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CRISPR Technology in Bacterial Gene Editing and Therapy

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Annotation: CRISPR technology is a part of a defense system found in bacteria and archaea that is used to destroy the DNA of foreign pathogens. A segment of DNA from the pathogen is incorporated into the bacteria's DNA in between repeats of palindromic sequences, and enzymes transcribe these encoded repeat elements and use the RNA molecules as a guide to finding and destroying the DNA. The system is surprisingly malleable and has been repurposed for many uses, including gene editing of a wide variety of organisms. Bacteria protect themselves by putting a piece of an invading virus's DNA inside a CRISPR cassette, essentially making themselves a wanted poster and keeping it in their genome so that more of the organisms can use it. One of the proteins made by the bacteria reads the DNA sequence from the wanted poster, and when the pathogen returns, it pairs to the DNA sequence and

destroys it. However, the bacteria have also made a few small changes in the repeated DNA sequences over time to prevent the bacteria from reading itself or from reading too closely related wanted poster designs.

When being used for gene editing, molecular biologists will design a wanted poster of their own and put it into the CRISPR/Cas9 system, but with a couple of tweaks to focus it on the desired DNA sequence. The encapsulated RNA and protein are injected into the target organism, where they are used to find and destroy the DNA. When the cell attempts to repair the double-strand break, it will use an introduced DNA sequence as a template for repair. When the CRISPR/Cas9 system locates the target sequence, the protein will bind and cleave the targeted sequence. Due to its simplicity, power, and precision, CRISPR/Cas9 has empowered researchers and large organizations to engineer genetic changes in mice, monkeys, and human cells. These changes—ranging from deletion or silencing of a specific gene to insertion of a gene bearing a variant allele or genetic material—hold tremendous therapeutic promise. But as the toolkit for engineering the genomes of living organisms has evolved, so has the risk of a doomsday bug or infotech nightmare looming over humanity.

1. Introduction to CRISPR Technology

The CRISPR technology is a gene editing tool recently discovered in bacterial systems and has found increasing application over the years owing to its user-friendly nature and effectiveness in precise gene editing. The system offers the possibility of precisely targeting genes to effect mutations intended for the repair or modification of disease-inducing mutant genes in organisms.

As a technology based on the prokaryotic antiviral defense system, different kinds of CRISPR systems exist. The first type identified was the CRISPR/type II system, and this has seen several improvements over the course of its application for gene editing purposes. The flexibility of the system has allowed for the development of derivatives of the native CRISPR system with improvements such as reducing off-target mutations and increasing the precision of gene editing. We present the development of the CRISPR system, including improvements that have been carried out by various researchers, such as optimization of reagents used in the system, research on factors affecting gene editing, and prevention of off-target mutations. We also consider some improvements to the CRISPR technology. As an example of the application of the CRISPR technology, we present the possibility of using the CRISPR system to manipulate the gene of a malaria-transmitting mosquito. In conclusion, possibilities for the application of CRISPR technology in disease therapy and effecting precise mutations in the genomes of either patients or organisms are discussed. [1][2]

1.1. Historical Background

In 2007, a mechanism of RNA-mediated gene expression regulation was found in archaea. By 2010, analogs of RNA-mediated interference were identified in bacteria, and the name CRISPR-Cas was proposed. Soon, the Cas9 protein was recognized as a useful RNA-directed DNA editing tool that, after modifications, became very popular and widely used in various species. PAM is the short DNA sequence adjacent to a target sequence necessary for the recognition and binding of the Cas protein. Because we are working with bacterial Cas proteins, the PAM-related topics do not relate to eukaryotic PAMs, which can introduce potential genomic biases into RNA-directed Cas protein binding. Other type II Cas proteins and CRISPR-Cas protein subtypes have different PAM recognition rules. For example, in the case of a sequence off of the target, the availability of roommates such as DNA incorporated Cy5 that is located just two nucleotides away can also lead to the recognition of a mismatched PAM. Subtypes also affect spacer-adjacent motifs.

All CRISPR-Cas proteins have other sequence requirements, including secondary structure preferences. These can potentially limit PAM activity. For some designs, such as multiple guide RNA-based editing with dCas proteins, the absence of a PAM requirement can be beneficial. The detailed ATP measurement methods and AFM studies of 70 families of end-realization revealed a range of off-target effects. Of course, none of the PAM requirements, especially the additional PAMs such as the Cm and base modifications to the crRNA, will eliminate the possibility of off-target effects. Clustered Regularly Interspaced Short Palindromic Repeats interfere with bacteriophages and mobile genetic elements, while CRISPR-associated enzymes are responsible for the interference. [3][4]

1.2. Mechanisms and Components

In typical CRISPR-Cas systems, the chromosomally encoded agronomical single guide RNA and the Cas9 nuclease are needed to effect the targeted sequence exchange. Core components common to all CRISPR-Cas systems are Cas proteins, including Cas9, and the functional crRNA or gRNA. The targeted DNA sequence should be identical or similar to the CRISPR RNA protospacer component that approaches the leader sequence and should end with the tracrRNA. Cas9 is an RNA-guided endonuclease that makes a double-stranded break 3 bp from the PAM in a sequence-specific manner. PGTS genes, however, show differences in their protospacer recognition motifs that lead to some of the key structural and functional characteristics of the bacterial DNA being modifiable in multiple parts of the organism.

Pairing of 5′ “seed” sequences at the 3′ end of the crRNA to the seed + proto-pan genome coding sequence controls the association of the PAM and mediates sequence recognition and specificity. Minor changes in the promoter efficiencies of CRISPR-Cas9 facilitate the modification of the efficiency of engineering in these two organisms. The unique sets of CRISPR genes provide in-depth information on the CRISPR gene family in a wider breadth of bacterial species. Such CRISPR views will not only help to understand the evolutionary pressures that govern the composition and organization of these genetic loci but also provide important guidance for the therapeutic use of

CRISPR-based approaches for genome modification. [5][6]

2. Applications in Bacterial Gene Editing

The main application of the CRISPR/Cas9 system in bacteria is gene editing. Bacteria possess their own CRISPR/Cas systems, which makes new gene insertion or deletion very efficient and low cost. The general steps of CRISPR-mediated gene editing in bacteria include: (1) designing sgRNA to target the gene to be inserted or deleted, and recombinationally mutated donors, (2) transforming the bacterial host with the Cas9-expressing plasmid and the candidates, (3) screening mutants with better genotypes regulated by the mutations in the cells, and (4) amplifying the genotypes using colony PCR. After obtaining the positive mutants, curing the Cas9-expressing plasmid can eventually realize the clean gene-editing effect.

Once programmed, the CRISPR/Cas9 can effectively knock out a gene of interest by plasmid-induced dissociation of the bacterial host along with a repair mutation in the genomic DNA. The main problem of the traditional homologous recombination method in secondary genome integration is the occurrence of cell death in the recombination steps, whereas the CRISPR/Cas9 system can generate mutant derivatives with genetic events. The CRISPR/Cas9 system shows advantages over processes, demonstrating significant agronomic trait improvements in the bacterial chromosomes. This system quickly repairs double-stranded breaks in the genome. Rocky sites of saturated mutagenesis are vital for modified host microorganisms. High-throughput combinatorial optimization, which can generate any desired mutant, usually takes a few steps with large numbers of DNA guides and donor templates to repair during bacterial transformations, followed by mutagenic recombination to the mutant. These nucleotide-free DNA sequences are constantly integrated into bacterial chromosomes with repair-like single-stranded repair cassettes. [7][8]

2.1. Gene Knockout

The simplest task for CRISPR is knockout, where the relevant gene is inactivated. Adjacent to the endonuclease genes, or within a separate batch of constructs, is the gene necessary for the expression of an appropriate gRNA. In the most common instances, these use the same small RNA molecule, showing how the method naturally targets its own expression. The requirement for complementary sequence between the two RNAs for intact enzyme function, but the lack of need for close sequence matching between the DNA and the gRNA, means that plasmid DNA or synthetic RNA encoding the gRNA can be transfected into host cells or embryos. This competes with the genomic locus' DNA, causing RNase activity by the gRNA. Around 3–5% repair events immediately restore the target cut, but those lacking an active or very derived gene produce no correct protein, effectively giving a population of null alleles.

2.2. Gene Insertion and Replacement

Gene Insertion and Replacement. The CRISPR–Cas system has been used to insert new genes into bacterial genomes as well as to replace existing ones. Originally described for gene disruption in certain bacteria, the CRISPR exonucleolytic activity of SpCas9 through the non-homologous end joining (NHEJ) pathway has been exploited to achieve gene insertion and replacement in different bacteria. This editing can occur in two ways: a double-strand break (DSB) at a genomic locus via the activity of the Cas9 nuclease followed by NHEJ repair in a non-cognate manner with an exogenously supplied DNA fragment; or an NHEJ-based modification in the absence of a DSB. Both strategies are useful to replace or add genetic traits in bacterial chromosomes without marker recycling and with no or few detectable off-targets.

The first of these gene editing strategies requires the use of donor sequences carrying short homologous arms when co-transformed together with expressing vectors for a sgRNA and CRISPR–Cas9 components. Originally termed precise multiplex gene replacement to distinguish it from error-prone methods to replace in certain bacteria the CRISPR-modified protospacer or PAM site using long, non-recombinogenic extensions of the protospacer DNA region, it relied on the induction of non-homologous end joining activity to achieve recombination between the

chromosomal DSB and the donor, without dependence on significant homology between them. In the absence of the CRISPR target, such correctly recombined alleles were found to be outnumbered by the amount of spontaneous recombinations that take place in this bacterium. [9][10]

3. Therapeutic Potential in Bacterial Infections

Widespread and increasing antimicrobial resistance suggests that different therapeutic strategies are needed. Bacteriophages have undergone a renaissance as possible therapeutics, but are not ideal for a number of reasons. Thus, the ability to engineer antiphage nucleotides into bacterial genomes is widely applicable. Here we describe work showing that the clustered regularly interspaced short palindromic repeat system can potentially prevent and cure some phage infections. The approach is based on the intrinsic self/non-self discrimination that most bacteria perform by using CRISPR. Indeed, numerous real-world applications have been demonstrated, and more are likely to be forthcoming.

A remarkable feature of CRISPR-Cas immune systems is their adaptive ability: the systems are able to learn from, and then remember and respond to specific insults. In this review, we discuss how adaptation results in several interesting possibilities, including adaptive antimicrobials and CRISPR-based diagnostics. Widespread bacterial drug resistance results in great difficulty in treating infections, with large socio-economic consequences. To alleviate this problem, it is imperative to decrease not only the frequency of non-self 'hits' and to minimize the acquisition of de novo mobile elements that may carry harmful genes, but also to develop new methods to prevent and cure bacterial infections. With this goal in mind, we have proposed that phage-resistant bacteria can be constructed by integrating antiphage sequences into bacterial genomes using the CRISPR-Cas system. [11][12]

3.1. Antibiotic Resistance

The use of CRISPR for combating the antibiotic resistance crisis arises from the ability to selectively identify and destroy undesired bacteria. CRISPR-based diagnostic platforms have been developed that can directly detect antibiotic-resistant bacteria in clinical samples. Since CRISPR systems were first discovered in bacteria, they have been remarkably useful in genetically manipulating bacteria. Thus, several CRISPR systems have been used to engineer novel antibiotics or to manipulate the antibiotic resistance gene to change the antibiotic resistance. CRISPR systems have also been used to restore antibiotic sensitivity in resistant bacterial strains. Co-opting the CRISPR systems from bacteria, or repurposing existing class 2 CRISPR systems, is a sensible step. Antibiotic resistance recently emerged as one of the most pressing global health crises. Ancient bacteria that managed to accumulate mutations or shunt their plasmid's coding regions have begun to circulate, leading many of the most common antibiotics to become increasingly useless. Moreover, there has been no major discovery of new major antibacterial classes in years. Just a few weeks ago, however, a CRISPR-based assay able to determine if specific bacteria are sensitive to specific antibiotics was published. Researchers have now used CRISPR to demonstrate how novel antimicrobials could be developed and to restore antibiotic sensitivity to resistant bacteria. The CRISPR systems from bacteria have been used to precisely manipulate bacterial genomes and engineer them to lose resistance genes. [13][14]

3.2. Novel Therapeutic Approaches

In the last few years, after its massive adoption in the field of therapeutic gene editing, CRISPR technology has emerged as a disruptive tool, laying out new frontiers in the research of genetic-based medicines to treat inherited genetic diseases. A multitude of rare inherited monogenic disorders are caused by different genomic mutations that lead to the loss of protein functionality. The idea at the basis of the therapeutic application of CRISPR technology is to rewrite the mutated genes or to insert correct ones, with a mechanism much less invasive, much more targeted, and precise compared to the canonical gene therapy methods, in which the editing is random and does not preserve the original gene context. It's worth mentioning the approaches that imply the delivery

of only a spot-modified gene copy, since they preserve the correct structure and function of the gene within the final products. The greatest advantages of this last category of gene therapy come from this feature that the patient's cells toward which the editing machinery has been targeted remain the starting version of themselves, with only a modification in the mutated gene.

Oligonucleotide-mediated gene correction: therapeutic oligonucleotides are single-stranded DNA that can be used as a template for HDR, promoting the correction of the pathogenic mutations in the cells. This approach has been largely used in the context of CRISPR-mediated editing, in view of promoting the therapeutic correction of a large number of pathogenic mutations, as proof of concept of the efficacy of this kind of approach. Therapeutically selective RNA base correction: the synthetic RNA/Cas complexes are used to correct a DNA base, to the detriment of the other competitors, with a substantial effect on the global target-to-nontarget specificity of CRISPR. This approach has the major con of causing a lot of off-target effects in some sequences of the genome, but is less costly against hypotension or other chromosomal aberrations due to trans-splicing events if compared with gene replacement strategies.

In a broader perspective, the therapeutic application of CRISPR technology in various models of monogenic disorders by the genetic correction of embryos may pass through the production of an in vitro monoclonal patient's embryo itself that starts to develop, but then the selection gets stopped at the embryonic stage in which the CRISPR machinery, specifically dedicated to gene repair, has been correctly delivered inside, repairing the mutated gene(s) and producing a selected number of in vitro karyotypically normal cells that will be re-injected in the patient's embryo to make it grow. This approach, which is still in its first steps but starts to give encouraging results, has been followed in the research of genetic diseases where it is relatively easier to generate single-cell human embryos, but is still subjected to a lot of ethical and legal issues. However, aiming at providing the foremost prerequisites of therapeutic safety and efficacy, the progressive increase in the use of CRISPR technology in this field will allow its relative techniques to meet these constant growths not only in terms of the number of patients or diseases that can be treated, but also in terms of the number of possible gene target sites that are breaking down the recurring barriers posed by the genome structure and its cell type-specific complexities. [15][16]

4. Challenges and Ethical Considerations

Whole-genome sequencing has never been easier to perform, and technology now allows laboratories without bioinformatics capabilities to perform this task, requiring only a basic understanding of computers. Thus, the financial cost of WGS compared to gene editing in full-GATC-containing bacteria suggests that WGS of human pathogens may become the method of choice when diagnosing important pathogenic strains, which can be combined with knowledge of their underlying mutation status to orient the strategy designed to eradicate them. However, discussions of ethical considerations in the context of a world full of enriched and empowered individuals who might employ these tools de facto and completely disregard any form of regulation seem to remain irrelevant, at least until the release of most genome editing tools to a wider community in the form of beginner-friendly kits.

The widespread adoption of simple-to-use and inexpensive portable in-home and on-gardening bioweapons will require the establishment and implementation of a dual-use research code of conduct. In contrast, whole genome sequencing is all but adopted by gardeners, but if we are not scared enough to lock down access even to viruses, we may want to instigate the widespread adoption of specific diagnostics of living entities, propose machines that are able not to produce genetically identical branches, and share with as many people as possible an inexpensive cure. Once informed consent and ethical considerations have been conquered, and the general population has learned more about themselves and about pathogens, treatment practice may force more immediate and ethical discussions of how the technology should be configured in terms of patented uses. We do, however, expect the stringent work conditions that are to be universally disregarded. [17][18]

5. Future Directions and Opportunities

As a fundamental layer of the stress response regulatory network, small RNAs can regulate the expression of multiple genes and shape the gene regulatory landscape for CRISPR technologies to take effect or perform any modifications. Given the broad applications, sRNA-based CRISPR technology has attracted great attention, especially with the discovery of a new type of dual trans-acting small interfering RNA in many bacteria. Moreover, as the medium of conversation between OTUD and phenotype-associated gene(s), small RNAs can easily link to genetic information and can be ingeniously applied in diverse biosensors for various purposes. This review presents an up-to-date summary of CRISPR-coupled sRNA-based technologies and applications and focuses on the potential of small RNAs in future developments of CRISPR technology.

It is important to develop precise methods to adjust the expression of CRISPR effectors in time and space. Optimizing the structure and expression of effectors is an effective way to improve gene editing and repress on-target activity. Developing inducible expression systems, transcriptional termination, protein stabilization, or image-guided effectors, among others, provides accurate spatiotemporal control. Single-cell RNA-seq, split fluorescent protein, and abdA expression correlation determine whether off-targeting occurs in the generation of cells with a competitive growth advantage in the genome. We also introduce the new fusion protein combined with immunolocalization. For cancer therapy, conditional gene ablation is controlled by light-induced dephosphorylation and spatiotemporal activation based on tumor-specific marker-directed on-demand expression systems. The split recombinase fragment system not only exhibits high recombination accuracy but also mediates tissue-restricted recombination. In addition, combined administration strategies achieve optimal gene editing through gene expression control and gene inactivation.

6. Conclusion

As shown in this paper, CRISPR technology in bacterial genome editing and therapy has progressed rapidly. There are several CRISPR-based gene editing tools and CRISPR-associated novel enzymes developed for precise bacterial editing. In addition, CRISPR technology also provides an efficient way to manipulate the bacteriophage to help develop new therapeutic agents. These tools can guarantee efficient and precise delivery of desired sgRNAs for controlling the activity of the Cas effectors. In the near future, CRISPR technology might be used in engineering or editing particular prokaryotes for human disease therapy such as autoimmune diseases. These approaches ensure more precise engineering and editing results, demonstrating the high potential of the bacteria-based treatment strategy. However, some questions regarding CRISPR technology need to be addressed before clinical experiments. Off-target effects are potential concerns in clinical prospects. Furthermore, other limitations including the immunogenicity of CRISPR proteins, sgRNA delivery, and precise gene editing efficiency need to be addressed. In future studies, conceivably, high-throughput identification methods in combination with deep sequencing and clustered regularly interspersed short palindromic repeats transgenic animals could be useful in performing a more comprehensive analysis of the off-target effects caused by the CRISPR-Cas system in bacterial editing.

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