

CRISPR-Cas Systems: Mechanisms, Applications, and Future Biotechnological Prospects

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Abstract. The CRISPR-Cas system is an adaptive immune mechanism in prokaryotes that has revolutionized genome editing technology in the field of biotechnology. Originally a natural defense against viruses in bacteria, the CRISPR-Cas system has been developed into a versatile tool widely used in gene editing, gene regulation, and disease detection. This review summarizes the molecular mechanisms by which the CRISPR-Cas system identifies and attacks foreign DNA, focusing on its three key steps: adaptation, expression, and interference. Cas9, Cas12, and Cas13 from Type II systems have become mainstream tools in current biotechnology due to their simple structure and diverse functions. This paper also focuses on a newer Cas protein, Cas14, which is small and capable of recognizing single-stranded DNA, showing great potential in the development of high-precision diagnostic tools. Beyond basic research, CRISPR technology has been widely applied in agricultural improvement, disease treatment, and synthetic biology. However, off-target risks, delivery challenges, and ethical issues remain major obstacles to its clinical adoption. Additionally, the paper emphasizes the integrated application of artificial intelligence in optimizing CRISPR systems and stresses the necessity of establishing rigorous ethical governance frameworks. By evaluating fundamental mechanisms and frontier applications, this paper highlights the importance of discovering new Cas proteins and advancing CRISPR technology for future therapeutic and industrial applications.

Keywords: CRISPR-Cas, gene editing, Cas proteins, biotechnology, genome engineering

1. Introduction

The CRISPR-Cas (clustered regularly interspaced short palindromic repeats) system is an adaptive immune mechanism in prokaryotes. It protects bacteria and archaea from invading genetic elements, such as bacteriophages and plasmids. This defense system captures short fragments of foreign DNA during infection and integrates them into the host genome as spacer sequences. In subsequent exposures to the same or similar invaders, these stored sequences guide CRISPR-associated (Cas) proteins to recognize and cleave the invading nucleic acids, thereby providing sequence-specific immunity. Through this process, the CRISPR-Cas system enables prokaryotes to adaptively remember and respond to genetic threats. Since its discovery in the late 20th century, the CRISPR-Cas system has become a central research topic in microbiology, molecular biology, and biotechnology. Over the past two decades, researchers have revealed its remarkable diversity,

including Cas9, Cas12, Cas13, and the recently discovered Cas14. This diversity underscores the system's continuous evolution and its ongoing competition with mobile genetic elements.

The discovery of the CRISPR-Cas system has triggered a technological revolution, transforming a naturally occurring microbial defense mechanism into a powerful genetic engineering tool. Among these, CRISPR-Cas9 has enabled precise gene editing across a wide range of organisms, offering transformative solutions for disease modeling, drug discovery, gene therapy, and agricultural improvement. Nevertheless, despite substantial progress, critical challenges remain. Off-target effects, delivery barriers, and ethical concerns continue to hinder clinical and widespread applications. Additionally, the current reliance on a limited set of Cas proteins highlights the urgent need to identify novel Cas variants with enhanced or specialized functionalities.

In this context, novel effector proteins such as Cas14 have attracted increasing attention. These proteins are smaller in size, possess unique structural characteristics, and exhibit functional specificity, such as the ability to recognize single-stranded DNA independent of PAM sequences. These characteristics make them highly potential for the development for more flexible, safer, and more precise gene-editing technologies. Additionally, studying these proteins provides deeper insights into the evolution and diversification of CRISPR systems. As CRISPR technology continues to advance and become an important platform for synthetic biology and medical research, the search for and study of new Cas proteins have become increasingly important.

This review aims to explore how bacteria utilize different CRISPR-Cas systems to defend against phage infection and to evaluate novel Cas effector proteins with biotechnological application potential. Through these studies, this review hopes to gain a deeper understanding of the role of the CRISPR system in nature while expanding its technological potential.

2. The mechanism of CRISPR-Cas

The CRISPR-Cas system consists of repetitive DNA sequences and unique spacer sequences derived from previously encountered external genetic elements. The spacer sequences serve as a molecular memory bank, enabling the host to recognize and neutralize similar genetic threats during future infections [1]. Meanwhile, the direct repeat sequences serve as structural scaffolds, facilitating the processing and organization of the CRISPR array.

The CRISPR-Cas immune system operates through a highly coordinated three-stage process: adaptation, expression, and interference. During the adaptation phase, when bacteria encounter invading viruses or plasmids, a small segment of foreign DNA is cleaved and integrated as a new spacer sequence into the CRISPR site of the host genome. This newly acquired spacer sequence expands the host's immune repertoire and records the history of past infections. Subsequently, during the expression phase, the entire CRISPR locus, including both repeat and spacer sequences, is transcribed into long precursor RNA (pre-crRNA). Cas endonucleases then process pre-crRNA into a series of small mature CRISPR RNA (crRNA) molecules, each containing a spacer sequence flanked by partial repeat regions. These crRNA molecules assemble into ribonucleoprotein complexes by binding to specific Cas proteins and serve as a guide sequence to recognize invading nucleic acids. Finally, during the interference phase, the crRNA-Cas complexes by binding to complementary sequences in the genome of invading viruses or plasmids. Upon successful recognition, Cas nucleases are activated to perform site-specific cleavage of target DNA or RNA, effectively neutralizing the threat and protecting the host cell [2].

The CRISPR-Cas system is organized into two main functional modules. The adaptation module primarily consists of highly conserved Cas1 and Cas2 proteins, responsible for acquiring and integrating new spacer sequences into the CRISPR array. Cas1 acts as both a DNA endonuclease and

an integrase, while Cas2 typically serves a structural or stabilizing role within the adaptation complex.

In contrast, the effector module forms the foundation of modern biotechnological applications. It mediates the detection and cleavage of foreign nucleic acids, exhibiting significant structural and functional diversity, reflecting its relatively independent evolutionary pathway. For example, Cas9 induces double-strand breaks at specific genomic loci, enabling targeted gene deletion or insertion through DNA repair pathways. Genomic studies suggest that the adaptation module may have originated from ancient mobile genetic elements, which are self-synthesizing DNA transposons that utilize Cas1-like enzymes (casposases) for mobility [3]. This evolutionary connection highlights the modularity and flexibility of the CRISPR system. Also, the effector module appears to have recruited components from multiple sources, such as toxin-antitoxin systems encoding HEPN domain nucleases and TnpB family transposases. Different CRISPR systems have repeatedly recruited these elements to perform key functions such as target recognition and cleavage. Due to this evolutionary mosaicism, CRISPR-Cas systems exhibit diverse targeting strategies and molecular structures, making them a versatile platform for natural defense and engineered genome manipulation.

3. Current research on CRISPR-Cas systems

The discovery of the CRISPR-Cas system can be traced back to 1987. At the time, the highly conserved nature of these repeat sequences attracted scientific attention, although their biological significance remained unknown for many years. It was not until the early 21st century that scientists identified similar repeat sequences in various bacteria and archaea and discovered that these sequences were often located near CRISPR-associated (cas) genes. In recent years, with a deeper understanding of the structure and function of the CRISPR-Cas system, it has become a research focus in multiple fields, including microbiology, genetics, molecular biology, and bioengineering. Researchers have revealed the complex mechanisms underlying CRISPR-mediated immune responses and successfully adapted these natural systems into powerful genome editing tools. CRISPR is no longer viewed simply as an immune defense mechanism in prokaryotes but has evolved into a multifunctional, efficient gene editing platform with broad scientific and clinical implications.

3.1. Applications of type II systems in biotechnology

Among various CRISPR systems, type II systems, particularly those involving Cas9, Cas12, and Cas13 effectors, have gained significant attention due to their relatively simple structure and flexibility. Unlike Class I CRISPR systems, which require multiple coordinated proteins, Class II systems can complete their functions using just a single large effector protein. This protein is guided by a small molecule called a single-guide RNA to precisely cut the target nucleic acid. The most well-known example is Cas9, the first CRISPR tool to be widely used for gene editing. Cas9 requires only an sgRNA to specify the target DNA site; if a protospacer adjacent motif (PAM) sequence is nearby, Cas9 can cleave the DNA at that site. This simplicity makes Cas9 very easy to “reprogram” to target different DNAs [3]. Cas12 builds on this foundation by adding new features: it preferentially targets single-stranded DNA and can perform additional collateral cuts in surrounding region, which is useful in molecular diagnostics. Cas13 does not cut DNA but instead cuts RNA, making it suitable for regulating gene expression, detecting RNA viruses, or performing RNA-level engineering without altering the DNA itself [4].

These CRISPR systems have been widely studied and applied in various fields, including medicine, agriculture, and environmental protection. They can all be paired with artificially designed guide RNAs and engineered into customized versions for specific functions, such as base editing, changing the expression status of genes, or amplifying molecular signals. Due to their structural similarities with Cas9, Cas12 and Cas13 have also been used in gene editing. Cas13-based platforms such as SHERLOCK and DETECTR have been developed as rapid and highly sensitive technologies for detecting viral RNA. These systems utilize the Cas enzymes' sidecut activity to generate detectable fluorescent or colorimetric signals. During the COVID-19 pandemic, CRISPR diagnostic technologies provided rapid, sensitive, and cost-effective methods for detecting viral RNA, even in resource-limited settings. For example, SHERLOCK enables attomolar-level detection without a thermal cycler, facilitating on-site viral [1]. These systems utilize the flanking cleavage activity of Cas13 to amplify detection signals without traditional nucleic acid amplification, thus demonstrating potential for diagnostics.

3.2. Applications of CRISPR-Cas9 systems in research and biotechnology

Among all current CRISPR systems, Cas9 is the most representative and widely used tool. Its high precision and efficiency in target recognition and cleavage make it ideal for gene editing across a broad range of organisms, from bacteria to humans. Cas9's programmability—through the simple design of a short guide RNA complementary to the target DNA sequence, significantly simplifies the genome editing process compared to earlier technologies such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs).

To expand the application range of Cas9, researchers have developed a series of engineered Cas9 variants with enhanced properties. For example, by fusing a Cas9 nickase (Cas9n) with impaired catalytic activity to a deaminase, a base editor enzyme was created that can precisely convert one DNA base to another without introducing double-strand breaks (DSBs). Similarly, Cas9 nickase, which only cuts single-stranded DNA, enhances editing specificity and reduces off-target effects by requiring two adjacent sgRNAs to perform double-strand breaks.

The broad application of Cas9 technology is not limited to basic research but also shows great potential in clinical research and gene therapy. In vitro gene editing therapies for genetic diseases such as sickle cell anemia and β -thalassemia have successfully entered clinical trial phases, involving the ex vivo editing of hematopoietic stem cells followed by autologous transplantation back into patients. These pioneering studies mark a significant milestone in transitioning CRISPR-Cas9 technology from the laboratory to clinical applications [1].

Additionally, CRISPR-Cas9 is being explored as a promising antiviral strategy. Researchers have demonstrated the ability to target and remove viral genomes integrated into host cell DNA (such as latent HIV proviruses), aiming to achieve functional cure of chronic viral infections [3]. Beyond HIV, efforts are underway to apply Cas9-mediated gene editing technology to combat other intractable viral pathogens, such as hepatitis B virus (HBV) and human papillomavirus (HPV) [3].

3.3. Development of CRISPR-Cas14

In recent years, continuous exploration of the structure and function of the CRISPR-Cas system has led to the development of various novel Cas proteins and their derivative technologies, greatly enriching the gene editing toolbox. For example, Cas14 is an ultra-compact, single-stranded DNA-specific nuclease that has shown promise in molecular diagnostics and targeted single-strand editing. Compared to traditional Cas9 (approximately 1,368 amino acids) or Cas12a (approximately 1,300

amino acids), Cas14 is much smaller, consisting of only 400–700 amino acids [5]. Its reduced size and simplified structure greatly facilitate the delivery of gene editing tools, particularly in therapeutic applications where viral vector packaging volume is limited.

Importantly, Cas14 does not require a protospacer adjacent motif (PAM) sequence to recognize targets, significantly expanding the range of potential targets in the genome and reducing editing blind spots caused by PAM restrictions [5]. Additionally, Cas14 specifically recognizes and cleaves single-stranded DNA (ssDNA), rather than double-stranded DNA or RNA. Its targeting specificity and high sequence sensitivity make it particularly suitable for high-resolution nucleic acid detection, including the identification of single-nucleotide polymorphisms (SNPs) in clinical diagnostics [5].

Recent studies have demonstrated that Cas14 can be integrated into CRISPR molecular diagnostic platforms similar to SHERLOCK or DETECTR. Through its trans-cleavage activity, Cas14 enables the generation of fluorescent or colorimetric detection signals without requiring target amplification [5]. This amplification-free detection method simplifies the complexity of traditional PCR-based methods and reduces the risk of contamination, enabling rapid, portable, and cost-effective diagnostic testing that can be deployed in point-of-care settings.

3.4. Other applications of CRISPR

Beyond biomedical applications, CRISPR technology has revolutionized the agricultural sector. It has been used to enhance crop traits, including improved nutritional value, increased disease resistance, herbicide tolerance, and greater environmental stress resilience. By introducing precise targeted mutations into endogenous genes, researchers have successfully improved major crops such as tomatoes, rice, wheat, and corn [4]. Crucially, CRISPR-mediated editing typically does not involve the insertion of foreign DNA sequences, meaning that the resulting plants are often classified as non-genetically modified organisms. This characteristic not only simplifies regulatory approval processes in certain regions but also enhances public acceptance compared to traditional genetically modified organisms (GMOs). As a result, CRISPR technology offers a promising pathway toward achieving global food security, sustainable agriculture, and the development of climate-adaptive crop varieties.

Meanwhile, the widespread application of artificial intelligence technologies such as deep learning in CRISPR research has brought unprecedented efficiency improvements to gRNA design and system optimization. Traditional gRNA screening methods heavily rely on trial-and-error experiments for validation, which are time-consuming and costly. In contrast, deep learning models can automatically extract complex nonlinear relationships between nucleotide sequence features and editing activity by leveraging large-scale training datasets, allowing accurate prediction of gRNA targeting efficiency and off-target risk [6].

Researchers have developed various models based on advanced neural network architectures, including convolutional neural networks (CNNs), recurrent neural networks (RNNs), and Transformer-based models such as DeepCRISPR, DeepCpf1, CRISPR-ONT, CRISPRon, and EpiCas-DL [6]. These AI-driven tools enable rapid in vitro screening of thousands of candidate gRNAs and assist in designing customized guide RNAs optimized for specific cell types, organisms, or experimental conditions. More importantly, deep learning-driven gRNA design has been validated across diverse biological systems, including plants, mammals, and microorganisms, demonstrating its broad applicability and robustness [6].

4. Challenges and future prospects

4.1. Technology challenge

Although CRISPR-Cas technology shows great potential in the field of gene editing, it still faces numerous challenges. One of the main limitations is the off-target effects associated with CRISPR-Cas9 and its related systems, which may lead to unpredictable unintended genetic modifications [2]. Off-target activity might trigger insertions, deletions, or chromosomal rearrangements at unintended genomic sites, posing significant risks in clinical applications that require high precision. Although strategies such as developing high-fidelity Cas9 variants, modifying sgRNA design, and pairing with nucleases have been developed to reduce off-target effects, these risks have not yet been completely eliminated [2].

Another major challenge is delivering CRISPR components to target cells, particularly in therapeutic applications. Although viral vectors such as adeno-associated virus (AAV) are widely used due to their high transduction efficiency and relatively low pathogenicity, inherent risks include immunogenicity, limited packaging capacity (~4.7 kb), and the potential risk of random genomic integration, which may lead to insertion mutations or tumorigenicity [7]. Non-viral delivery methods, such as liposomal nanoparticles and electroporation, offer alternative approaches but often face challenges related to delivery efficiency, tissue specificity, and cytotoxicity [7]. Therefore, developing safe, efficient, and multifunctional delivery platforms remains a priority for the clinical translation of CRISPR technology.

These unresolved challenges also limit the development of CRISPR-based diagnostic technologies. Many CRISPR diagnostic platforms, such as systems utilizing Cas12 or Cas13, rely on nucleic acid amplification (e.g., PCR, RPA) to enhance sensitivity. However, amplification steps introduce sample contamination and false-positive results, hindering the application of CRISPR diagnostics in point-of-care testing or resource-limited settings [8]. Currently, amplification-free detection strategies are being developed by leveraging the bypass cleavage activity of Cas proteins and optimizing signal amplification methods; however, sensitivity and specificity still need to be improved [8].

Future research should focus on discovering more novel, customizable Cas proteins while gaining a deeper understanding of the evolutionary drivers behind the diversity of the CRISPR system. Through targeted design and modification, we can also make these Cas proteins safer and more efficient. Furthermore, integrating CRISPR technology with other frontier fields such as synthetic biology, nanotechnology, and computational biology will facilitate the development of next-generation gene editing platforms. This integrated system will enable more precise and regulatable gene-editing technologies, driving the widespread application of CRISPR in medicine, agriculture, and environmental science.

4.2. Ethical challenge

In addition to technical challenges, the application of CRISPR technology has raised complex ethical and regulatory issues. The ability to edit human embryos, germ cells, or even entire species has raised serious ethical questions about unintended ecological consequences, genetic fairness, and the long-term impact on human evolution [9]. A few striking events, such as the controversial case of gene-edited babies, have led to intense global discussions about the boundaries of acceptable genetic intervention and the necessity of collective regulation.

To address these concerns, many scientific institutions and governments have called for the establishment of rigorous ethical frameworks. International efforts, such as initiatives led by the World Health Organization (WHO) and the International Committee on Human Germline Genome Editing, emphasize the necessity of achieving global consensus and ensuring transparency when defining the boundaries of CRISPR applications [9]. This means we need to establish stricter regulatory frameworks, introduce long-term follow-up monitoring in clinical trials, and clearly distinguish between somatic cell editing, which only affects individuals, and germline editing, which may affect future generations. Additionally, ethical guidelines must be developed, and public education initiatives launched to ensure the safe and responsible use of CRISPR technology in medicine, agriculture, and the environment.

5. Conclusions

The CRISPR-Cas system originated from the innate immune mechanism of prokaryotic organisms and has evolved into a powerful molecular biotechnology platform with diverse functions and broad applications. This paper explores its fundamental mechanisms, structural and functional differences among representative Cas proteins, significant achievements in biomedical and agricultural fields, and current research challenges that require urgent attention. Through a comparative analysis of systems such as Cas9, Cas12, Cas13, and the emerging Cas14, it is evident that each Cas protein exhibits unique recognition patterns, target specificity, and enzymatic characteristics, making them suitable for a diverse range of biotechnological applications.

However, despite significant progress, several challenges must be overcome before CRISPR-Cas systems can be applied to clinical therapy, disease diagnosis, precision breeding, and environmental applications. Key challenges include enhancing the specificity and stability of Cas effectors to minimize off-target effects, improving the efficient delivery of CRISPR components to target cells or tissues, and addressing bioethical issues associated with gene editing, particularly in human germ cells and ecosystems.

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