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REVIEW ARTICLE **OPEN ACCESS**

CRISPER-CAS System and Its Applications

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ABSTRACT
The CRISPR-Cas focuses on adaptive immunity systems of many bacteria and nearly all archaea. Its discovery has proven it as a simple tool and its effectiveness for many aspects like Transcription Activator. It can be used to add desirable and remove undesirable alleles simultaneously in a single event. Presently CRISPR-Cas has emerged as a engineering tool to controlled genetic modification in many organisms like Staphylococcus aureus, Escherichia coli, Saccharomyces cerevisiae etc. CRISPR-Cas9 system has revolutionized the area of genome editing because of easy laboratory construction, targeting multiple genomic sites. The Cas9 enzyme has been used as a popular tool for directed gene editing used in medical research areas. It can be used as an effective gene therapy method in various genetic disorders where no curative treatment is available. Mainly it is used in microbial adaptive immune system, gene regulations, functional genomics, genome editing,
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INTRODUCTION

In 1970, recombinant DNA technology development marked the beginning of new era in biology. Molecular biologists acquired the power to manipulate the blue print of life "DNA", making it possible to study genes and harness novel medicines. It has given us a creation to get the desired change in the genome either by gene insertion or gene deletion at target sites. One such development of novel molecular tool is CRISPER.

The clustered regularly interspaced short palindromic repeats (CRISPER)/CRISPER-associated (Cas) systems are found in many bacteria and nearly all archaea and constitute an adaptive immune system that defend them from phages and plasmid by recognizing them and then degrading their DNA[1]. The basic understanding of the immunity mechanism was given by Barrangou and coworkers, who showed that *Streptococcus thermophilus* can acquire resistance against a bacteriophage by integrating a genome fragment of an infectious virus into its CRISPER locus.[2] Almost all bacterial genomes have CRISPER-Cas9 loci.

The loci consist of clustered direct palindromic repeats and each repeat is followed by short segments of spacer DNA from previous exposures to foreign DNA [3][4]. The repeats are typically 28 to 37 nucleotides long and identical in a single locus. The interspersed nucleotides sequences are called as spaces and are derived from foreign viruses. Spacer sequence is transcribed into CRISPERRNA (crRNA). CRISPER locus also contains DNA sequences which code for a complementary transactivating Crisper RNA (tracrRNA) and various Crisper Associated genes (cas) which code for nucleases. These crRNA hybridize with complementary transactivating Crisper RNA (tracrRNA) and together, as a double strand, they recognise the complementary foreign nucleotide sequences.

Structure of CRISPER/Cas9 system in figure 1.

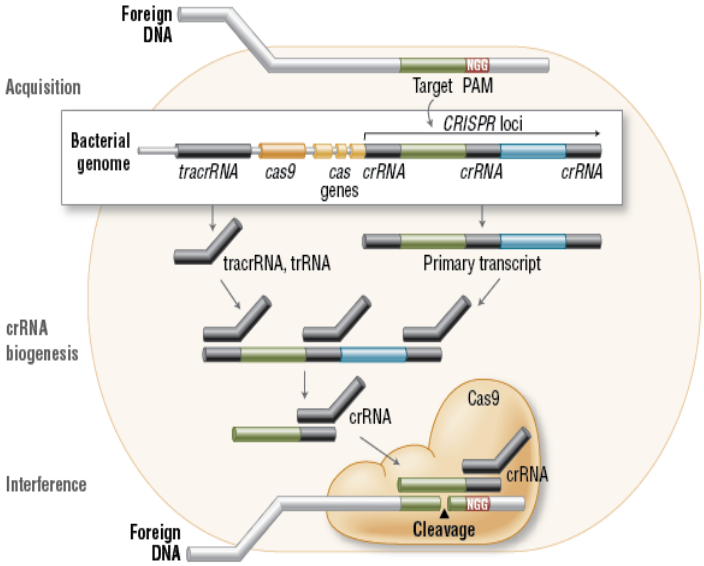


Figure 1: Foreign DNA is incorporated into the bacterial genome at the CRISPER loci. CRISPER loci is then transcribed and processed into crRNA. Cas9 endonuclease cleaves foreign DNA containing a 20-nucleotide crRNA complementary sequence adjacent to the PAM sequence**.**

There are 3 types of CRISPER-Cas immune systems. Out of them the type II system (adapted from Streptococcus pyogenes), which uses Cas9 as a nuclease, is used as a method of genome editing, which is why it is known as the CRISPER-Cas9 system. Cas9 nuclease has HNH nuclease domain and the RuvC-like domain which generate Double stranded breaks (DSBs).Cas9's functions in the presence of two nuclease domains, a RuvC-like nuclease domain located at the amino terminus and a HNH-like nuclease domain that resides in the mid-region of the protein(7). Hence the system utilizes Cas9, crRNA, tracrRNA and a section of DNA repair template that is utilized in either non-homologous end joining (NHEJ) or homology directed repair (HDR).

HISTORY

The story of CRISPER began in 1987 by the researcher Yoshizumi Ishino and his colleagues in Osaka University. While studying the *iap* enzyme involved in conversion of alkaline phosphatase in *E.coli* , they accidently They accidentally cloned part of a CRISPER together with the *iap* gene, the target of interest. They found a strange set of 29 nt repeats downstream of the *iap* gene which were interspaced by five intervening 32 nt non repetitive sequences [8][9]

Over the next decade, more repeat elements were reported from genomes of different bacterial strains. Meanwhile repeats were observed in the archaeal organisms of *HAloferax and Haloarcula* spcies for the firwst time transcription of the interrupted repeats were noted by Mojica. [10]IN 2001, Jansen and Mojica coined the acronym CRISPE (clustered regularly interspaced short palindromic repeats) to avoid the confusion used to describe the sequences in scientific literature. [11] At the same time, several clusters of CRISPER-associated (***Cas***) genes were identified which were conserved and typically adjacent to the repeat elements [11] serving as a basis for the eventual classification of three different types of CRISPER systems (types I–III) [12]. Types I and III CRISPER loci contain multiple Cas proteins known as Cascade and form complexes with crRNA for recognition and destruction of target nucleic acids.[5] In contrast, the type II system has a significantly reduced number of Cas proteins. Despite increasingly detailed mapping and annotation of CRISPER loci across many microbial species, their biological significance remained elusive.

Until about three years ago, improvements in the ***in vivo*** genetic engineering of specific genes in plants and animals have relied on technologies involving the combination of a protein that has been engineered to bind to a specific DNA sequence with a nuclease that introduces a double-strand break at the target site. These technologies rely on zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) to create the necessary DNA binding specificity.[10a][10b] A research was done for CRISPER system on *Streptococcus pyogenes* that relies on the protein Cas9.[12] The Cas9 endonuclease is a four-component system that includes two small RNA molecules named CRISPERRNA (crRNA) and trans-activating CRISPERRNA (tracrRNA)[12]. Jennifer DouDNA and Emmanuelle Charpentier re-engineered the Cas9 enzyme into a two-component system by fusing the two RNA molecules into a "single-guide

RNA" that, when combined with Cas9, could find and cut the DNA target specified by the guide RNA[13]. By manipulating the nucleotide sequence of the guide RNA, the artificial Cas9 system could be programmed to target any DNA sequence for cleavage.[13] Another group of Šikšnys together with Gasiūnas, Barrangou and Horvath showed that Cas9 from the *S. thermophiles* CRISPER system can also be reprogrammed to target a site of their choosing by changing the sequence of its crRNA[14]. These advances fueled efforts to edit genomes with the modified CRISPER-Cas9 system. For the first time, Feng Zhang's and George Church's groups did genome editing in human cell cultures using CRISPER-Cas9 [9][15][16]. Then it has since been used in a wide range of organisms, including baker's yeast (*Saccharomyces cerevisiae*)[17][18], the zebrafish (*D. rerio*)[19], fruit flies (*Drosophila melanogaster*)[20], nematodes (*C. elegans*),[21] plants,[22] mice,[23] monkeys[24] and human embryos.[25]

LOCUS STRUCTURE

A CRISPER locus comprises of AT-rich leader sequence followed by CRISPER array, a short series of repetitive DNA called repeats and non- repetitive sequences are called unique spacers.[26]CRISPER repeats range in size from 28 to 37 base pairs. The size of spacers in different CRISPER arrays is typically 32 to 38 base pairs (range 21 to 72 base pairs)[27]as shown in figure 2. New spacers can appear rapidly as part of the immune response to phage infection.

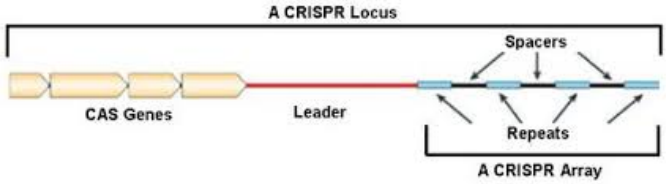


Figure 2: CRISPER locus - Three components of CRISPER locus are : Cas genes ,a leader sequence ,a repeat and spacer array.

Small clusters of CRISPER-associated *Cas* genes are located next to CRISPER repeat-spacer array or sometimes located upstream. There are 93 *Cas* genes which are grouped into 35 families based on sequence similarity of the encoded proteins. 11 of the 35 families form the *Cas* core, which includes the protein families Cas1 through Cas9[28]. A complete CRISPER-Cas locus has at least one gene belonging to the *Cas* core. Based on CRISPER locus organization and ***Cas*** gene content, CRISPER-Cas systems are classified into three main types (I, II and III) and further subdivided into 11 subtypes (I-A to I-F, II-A to II-C, and III-A to III-B)[29] . Class 1 systems use a complex of multiple Cas proteins to degrade foreign nucleic acids. Class 2 systems use a single large Cas protein and RNAse III for the same purpose. Class 3 uses Cas6 proteins for the editing activity. [29]

MECHANISM

CRISPER system is a type of adaptive immunity system. It defends the bacteria and archea from from viral gneome .

It is a three step mechanism:

1. Spacer Acquistion
2. crRNA Processing / Biogenesis
3. Interference

1) **Spacer Acquistion** : The first step is same in all the three types of different CRISPER system. In this when a bacteriaphage infects a cell for the first time the bacterial cell chop up the viral genome and take a piece of it and integrate it into spacer DNA. These spacers DNA are pieces of different viral genomes that have infected the cell previously so each time a bacteriophage infects the cell the bacteria takes it and insert It into the spacer DNA.[30][31][32]

Near the CRISPER locus there are Cas genes which translates into the Cas enzymnes . these Cas enzymes are mostly nucleases or heliCases. Nucleases cut the DNA into blunt ends i.e between the nucleotides while the heliCases destroy the hydrogen bonds between the two strands of DNA and then separate them. The two Cas enzymes Cas1 and Cas2 are involved in spacer acquisition. Both Cas1 and Cas2 are dimers and form a complex where a Cas2 dimer bridges two Cas1 dimers.[33] In this complex Cas2 performs a non-enzymatic scaffolding role binding double-stranded fragments of invading DNA, while Cas1 binds the single-stranded flanks of the DNA and catalyses their integration into CRISPER arrays.[33] New spacers are added next to the leader sequence at the beginning of the creating CRISPER.

2. **CrRNA processing or Biogenesis**: The crRNA is acronym for CRISPERRNA processing. In the second step, CRISPER sequence having the different pieces of bacteriophage genome is going to be transcribed into mRNA (crRNA) (one of the two strands of DNA is being transcribed). This transcript is then cleaved

by Cas proteins to form crRNAs.[3] The mechanism to produce crRNAs differs among CRISPER/Cas systems.

In type I the CRISPER repeat forms loops (figure 3) and cRNA will be cleaved using the enzymes Cas6e or Cas6f. These Cas proteins cleave the longer transcript at the edge of the paired region, leaving a single crRNA along with a small remnant of the paired repeat region.[34]

In Type II system it encodes an extra small RNA that is complementary to the repeat sequence, known as a trans-activating crRNA (tracrRNA) such that the primary CRISPER transcript results in base pairing and the formation of dsRNA at the repeat sequence, which is subsequently targeted by Cas9 and RNAseIII for cleavage.[35]

Type III systems use Cas6 and cleave the longer transcript wrapping around the Cas6 to allow cleavage just upstream of the repeat sequence. In this repeatsdo not produce stem loops[36][37].

3. **Interference**: In the third step, the CRISPERRNA will now be integrated within the Cas proteins. In type I systems, Cas3 is conserved marker protein in the interference reaction which contains a HD phosphohydrolase domain and a DExH-like heliCase domain[38]. Cas3 interacts with a complex of different Cas proteins and a complex is formed termed as Cascade that binds and deliver the crRNA. The PAM sequence is recognized on the crRNA-complementary strand and is required along with crRNA annealing. When the correct base pairing between the crRNA and the protospacer signals a conformational change in Cascade that recruits Cas3 for DNA degradation.[38]

Type II systems only a single multifunctional protein Cas9 is used for the interference step[38]. Cas9 requires both the crRNA and the tracrRNA to function and cleaves DNA using its dual HNH and RuvC/RNAseH-like endonuclease domains. The double-stranded endonuclease activity of Cas9 also requires that a short conserved sequence, (2–5 nts) known as protospacer-associated motif (PAM), follows immediately 3′- of the crRNA complementary sequence [39]. In fact, even fully complementary sequences are ignored by Cas9-RNA in the absence of a PAM sequence Also base pairing between the PAM and the phage genome is required in type II systems. However, the PAM is recognized on the same strand as the crRNA (the opposite strand to type I systems).[39]

Type III systems, like type I require six or seven Cas proteins binding to crRNAs.[40] the systems targets the mRNA of phages rather than phage DNA genome, which may make these systems uniquely capable of targeting RNA-based phage genomes.[41][16]

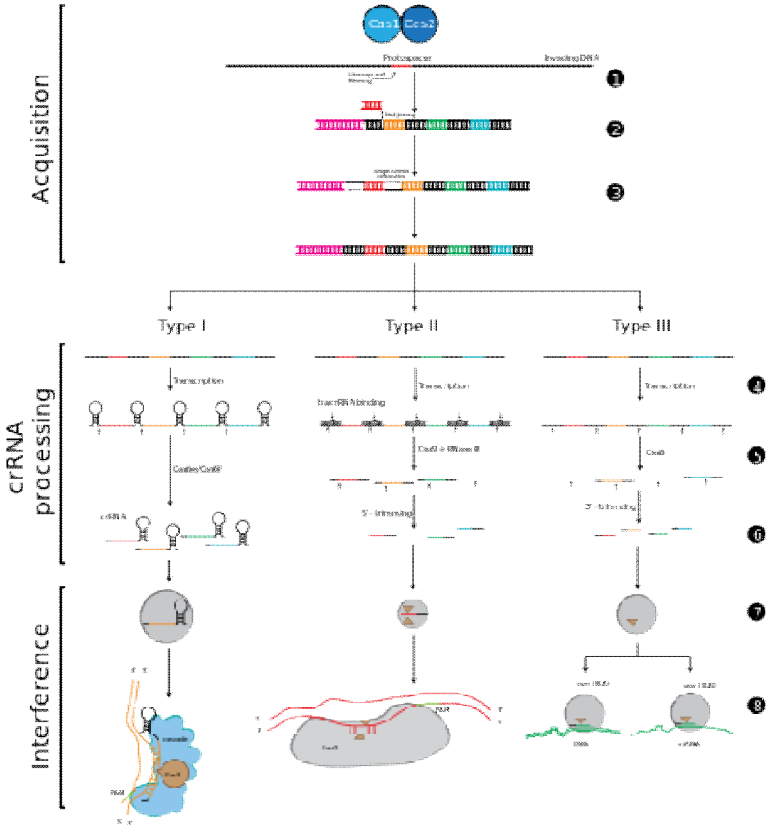
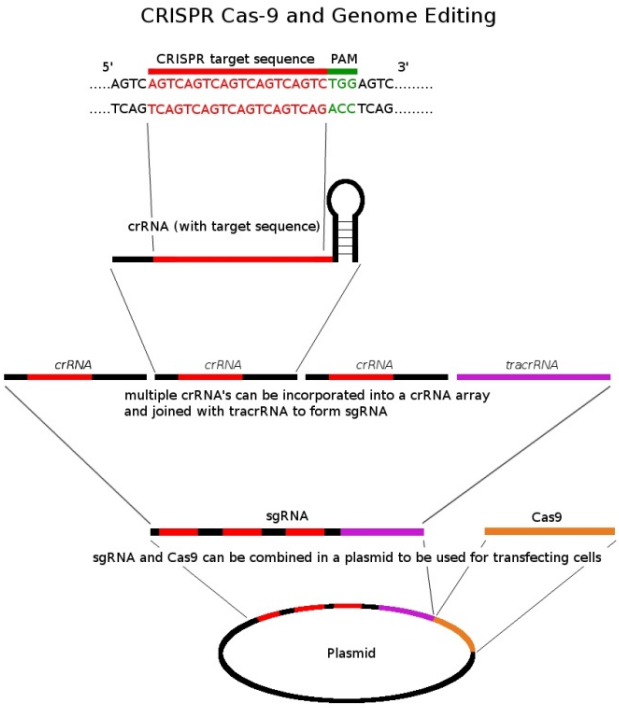


Figure 3 : Stages of CRISPER immunity including all the three types of adaptive immunity

Applications

In 2000, researchers developed zinc finger nucleases (ZFNs), synthetic proteins whose DNA-binding domains allows creating double-stranded breaks in DNA at specific points. In 2010, synthetic nucleases called transcription activator-like effectors nucleases (TALENs) provided an easier way to target a double-stranded break at a specific location on the DNA strand. Both zinc finger nucleases and TALENs require the creation of a protein for each targeted DNA sequence, which is difficult and time-consuming process than that for guide RNAs. CRISPERs are much easier to design because the process requires making only a short RNA sequence[43] CRISPER can target several DNA sites simultaneously by simply introducing different gRNAs and also CRISPER costs are relatively low. This technology had been used to functionally inactivate genes in human cell lines and cells, to study *Candida albicans*, to modify yeasts used to make biofuels and to genetically modify crop strains.[44] CRISPER is also used to change mosquitoes so they cannot transmit diseases such as malaria.[45] CRISPER/Cas9 system carries genome editing with a Type II CRISPER system. The system utilizes Cas9, crRNA, tracrRNA and an section of DNA repair template that is utilized in either non-homologous end joining (NHEJ) or homology directed repair (HDR).CRISPER/Cas9 system employs a plasmid to transfect the target cells. The main components of the plasmid are shown in figure 4.



1. GENE SILENCING

The Cas9 enzyme has been used as a popular tool for directed gene editing in eukaryotic systems.[16]With the use of a target-specific CRISPERRNA (crRNA) and trans-activating crRNA (tracrRNA), or a fused format called a single guide RNA (sgRNA), locations within complex mammalian genomes can be targeted by the Cas9 endonuclease for a double stranded break [46]. These breaks can be repaired by endogenous DNA repair mechanisms through a process known as non-homologous end-joining (NHEJ). Because NHEJ is error prone, genomic deletions or insertions can result that create frame shifts and premature termination to permanently silence target genes. The insertions and deletions resulting from NHEJ are random and differ from cell to cell. The exact genomic changes that result can be determined by additional experiments on clonal cell lines.[47]

2. HOMOLOGY DIRECTED REPAIR

Due to its versatility, CRISPER/Cas9 genome editing tools have been used in medical research areas and show promising results. One method of CRISPER/Cas9 that has shown great potential in the gene therapy research area is homology directed repair (HDR). CRISPER/Cas9 is used to create a double stranded break in the DNA and ideally, one could insert a donor template to alter the coding region to potentially correct a mutation[47]. There have been several studies discussing HDR's potential to "fix" different genetic mutations.

2. DISEASES

CRISPER/CAS9 IN REPAIRING OF CFTR DEFECT

A study performed by Schwank et al. used the CRISPER-Cas9 genome editing approach to correct the d508 mutation in the cystic fibrosis transmembrane conductance regulator gene (CFTR). They isolated and expanded adult intestinal stem cells from two patients affected with cystic fibrosis.[48] Hence Genomic editing was done using CRISPER-Cas9 system and homology mediated repair of d508 mutation. Thus providing a proof that the CRISPER-Cas9 system can be used as an effective gene therapy method in various genetic disorders where no curative treatment is available.

CRISPER IN HIV THERAPY

After the introduction of highly active antiretroviral therapy (HAART), human immunodeficiency viral (HIV) infection is considered a chronic disease requires prolonged treatment but due to high cost of treatment, commitment of the family and treating clinicians . CCR5 is a co-receptor on CD4+ cells which is essential for HIV entry inside these cells. A 32 base pair deletion in single exon of CCR5 gene produces a frame shift and disrupts the gene function. A patient from Berlin who had HIV with lymphoma was cured by bone marrow transplantation from a person who was harbouring this homozygous deletion in CCR5 gene. Hence disruption of CCR5 gene by gene silencing methods appears to be an attractive model of gene therapy for HIV.[49] The first reported genome editing method was based on using ZFN and then other methods such as TALEN and CRISPER-Cas9 system began to be used. Genome editing system has been used to eliminate integrated HIV proviral DNA. Ebina *et al*. in 2013 in their study targeted the long terminal repeats (LTR) of the integrated HIV viral genome using CRISPER-Cas9 and showed the blocked expression of genes.

CANCER

In 2016, CRISPER had been studied in animal models and cancer cell lines, to learn if it can be used to repair or thwart mutated genes that cause cancer [50] and involving the first clinical trial of CRISPER. It involved removing immune cells from people with lung cancer, using CRISPER to edit out the gene expressed PD-1, then administrating the altered cells back to the same person. In 2017, 20 other trials were done on human in China.[51]

In 2016 the United States Food and Drug Administration (FDA) approved a clinical trial in which CRISPER would be used to alter T cells extracted from people with different kinds of cancer and then administer those engineered T cells back to the same people[52].

4. PRODUCING FOOD

Researchers at Tokushima University created seedless tomatoes using CRISPER. Seedless fruit could be a vital step towards more sustainable food production, as they can be grown from scratch in laboratories. This avoids environmental compilations like insufficient pollination, rain or sunlight, potentially leading to higher crop yields. It is believed that CRISPER could be used to remove allergens from food, as well as improving shelf life.

5. CREATING BIOFUEL

A partnership between J. Craig Venter and Exxon Mobil has used CRISPER to improve the energy production of algae. After eight years of research, their joint venture Synthetic Genomics Inc. has successfully doubled the amount of oil produced by the aquatic organism via CRISPER gene editing. Thus representing a significant development in alternative energy solutions.

6. EMBRYONIC STEM CELLS AND TRANSGENIC ANIMALS

CRISPER-Cas systems can be used to efficiently engineer one or multiple genetic changes to murine embryonic stem cells for the generation of genetically modified mice [53]. A similar approach has been used to genetically modify primate single cell embryos[54]

CONCLUSION

CRISPR-Cas9 system has revolutionized the area of genome editing because of easy laboratory construction, targeting multiple genomic sites simultaneously with high precision and accuracy and a wide range of applications. Technological and ethical issues still stand between us but we are only at the beginning of genome-editing tool “CRISPR-Cas9” so a lot of progress still has to be made.Future challenges involve invention of efficient delivery systems, reducing off-target effects and increase in efficient homology mediated repair.

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