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Progress in The Application of CRISPR-Cas9 Combined With Microbial Engineering

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Abstract. CRISPR - Cas9 as the third generation of gene editing techniques, widely exists in prokaryotes, and has been widely applied to clinical and pharmaceutical fields. This article reviews the discovery, immune defense process and mechanism of CRISPR-Cas9 and summarizes its application in microbial cells. Advances include achieving gene editing in E. coli, improving defense against phages, and stabilizing chemical production; It is used to modify the characteristics of lactic acid bacteria and precise genome editing in algae; In terms of viruses, it can be used to treat HIV-1 infection, hemoglobinopathy, etc. Research gaps include less research on the effect of optimal length and selective markers of donor DNA homologous arms on efficiency in bacterial gene editing and insufficient evaluation of potential off-target effects and long-term effects of CRISPR/Cas9 systems in algae. This paper mainly analyzed the application of CRISPR-Cas9 in bacteria, lactic acid bacteria, algae, and viruses, and obtained corresponding research results. It provides a theoretical basis and a new idea for the research in the field of microbiology, but there are still some problems to be solved, and future research can focus on optimizing the system, reducing the off-target rate, and improving the editing efficiency.

Keywords: CRISPR - Cas9; microbial engineering; E. coli; algae.

1. Introduction

The CRISPR-Cas9(clustered regular interspaced short palindromic repeat-associated protein 9) system, as a third-generation gene editing technology, is widespread in prokaryotes, it is used in the acquired immune defense system lower than the invasion of exogenous genetic materials and has been widely used in clinical and pharmaceutical fields. The field has developed rapidly under the broad wave of research on CRISPR-Cas9. This paper reviews the recent literature on the discovery of CRISPR-Cas9, immune defense process and mechanism of action, and summarizes the research progress of its application in microbial cells combined with microbial engineering.

The CRISPR-Cas9 consists of a complex composed of tracrRNA, crRNA and Cas9 proteins to recognize PAM sequences and perform the cutting function, and the PAM sequences of CRISPR-Cas9 from different microbial sources are different [1]. On the bacterial side, the researchers used CRISPR cas9 technology to edit the genes of E. coli, and precise gene editing is critical to understanding its metabolic pathways and physiological functions. At the same time, the technology has also been used to modify lactic acid bacteria so that we can more precisely explore the function of their active ingredients, such as the mechanism of action of exopolysaccharides (EPS) in antiviral infection [2]. In algae research, CRISPR cas9 technology helps to optimize the nutrient composition of algae, gain insight into the biology of microalgae, and tap its potential for biological production and biotechnology applications. In addition, for the study of viruses, CRISPR cas9 technology can be used to edit the viral genome, inhibit viral infection and reactivation opens new ways. The combination of CRISPR cas9 and microbial engineering has important research significance.

When it comes to bacterial gene editing using CRISPR gene editing, there is relatively little research on the optimal length of the homologous arm of donor DNA and the effect of selected markers on the efficiency of gene editing [3]. More research is also needed to assess the potential off-target effects CRISPR/Cas9 systems may cause in Marine algae and the long-term effects on algal ecosystems [4].

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In this paper, the application of CRISPR cas9 technology in bacteria, algae and viruses will be discussed in detail. In the future, combined with the big data of microbiome, continue to delve deeper into CRISPR/Cas systems of different microbial sources and other novel gene editing tools. Existing CRISPR/Cas9 systems are optimized and upgraded to reduce off-target rates, improve editing efficiency, and expand CRISPR/Cas9 applications.

2. Mechanism

First discovered in bacteria and archaea, the CRISPR/Cas system is an acquired immune system that uses CRISPR RNA (crRNA) to recognize DNA and Cas nucleases to mediate DNA cleavage to fight viruses and phages. The system is present in about 40% of sequenced bacteria and nearly 90% of sequenced archaea genomes. CRISPR loci are composed of conserved repeats, including non-repeating spacers. Cas nucleases are critical for processing foreign DNA into small fragments. The system can be divided into types I, II and III according to the sequence and structure of Cas protein. Type II, dominated by Cas9 protein, recognizes the DNA target sequence containing PAM under the guidance of guide RNA (gRNA), site-directed cleavage breaks the double strand, and self-repair through non-homologous end junction (NHEJ) or homologous recombination (HR), resulting in insertion, deletion, repair, or replacement of mutation sites [5].

CRISPR-Cas9 consists of several components. The Cas9 enzyme is a large (1,368 amino acid) multi - domain and multifunctional DNA endonuclease, which can cut DNA through its two distinct nuclease domains (HNH and RuvC). The guide RNA can be either a natural crRNA - TracrRNA or a synthetic single guide RNA (sgRNA), guiding the Cas9 enzyme to recognize specific DNA sequences. When Cas9 binds to the guide RNA (crRNA - TracrRNA or sgRNA), it forms an active DNA monitoring complex. The 20 - nt interval sequence of crRNA confers DNA target specificity, and tracrRNA plays a key role in Cas9 recruitment. After sgRNA binds to Cas9, it drives Cas9 to change from an inactive conformation to an effective conformation for DNA recognition. The most significant conformational change occurs at the REC lobe, specifically Hel - III, which shifts about 65A toward the HNH domain upon sgRNA binding [6]. The Cas9 - guide RNA complex searches for complementary target DNA sites by recognizing Protospacer adjacent motif (PAM) sequences. PAM sequences are critical for distinguishing between self and non - self - sequences, and a single mutation in these sequences may invalidate Cas9 cleavage activity. Cas9 starts the target DNA search process through three - dimensional collision, first detecting the appropriate PAM sequence, and then checking the complementarity of flanker DNA and guide RNA. Once a target site with the appropriate PAM is found, Cas9 triggers local DNA melting at nucleation sites adjacent to PAM, followed by RNA strands invading DNA strands to form RNA - DNA hybridization and replacement (called R - loops) [7].

3. Progress in the Application of CRISPR-Cas9 in the Field of Microbiology

3.1.Escherichia Coli

E. coli can be grown and cultured easily and inexpensively in a laboratory setting and is widely used as a host for gene replication and expression in biological experiments. In 2013, Jiang et al. used the CRISPR/ CEA s9 system in Streptococcus pyogenes for the first time to achieve genome editing in E. coli, and the repair rates of cells containing target mutation sites reached 100% and 65%, respectively [8]. Compared with the first- and second-generation gene editing technologies, c can edit single or multiple genes in microbial genomes more simply and efficiently. For example, through the construction of pEcCas/pEcgRNA system, the efficient editing of cadA and maeA genes in BL21(DE3) strain was successfully achieved, solving the problem that the original pCas/pTargetF system could not perform gene editing in this strain. The system can also edit non-essential genes of many E. coli strains, such as BL21 StarTM (DE3), MG1655, DH5α, CGMCC3705, Nissle1917 and ATCC9637, and the efficiency is close to 100%. In addition, CRISPR-Cas9 was successfully used in Citrobacter Tuttum (T. citrea DSM13699) to efficiently edit its non-essential genes gdh1, gdh2 and

gdh3. During the experiment, the pEcCas/pEcgRNA system showed the characteristics of simplicity and high efficiency, such as shortening the experiment time and improving the editing efficiency by optimizing the plasmid construction and removal process [9]. By constructing a CRISPR/Cas9 system targeting the T7 phage genome, the defense capability of E. coli BL21 against phages can be improved. In 2020, researchers successfully improved E. coli BL21's defense against phage infection by designing different N20 genes targeting the caudate protein gp12, capsid assembly protein, and 3.8 protein in the T7 phage genome, respectively. Specifically, OD600 of BL21(pcas9, pTgRNA) and BL21(pcas9, p3gRNA) was significantly higher than that of BL21(pcas9) infected phages, indicating that CRISPR/Cas9 targeting phage genome can enhance the defense capability of BL21 against phage infection. And the target site plays a key role. Meanwhile, by transferring the prfp plasmid into a CRISPR/Cas9 strain induced by a T7 promoter, it was found that the CRISPR/Cas9 defense system could protect BL21 and increase its protein production during the biological process of phage infection, although there was still a gap compared to control strains without phage infection. But it provided the basis for engineering industrial E. coli strains resistant to phage infection [10].

Using CRISPR-Cas9, stable strains can be integrated to produce the desired chemicals. In 2020, By constructing plasmid pCas that incorporate -Red and CRISPR/Cas9 systems used to generate double-strand breaks (DBS) at universal N20PAM during Escherichia coli genome editing, the researchers assisted in -Red facilitated homologous dependent recombination (HDR). To remove redundant sequences. When genome editing is performed, the plasmid pRC-IS5, which contains large synthetic pathways and gRNA recognition regions, is first inserted into the E. coli W3110 genome through -Red-promoted HDR, and then the CRISPR/Cas9 system in pCas is used to generate DBS at N20PAM. Homologous recombination is achieved through in-RED mediated deletion of the lag chain in the replication fork, and the redundant sequences including IS5 sequence, chloramphenicol labeling and R6K are finally deleted. This process allows the large synthetic pathway to be more efficiently integrated into the E. coli genome, providing strong support for stable chemical production [11]. CRISPR-Cas9 can also enable phage defense and elimination by targeting the phage genome and inducing DNA breaks, and a study has constructed a CRISPR-Cas9-based mobile Anti-phage system (MCBAS) for phage defense in E. coli. The results show that MCBAS can effectively clear the DNA of MKR phages infected with E. coli. For example, both pGM1 and pGM2 can effectively block the replication of MKR phages in the body, and pGM2 has a more significant effect, ultimately reducing the infection rate of E. coli to about 0.2%. In addition, MCBAS can also prevent the infection of MKR phage to E. coli, such as the infection rate of pGM2 at all time points is less than 1%, while the infection rate of pGM1 increases in the later period. It provides an effective means to protect Escherichia coli from bacteriophage infection [12]. In summary, the application of CRISPR-Cas9 in E. coli can enhance editing efficiency by constructing specific systems, such as pEcCas/pEcgRNA system, CRISPR/Cas9 system targeting T7 phage genome, and binding -Red and CRISPR/Cas9 systems. Its main application areas include gene editing, such as achieving efficient editing of cadA and maeA genes and editing of non-essential genes; Improve the defense ability to phage and enhance the resistance ability to phage infection; Achieve stable chemical production and integrate large synthetic pathways into the genome more efficiently by optimizing plasmid construction and removal processes.

3.2.Lactic Acid Bacteria

Relevant studies have shown that lactic acid bacteria have good resistance to harsh environments, which reveals the great potential of lactic acid bacteria as cell factories for biorefining and production of bioactive substances [13]. At the same time, lactic acid bacteria and their metabolites have been proven to have beneficial effects on the host, which can play a role in regulating intestinal flora, improving the gastrointestinal environment and lowering cholesterol. However, the bioengineering technology of lactic acid bacteria applied in the biomedical field is not perfect, and lagging genome editing technology has always hindered the transformation of lactic acid bacteria at the genetic level.

In the application of probiotics, the molecular mechanisms by which probiotics and LAB interact with the host are not well understood, resulting in no approved health claims for probiotics in the European Union. The combination of genome editing, and omics studies helps to gain insight into the function of probiotics, and it is hoped that genome editing can be used to create genetically modified or modified probiotics in the future. Compared with traditional genome editing based on integrating plasmids, CRISPR-Cas9 technology achieves gene insertion or deletion through two cross-events, but the process of screening integrators and removing integrators is time-consuming. Recombinant engineering can directly integrate linear single - or double-stranded DNA oligonucleotides into the genome but requires identifying phage-derived proteins for each new strain and optimizing the system. CRISPR-Cas9 technology can be used as a powerful anti-selection tool to improve editing efficiency, but there are challenges in the application of bacteria, such as strict control of activity, strains specific differences, and possible toxicity. In addition, reengineering methods and CRISPR are established [14].

CRISPR/Cas9 technology can modify the characteristics of lactic acid bacteria. Through gene editing technology, Lactobacillus paracasmodium can be transformed into L-lactic acid production strains with high optical purity, and the significance of different bsh genes in the gall salt resistance of Lactobacillus plantarum can be determined, to reduce the death of lactobacillus after the formation of DSBs and create nutrition-deficient lactobacillus. To improve the efficacy of anti-porcine rotavirus (PoRV) vaccine, improve the efficiency of CRISPR/ Cas9-assisted ssDNA recombination, and explore the off-target effect and escape mechanism of CRISPR/Cas9 system. Transcriptional regulation based on dCas9: dCas9 can inhibit the enzyme activity of RuvC and HNH domains of Cas9 nuclease, thereby inhibiting the binding of RNA polymerase to target genes and achieving gene silencing. CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi) techniques developed based on the dCas9 principle can be used for transcriptional activation and transcriptional inhibition, respectively [15].

3.3.Algae

CRISPR/Cas9 technology enables precise editing of algae genomes, providing a new tool for genetic improvement of algae. Gene knockout has been successfully achieved in some algae, such as multiple gene knockout in chlamydomonas via the CRISPR/Cas9 system mediated by electroporation [16]. The CRISPR/Cas9 system has been used to edit specific genes in diatoms.

In 2021, a study demonstrated for the first time the feasibility of inducing targeted mutations by directly introducing CRISPR-Cas9 RNP into brown seaweed cells,[17]. providing an effective reverse genetics tool for basic and applied studies of Ectocarpus, and the method has the advantages of high efficiency, strong repeatability, and low frequency of off-target mutations. It is expected to be applied to other brown seaweed species.

Related studies have shown that when CRISPR-Cas9 gene editing was first performed in chlamydomonas, the efficiency was low, which may be related to the cytotoxicity of Cas9. Using hybrid versions of Cas9 and RNP (ribonucleoprotein) delivery methods, the efficiency of Cas9 has been improved, but there are still some problems, such as the toxicity of Cas9 and low homologous recombination frequency. In Phaeodactylum tricornutum, the mutation of CpSRP54 gene was successfully realized by converting the Cas9 and sgRNA modules with optimized coding, and the mutation frequency was up to 31%. For Thalassiosira pseudonana, a colony with a specific mutation was successfully obtained by transforming Cas9 and sgRNA by microparticle bombardment, and efficient HDR (homologous directed repair) was achieved by cotransformation [18].

3.4.Virus

As a class of pathogenic microorganisms, viruses play an important role in it. In-depth study of viral genes contributes to a better understanding of the pathogenesis of diseases, and viral genome

engineering also plays an important role in many aspects of virology research, including virus-host interaction, viral gene function, gene-edited animals, and the development of recombinant vaccines.

For DNA viruses, studies have shown that the CRISPR/Cas9 system can effectively confer resistance to twin viruses, reduce viral replication, lower viral titers, and reduce disease symptoms. By targeting conserved non-coding sequences, virus escape can be avoided, providing an effective strategy for providing potentially long-lasting resistance. For RNA viruses, there are currently no reports of direct targeting and cutting RNA viruses, but Cas9 can be programmed to cut RNA, which may change in the future. Resistance to RNA viruses can be created by mutating translation initiation factors in plants associated with RNA virus infection, such as eIF4E and elF(iso)4E. [19].

In clinical applications, CRISPR-Cas9 can treat HIV-1 infection by disrupting the CCR5 gene. CRISPR-Cas9 was used to modify patient-derived hematopoietic stem cells and progenitor cells (HSPCs) to treat sickle cell disease and B-thalassemia, and some patients have achieved good results [20]. By editing T cells with CRISPR-Cas9, it can inhibit the regulatory genes at certain checkpoints and promote the anti-tumor response of cytotoxic T cells, and relevant clinical trials have achieved certain results.

4. Conclusion

This paper mainly summarizes the application progress of CRISPR-Cas9 in the field of microbial engineering. In bacteria, the technology can be used for gene editing of Escherichia coli, improve its defense against phages, and achieve stable chemical production; In terms of lactic acid bacteria, CRISPR-Cas9 technology provides insight into the function of probiotics and can be used to modify the characteristics of lactic acid bacteria, but there are some challenges in application; In algae, the technology can realize the precise editing of algae genome; In terms of viruses, CRISPR-Cas9 can be used to treat HIV-1 infection, hemoglobinopathy, etc., and has different mechanisms of action for DNA viruses and RNA viruses. These results are of great significance for the in-depth understanding of the physiological functions and metabolic pathways of microorganisms and the development of novel microbial products. Through gene editing technology, we can explore the function of microorganisms more precisely, supporting biological production and biotechnology applications.

However, there are few studies on the effect of the optimal length of the donor DNA homologous arm and the selection of markers on the gene editing efficiency in bacterial gene editing. In algae research, there has been insufficient assessment of the potential off-target effects that CRISPR/Cas9 systems can cause and the long-term effects on algal ecosystems. Delivery challenges, off-target effects, PAM restriction, and immune responses for CRISPR/Cas9 applications in vivo require further optimization of sgRNA design, improvement of Cas9 protein, and development of new gene-editing technologies. Future research can further optimize the CRISPR/Cas9 system, which is expected to treat gene-related diseases by deleting, inserting, regulating, and blocking related genes, and needs to solve some problems, such as improving the efficiency of gene editing, reducing off-target effects and immune responses, to advance its clinical application. At the same time, further studies of CRISPR/Cas systems of different microbial sources and other novel gene editing tools are also needed

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