CRISPR-Cas13 Applications in Antibiotic Development and Bacterial Screening: A Literature Review

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Antibiotic resistance is a major concern for public health worldwide. The CRISPR-Cas13 system is a gene editing technology derived from an acquired defense mechanism in bacteria. CRISPR-Cas13 is a potential candidate for the development and screening of novel antimicrobials that could curb antibiotic resistance and help to identify harmful pathogens. This literature review examines the history of antibiotics and antibiotic resistance, the CRISPR-Cas13 mechanism, and notable applications of CRISPR-Cas13 in antibiotic development and bacterial screening. In addition, the review discusses the drawbacks of this technology and makes comparisons of CRISPR-Cas13-based applications to traditional antimicrobials and screening tests such as reverse transcription polymerase chain reaction (RT-PCR).

I. Introduction

Antibiotic resistance is an increasingly critical threat to public health. It is caused by the evolution of bacteria in response to antibiotic use. In 2019, antibiotic resistance was responsible for 1.27 million deaths worldwide.[1] Other adverse outcomes of antibiotic resistance include treatment failures, increased illness severity, and surplus medical costs.[2]

As more antimicrobial drugs were deemed unusable, researchers began to seek alternative solutions to combat antibiotic resistance.[3] Gene editing technologies and the CRISPR-Cas systems have become a promising candidate for this matter. Clustered regularly interspaced short palindromic repeats (CRISPR) combined with CRISPR-associated proteins (Cas) are a defense mechanism first discovered in Escherichia coli (*E. coli*) genomes in 1987.[4] CRISPR-Cas systems are being used and researched as a novel gene modification technology in many fields of study.

There is great potential for CRISPR-Cas13, a Class 2, Type VI categorized CRISPR-Cas system, in combating bacterial superbugs.[5] A CRISPR-Cas13 system includes a guide RNA to identify a specific gene and Cas13, which functions as a pair of "RNA scissors" to perform single-stranded RNA breaks.[6] Research on CRISPR-Cas13 has produced results proving that this system could kill antibiotic resistant bacteria and be applied to several methods of bacterial diagnosis.[5]

This paper focuses on the applications of CRISPR-Cas13 in antibiotic development, namely from its ability to kill antibiotic resistant bacteria and detect pathogens. The paper first introduces antibiotic resistance and CRISPR-Cas13, and then examines notable studies using CRISPR-Cas13 systems to alter or identify bacterial genetics. The paper will conclude with prospects for gene editing technology to be used in future antimicrobials.

II. Antibiotic Resistance

A. The History of Antibiotic Resistance

The discovery of antibiotics is considered one of the most ground-breaking scientific advancements in the 20th century.

Antibiotics bind to their receptors, microbial proteins. As defined by Brunton et al. (2008), the mechanisms behind antibiotics can be split into six main categories: (1) preventing the synthesis of bacterial cell walls, (2) acting on the cell membrane in order to increase permeability and induce leakage of intracellular compounds, (3) distorting the function of ribosomal subunits to inhibit protein synthesis, (4) binding to the 30S ribosomal subunit to inhibit protein synthesis, (5) inhibiting RNA polymerase to affect the metabolism of bacterial nucleic acids, and (6) using antimetabolites to stall vital enzymes of folate metabolism.[7] As a result, bacteria are killed or prevented from spreading within the body.

However, this discovery has given rise to problems regarding antibiotic resistance – the bacteria's ability to protect themselves from antimicrobial effects. Antibiotics may only be effective in fighting infections for a period of time before bacterial resistance is reported.[8] Thus, while other drug groups such as cardiovascular and anti-inflammatory can last for a lifetime, there is a constant demand for novel antibiotics on the market.[8]

B. Negative Impact Of Antibiotic Resistance

There are several adverse outcomes to antibiotic resistance. In 2019, there were an estimated 1.27 million deaths worldwide caused by failure of antibiotic drugs due to antibiotic resistance developed by bacteria.[1] In addition, because of resistance, entire hospital units or departments can be forced to shut down, and on average, an additional $10,000 to $40,000 is spent for each patient infected by a multidrug-resistant (MDR) organism.[2]

C. Novel Solutions to Antibiotic Resistance

There is an urgent need for effective solutions to combat this global issue. In July 2020, the AMR Action Fund was organized to fund companies that are developing new antibiotics. As of 2022, 140 countries have developed action plans on antimicrobial resistance.[9]

In recent years, CRISPR-Cas has become a popular research topic within the scientific community. This genetic modification technique has been applied to several medical settings. Recent research has produced promising results for the application of CRISPR-Cas systems in the fight against antibiotic resistance.

III. CRISPR-Cas13

CRISPR is an array of short repeated sequences separated by spacers termed protospacers which contain sequences acquired from bacteriophage invasions into the bacterial cell [10]. CRISPR combined with Cas is an acquired defense mechanism found in prokaryotes such as bacteria and archaea.

Adaptation, expression, and interference are the three stages involved in a CRISPR-Cas immune response.[11] During adaptation, Cas proteins bind and cleave the target DNA, usually after identifying a short motif.[11] The CRISPR array is transcribed into pre-CRISPR RNA (pre-crRNA), then processed into mature CRISPR RNAs (crRNAs) in the expression stage. During interference, the crRNA guides the Cas complex to detect the protospacer in the invading organism. Once detected, the Cas nuclease cleaves and deactivates the genome of the invading virus or plasmid.[11] Once the three phases are completed, the Cas nuclease cleaves and deactivates the genome of the invading virus or plasmid.[11]

As of 2020, CRISPR-Cas systems have been categorized into two classes, six types, and 33 subtypes.[11] The two CRISPR-Cas classes mainly differ in that class 1 systems have effector modules with multiple Cas proteins, which may function as a complex. On the other hand, class 2 consists of a single, multi-domain crRNA-binding protein.[11]

Due to its relatively simple structure, class 2 CRISPR-Cas systems have been the primary focus in gene editing technology research [4]. Class 2 systems are further divided into three types: type II, V, and VI. Both type II and type V systems induce double-strand DNA breaks, while type VI effectors specifically target the RNA.[11] While Cas9 is the most well-known effector amongst other class 2 effectors, it is crucial to seek innovations in CRISPR not only in Cas9 proteins, but also other potential effectors such as Cas13.

Cas13 was first identified in 2015.[12] Unlike previously discovered type II CRISPR-Cas systems such as Cas9 or Cas12, Cas13 exclusively binds and cleaves RNA. The CRISPR-Cas13 complex consists of two Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) domains, and functions similarly to CRISPR-Cas9.[6] Contrary to the Cas9 mechanism, however, Cas13 executes collateral cleavage activity on non-complementary RNAs, resulting in the breakdown of any nearby transcripts after the target sequence has been cleaved.[6] The collateral activity of Cas13 is the basis for its diagnostic applications.[6] Cas13 can also be applied to directly kill bacteria with specific antibiotic resistant genes.[13]

IV. Application of CRISPR-Cas13 in Antibiotic Development

A. Direct Killing of Bacteria

In 2020, a study demonstrated the capability of CRISPR-Cas13a in killing E. coli containing carbapenem-resistant genes.[13] The results showed that while CRISPR-Cas9 can only target *blaIMP-1* on the chromosome, the Cas13a system can act on both chromosomes and plasmids carrying antimicrobial-resistant genes.[13] As several clinically important antibiotic resistant genes are located on the plasmid, CRISPR-Cas13a systems can be seen as possessing higher bactericidal activity than Cas9.

After determining the inhibition ability of CRISPR-Cas13a, CRISPR-Cas13a was packaged into an *E. coli* phage M13 capsid to synthesize constructs termed EC-CapsidCas13a_*blaIMP-1*. This process allows the delivery of *blaIMP-1* –targeting CRISPR-Cas13 to bacteria genes.[13] Through examinations, the study indicated that the constructs could selectively kill their target among a population of antimicrobial resistant bacteria, making them a potential microbial control agent which will not exhibit unfavorable effects on other bacterial populations.[13]

B. Diagnostic Applications of CRISPR-Cas13

1. SHERLOCK

Several studies have indicated that CRISPR-Cas13 can be used in medical diagnosis due to its pathogen recognition ability.

Specific High-sensitivity Enzymatic Reporter unLOCKing, or SHERLOCK, is the first Cas13a-based molecular detection platform.[6] Developed in 2017 by Gootenburg and colleagues, SHERLOCK has been used for many different purposes, including the identification of bacterial pathogens.[14]

SHERLOCK uses a detection mix containing sample RNA, Cas13a, designed crRNA, and reporter RNA. The diagnosis process is performed in two steps, which include amplification of DNA or RNA, T7 RNA polymerase transcription, and the addition of RNA into a CRISPR-Cas13 target sequence detection reaction.[6] As a result, Cas13 can cleave the sample RNA and reporter RNA to release fluorescence signals indicating the presence of the targeted gene.[6]

2. APC-Cas

Salmonella is a genus of rod-shaped, Gram-negative bacteria that belongs to the *Enterobacteriaceae* family.[15] *Salmonella* can cause gastroenteritis, typhoid fever, and other extraintestinal problems, and is responsible for 155,000 global deaths annually.[15] One of the most common *Salmonella* serotypes worldwide is the *Salmonella* Enteritidis (*S*. Enteritidis).[16]

In 2020, a CRISPR-Cas13a-based detection system termed “allosteric probe-initiated catalysis and CRISPR-Cas13a” (APC-Cas) was developed. The system could identify low numbers of *S*. Enteritidis in mice serum and several samples such as milk.[16] Moreover, APC-Cas was found to successfully differentiate early stage from late stage-*S*. Enteritidis-infected mice, indicating promising future clinical applications.[16]

APC-Cas functions with the combination of an allosteric probe (AP) and a CRISPR-Cas13a system.[16] Allosteric transformation is activated by the identification of the target pathogen. The double-stranded DNA produced via the AP then undergoes amplification by T7 RNA polymerase, resulting in numerous single-stranded RNA (ssRNA). In the final step, Cas13 containing crRNA complementary to the

transcripted ssRNA binds to the aforementioned ssRNA. This will activate Cas13 collateral cleavage activity, enabling Cas13 to cleave RNA reporter probes and spawn fluorescence signals.[16]

In an evaluation of *S.* Enteritidis in different milk samples, APC-Cas could effectively differentiate *S.* Enteritidis-contaminated milk from pasteurized milk and yielded more accurate results than real-time polymerase chain reaction (PCR).[16] An increased intensity of fluorescence was observed with increased amounts of *S.* Enteritidis.[16] Similarly, an experiment detecting *S.* Enteritidis in mice serum indicated the ability of the system to identify mice in early stages of infection.[16]

3. CRISPR-Cas13a to Detect the lcrV Gene

In 2021, Schultzhaus et al. applied CRISPR-Cas13 to detect the *lcrV* gene in *Yersinia pestis* and contributed several possible improvements in CRISPR-Cas detection protocols.[17] The *lcrV* gene assists the production of virulence factors and host intrusion during the *Y. pestis* infection cycle. *lcrV* has been a common target in studies on antibody-based detection and treatment methods.[17]

crRNAs were designed according to the most updated SHERLOCK protocol to target each part of the *lcrV* coding sequence. Observations from the 35 synthesized crRNAs indicated that while some crRNAs could produce responses quite rapidly, there is still a chance of detection failure.

The study looked into devising an efficient in vitro CRISPR-Cas13 screening testing protocol, and concluded several suggestions, including the possibility that purification steps could be omitted while still maintaining effective testing.[17]

V. Challenges and Future Prospects

A. CRISPR-Cas13 Challenges

1. CRISPR-Cas Antimicrobial Challenges

Though a promising gene editing technology for antibiotic advancements, CRISPR-Cas systems still face several obstacles.

Current studies on CRISPR-Cas-based antimicrobials have been conducted in near-clonal bacterial populations.[10] To be successfully used on a large scale, these antibiotics must also be effective in real-world environments.[10] Due to the complexity of these environments, it is much more difficult to effectively identify and target specific bacterial hosts carrying resistant genes. Using CRISPR-Cas-based antibiotics in diverse and complicated environments may also cause unwanted knock-on effects. However, to achieve this, more time-consuming or novel methods must be adapted, which can be especially challenging.[10]

Another problem surrounding CRISPR-Cas antimicrobial applications is the delivery of the system to targeted genes. Phages used to deliver CRISPR-Cas usually have narrow host ranges, and the use of CRISPR-Cas nucleases in certain organisms may lead to cytotoxicity, thus making CRISPR-Cas systems ineffective.[10] With the addition of a loosely structured and complex bacterial environment, the effective transportation of CRISPR-Cas systems will further be a great concern.

It is predicted that bacteria will evolve to defend themselves against CRISPR-Cas attacks. Over 20 families of anti-CRISPR genes have been identified, and through further discovery and research, many more may be found in the future.[10] This may pose as a threat to the efficiency of CRISPR-Cas-based antimicrobials, similar to how many traditional antibiotics have become ineffective after reports of antibacterial resistance. Thus, the problem of bacterial resistance to CRISPR-Cas must be tackled to provide long-term opportunities for this technology to develop and thrive.[10]

Finally, legislative and social issues, such as the lack of governmental CRISPR-Cas regulations and debates on the ethical aspects of using CRISPR-Cas need to be considered in bringing CRISPR-Cas antibiotics into clinics and hospitals. Public support is indelible in refining and maintaining usage of this technology in future decades.[10]

2. Diagnosis issues

Many diagnosis processes using CRISPR-Cas and CRISPR-Cas13 systems, including SHERLOCK and APC-Cas, require purification of samples or multiple steps before Cas13 is activated.[6,16] These issues may decrease the convenience of CRISPR-Cas13 applications as they enter public use. Several studies have examined methods to resolve these drawbacks, such as by using HUDSON (heating unextracted diagnostic samples to obliterate nucleases) to enable efficient SHERLOCK detection from raw patient samples, thus decreasing the time needed to perform a diagnosis. However, most of these studies have been conducted individually from one another.[6] The combination of these improvements is needed to optimize the performance capability of Cas13 in pathogen detection.[6]

3. Immunogenicity to CRISPR-Cas

Several recent studies have identified antibodies against the Cas9 nuclease.[18] While specific studies on the immunogenicity of Cas13 nucleases have not been conducted widely, it is likely that our body will also react to the appearance of CRISPR-Cas13 systems, as CRISPR-Cas13a systems for bacterial killing are derived from *Leptotrichia shahii*, which are present in several parts of the human body.[19]

B. Resolving Drawbacks

Researchers are constantly developing methods to resolve CRISPR-Cas drawbacks. Careful monitoring of CRISPR-Cas antimicrobial effects on a variety of bacteria and their associated plasmids can better predict the performance of CRISPR-Cas systems in real-world environments.

Phage host range can be improved through phage engineering.[10] Several studies have taken advantage of the relation between host range and tail fiber composition of some phages to genetically modify phages with broadened host ranges.[20] For example, by exchanging the tail fiber gene of phage T3 with that of phage T7, Lin et al. formed a

newly engineered phage with a wider host range and higher absorption efficiency.[20]

To prevent resistance caused by the mutation of targeted genes, multiplexing emerges as a promising solution.[10] By using multiplexing, several sequences are targeted at once and the chances of resistance are thus reduced.

In order to solve legislative issues, guidelines on CRISPR-Cas and other gene editing techniques should be developed and constantly updated.[10] It is also vital that citizens around the world are informed of and can deliver feedback on CRISPR-Cas gene editing technology.[10]

The involvement of multiple steps to perform a CRISPR-Cas-based diagnosis could prevent further applications of this technique in healthcare facilities. However, numerous studies have proposed modifications to original CRISPR-Cas and specifically CRISPR-Cas13-based diagnosis guidelines to simplify and shorten the process while still maintaining its high efficiency. Though complex to program, APC-Cas does not require bacterial isolation and washing steps to be performed.[16] Following the development of SHERLOCK, several innovations including HUDSON and CARMEN (Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acid) have been dedicated to increasing the convenience of this diagnostic method.[6] Moreover, Schultzhaus and colleagues have hypothesized and tested that SHERLOCK could be run without crRNA purification steps and still produce highly specific results.[17] This relieves the concern of additional reaction components diverting diagnosis outcomes and decreases the overall time required for the process.[17]

Finally, immunogenicity to CRISPR-Cas systems can be minimized using current methods such as the modification of Cas proteins to avoid recognition by the immune system.[18] The use of Cas proteins from non-pathogenic bacteria orthologs can also be applied to circumvent pre-existing antibodies.[18] Selecting immune privileged organs such as the eye could be another possible solution to tolerate immune responses.[18]

TABLE I. CRISPR-Cas drawbacks and proposed solutions. Adapted from E. Pursey et al, 2018 [10]

Drawbacks	Solutions
CRISPR-Cas-based Antimicrobials	
Microbial environment complexity	Assess microbial environments Monitoring of antimicrobial effects on bacteria and associated plasmids
Delivery of CRISPR-Cas	Phage host-range engineering Application of conjugative plasmids
Evolution of CRISPR-Cas resistance	Application of multiplexing Use of alternative Cas effector proteins
Legislation and social issues	Update of guidelines Spread of public awareness and support
CRISPR-Cas-based diagnosis	
Process complexity	Omission of purification and isolation steps
Immunogenicity	
Immune response to CRISPR-Cas	Modification of Cas proteins Use of Cas proteins from non-pathogenic bacteria orthologs Targeting of immune privileged organs

C. CRISPR-Cas13 in Comparison to RNAi

Due to its affordability and specificity, CRISPR-Cas13 has potential to become a better alternative to current RNA-targeting methods in the development of antibiotics. One of these methods is RNAi (RNA interference), which uses regulatory RNAs.[21]

RNAi and CRISPR-Cas13 are both RNA-targeting antimicrobial candidates with similarities. RNAi can control gene expression through Watson–Crick base-pairing of sRNAs and mRNAs containing complementary sequences. CRISPR-Cas13 systems also apply Watson–Crick base-pairing to the guide RNA and the targeted sequence.[22] While RNAi can silence specific genes and degrade mRNA, CapsidCas13a can induce bacterial growth in E. coli. RNAi has already entered multiple clinical trials while CapsidCas13a research is still in its earlier stages.[23]

However, studies have indicated that Cas13 platforms can perform knockdown as efficiently as RNAi.[22] In addition, the use of CRISPR-Cas13 can reduce RNAi obstacles: current RNAi processes have higher off-target effects than CRISPR-Cas13 applications; research has shown that as many as 900 genes could be affected by RNAi.[22] In comparison, CRISPR-Cas 13 does not have such off-target effects. There are only minimal effects on the rest of the transcriptome when Cas13 is used for knockdown of a reporter transcript.[22] A high effectiveness at RNA knockdown (over 90% for each site targeted) was also recorded in analyzing Cas13b, another Cas13 enzyme.[22]

Production of RNAi-based applications remains laborious and expensive, even after the development of more cost-effective procedures.[24] This could become an obstacle in making the technology available to a wider population.

These findings indicate that though similar to RNAi in certain aspects, CRISPR-Cas13 could potentially perform with higher efficacy as it is refined and optimized in future studies.

D. CapsidCas13a in Comparison to Traditional Antibiotics

While numerous questions such as ethical issues and phage capsid packaging efficiency remain before this application can come into clinical use, CapsidCas13a is a promising candidate for a novel line of antibiotic drugs.

Bacteria can resist antibiotics through the ejection of antibiotic-destroying enzymes.[7] This can be avoided by applying CapsidCas13a and other CRISPR-Cas-based antimicrobials in disease treatment. Unlike traditional antibiotics, these antimicrobials target specific bacterial genes instead of enzymes or the cell wall or membrane.[7]

Traditional antibiotics could unwantedly eliminate larger bacterial populations than desired. Using CapsidCas13a could avoid this situation as not only does CapsidCas13a selectively destroy targeted bacteria and avoid damage to surrounding populations, it also aims at genes on both the chromosome and plasmid.[13] This is vital as with CapsidCas13a, better antimicrobials with higher specificity and with a wider variety of targets could potentially be developed. Moreover, as CapsidCas13a cleaves messenger RNA (mRNA), which has lower mutation activity than DNA, this novel application could potentially have higher efficacy than Cas9-based antimicrobials.[5]

E. SHERLOCK and APC-Cas in Comparison to RT-PCR in Bacteria Diagnostics

Reverse transcription polymerase chain reaction (RT-PCR) is currently one of the most used methods to detect pathogens. Though RT-PCR is frequently used for its ability to detect antimicrobial resistance and accuracy compared to traditional culture and staining methods, this common tool still has several disadvantages.[22]

The detection accuracy of RT-PCR remains an issue, especially when faced with wide scale public health events such as pandemics. RT-PCR tests in detection of the SARS‑CoV‑2 mRNA have a sensitivity of about 63%–78%.[6] This means false-negative results are possible, typically due to the low viral load of patients during early or late stages of infection.

CRISPR-Cas13-based bacterial diagnosis technologies, however, are comparably more sensitive and specific than RT-PCR. SHERLOCK and APC-Cas can both detect low numbers of pathogens. SHERLOCK recorded a 100% in SARS-CoV-2 detection specificity.[6] In identifying S. Enteritidis in mice serum, APC-Cas could effectively detect low numbers of pathogenic cells and distinguish between early and late stages of bacterial infection.[16]

RT-PCR also remains time-consuming, laborious, and expensive.[16] A complete RT-PCR needs to be performed in 4 to 8 hours, requires training and numerous equipment fees.[25]

In contrast, CRISPR-Cas13-based applications are more convenient and affordable. SHERLOCK does not require additional machinery to function, and results can be returned in approximately two hours.[6] For APC-Cas processes, bacterial isolation, nucleic acid extraction, and washing steps are not required. Results are also returned within just 140 minutes..[6,16]

TABLE II. COMPARISON BETWEEN RT-PCR, SHERLOCK, AND APC-CAS PATHOGEN SCREENING METHODS

	RT-PCR	SHERLOCK	APC-Cas
Completion time	4-8 hours	~2 hours	140 minutes
Detection limit	High	Low	Low
Price	High	Low	Low
Specificity	Lower specificity	Higher specificity	Higher specificity Can differentiate between stages of *S.* Enteritidis infection
Purification steps required	Yes	Can be omitted	Washing steps can be omitted

F. APC-Cas in Comparison to Other Salmonella Detection Methods

The diagnosis of *Salmonella* is vital in issuing more efficient treatment methods for patients. Late identification or misdiagnosis of *Salmonella* may increase death risks and induce other problems such as antibiotic resistance.[26] Thus, there is a great need for rapid, sensitive, and convenient *Salmonella* diagnostic methods in order to effectively prevent further communal outbreaks and other undesirable outcomes for *Salmonella*-infected patients.

Over the years, methods ranging from molecular-based techniques and immunological-based techniques to mass spectrometry and biosensors have been developed to detect *Salmonella*.[26] APC-Cas has been able to efficiently skip bacterial isolation and washing steps. As APC-Cas can detect early and late infection stages through examining fluorescence growth rates of *S.* Enteritidis exhibited in infected mice serum, this technology may be extremely valuable for better treatment options.[16]

II. CONCLUSION

CRISPR-Cas13 is a novel type of CRISPR-Cas system that could contribute significantly to the development of antibiotics. Several studies have suggested the potential of CRISPR-Cas13 systems in creating antimicrobials that can effectively target bacteria with resistant genes with less effects on other bacterial populations.

The application of CRISPR-Cas13-based technologies are hindered by some limitations such as delivery methods, lack of testing in real-world environments, and social issues. Bacterial resistance to Cas proteins and immunogenicity are also problems that need to be taken into consideration while developing these technologies.

Numerous promising studies on CRISPR-Cas13 antibiotics and screening have been conducted. Further research into this RNA-targeting system could unravel more fascinating findings about CRISPR-Cas13. With its relatively simple structure and ease in programming, CRISPR-Cas13 could become a novel class of antibiotics and a valuable screening tool that could be used long-term against organisms which constantly threaten the lives of thousands around the globe.

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