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CRISPR-CAS9: A Revolutionary Tool for Genome Editing

M. Yaswanth Babu[1] and Y. Bindu Sai[2]

[1]M.F.Sc., Fish Biotechnology Research Scholar, Central Institute of Fisheries Education, Panch Marg, Off. Yari Road, Versova, Andheri (West), Mumbai, Maharashtra

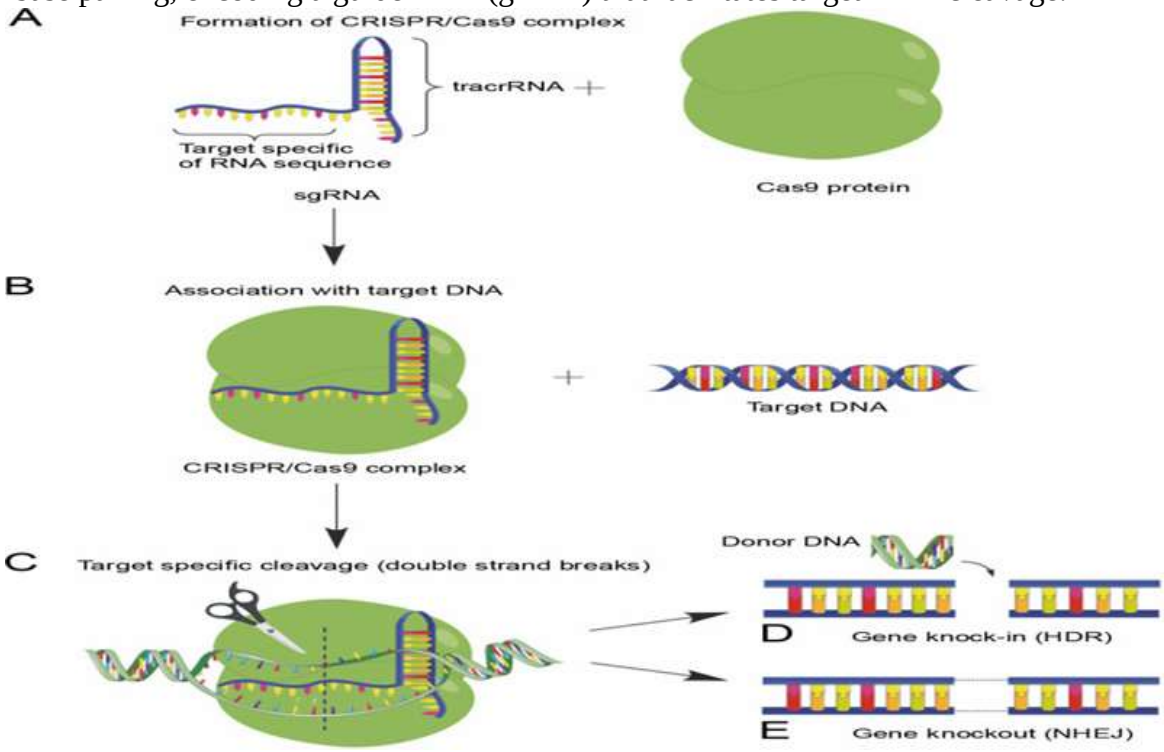
[2]M.F.Sc., Aquatic Animal Health Management Research Scholar, College of Fisheries Science, Muthukur, Nellore, Andhra Pradesh

SUMMARY

The technology known as CRISPR-CAS9 has gained widespread recognition in recent years for its significant uses in the field of biotechnology, including the ability to modify any organism's genome for the goal of treating a wide range of complicated disorders. The RNA-guided gene editing tool known as clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9 (CRISPR-CAS9) has a number of benefits over traditional techniques (such as zinc finger nucleases and transcription activator-like effector nucleases), including affordability, adaptability, and user-friendliness. The CRISPR/Cas-9 system requires two critical components: guide RNA (gRNA) and CRISPR-associated (CAS-9) proteins. The most practical technique for gene editing is still CRISPR-CAS9, despite certain drawbacks like safe and effective delivery. The CRISPR-CAS9 system has the potential to be a therapeutic system for treating problems related to genome mutations, specifically cancer treatment, due to its capacity to edit the genome and correct accidental mutations. This will cover the categories and mechanisms of action of CRISPR-CAS-based gene editing.

INTRODUCTION

A sort of genetic engineering known as "genome editing" involves purposefully adding, removing, or changing DNA in living cells. The term CRISPR describes the distinct arrangement of brief, partially repetitive DNA sequences seen in prokaryotic genomes. Prokaryotes can protect themselves against viruses or bacteriophages by using CRISPR and the protein that it is linked with, called Cas-9, as mechanisms of adaptive immunity. The term "RNA-mediated adaptive immune system defense" refers to clustered regularly interspaced short palindromic repeats, which are found in bacteria and archaea. This system keeps plasmids and viruses from invading these species. As a member of the Type II CRISPR system, Cas9 binds directly to target DNA via Watson-Crick base pairing, encoding a guide RNA (gRNA) that facilitates target DNA cleavage.



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In response to this double-strand break, the host cell uses two distinct mechanisms: (a) homology-directed repair (HDR) and (b) non-homologue end joining (NHEJ), which result in frameshift mutation and insertion/deletion in the target DNA, respectively, and HDR, which provides a donor DNA template for homologous recombination. Cas9 is useful in genetic engineering for a wide range of tasks, including gene expression, gene editing, and gene functional research.

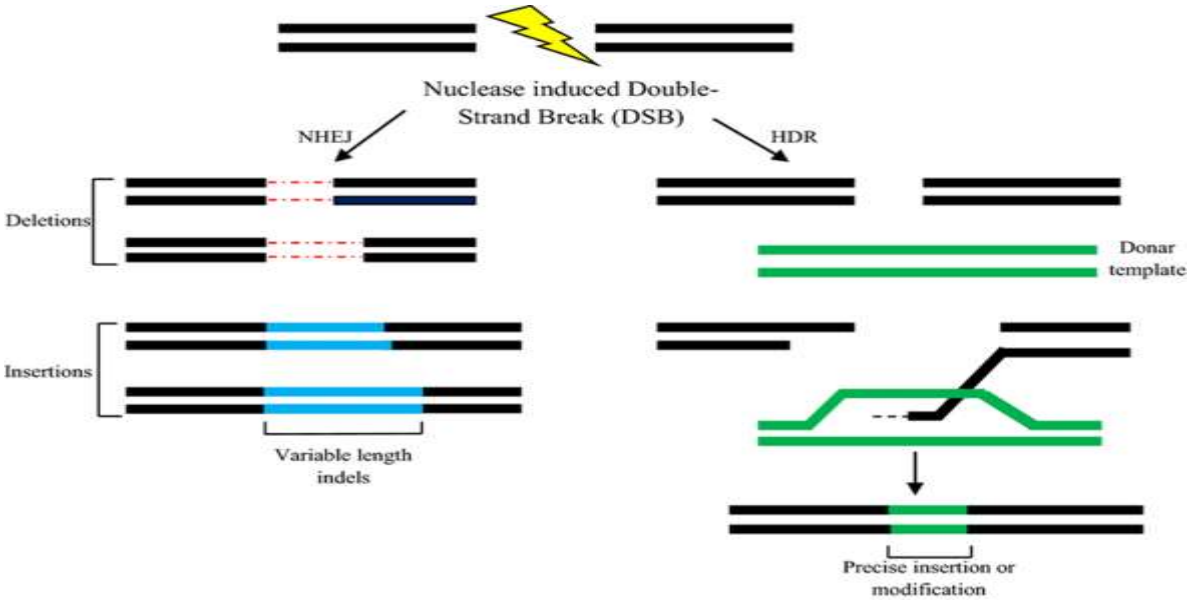
CRISPR-Cas SYSTEM CLASSIFICATION:

A CRISPR array and a clustered collection of CRISPR-associated (Cas) genes make up the CRISPR-Cas system. Effector complexes, which are functional proteins, are encoded by the Cas genes. There are **two classes** of CRISPR-Cas systems, and there are several varieties and subtypes within each class. Class 2 is found exclusively in bacteria (not in hyperthermophiles), whereas Class 1 is found in both bacteria and archaea. Type I, Type III, and Type IV are found in CRISPR-Cas Class 1, whereas Type II and Type V are found in Class 2. In general, Class 2 functional protein structures are less complex than Class 1 ones. Thus, Cas9 and Cpf1, respectively, perform the role of functional proteins (Cas proteins) in Type II and Type V. Both Cpf1 and Cas9 are big, solitary proteins. On the other hand, Class 1's functional proteins are multi-subunit assemblies of multiple proteins. With the exception of Type IV, all kinds exhibit both the Cas1 and Cas2 genes. The CRISPR-Cas system assigns distinct responsibilities to distinct Cas proteins. A well-known integrase enzyme called Cas1 is necessary for the precise breaking of a CRISPR array in order to insertion a recently discovered spacer. Although its function is unknown, the Cas2 protein is required for the adaptation stage in *Escherichia coli* and possesses RNase and DNase activity.

Mechanism involved in CRISPR-Cas system:

The mechanism behind CRISPR-Cas organization operates in **three** noticeable phases:

(1) Adaptation
(2) Expression and Maturation
(3) Interference



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Adaptation of CRISPR-Cas spacer sequences

The adaptation phase occurs in **two** steps:

- The bacterium's Cas proteins recognize the foreign nucleic acid and get certain sequences from it, which are referred to as "protospacer" sequences.
- The protospacer is integrated as a "spacer" at the end of the leader sequence in the CRISPR array, extending the first repetition of the array. These spacers are in charge of giving bacteria and archaea an immunological memory that will help them defend themselves if they come into contact with MGEs again. This step primarily involves Cas1 and Cas2.

Expression and maturation of the CRISPR-Cas system

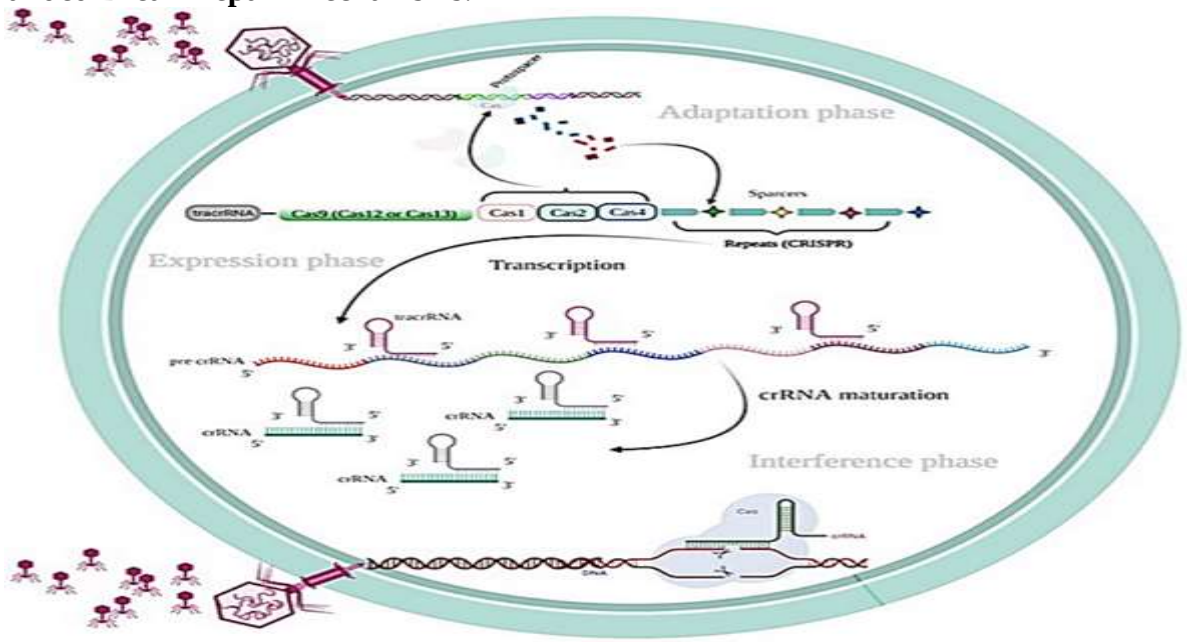
- The leader sequence, which is located upstream of the CRISPR loci, functions as a promoter and starts transcription of the loci, resulting in the production of lengthy precursor CRISPR RNA, or pre-crRNA. This pre-crRNA is then processed into tiny and mature units, or crRNA.
- A spacer region (a sequence demonstrating complementarity to the foreign nucleic acid) at the 5′ end is joined to a repeat sequence at the 3′ end to represent crRNA.

Interference of the CRISPR-Cas system

- The foreign MGEs are identified via the Watson-Crick base pairing of sequences that are complementary to the crRNA, which results in the formation of the Cas-crRNA complex. The targeted element is then exposed to cleavage.

Protospacer adjacent motif (PAM), a small conserved sequence of 2-5 bp, must be present in order for the Cas-crRNA complex to distinguish between self and non-self-nucleic acids at the target location of the invader.

Double-Stranded Break Repair Mechanisms:



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The two methods for repairing double-strand breaks (DSBs) produced by the Cas-9 protein in the CRISPR/Cas-9 mechanism are homology-directed repair (HDR) and non-homologous end joining (NHEJ). In the absence of external homologous DNA, NHEJ unites DNA fragments enzymatically to aid in the repair of double-strand breaks (DSBs). NHEJ is active throughout the whole cell cycle. It is the most common and effective cellular repair mechanism that is most active in cells, although it is prone to mistake and can produce premature stop codons or frameshift mutations due to tiny random insertions or deletions (indels) at the cleavage site. Utilizing a homologous DNA template is necessary for HDR, which is extremely accurate. It is primarily active throughout the cell cycle's late S and G2 phases. HDR needs a lot of donor (exogenous) DNA templates with a sequence of interest in order to do CRISPR-gene editing. By inserting a donor DNA template at the anticipated DSB site that has sequence homology, HDR carries out the precise gene insertion or replacement.

Strategies for Genome Editing:

Three main strategies for genome editing using CAS9.

- Using a **plasmid encoding Cas9 protein and sgRNA**, known as the plasmid-based CRISPR-Cas9 strategy, in this strategy, both the Cas9 gene and sgRNA are assembled in the same plasmid; thus, applying this strategy prevents multiple transfections. However, in this system, plasmids are required to be transferred into the nuclei of target cells, which is the most important challenge in the plasmid-based CRISPR-Cas9 system.

- The direct transfer or delivery of the **Cas9 messenger RNA (mRNA) and sgRNA** combination into host cells. The most important characteristic of this strategy is the poor stability of mRNA, which causes transient expression and a short duration of genome editing.
- The direct delivery of the **Cas9 protein and sgRNA** combination. This strategy has several advantages, including fast action, high stability, and poor inducing antigenicity responses.

Delivery Systems:

For the CRISPR-Cas9 protein, two significant delivery methods were presented: the viral and nonviral delivery systems (the physical delivery system). Nonviral vectors are easier to use and safer than viral vectors. Viral vectors, on the other hand, have a high delivery efficiency.

Viral delivery

Adeno-associated viruses (AAV)

Belong to the **parvovirus** family, which is dependent on herpes or adenoviruses (helper viruses) for infection and replication in host cells. AAVs are nonenveloped, tiny viruses (20–25 nm) that can attach to various receptors. For instance, AAV2, AAV3, and AAV6 bind to heparin, while AAV4 and AAV5 primarily bind to acid sialic. AAV use as a vector for the CRISPR-Cas9 system has a number of benefits, including high infection efficiency, broad cell tropism, minimal immunogenicity, and non-pathogenicity for humans. AAVs have a tiny genome made of single-stranded DNA. One of the restrictions on using AAV vectors is the size of the genome. The 4.7 kb genome is made up of the promoter and polyadenylation signal in addition to the 4.3 kb genome encoding for Cas9 and a sgRNA that was isolated from *Streptococcus pyogenes* (SpCas9).

Lentivirus

Lentivirus is a key viral vector for delivering the CRISPR-Cas9 system. The Retroviridae family, which includes lentivirus, is capable of integrating into the host genome. Compared to the AAV, this enclosed virus is bigger and has a single-stranded RNA genome that can include a genome as large as 9 kb. Numerous benefits of the lentivirus vector include its large packing size, lengthy expression duration, low immunogenicity, and high infection effectiveness. Additionally, lentivirus vectors have been shown in certain trials to be effective instruments for the treatment of viral illnesses.

Nonviral delivery

Electroporation

Electroporation is the process of creating a competent cell (higher permeability of cell membrane) using electrical current. Target cells can be penetrated by RNA, DNA, and proteins due to the transient increase in cell membrane permeability. It has recently been determined that electroporation is an appropriate method for delivering CRISPR-Cas9 systems. All three strategies—the Cas9 protein and sgRNA combination, the plasmid-based CRISPR-Cas9 system, and Cas9 mRNA and sgRNA—are electroporated. Electroporation proved to be more effective for RNP than the other two techniques, according to a prior study. Additional benefits for electroporation were identified, including excellent efficacy and in vitro and in vivo application. However, the main drawback of electroporation is the triggering of cell death.

Hydrodynamic delivery

This procedure involves quickly injecting a sizable volume of DNA-containing solution (10% of the blood weight) into mouse veins. The injected nucleic acid is then taken up by the liver cells. Hydrodynamic delivery has been shown to be a useful technique for delivering the CRISPR-Cas9 system to preclinical mice' livers. This approach is quick, easy to use, affordable, and compatible with all CRISPR-Cas9 system techniques. However, its effectiveness is restricted to liver tissue, it has negative effects on the host's heart and liver, and it can only be employed in small, preclinical animals (mice).

Lipid transfection

RNA and DNA can be delivered to target cells via lipid nanoparticles or complexes, which are widely used techniques. Distinct charges between lipids (positive charge) and nucleic acids (negative charge) enable the formation of an appropriate complex for cell entry (endocytosis and macro-pinocytosis). Furthermore, nucleic acids are shielded from host nucleases by this encapsulation. This technique was first used for small interfering

RNA (siRNA) therapy, but it is currently being used to deliver CRISPR-Cas9 to cells. Despite its low effectiveness, this approach is easy to use, safe, and suitable for all CRISPR-Cas9 system methods.

Gold nanoparticles

RNPs are defined for this unique in vitro delivery strategy. The membrane fusion mechanism that is dependent on cholesterol facilitates the uptake of these nanoparticles into target cells, not cellular endocytosis. Several entrance pathways are likely linked to high delivery efficiency. However, one restriction in vivo is the toxicity of these nanoparticles at high quantities.

Applications of CRISPR-Cas technology:

Gene therapy

CRISPR-based genome editing tests have shown that this technology offers great promise for gene therapy, specifically for modifying or removing disease-causing genes. A study showed that homologous recombination of the CFTR (cystic fibrosis transmembrane conductance regulator) locus, a gene locus responsible for the disease, might be used to treat cystic fibrosis in vitro utilizing organoids derived from cultivated intestinal stem cells. Genome editing using CRISPR-Cas has shown effective in treating conditions like Fanconi anemia and crystalline gamma-c (Crygc)-associated cataracts.

Drug discovery and target

The development of therapeutic medications to treat genetic illnesses and the field of drug discovery both stand to benefit greatly from the application of CRISPR-Cas9 technology. The CRISPR-Cas9 system can be used to run genomic screens to find drug-resistant mutations in genes. CRISPR-Cas9 genomic screening has also been used to examine the effects of medications on cancer cells and infectious agents, as well as the proteins or genes that are implicated.

Role in Gene Activation and Silencing

Beyond its potential for genome editing, CRISPR/Cas-9 can be utilized to precisely control (repress or activate) a particular gene target by sophisticated Cas-9 protein modification. By deactivating its HNH and RuvC domains, researchers created dCas-9 nuclease, an advanced modified Cas-9 endonuclease. While the dCas-9 nuclease does not exhibit DNA cleavage activity, it does exhibit DNA binding activity. Then, the CRISPR/dCas-9 complex can be created by fusing dCas-9 with transcriptional activators or inhibitors. Consequently, the expression of a particular gene of interest can be silenced (CRISPRi) or activated (CRISPRa) using catalytically inactive dCas-9. Furthermore, by combining the dCas-9 enzyme with a marker like green fluorescent protein (GFP), CRISPR/dCas-9 can also be used to see and identify the precise location of the gene of interest inside the cell (subcellular localization). This makes it possible to image and mark endogenous loci in living cells with specificity for later use.

Microbiology

In yeasts, molds, and filamentous fungi, the CRISPR-Cas9 system has also proven successful in deleting or altering genes, which may have both pathogenicity and industrial significance. This approach modified the genomes of industrially relevant bacteria, including Streptomyces and Clostridium species, to produce biofuels, antibiotics, and anticancer drugs efficiently. *Saccharomyces cerevisiae* and other yeast species have an HDR repair process that enables precise genome editing. These eukaryotic organisms have had their strains modified using CRISPR-Cas9 technology in order to make them useful for metabolic engineering and synthetic biology. With the use of this technology, genetic interaction screens for the production of diverse mutant yeast strains might be achieved.

Cancer

Somatic genome editing using the CRISPR-Cas9 system has cleared the path for cancer modeling and the creation of model organisms with hematopoietic malignant tumors. Because Cas13a can discriminate between mutations that can lead to cancer, the CRISPR system can also be used to detect cancer in an individual during the early stages of the disease. Genome editing utilizing multiplexed CRISPR-Cas9 has enormous potential for assessing susceptibility in cancer-causing cells. Because the CRISPR system enhances efficaciousness and reduces unfavorable variables, it offers enormous potential for cancer immunotherapy.

Challenges for CRISPR/Cas-9 Application:

Immunogenicity, a lack of a safe and efficient delivery route to the target, off-target effects, and ethical concerns have all been key impediments to expanding the technique in clinical applications. Due of the bacterial origin of the CRISPR/Cas-9 system's components, host immunity may trigger an immunological reaction against them. In healthy humans, there were preexisting cellular (anti-Cas-9 T cells) and humoral (anti-Cas-9 antibody) immune responses to the Cas-9 protein.

The designed sgRNA will mismatch with the non-target DNA and can result in nonspecific, unexpected genetic modification, which is called the off-target effect. The 20-nucleotide sgRNA sequence and the **PAM** sequences next to the target genome define the CRISPR/Cas-9 target efficiency. It has been demonstrated that off-target effects may arise from more than three mismatches between the target sequence and the 20-nucleotide sgRNA. The use of the CRISPR/Cas-9 editing system for therapeutic reasons is restricted due to the potential for detrimental events such sequence mutation, deletion, rearrangement, immunological response, and oncogene activation caused by the off-target effect.

CONCLUSION:

The CRISPR/Cas-9 system in nature is used to protect prokaryotes from invading viruses by recognizing and degrading exogenous genetic elements. The two components of CRISPR/Cas-9 gene editing are Cas-9, a protein that essentially cuts DNA at the position indicated by guide RNA, and guide RNA, which is utilized to locate (bind) the target DNA to be edited. This technique was adapted from acquired immunity in prokaryotes. Finding the target gene that controls the desired phenotype and creating the guide RNA are essential steps in the CRISPR/Cas-9 gene-editing process. Molecular biology is entering a new era with a wide range of applications, from fundamental molecular research to therapeutic uses. There are still certain problems to overcome in practical applications, and numerous improvements are required to overcome impediments and ensure maximum benefit while reducing danger.

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