MINI REVIEW

# RNA-Mediated Nuclease Genome Editing System Type II: A Mini Review

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**Abstract:** The CRISPR /CAS system is a modern genome editing tool derived from a bacterium as it functions as an active immune system to prevent the bacteria from the viruses and plasmids. In the earlier years before the discovery of the CRISPR system, there were many other tools to perform genome editing but due to their low specificity and reliability, they were not considered an efficient tool. The discovery of CRISPR/CAS9 system overcomes these limitations and considered as a highly specific and efficient technique in the field of genome editing or DNA alteration. The purpose of studying the CRISPR/CAS system is to develop a powerful gene-editing tool that can make every possible gene editing and also to prevent the genomic defect in a certain group of organisms. The CRISPR/CAS9 system uses an RNA molecule which is a main functional part of the CRISPR through which a bacterial cell can recognize and cleave/destroy the foreign viral elements that enter the bacterial cell using a certain group of restriction nuclease enzymes isolated from different bacteria. Several repairing mechanisms in the cell have been used to ligate the degraded viral sequences as these repair mechanisms are error-prone and generate frame shift mutations in the sequence. As a result, the foreign viral components and their expressions were reduced or deleted. The purpose of this review is to understand the mechanism of bacterial CRISPR/CAS9 system and also to know the use of this technique to prevent single genomic defects. In this review discuss the role of the CRISPR/CAS9 system as an active immune system of bacterial cells, classifications, and the CRISPR/CAS9 system (type II) use in the genome-editing mechanism.
**Keywords:** CRISPR/CAS system, CRISPR/CAS9 genome editing, the DNA repair mechanism, the Vector system in CRISPR, application

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## 1 Introduction

The mechanism of gene editing allows the changes in the genes by knocking-out or knocking-in the nucleotide bases in a particular gene sequence (Pandey et al., 2022). The first two methods for genome editing are (1) Zinc-Finger nuclease (ZFNs). This ZNF binds the DNA-binding domain with a FOKI nuclease enzyme (act as a restriction enzyme) for the recognition and cleaving of the target site. The ZFN mechanism involves some arrays ($C_2H_2$/Cys2 OR HIS2) that recognize by 3BPof DNA target sequence (Nasrallah et al., 2022). (2) Transcription activator-like effector nuclease (TALENs). The TALENs are the naturally occurring proteins bounded with a DNA as DNA-binding protein. In the TALENs there are some domains (DNA-binding domains) having 34 amino acid modules (Zheng et al., 2020). This system degrades the target DNA from a specific site and makes double-strand break (DSB). This DNA breaks is than repaired by the DNA repair mechanism like (NHEJ/HDR). These repair mechanisms are error-prone repair mechanism and make alteration/mutation in the different bases present in the sequence. But these methods have some limitations with their efficiency and low specificity to recognize the target sequence and to degrade a specific target sequence. To overcome these limitations a new method having high accuracy of recognizing the target genome sequence and make a specific cut in the DNA strand have been introduced called CRISPR/-CAS system. This was first reported in the bacteria (*streptococcus thermophiles*) and *Achaea* generating a defence mechanism for the bacteria against the phage and plasmids infections. In the bacteria genome, there are certain CRISPR loci present at different sites in their chromosome. These loci have a short palindromic repeater sequence (29BP) along with a spacer sequence of (32BP) (Mohanraju et al., 2022). In CRISPR locus-specific genes named CAS genes were located upstream on the CRISPR loci along with tracr RNA (trans-activating CRISPR RNA) responsible for producing tracr RNA molecules. The crRNA helps in the recognition of target sequence. CAS genes play a role in the production of CAS nuclease proteins that helps in the cleavage of the target DNA sequence. In the CRISPR/CAS9 system, CAS9 nuclease is responsible to cleave the DNA by the guidance

of a short 10-12BP sequence of RNA molecule that has the complementary sequence to the target sequence of the foreign/viral genome. A specific sequence which is present near to the targeted sequence and play a significant role in the recognition of the target site named PAM (Protospacer adjacent motifs). This PAM sequence is complimentary of the sequence present in the spacer unit of CRISPR loci (Miyaoka et al., 2016). The CAS9 nuclease attached to the PAM and guide RNA to recognize the target site. It joins their complementary bases with the PAM sequence and cut/degrade the target sequence using two types of domains named as (RuvC and HNH nuclease domains). These domains cut the target strands from different positions and make a double-strand break in the sequence (DSBs). These DSBs are than repaired through an error-prone. This method is better to use as it can insert bases and help in repairing the DNA damage and to modify the DNA (Miyaoka et al., 2016).

## 1.1 The bacterial CRISPR system

There are several groups of viruses and plasmids infections that infect the bacterial cells. As to respond against these infections bacteria develop a system which enhances their immunity to take defence against these agents and such type of system is known as CRISPR/CAS system. It is an effective and highly accurate mechanism that is able to recognize the viral/phage genome and bacterial own genome that destroys only the foreign viral fragments. So, this specification makes the CRISPR system high accuracy tool to destroy the foreign viral elements (Rath et al., 2015). From previous studies on *Streptococcus thermophiles*, that the bacterial CRISPR locus has some additional strains having similarities to the viral strains in the spacer unit of the CRISPR locus. This happened due to the recognition of specific motifs associated with the spacer unit of the bacteria. The phage sequence that is adjacent to the spacer region is known as protospacer and elements or the motifs that present on the target strand and used in the recognition of the target site are known as protospacer-adjacent motifs (Zhang et al., 2021) and their base pairs joining result in generating an active response against the phage. The bacterial CRISPR/CAS mechanism is a three-step process (Liu et al., 2021) (Figure 1).

### 1.1.1 Adaptation

The adaptation process initiates with the first attack of the phage/virus on the bacterial cell and injects its components inside the bacteria. After that the bacterial defence system become lysed and viral sequence break into small fragments using restriction enzymes (nuclease/helicases). These small fragments are then attached at a particular position in the spacer unit of the CRISPR locus. This joining adapts the foreign/viral components as a part of the bacterial genome and bacteria store these sequences as an active memory responder sequence. If the virus/plasmids again attack on bacterial cell than it can easily recognize and generates an active immune response against the infections.

### 1.1.2 Expression

The expression step involves the transcription of the pre-crRNA to form a CRISPR RNA (crRNA). The crRNA is a short RNA molecule having the viral sequence (in the spacer unit). The pre crRNA joins with a different group of RNA known as tracr RNA. This pre-crRNA and tracr-RNA join together to make a pre crRNA: tracr RNA complex to form RNase III (with the help of Nuclease). It cleaves pre-crRNA and tracr-RNA complex into small fragments. These small fragments of pre-crRNA and tracr-RNA strand known as crRNA. It is the functional RNA molecule for the target site recognition. crRNA and tracr RNA both attached with the CAS nuclease and these three main components of the CRISPR recognize and cleave the target DNA sequence. The crRNA and tracr-RNA complex are simply modified to form a single guide RNA molecule (sgRNA) that is a simple and faster recognition tool used in the CRISPR system.

### 1.1.3 Interference

The interference is the major step in the process where the sgRNA having the viral sequence recognize and joins with the complimentary PAM sequence present on the 3bp away from the target site. This recognition directed the CAS nuclease to attach with the PAM sequence and start cleaving the target site by using its CAS domains and make double-strand brakes in the target sequence (Zakrzewska and Burmistrz, 2023).

After the target site cleavage the sequence and the genome expression will be changed so the effectiveness of the viral sequence will be completely reduced.

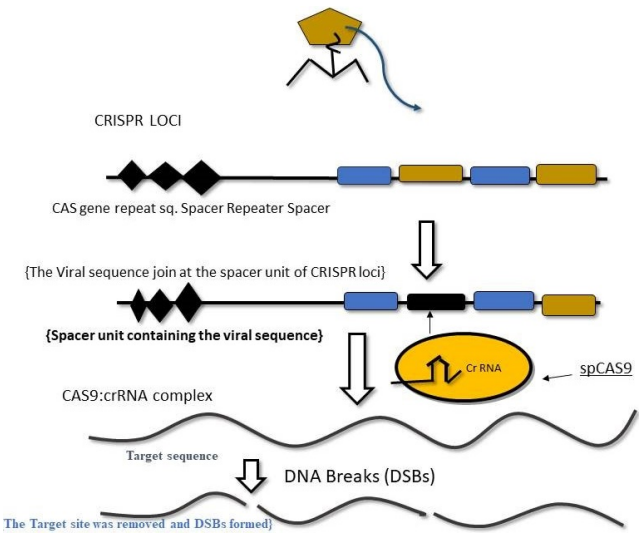


**Figure 1.** Showing the mechanism of bacterial CRISPR/CAS system.

**Table 1.** Showing classification of CRISPR system

| Class | Type | Signature Gene | Pre-crRNA Processing gene | Recognition site | Target strand | Sub type | Domains |
|---|---|---|---|---|---|---|---|
| CLASS-I | Type-I | CAS 3 | CAS 6 | RRM (RNA recognition motifs) | DNA | I-A, I-B, I-C, I-E and I-F | HD Domain (Bhatia et al., 2023) |
| CLASS-I | Type-III | CAS 10 | CAS 6 | CRISPR repeat | DNA or RNA | III-A and III-B | HD, Two Alpha Helix and Two Palm Domain (Kazlauskiene et al., 2017) |
| CLASS-I | Type-IV (Pinilla-Redondo, 2020) | CAS 8 (csf1) | CAS 6 | PAM | DNA | IV-A and IV-B | - |
| CLASS-II | Type-II | CAS 9 | RNase III | PAM | DNA | II-A, II-B and II-C | Ruvc and HNH |
| CLASS-II | Type-V (Bandyopadhyay et al., 2020) | FnCAS 12a (cpf-1) | CAS 12a | T-dependent PAM | RNA and DNA | - | Two (2) Ruvc Domain |
| CLASS-II | Type-VI (O' Connell et al., 2019) | CAS 13 (C2c2) | RNase | Direct-repeats (DR) | DNA | VI-A to VI-D | Two (2) HEPN Domain |

## 1.2 Classification of CRISPR/CAS system

The CRISPR/CAS system is divided into two classes (Class 1 and 2). These classes further divide into three different types based on the CAS genes expressing nuclease proteins. Class1 CRISPR system is divided into three types (Type I, III, and IV) while class 2 is divided into types (II, V, and VI). In every type of CRISPR system, there are different signature genes (CAS genes producing nuclease) and divided into various subtypes. These CAS proteins use different types of nuclease domains that cleave the DNA (Makarova et al., 2020). Further, these six types of the CRISPR system is divided into various subtypes. These subtypes have a specific CAS gene producing nuclease and different functions. The classification of the CRISPR system was shown in the Table 1.

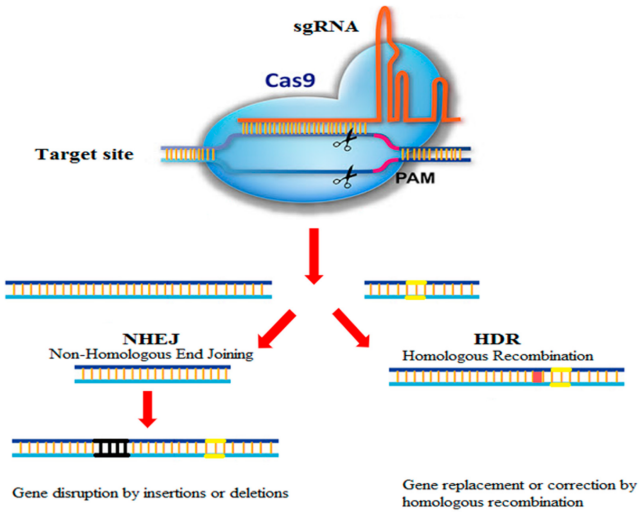


**Figure 2.** Showing the CRISPR genome editing mechanism (Tavakoli et al., 2021).

# 2 The CRISPR/CAS9 Genome Editing

The CRISPR /CAS9 bacterial immune system is useful tool for genome editing. Due to its high specificity, low cost, and ease to perform the CRISPR is considered a very useful method in gene alteration and genetic modification. The CRISPR makes it easy to add or delete the genes from a specific position in the genome sequence. These alterations help scientist to make alterations in the genome and study to find different genome therapies to get the knowledge about different genomic expressions. The first genome editing was done by using the CRISPR/CAS9 (type II) system of *S. pyogenes* was reported in the year 2012. A crRNA was used to guide the CAS9 nuclease to make a site-directed double-strand break (DSB). The cleaved DNA then joins by using the donor template in homology-direct repair mechanism (HDR) or without the use of a donor template (non-homologous end joining) (Zakrzewska and Burmistrz, 2023). The mechanism of CRISPR genome editing (Figure 2) is as follows:

1) In the first step CRISPR/CAS9 genome editing is to prepare the desired crRNA (CRISPR RNA). This crRNA used to perform complementary sequence for the sequence to be targeted.

2) This crRNA along with its tracr RNA or the modified sgRNA is then transferred to the desired target site using various types of vector delivery system. The three main components (sgRNA, CAS genes, and tracr RNA) were transferred using a vector system.

3) After the sgRNA and CAS genes enter the host genome the sgRNA binds to the target site in the nucleus as a result the CAS enzymes recognize and cleave the target site by using their nuclease domains (Mohanraju et al., 2022).

4) The cleaved DNA is then repaired (using a DNA repair mechanism) that inserts the indels which cause the mutations in the sequence.

5) These mutations and gene alteration help the scientists to study the different gene expression and their clinical applications for the treatment of genomic defects (Thakral et al., 2020).

## 2.1 CAS 9 Nuclease

The CAS 9 nuclease is the main functional restriction proteins found in the type II CRISPR system. It is a bilobed structure protein and commonly derived from *Streptococcus pyogenes*. These proteins are produced by the different types of CAS9 genes present in the CRISPR locus. The CAS9 nuclease has two nuclease domains (HNH) and (RuvC). There are three variants of CAS9 nuclease which are reported and use in the genome editing process.

1) In the first variant the CAS9 nuclease is simply wild-type nuclease which mainly used in the cleaving of double-stranded DNA, resulting in the formation of double strands brakes (DSB) (Gao et al., 2020).

2) The second variant of CAS9 is an artificially mutated nuclease known as CAS9D 10A. This type of nuclease only has a nickase activity. That means this nuclease can only cleave the single strand of DNA as a result the NHEJ repair mechanism doesn't activate only the HDR repair mechanism works. This helps in reducing the indel formation or mutations which is the end result of the NHEJ repair mechanism.

3) The third variant of CAS9 is a nuclease-deficient CAS9 (dCAS9) which is an inactivated form of CAS9. This type of nuclease can only bind to a specific site but don't able to cleave the DNA strands as the mutation in HNH and RuvC domain inactivates their degrading activity. This type of CAS9 (dCAS9) nuclease is used as a visualizing tool or activation tool which helps in recognition and can only bind to the specific location in the DNA (Javaid et al., 2022).

## 2.2 RuvC

The RuvC domain cuts the DNA strand which is a non complimentary to the target strand. There are three segments (RuvC I, II, and III) in RuvC domain in which the HNH domain is present between the second II and the third III segment of RuvC (Fang et al., 2019). RuvC domain has folded structure which is similar to RNASE H. It degrades the DNA strands. The RuvC domain uses its active site to cleave the single strand and the cleaving mechanism is similar to the two-metal mechanism of RuvC (Jinek et al., 2012) (Figure 3).

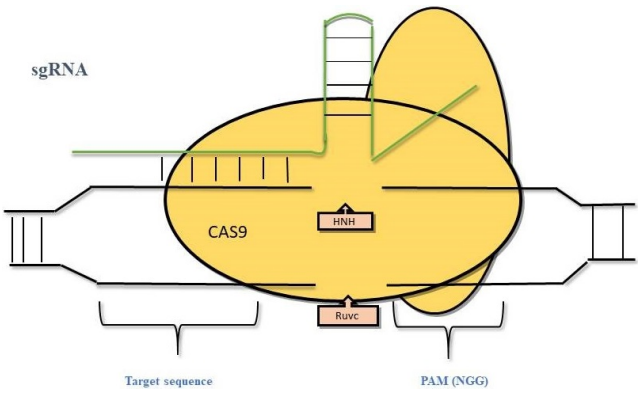


**Figure 3.** Showing CRISPR/CAS9 showing the cleaving site of RuvC and HNH.

## 2.3 HNH Domain

The HNH domain cleaves the 20-mer complementary target sequence to the sequence in the spacer unit of crRNA. The domains are characterized on the basis of their structure in the helix DNA (Watson's DNA structure) (Schulze et al., 2020). The active site of the HNH domain has 2 Beta-Alpha metal folds and these folds in the HNH domain show a similarity with the other nuclease such as phage T4 endonuclease 7 and *Vibrio vulnificus* nuclease. The HNH nuclease domain is also used to make a CAS9 nick by mutation in its the Histidine residue with the alanine which makes the nuclease inactivated so it only binds with the specific site but do not cleave the target region (Shin et al., 2022).

The lobes of CAS9 are (1) recognition lobe (REC) and (2) nuclease lobes (NUC). The gRNA and target DNA both are present in between the central position in these two lobes (Wang et al., 2022). The role of the recognition lobe is to bind DNA and sgRNA at the specific position whereas the nuclease lobe contains the HNH and RuvC domain which help to cleave the DNA strands. In the nuclease domain, there is a carboxyl-terminal that helps the CAS9 to join with the PAM (protospacer adjacent motifs). The crRNA have the protospacers and repeater sequence which joined to the complementary target sequence (with first 17-20 nucleotides) (Karvelis et al., 2015). The CAS proteins recognize the PAM sequence near the target site (Xu et al., 2015). The mechanism of CAS9 nuclease is shown in Figure 2.

## 2.4 Protospacer Adjacent motifs sequence (PAM)

The PAM sequence is a short 2-3 bp sequence present downwards to the target DNA sequence. This is a site used for recognition of the target site present at the spacer region in sgRNA. The sgRNA recognizes the target sequences and joins them with their complementary bases. The CAS nuclease is directed by the sgRNA which can easily recognize PAM sequence and bind with PAM sequence (Karvelis et al., 2015). The main function of the PAM sequence is to recognize target sequences. It helps the CAS proteins to identify the viral/foreign DNA sequence and cleave the foreign sequence. The classification of the PAM sequence is based on their structural organization. The spCAS9 in the CASe of Type II CAS nuclease recognize the 5'NGG3' ('N' represents the Nuclease and 'G' represents Guanine) as a PAM. Different/specific CAS protein recognizes specific structural PAM sequences along with their gRNA34. The specificity of CAS nuclease is to recognize the PAM sequence in the genome is different for every different CAS enzyme (Di Cesare et al., 2016). Few examples of the specificity of different CAS nuclease to recognize the specific PAM structure is discussed in the Table 2.

## 2.5 The Repair mechanism

In the cell certain respond are generated by different DNA damage response pathways to repair the damaged or to re-

**Table 2.** Showing specific PAM recognition site for specific CAS nuclease

| Types of CAS Nucleases | PAM Recognition site | References |
|---|---|---|
| *Neisseria Meningitidis* (NmeCAS 9) | 5'NNNNGATT3' | Harrington et al., 2017 |
| *Streptococcus aureus* (SaCAS9) | 5'NGRRT3'/ 5'NGRRN3' | Di Cesare et al., 2016 |
| *Streptococcus Pyogenes* (spCAS 9) | 5'NGG3' | Esquerra et al., 2023 |
| *Streptococcus thermophilus* (StCAS 9) | 5'NGGNG3' | Bao et al., 2020 |

move these damaged DNA from their genome. When target DNA cleaved by the CAS nuclease results in the formation of double strand. To ligate or to repair this cut DNA the repair mechanism/pathways is used which include five types of repairs; non-homologous end joining (NHEJ), (HDR) Homology-direct repair, base-excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR). These repair systems are error-prone at the time of ligation. The bases can be knock-in or knock-out from the sequence and generates errors and mutations (Miyaoka et al., 2016).

### 2.5.1 NHEJ

The non-homologous end-joining pathway is an error-prone pathway that joins the braked strands without using a donor or a template strand. It joins the bases according to their arrangements in the DNA helix. The advantage of this mechanism is the indels formation which is a useful way for the researchers to either add or delete the genes from the sequence in a single time and consider as a highly useful repair mechanism to perform the mutations or the gene alterations. As compare to HDR, this method is fast and highly flexible for generating the mutations in different genome sequence that helps to identify the different expressions of the genes in a particular sequence. The components use in HNEJ are heterodimers (KU 30-40 in prokaryotes), (KU 70-80 in eukaryotes) (Bhatia, 2020) dependent protein kinase, Artemis/DNA polymerase (for repairing the ends), DNA ligase (LIG4) to bind the DNA strand, X-ray cross complementation group 4 (XRCC4) (Chatterjee and Walker, 2017).

### 2.5.2 HDR (homology-direct repair)

The homology direct repair mechanism use a donor or template strand to ligate the two single strands of the braked ds-DNA. The HDR is restricted and can't use at the 'G' and S1 phase of the cell cycle as it requires a donor template (Burgio and Teboul, 2020). When a donor/ template strand attached between two single-stranded DNA brakes it start adding the complementary bases to join the break DNA strands. This repair mechanism results in the knock-in (addition) of the bases in the sequence as a result it shows a high potential to cure the DNA damages and in DNA modifications. Components of HDR repair mechanisms are MRN-complex (catalysing single strand 5'-3' restriction) (Ye et al., 2021), CTP -1(cell-cycle regulated gene), Replication protein A. The HDR repair mechanism initially starts with the formation of 3'-single strand template DNA using the MRN complex. It restricts the DSB along with the ctp1 which is a simple process to prepare a donor template strand. After the ssDNA (Donor template) strand formed than strand is coated by replicating proteins A (single-strand binding protein) from its tail (Ye et al., 2021). This RPA is replaced with RAD51 catalyzed to form a nucleoprotein. It searches similar homologous chromatin and the small sequence is elongated by pairing sequence to make a template DNA strand (Provasek et al., 2022). Overall, the DSB by restrict from 3' end with the MRN complexes and make a small template strand homologous to the strands in the bases present in the end positions of the DSBs. These templates are joined between the braked strands and repair the DNA strand.

## 3 Conclusion

The CRISPR/CAS9 system is a very useful tool for genome editing. However, the accuracy is still low because of the off-targeting effects and the low specificity of CAS nuclease. The main challenge for the researchers is to find a way through which enhancement of the specificity of CAS enzymes which reduces the off-targeting defects and makes CRISPR a highly useful technique for genome editing. Nowadays, there are very small ranges of spacer sequences that are known or discovered so research should be urgently needed for CRISPR mechanism as Spacer sequences is a main component in the process. In the future, as the diversity of these sequences would be discovered it helps scientists to make every possible gene editing in almost every possible organism. This developments or discoveries in the CRISPR system makes it easier, effective and increase its accuracy towards targeting the sequences. However, from the current status of CRISPR technique it helps in different fields from the genetic treatments of plants which make them disease resistant and increase their production.

## Author contributions

Writing, visualization: Nitish Bhagle; Visualization, writing and editing: Jebi Sudan, Himanshi Mangla, Aruna Sharma; Conceptualization, supervision: Saurabh Dave, Hardik Pathak.

## Conflicts of Interests

Authors have reported no potential conflict of interest.

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