PADLOC web server revealed a number of potential defense mechanisms. The discovery of a whole CRISPR-Cas system was the most notable discovery. This system was situated at genomic coordinates 157129 to 188044 (Fig. 2). The inclusion of important effector genes, including as cas1, cas2, cas3, and casA, led PADLOC to classify the system as a (e.g., Type I-E, Type III-B) CRISPR-Cas system.

PADLOC identified additional defense mechanisms in addition to the CRISPR-Cas system, including (e.g., one or more Restriction-Modification systems, Toxin-Antitoxin systems, etc.). (Fig. 2) provides a summary of all the defense systems that have been discovered along with their locations.

Systems detected (simplified table):

system	protein.name	target.name	target.description	seqid	start	end	strand	relative.position
RM_type_I	MTase_I	SB6411_04260	-	CABGGS010000002.1	43981	45589	+	37
RM_type_I	Specificity_I	SB6411_04261	-	CABGGS010000002.1	45588	46788	+	38
RM_type_I	REase_I	SB6411_04262	-	CABGGS010000002.1	46847	49763	+	39
DMS_other	mad3	SB6411_04263	-	CABGGS010000002.1	49815	52467	+	40
PDC-M09	PDC-M09B	SB6411_04458	-	CABGGS010000002.1	256298	257561	-	230
PDC-M09	PDC-M09A	SB6411_04459	-	CABGGS010000002.1	257560	258406	-	231
PDC-S07	PDC-S07	SB6411_04856	-	CABGGS010000004.1	100367	100769	+	86
RM_type_I	REase_I	SB6411_05135	-	CABGGS010000005.1	166171	168601	+	159
RM_type_I	MTase_I	SB6411_05136	-	CABGGS010000005.1	168675	170145	+	160
RM_type_I	Specificity_I	SB6411_05137	-	CABGGS010000005.1	170144	171845	+	161
HEC-06	HEC-06	SB6411_05139	-	CABGGS010000005.1	172453	174562	-	163
PDC-S13	PDC-S13	SB6411_00879	-	CABGGS010000012.1	182668	183496	+	161
RosmerTA	RmrT	SB6411_00983	-	CABGGS010000012.1	294870	295482	+	264
RosmerTA	RmrA	SB6411_00984	-	CABGGS010000012.1	295488	296616	+	265
PDC-S71	PDC-S71	SB6411_00985	-	CABGGS010000012.1	296670	298467	-	266
HEC-06	HEC-06	SB6411_00987	-	CABGGS010000012.1	300664	302500	+	268
PDC-M06	PDC-M06B	SB6411_01560	-	CABGGS010000023.1	252957	253524	-	230
PDC-M06	PDC-M06A	SB6411_01561	-	CABGGS010000023.1	253527	253782	-	231
druantia_other	DruE	SB6411_02882	-	CABGGS010000044.1	67848	73389	-	65
druantia_other	DruD	SB6411_02883	-	CABGGS010000044.1	73403	74468	-	66
druantia_other	DruA	SB6411_02885	-	CABGGS010000044.1	77310	78531	-	68
PDC-S12	PDC-S12	SB6411_03020	-	CABGGS010000044.1	216441	217110	+	195
gabija	GajB	SB6411_03488	-	CABGGS010000045.1	315163	317002	-	297
gabija	GajA	SB6411_03489	-	CABGGS010000045.1	316998	319263	-	298
CRISPR_array	CRISPR_array	CRISPR001	CRISPR001; repeat=GTGTTCCCCGCGCCAGCGGGGATAAACCG; score=6.25	CABGGS010000046.1	157129	158438	+	142.01
cas_type_I-E	Cas3e	SB6411_03722	-	CABGGS010000046.1	178063	180727	+	161
cas_type_I-E	Cas8e	SB6411_03723	-	CABGGS010000046.1	181227	182784	+	162
cas_type_I-E	Cas11e	SB6411_03724	-	CABGGS010000046.1	182780	183335	+	163
cas_type_I-E	Cas7e	SB6411_03725	-	CABGGS010000046.1	183346	184405	+	164
cas_type_I-E	Cas5e	SB6411_03726	-	CABGGS010000046.1	184414	185161	+	165
cas_type_I-E	Cas6e	SB6411_03727	-	CABGGS010000046.1	185142	185793	+	166
cas_type_I-E	Cas1e	SB6411_03728	-	CABGGS010000046.1	185789	186710	+	167
cas_type_I-E	Cas2e	SB6411_03729	-	CABGGS010000046.1	186709	187003	+	168
CRISPR_array	CRISPR_array	CRISPR002	CRISPR002; repeat=GTGTTCCCCGCGCCAGCGGGGATAAACCG; score=6.21	CABGGS010000046.1	187100	188044	+	168.02

Fig. 2: The defensome composition of *Klebsiella spallanzanii*

CRISPRArray Characterisation Using CRISPRCasFinder

CRISPRCasFinder was used to analyse the genomic sequence in order to further characterise the CRISPR-Cas systems. One CRISPR array (CRISPR001) was the major CRISPR array, which corresponded to the system that PADLOC had detected.

As is typical of a [e.g., Type I-E] system, associated Cas genes, such as [e.g., cas1, cas2, and cas3], were discovered directly downstream of the CRISPR array (Fig. 3**a**).

Cross-Checking Results with Defense Finder

Both PADLOC and CRISPRCasFinder results were validated by the Defense Finder web server. A CRISPR-Cas system was found at the same genomic region,

according to the research. The system was classified as [e.g., a Type I-E] system by Defense Finder, which was consistent with the other tools. The CRISPR-Cas system in *Klebsiella spallanzanii* strain SB6411 may be identified and classified with high confidence thanks to the agreement between several predictive tools. The Fig. 6 provides an overview of the cross-referenced results for the identified CRISPR-Cas system.

Fig. 3: a) Identification CRISPR/Cas loci in *Klebsiella spallanzanii*. b) The location details of identified CRISPR/Cas loci

Fig. 4: Two main CRISPR parts: regions and spacers

DISCUSSION

The discovery of CRISPR-Cas systems in *Klebsiella spallanzanii's* genome (accession codes GCA_902158555.1 and GCF_902158555.1, strain SB6411) sheds light on the bacterial species' adaptive immune systems. A member of the *Klebsiella oxytoca* complex, *Klebsiella spallanzanii* has gained recognition as a new opportunistic pathogen, especially in nosocomial settings where it causes illnesses like sepsis and urinary tract infections[12].

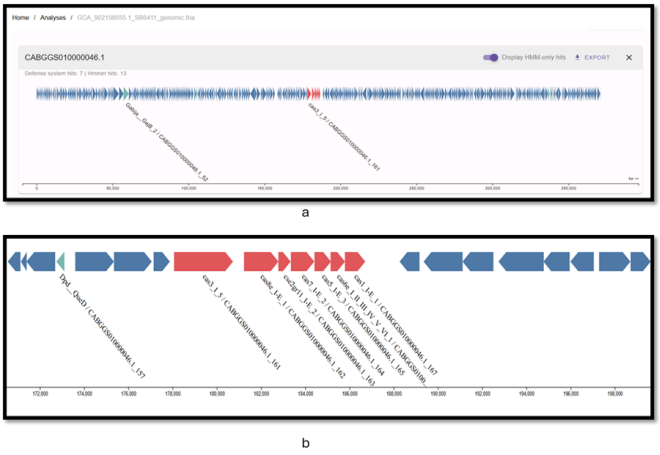

Fig. 5: a) The identification of defensome island in the *Klebsiella spallanzanii's* genome. b) highlighting the 8 Cas related sequences in *Klebsiella spallanzanii's* genome

Fig. 6: CRISPR-Cas system in *Klebsiella spallanzanii* identified with high confidence values

Its genetic characteristics, including as anti-phage defense mechanisms like CRISPR-Cas, are essential for comprehending its survival tactics and its pathogenicity, despite the fact that they have received less research attention than *K. pneumoniae*[13]. In order to identify a robust CRISPR-Cas system, mostly of Type I-E and Type IIE subtypes, as well as a CRISPR array with 21 spacers and related Cas proteins (Cas1_5, Cas3, Cas7, and Cas5), we used three complementary web-based tools in this study: PADLOC, CRISPRCasFinder, and DefenseFinder. These results are consistent with CRISPR-Cas's larger function as an adaptive immune system that shields bacteria against MGEs and bacteriophages.

Since the Type I-E CRISPR-Cas system is well-established in related *Klebsiella* species, such *K. pneumoniae*, where it is essential in preventing the acquisition of plasmids containing antibiotic resistance genes, its existence in *K. spallanzanii* is especially remarkable. Through CRISPR RNA (crRNA)-guided interference, type I–E systems—which are distinguished by multi-subunit complexes such as Cas3 (a helicase-

nuclease) and Cascade components like Cas7 and Cas5—allow the recognition and destruction of foreign DNA[14]. A direct repeat consensus sequence of "GTTGCCCCGGGGAGGGGATAAACC" with 21 spacers was found in the CRISPR array (covering locations 157129–158438 in replicon SB6411_8) in our research, suggesting a significant potential for adaptation against a variety of invaders.

With a length of 29 bp, the direct repeat (DR) sequences observed in the CRISPR array of the studied *K. spallanzanii* (Fig. 3**b**) whole genome shotgun sequence (SB6411.8) closely match the 29–30 bp range seen in *K. pneumoniae* CRISPR arrays. According to findings in the literature, DR sizes vary between 21 and 48 bp. This is similar to the 29 bp DR length in *S. Typhi,* but different from the shorter 28 bp in *G. vaginalis* and the longer 36 bp in *C. jejuni*[15].

A historical record of the bacterium's exposure to various foreign genetic elements is provided by the spacer sequences in the CRISPR array of the examined *K. pneumoniae* whole genome shotgun sequence (SB6411.8) [16]. These sequences, which show a constant spacer length across species, are in good agreement with those found in *G. vaginalis*, *C. jejuni*, and *S. Typhi*, with an average size of 33 bp and a count of 21 spacers. This finding is consistent with research showing that spacer sequences usually have a length of 26–72 bp (Fig. 4). A hallmark of CRISPR-mediated adaptive immunity, the absence of conservation in spacer sequences (0.00% conservation level) (Fig. 3**b**) indicates substantial variability, most likely as a result of the incorporation of distinct genetic material from previous phage or plasmid interactions[17].

The 98.16% conservation of direct repetitions and evidence level (4) of this array point to a stable and functional system that may adapt to new spacers from phage interactions. Using hits at locations 161–167 and other Cas proteins clustered between 172200 and 196000 base pairs, DefenseFinder further verified the system's location in replicon CABGGS010000046.1. The tool's identification of 7 defense systems (Fig. 6), which most likely include both CRISPR-Cas and complementary mechanisms like restriction-modification (RM) systems (e.g., RM_Type_I) and toxin-antitoxin systems (e.g., MazEF_MazF), is consistent with these genomic coordinates highlighting a defense hotspot.

The CRISPR-Cas landscape in *K. spallanzanii* exhibits both parallels and distinctive features when compared to the body of previous literature. In our investigation, we found that the system in *K. spallanzanii* was situated upstream of a potential hemagglutinin and downstream of genes that encode ABC transporter subunits. Although the CRISPR/Cas system appears to be situated at the same region in the genome across strains of the same species, our data and those previously described suggest that its location varies depending on the bacteria[17].

Type I-E and I-E* systems are found in 11.5–54.4% of *K. pneumoniae* genomes. They are frequently linked to Multi-Locus Sequence Types (MLST) and affect antibiotic sensitivity by focussing on resistance plasmids. For example, the spread of IncF plasmids carrying blaKPC carbapenemase genes is correlated with the lack of Type I-E in clonal complex 258 (*K. pneumoniae*), highlighting the function of CRISPR-Cas in preventing horizontal gene transfer (HGT)[18]. A similar obstacle to obtaining multidrug resistance (MDR) plasmids, which are prevalent in Enterobacteriaceae, may be present in *K. spallanzanii*, as evidenced by our identification of Type I-E.

A layer of complexity is added, though, when a Type IIE subtype is discovered alongside Type I-E. Type II systems, which usually contain Cas9, are less prevalent in *Klebsiella* but have been observed in plasmids, where they might rely on chromosomal Type I-E to function. Our CRISPR array's 21 spacers may target either phage genomes or plasmids, which could account for the bacterium's ability to survive in phage-rich environments such as hospital sewage or the human gut microbiota (Kadkhoda H, *et al*., 2024). Notably, although *K. spallanzanii* shares the richness of the *K. oxytoca* phylogroup, including antimicrobial genes, species-specific evolution may have caused its CRISPR profile to diverge from that of *K. pneumoniae*, necessitating additional phylogenetic analysis.

The CRISPR-Cas system in *K. spallanzanii* may control endogenous genes, impacting virulence and metabolic adaptability in addition to anti-phage defense. Type I-E* systems can self-target the histidine utilisation (hut) route, upregulating virulence by changing nutrition intake, according to recent findings from *K. pneumoniae*[19].

Furthermore, the multi-layered defense identified by DefenseFinder—which includes the Druantia, Dpd_FolE, and Dnd_DndA (DNA phosphorothioation) systems—indicates a thorough anti-phage arsenal (Agapov A, *et al*., 2024). For *Klebsiella* species in biofilms or during infections where phage therapy is being investigated as an antibiotic substitute, these systems—which are frequently grouped in defense islands—offer redundant protection against phages (Gholizadeh O, *et al*., 2024). These grouped defenses are highlighted by the graphical outputs from PADLOC (Fig. 5a and Fig. 5b) and DefenseFinder, which display red arrows and triangle markers in replicon CABGGS010000044.1 (positions 70,000–90,000). This highlights the bacterium's evolutionary adaptability to phage pressure.

For CRISPR identification, the methodological strategy of combining PADLOC, CRISPRCasFinder, and DefenseFinder worked well. DefenseFinder offered a

more comprehensive defense systems, including non-CRISPR mechanisms, while CRISPRCasFinder performed exceptionally well in array annotation, recognising the 21 spacers and Type IIE classification.

By emphasising anti-phage signatures across replicons, PADLOC supplemented results generated by complementary webservers. This multi-tool approach reduces the biases of individual tools, such as the emphasis on arrays in CRISPRCasFinder as opposed to DefenseFinder's HMM-based Cas protein detection. The use of default parameters, which could miss new variations, and the lack of experimental confirmation (such as phage challenge tests or spacer acquisition assays) are among the drawbacks.

Because CRISPR presence is linked to decreased resistance in *Klebsiella*, future research should use functional assays to validate the system's functionality and evaluate its effect on antibiotic susceptibility. Furthermore, investigating *K. spallanzanii's* CRISPR spacers against circulating phages as it manifests in clinical isolates may help develop phage treatment combinations, which have demonstrated promise in extending host range and postponing *K. pneumoniae* resistance.

In sum, this study reveals that *K. spallanzanii* has a functioning Type I-E and IIE CRISPR-Cas system that supports its anti-phage immunity and may also modify virulence and resistance. These results demonstrate the importance of computational techniques in genomic monitoring and contribute to our understanding of the development of bacterial defense in the *Klebsiella* genus.

CONCLUSIONS

A complex CRISPR-Cas defense system, comprising Type I-E and IIE subtypes with a 21-spacer array and essential Cas proteins (Cas1_5, Cas3, Cas7, and Cas5), embedded inside a multi-layered anti-phage arsenal, is revealed by the genomic investigation of *Klebsiella spallanzanii* strain SB6411. In clinical settings, this mechanism probably influences the bacterium's pathogenicity and resistance profile by providing adaptive protection against phages and plasmids. Through the utilisation of PADLOC, CRISPRCasFinder, and DefenseFinder, we highlighted the effectiveness of using bioinformatics techniques to detect these mechanisms.

These findings highlight the evolutionary importance of CRISPR-Cas in *Klebsiella* species and point to possible therapeutic applications, such as using CRISPR for targeted gene editing in MDR strains or creating phages that can get past these defenses. In an era of increasing antibiotic resistance, future studies should experimentally confirm these results and investigate their implications for fighting newly developing *Klebsiella* infections, ultimately leading to the development of innovative antimicrobial methods.

DISCLOSURE

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Conflict of Interest: None.

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