Expanding application of CRISPR-Cas9 system in microorganisms

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\textbf{A B S T R A C T}

The development of CRISPR-Cas9 based genetic manipulation tools represents a huge breakthrough in life sciences and has been stimulating research on metabolic engineering, synthetic biology, and systems biology. The CRISPR-Cas9 and its derivative tools are one of the best choices for precise genome editing, multiplexed genome editing, and reversible gene expression control in microorganisms. However, challenges remain for applying CRISPR-Cas9 in novel microorganisms, especially those industrial microorganism hosts that are intractable using traditional genetic manipulation tools. How to further extend CRISPR-Cas9 to these microorganisms is an urgent matter. In this review, we first introduce the mechanism and application of CRISPR-Cas9, then discuss how to optimize CRISPR-Cas9 as genome editing tools, including but not limited to how to reduce off-target effects and Cas9 related toxicity, and how to increase on-target efficiency by optimizing crRNA and sgRNA design.

1. Introduction

At present, three generations of nuclease-based genome editing technologies including ZFN, TALEN and the CRISPR-Cas systems have been developed \cite{1}. All these technologies rely on double-strand break (DSB) induced DNA repair system at specific site for sequence modification \cite{2}. However, ZFN technology generates cytotoxicity in cells and the production cost is high \cite{3}. TALEN has advantage such as higher fidelity and less off-target, but it's module assembly is complicated \cite{4}. Compared with the first two technologies, the CRISPR-Cas system has a larger target selection than the first two, avoid problems such as difficulty in assembly and off-target, and can effectively cleave any DNA site to achieve more accurate gene editing and modification \cite{5}.

CRISPR (clustered regularly interspaced short palindromic repeats) is an acquired immune system that protects against foreign virus or plasmid DNA in bacteria (> 50%) and archaea (> 90%) \cite{6}. The system consists of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas). CRISPR sequences are composed of short, highly conserved repeats and different spacers. Repeat mostly has a palindrome structure. Spacer is homologous to foreign DNA (such as plasmid or virus), and can form crRNA, it can form a complex with Cas functional protein to specifically recognize and eliminate foreign plasmid or virus that invaded cells after being transcribed and processed with repeat to form crRNA. In 1987, when Ishino and colleagues studied the \textit{iap} gene responsible for alkaline phosphatase isozyme conversion in \textit{E. coli}, they found a 29 nt tandem repeat sequence downstream. Interestingly, the repeat units are separated by 32 nt non-repetitive sequences, which is the earliest report of CRISPR \cite{7}.

Cas and CRISPR sequences of the CRISPR-Cas system are highly diverse and dynamic. In 2015, Makarova et al. updated the CRISPR system classification and divided the CRISPR system into two categories (Class I and Class 2), 5 types and 16 subtypes \cite{8}. The Class 1 system exists in bacteria and archaea including types I, III, and IV. And Class 1 includes effector complexes composed of four to seven Cas protein subunits. Most of the subunits of the Class 1 effector complexes contain RNA recognition motif domain \cite{9,10}. Class2 including types II, V, VI system mainly in bacteria, requires only a single Cas protein forming effect module \cite{11}. The characteristic effector molecule in Class 2 is Cas9, which has RNA-dependent endonuclease activity and contains two separate nuclease domains, HNH and RuvC, that function to cleave complementary and noncomplementary strands \cite{12}. In the Cas9 protein, the HNH domain inserted between two similar RuvC domains in responsible to cleave the target strand, which is the base that matches the gRNA base.

Among the many types of CRISPR systems, the CRISPR-Cas9 system
is relatively simple and more commonly used. In 2012, Doudna laboratories found that purified Cas9 from *S. thermophilus* can target and cleave gene by crRNA [13]. In addition, the Charpentier and Doudna labs also simplified the system. They combined tracrRNA (*trans*-activating CRISPR-associated RNA) and crRNA into a single guide RNA (gRNA). The combination of gRNA and Cas9 proteins can target specific sequences. In 2013, Zhang and Church groups applied this genome editing technology to eukaryotes almost simultaneously. Zhang’s laboratory used *S. thermophilus* and *S. pyogenes* type II systems and found that Cas9 can cleave both mouse and human genomes under the guidance of gRNA [14]. When an exogenous donor is provided, Cas9 can also precisely edit target sites by homologous recombination. In addition, when multiple leader sequences are added to the CRISPR sequence, the system can simultaneously edit multiple sites in the mammalian genome. The Church laboratory also used the Type II system to edit the human genome and obtained similar results. These two articles created a new era of CRISPR genome editing technology, and then the world of life sciences blew up the CRISPR storm. Since then, the CRISPR-Cas9 system has been used by thousands of laboratories for genetic editing of various biological models.

Based on the CRISPR-Cas9 principle, many CRISPR-Cas9 technologies have been developed, for example, multiple genome editing technology CMGE and GTR-CRISPR for *E. coli* and *Saccharomyces cerevisiae*; convert Cas9 to DNA nicking enzymes for precise mediated editing; fuse dCas9 with EGFP and other fluorescent proteins to perform fluorescent localization at specific sites in the genome; and DNA-methylated or acetylated proteins through CRISPR-Cas technology introduced into the target genome for epigenetic regulation, etc. At present, the CRISPR-Cas9 system has been widely used in industrial microorganisms to increase the output of target products and other model or non-model microorganisms, animals and plants as gene editing tools. However, problems such as toxicity of Cas9 and off-target effect restricts its exploitation in new microorganisms.

In order to more efficiently apply the CRISPR-Cas9 system to bacteria based on the foregoing basic introduction, this article introduces the mechanism and application of the CRISPR-Cas9 system, this review details how to optimize CRISPR-Cas9 as genome editing tools, including but not limited to how to reduce off-target effects and Cas9 related
toxicity, and how to increase on-target efficiency by optimizing crRNA and sgRNA design.

2. Mechanisms of CRISPR-Cas9 system

The three essential components of CRISPR-Cas9 system are the CRISPR locus composed of spacer and repeat sequences, tracrRNA, and Cas9 endonuclease. The Cas9 endonuclease including the RuvC domain and sgRNA design. 

Table 1

| Industrial strains | Applied CRISPR–Cas9 gene editing technology | Product | Product output | references |
|--------------------|--------------------------------------------|---------|----------------|------------|
| Synechococcus elongatus | Knock-out of pgs, knock-in of glnA | sucinic acid | 0.58–0.63 mg/L | [22] |
| Saccharomyces cerevisiae | Knock-out of FAA1, FAA4, POX1, are1, ARE2, FAA1, LPP1and DDP1 | free fatty acid | 30-fold | [23] |
| E.coli | Modification of glnA S-UTR | n-butanol | 0.82 g/L | [24] |
| Corynebacterium glutamicum | inactivated pyk&ldh | glutamic acid | 3-fold | [25] |
| Saccharomyces cerevisiae | Knock-down of Erg9, knock-out of ROX1, CRISPRa of the HMG1 | β-carotene | 3-fold | [26] |
| Bacillus subtilis | Repression of Bpr and Vpr | BLA | 260-fold | [27] |
| Clostridium tyrobutyricum | Integration of adhE1 or adhE2 | n-butanol | 26.2 g/L | [28] |
| Corynebacterium glutamicum | Knock-out of NglL221, gdh1, gdhP | γ-aminobutyric acid | 28.7 g/L | [29] |
| E.coli | Knock-in of fadR, dehE9 and acc | fatty acids | 13% | [30] |
| E.coli | Knock-out of sucCD, hemB | S-aminolevulinic acid | 2.81 g/L | [31] |
| E.coli | Knock-in and RBS substitution of thi, antOA, adc, adh | iso propiole | 17.5 g/L | [32] |
| E.coli | Knock-in of atmgs | β-carotene | 19.6 mg/L | [33] |
| Bacillus subtilis | Combined regulation of Rbb, RIBA and Rbb | riboflavin | 1.39 mg/L | [34] |
| Myceliophthora thermophila | Δare1Δalp1Δada1 | cellulase | 5.1–13.3 fold | [35] |
| Filamentous fungus | Multiplex gene editing | mucic acid | 12.05 g/L | [36] |
| Aspergillus niger | Knock-out of aAa | galactaric acid | 4 g/L | [37] |

CRISPR structure is maintained. This sub-stage can be called the “spacer integration process” [19].

Expression: The newly obtained spacers need to be transcribed and processed into mature small molecule crRNA to mediate specific immunity. This process is the second stage of CRISPR immunity, crRNA biosynthesis. Most type II systems encode a tracrRNA that is partially complementary to the repeat sequence. Generally, the leader sequence of a CRISPR structure contains promoter and other transcription elements. When the same virus or plasmid invades the bacteria again, the inserted new spacer sequence will be transcribed together with the repeat. Under the guidance of tracrRNA, the host-derived RNase III and the crRNA, the CRISPRa of the HMG1.

Interference: A functional crRNA directs Cas9 nucleases to specifically recognize and cleave the viral DNA/RNA that is homologous to the spacer to achieve a specific immune process. After processing by RNase III, the mature crRNA and tracrRNA continue to maintain RNA duplex. RNA duplex and Cas9 rapidly scan the target DNA. The bases of the crRNA and protospacer DNA match and further extend the base match to the entire protospacer region, forming a stable R-loop structure [21]. The HNH domain and the Ruv-C domain cleave the two strands of the target DNA, resulting in a double-strand break.

3. Applications of CRISPR-Cas9 in microbes

The CRISPR-Cas9 system can transform strains and increase the output of industrial products through various editing methods, such as knockout, integration, CRISPRi, precise editing, etc., as shown in Table 1.

3.1. Genome editing

With the continuous development of genome sequencing technology and the progress of the human genome sequencing project, scientists have obtained a large amount of genome information, which brings opportunities for the development of basic science and industrial biology. The laborious traditional genome editing technologies via homologous recombination (HR)-restrict their use in numerous organisms. New evidence that DNA double-strand breaks (DSBs) can stimulate error-prone non-homologous end-joining (NHEJ) or homology-directed repair (HDR) at specific gene position, lays the foundation for the
emergence of genome editing technology. CRISPR-Cas9 gene editing technology has significant advantages such as high efficiency, specificity, and simple design, which brings a huge breakthrough to gene editing technology.

Targeting gene sequences with double RNA (tracrRNA: crRNA) and introducing donor templates makes CRISPR-mediated insertions and deletions possible. Subsequently, replacing the double RNA (tracrRNA: crRNA) with sgRNA further simplified the system [12]. Song et al. developed the CRISPR-Cas9 D10A system for genomic engineering of Lactobacillus casei, which has been shown to mediate fast and efficient in-frame chromosome deletions and site-specific insertions/replacements [38]. As we all know, cyanobacteria are limited in their application as cell factories to produce biofuels and various biochemical products due to their oligoploidy nature and long-term instability of the introduced gene. Li et al. used the CRISPR-Cas9 system to effectively trigger a programmed DSB on the chromosome of the Synechococcus elongatus PCC7942, and achieved precise gene integration by co-formation with a template plasmid harboring the gene cassette and flanking homology arms. Subsequently, Li et al. further used CRISPR-Cas9-assisted simultaneous glc knockout and gltA/ppc knockout to modify the cyanobacteria to increase the succinic acid titer to 435.0 ± 35.0 μg/L, which is 11-fold increase compared with wild-type cells [22]. In order to develop microbial cell factories, multiple genome targets often need to be modified. Feng et al. developed a CRISPR-Cas9-assisted E. coli multiple genome editing technology (CMGE) based on a modular assembly strategy. The modification efficiencies of 2, 3 and 4 sites are 100%, 88.3% and 30% [39]. Zhang et al. developed a system for multiple genome editing of Saccharomyces cerevisiae, GTR-CRISPR, which uses reported effective gRNA to interrupt 8 genes simultaneously with efficiency up to 87%. They further developed an accelerated Lightning GTR-CRISPR system which can knock out 6 genes in 3 days. Using Lightning GTR-CRISPR to simplify the yeast lipid network, they modified the system [12]. Song et al. developed a CRISPR-Cas9 system to inactivative genes [12,14,42], the single-strand gap was increased by 2.5-fold [26]. And by combining and optimizing multiple metabolic targets, the display of endoglucanase on the yeast surface was increased by 2.5-fold [26].

The high specificity and efficiency of the CRISPR-Cas9 system has led to rapid development of precise gene editing technology. Cas9-induced DSB can be repaired by NHEJ or HDR. In the error-prone NHEJ pathway, the ends of DSBs are processed and recombined through endogenous DNA repair mechanisms, resulting in random mutations in the junction site. HDR is another major DNA repair pathway, which can produce precise modifications at the target location in the presence of exogenously introduced repair template [14]. While its efficiency may vary greatly depending on cell type and status, as well as genomic sites and repair template [40]. The repair template in the form of plasmid or ssODN can be high fidelity precise editing using HDR method. Single-strand nicks in DNA can induce HDR [41]. When Cas9 is converted to a DNA nickase (catalytic residue mutation, D10A in RuvC and H840A in HNH) [12,14,42], the single-strand gap is first repaired by a high-fidelity HDR pathway. In order to improve the CRISPR-Cas9 system, according to Ran et al., two Cas9 nickases guided by a pair of sgRNAs targeting opposite strands of a target locus mediate DSB. DSB can stimulate homology-directed repair (HDR) to achieve high-precision editing of target sites in genome [43]. Heo et al. used the CRISPR-Cas9 system to precisely edit the 5’-untranslated region sequence of gltA encoding citrate synthase to reduce its expression level, thereby carbon flux from acetyl-CoA to citric acid cycle was redirected to acetocetate-CoA. Finally, the butanol output reached 0.82 g/L [24].

3.2. Transcription control

CRISPR-Cas9 technology can also be applied to regulate gene expression at the level of transcription and translation, besides genome editing. Dcas9 (Cas9 mutated to inactivate nuclease activity) can bind to specific DNA sequences and regulate the expression of specific genes under the guidance of sgRNA. The method that dCas9 is used to inhibit gene expression under the guidance of sgRNA, called CRISPR interference technology (CRISPRi) [44]. According to the results of Bikard et al. [45] and Qi et al. [46], dCas9 mainly inhibits gene transcription in two ways: (1) restricting RNA polymerases binding to promoters to inhibit transcription initiation; (2) preventing RNA polymerases sliding on the DNA duplex to inhibit transcriptional extension. In order to increase the production of glutamic acid and fully transfer the carbon source to the final product, Wang et al. used the CRISPR-Cas9 system to inactivative genes pyk, ldhA, odhA involved in competitive pathway in Corynebacterium glutamicum. Finally, it was found that pykldhA double-inactivated strain can increase glutamic acid production by 3 times [25]. In addition to transcriptional repression, dCas9 protein fused with a transcriptional activation domain can also activate transcription of specific genes. namely CRISPR activation (CRISPRa) [47]. The position of the target sequence affects the activation efficiency of CRISPRa. When the distance between the target sequence and the promoter is appropriate, the activation efficiency is higher; when the target sequence is further from the upstream of the promoter, the activation efficiency will be reduced to a certain extent. Inhibition occurs when the target sequence and the promoter are close or the target sequence is in an open reading frame. However, due to the lack of effective gene activators, methods for regulating bacterial cell gene expression programs are limited. To cope with this challenge, Chen et al. identified a variety of synthetic transcription activators compatible with CRISPRa in E. coli, including Sox, Tet and ASIA. In particular, SOX interacts with the surface on the α subunit of RNA polymerase which is highly conserved among gammaproteobacteria, alphaproteobacteria, bacteroides, gram-positive bacteria. This conservative interface may allow the CRISPRa system developed in E. coli to be ported to non-model bacteria with a wide range of useful biological functions. It provides a basis for designing synthetic bacterial cell devices with applications in diagnostics, therapeutics and industrial biosynthesis [48].

As more and more microbial cell factories are used to produce fuels, chemicals, etc., it is necessary to carry out metabolic engineering of microorganisms to maximize yield and productivity. In this case, researchers often need to modify multiple metabolic engineering targets with different regulatory modes, such as increasing expression of genes encoding rate-limiting enzymes, decreasing expression of essential genes, and removing expression of competing pathways. However, due to our limited understanding of cell metabolism regulation, developing a combinatorial metabolic engineering strategy to modify the host genome in a modular, parallel and high-throughput manner will be the key to optimize microbial cell factories. Lian et al. designed a combinatorial metabolic engineering strategy (CRISPR-AID) based on an orthogonal three-function CRISPR system, which combines transcription activation, transcription interference and gene deletion. Through CRISPR-AID technology, the production of β-carotene was increased by 3-fold in one step. And by combining and optimizing multiple metabolic engineering targets, the display of endoglucanase on the yeast surface was increased by 2.5-fold [26].

The fine-tuning of gene expression is essential for protein expression and pathway construction, but it still faces huge challenges due to the hierarchical gene regulation at multiple levels in a context-dependent manner. Lu et al. co-expressed dCas9 with transcriptional regulators α and ω, and designed position-specific gRNA to activate or repress the expression of different genes. Finally, by controlling the time of dCas9 expression, the expression of target genes can be efficiently regulated in multiple dimensions. By combining OAPS and dCas9-α, Lu et al. systematically evaluated the effects of promoter-based transcription, chaperone-assisted protein folding and protease-mediated degradation on the expression of amylase BLA in Bacillus subtilis. Finally, the production of BLA was increased 260-fold [27]. In order to explore the optimal intermediate level of gene expression, Matthew et al. established a method in Saccharomyces cerevisiae for rapid fine-tuning and hierarchical expression of enzymes through the regulation of dCas9. In this method, changing the position of the sgRNA target is regarded as the
dominant parameter. By using the repressor and activator fused with dCas9, the medium-strength glycolytic promoter was modified to cover a nearly 40-fold expression range from near deletion to over-amplification. They finally achieved a 5.7-fold increase in titer applying this method to the glycerol biosynthetic pathway. They then identified and alleviated pathway bottlenecks resulting in a 7.8-fold increase in 3-dehydroshikimate titer and the identification of 3 unique targets for xylose catabolism by applying it to the pentose phosphate pathway in two distinct strain backgrounds. In addition, with the rapid decrease in the cost of DNA synthesis and the development of massively parallel sequencing technology, this technology may be used to complement genome-scale metabolic models in the future [49].

3.3. Other applications

DNA imaging: studying the interaction of specific genes with chromatin states requires a reliable method to visualize DNA in living cells. Traditional labeled DNA techniques, such as fluorescence in situ hybridization (FISH), require fixed samples and cannot capture live processes. Recently developed fluorescent marker Cas9 for specific DNA loci by Chen et al., is a viable alternative to DNA-FISH for live cell imaging [50]. When dCas9 is fused to EGFP and other fluorescent proteins, fluorescent localization can be realized at specific sites in the genome.

Instantaneous control: decomposing the two domains of Cas9 into two separate proteins and using chemical or light-induced dimerization methods can achieve instantaneous control of various genomic or epigenomic operations. Small molecule induction will facilitate the systematic control of Cas9, while optical regulation will allow more precise spatial perturbations. Konermann et al. have successfully used the light-induced dimerization domains CIB1 and CRY2 or the chemically-induced analogues ABI and PYL to construct inducible TALEs [51].

4. Optimization of CRISPR-Cas9 toolkit to improve editing efficiency

4.1. Improve editing efficiency by reduce off-target effects

Off-target effects occur when sgRNA is selected at a position with low specificity or the temporal, locus-specific and spatial control of Cas9 protein expression is not mastered, especially when Cas9 and gRNA are co-expressed on the same plasmid. At present, scientists have developed multiple CRISPR-Cas9 optimization schemes to increase the specificity of CRISPR-Cas9.

4.1.1. Reducing off-target effects by sgRNA design

The specificity of the CRISPR system can be improved by the modification of gRNA. Studies have shown that introducing deoxy nucleotides to create RNA-DNA hybrids in the CRISPR system can obtain higher specificity [52]. Chemical modifications, such as 2’-O-methyl-3’-phosphonooacetate introduced at specific sites on the DNA recognition sequence of the gRNA ribose phosphate backbone also increases specificity [53]. Addition of nucleotides [54,55] or truncation of nucleotides [56,57] can reduce CRISPR system tolerance to base mismatches to reduce off-target rates. In addition, several algorithm-based tools, such as ChopChop and CRISPR Design, based on a range of factors including sequence similarity, number and location of mismatches, have been developed to avoid off-target effect [58,59]. The sgRNA that reduces the off-target efficiency depends on the selection of a target site with none or few similar genes. Furthermore, Ran et al. plotted the relationship between gRNA and Cas9 ratios and the number of off-target effects [56]. All of the above have provided a powerful effect for reducing off-target rate by modifying sgRNA.

4.1.2. Reducing off-target effects by Cas9 modifications

In addition, Cas9 can be transformed to reduce off-target effects. As mentioned earlier, using two sgRNAs for directing two copies of Cas9n (either HNH or Ruv-C active site was mutated) to two adjacent target sites, the simultaneous of double-strand breaks can greatly improve the specificity of gene targeting [43]. Although a single Cas9n can create gaps in DNA and introduce mutations much less efficiently than wild-type Cas9 theoretically. There are reports that high frequent insertions/deletions may still be caused at certain genomic loci, so the introduction of two copies of Cas9n: sgRNA may inhibit off-target effect. In order to overcome this problem, some research teams modified the above scheme. They replaced Cas9n with Cas9d (both HNH and Ruv-C active sites were mutated), which fused with FokI nucleosome, to form RNA-guided FokI-Cas9d nuclelease (RFN) under the guidance of gRNA. Because FokI needs to form a dimer to exert nuclease activity, two gRNAs can be used to direct two copies of RFN to adjacent sites to activate FokI dimerization and nuclease activity in the above scheme, thereby improving specificity and efficient cutting [60,61].

Studies have found that the non-catalytic REC2 domain of Cas9 nucleases plays a crucial role in off-target recognition. Keewon et al. [62] used a single-molecule fluorescence method to study the conformational kinetics of the interaction between non-target DNA strands (NTS) and Cas9, and found that REC2 regulates NTS rearrangement through positively charged residues on its surface to perform cleavage reactions. This study promoted rationally designed highly specific Cas9 variants for genome editing.

4.2. Improve editing efficiency by reduce Cas9 toxicity effects

Due to the unique nature of the prokaryotic genetic profiles, the CRISPR-Cas9 system shows toxicity in a large number of microorganisms, which can easily lead to fatal chromosome breaks, resulting in low transformation efficiency and failure of gene editing. In the process of genome editing of Clostridium, Wang et al. [63] combined inducible expression of Cas9 and plasmid-borne editing templates. They observed severe vector integration (VIE) event, which has never been reported by other researchers in bacterial genetic engineering based on plasmid-edited templates for homologous recombination. This study offers two methods to reduce the toxicity of Cas9. On the one hand, the toxicity of this system is reduced by regulating the expression of cas9 gene; on the other hand, since most prokaryotes contain a natural CRISPR-Cas system, genome engineering can be achieved by using these endogenous immune systems to relieve Cas9 issues related to toxicity and low transformation efficiency.

4.2.1. Reducing Cas9 toxicity by regulates Cas9 protein expression

Due to absence of endogenous non-homologous end-joining (NHEJ) system, or deficiency of NHEJ, Cas9-induced double-strand breaks (DSB) is fatal to lots of bacteria. Therefore, regulating Cas9 expression is a crucial step for gene editing. Liu et al. developed a CRISPR-Cas9 genome editing toolbox for Streptomyces glutamine, in which Cas9 and gRNA expression cassettes were reconstituted to combat Cas9 toxicity and facilitate effective termination of gRNA transcription. Co-transformation of Cas9 and gRNA expression plasmids was exploited to overcome highly frequent mutation of Cas9, allowing not only highly efficient gene deletion and insertion with plasmid-borne editing templates (efficiencies up to 60.0 and 62.5%) but also simple and time-saving operation [64].

4.2.2. Reducing Cas9 toxicity by exploiting endogenous CRISPR-Cas

The CRISPR-Cas system has been extensively used for multifunctional genome editing and transcription regulation in various species since 2013. Most of these applications are based on the type I CRISPR-Cas9 system derived from Streptococcus pyogenes. However, the expression of heterologous Cas9 is highly toxic to a multitude of microorganisms [65,66], resulting in low transformation efficiency and failure of genome editing. Therefore, researchers proposed to use endogenous CRISPR-Cas system to reduce the toxicity of the CRISPR-Cas9
4.4.1. Optimizing sgRNA promoter

Construction of the sgRNA expression cassette.
moter and structure of sgRNA are two important constraints during the
system, the sgRNA expression cassette needs to be carefully designed to
result in a total transformation efficiency of 1.7 cfu/mL donor. In addition, any plasmid
containing these heterologous nucleases (or nicklase) proteins such as
Cas9 or nCas9 driven by the same inducible promoter could not be
successfully transformed. This indicates that the endogenous CRISPR-Cas
system can be applied to avoid toxicity caused by heterologous
CRISPR-Cas9/nCas9 system. Finally, they replaced cat+ by the alcohol
dehydrogenase gene adhE1 or adhE2 using the endogenous CRISPR-Cas
system, resulting in a butanol production mutant with a butanol titer of
26.2 g/L, which is the highest level been reported.

4.3. Improve editing efficiency by optimizing crRNA

4.3.1. SOMACA

Wu et al. established a synthetic oligonucleotide-mediated assembly
method (SOMACA) [68] for the construction of crRNA arrays, and
achieved 100% efficiency of double genes in-frame knocking out,
multiple point mutations (up to six), or single gene insertion using this
tool. Finally, the authors used this method to perform nonsense muta-
tions in the four genes (NAGA, NAGB, NAGP, GAMA) and the
products of the lactic acid (LDH) and acetic acid (PTA) synthesis
pathways in the decomposition of N-acetylglucosamine, increasing N-
acetylglucosamine titer by 50.9%.

4.3.2. Optimize crRNA length to improve editing efficiency

Liu et al. applied the bifunctional cluster regular spaced short pa-
linidromic repeat system (RE-CRISPR) to simultaneously edit and reg-
ulate genes in Corynebacterium glutamicum [69]. They used rfp, chro-
mosome-integrated rfp, and lacZ as reporter genes to study the effect of
the crRNA length on the efficiency of transcriptional repression. It was
found that crRNAs of 15 and 16 nt showed much higher repression
efficiency than crRNAs of other lengths. Finally, the application of the
RE-CRISPR system in the high-cysteine and serine metabolism en-
engineering of Corynebacterium glutamicum was demonstrated. The
combined use of the RE-CRISPR system simultaneously caused the deletion of
AECID and the inhibition of mcbR, thereby cysteine titer further increased
by 3.7-fold (42.8 mg/L). Simultaneous deletion of SDAA
and inhibition of GlyI increased serine production by 2.5-fold.

4.4. Improve editing efficiency by optimizing sgRNA

The CRISPR-Cas9 system is a revolutionary genome editing tool in
which the endonuclease Cas9 is guided to the genome target site by
complementary base pairing of sgRNA. The sgRNA includes a 20 base
targeting sequence at the 5’end. Cas9 mediates double-strand breaks
after targeting the receptor genome with the sgRNA. Therefore, in this
system, the sgRNA expression cassette needs to be carefully designed to
ensure the formation of a functional Cas9–sgRNA complex. The
promoter and structure of sgRNA are two important constraints during the
construction of the sgRNA expression cassette.

4.4.1. Optimizing sgRNA promoter

Gene editing efficiency is connected with sgRNA promoters. In some
cases, such as gene knockout, knockdown etc., sgRNA does not
need continuous expression. So when the system is first established in
an organism, it is necessary to choose a proper sgRNA promoter. Gene
editing efficiency of some sgRNA promoters varies with species. For
example, the RNA Pol III promoter of the spliceosome U6 snRNA is
widely used in the fungi CRISPR-Cas9 system, but useable U6 promoter
has not been found in Aspergillus niger. Therefore, Zheng et al. estab-
lished a new CRISPR-Cas9 system expressing sgRNA based on one en-
dogenous U6 promoter and two heterologous U6 promoters in
Aspergillus niger. All three U6 promoters can interrupt the Aspergillus
niger polynucleotide synthase alba gene and efficiently insert the gene at the
target genomic locus with 40 bp donor DNA [70]. Although the U6
small nuclear RNA (snRNA) promoter is one of the most commonly used
promoters in eukaryotes, the low sequence conservation of the U6
promoter restricts its identification in many species [71,72]. Moreover,
the U6 promoter requires guanosine nucleotides to initiate tran-
scription, thereby reducing the available CRISPR-Cas target sites [73,74].
Because the SS rRNA gene is highly conserved and expressed in eu-
karyotes, Zheng et al. used it as a promoter of sgRNA, and the editing
efficiency reached 100% in dozens of gene editing system established in
Aspergillus niger [75], and the system can also be used for simultaneous
mutation of multiple genes in Aspergillus niger. Therefore, the SS rRNA
gene can be widely used as a guide RNA promoter in the eukaryotic
CRISPR-Cas9 system.

4.4.2. Optimizing sgRNA structure

The secondary structure formed by partial sgRNA is necessary for
Cas9 activity. There are two interdependent variable regions in the
sgRNA gene. One region contains a 20 bp protospacer and the other is a
6 bp inverted repeating region that repeats the 5’end of the protospacer.
The first 20 nucleotide sequence of the sgRNA is used to guide targeted
DNA cleavage. Additional bases or other modifications at the 5’end of
sgRNA may cancel the ability of gRNA to guide Cas9 to cut DNA [76],
because eukaryote RNA polymerase II process and modify at both ends
of DNA during transcription, so the transcribed RNA cannot be used
directly as sgRNA. However, most of the well-characterized promoters
are currently transcribed by RNA polymerase II, so post-transcriptional
modification of sgRNA is important. In order to develop CRISPR-Cas9
systems which can perform directional mutations with spatial and
temporal control in a wide range of organisms, methods for producing
gRNA need to be improved. For example, when tending to sequentially
interfere with different genes, it is necessary to use various promoters,
such as hormone-responsive promoters and environmental signal-
regulated promoters to control the timing of gRNA production. Guo et al.
proposed a general method for efficient production of gRNA in vitro
and in vivo. They designed an artificial gene that produces an RGR with
a ribosome sequence at both ends of the gRNA after transcription.
Through the action of two ribozymes (the 5’ end hammerhead and the
3’ end hepatitis D virus) on both sides of the gRNA, the mature gRNA
self-catalyzed by primary transcript of RGR driven by any promoter can
effectively guide the specific cleavage of the target gene in vitro and
yeast [77]. Nodvig et al. also used this system to improve the efficiency
of gene editing in filamentous fungi [78]. Therefore, if a suitable pro-
moter is selected, cell and tissue specific gene expression can be
achieved.

4.5. Improve editing efficiency by increasing recombination rates

Due to low efficiency of endogenous recombination system in many
microorganisms, introducing a heterologous recombination system is a
crucial step to apply CRISPR-Cas system for genome editing. Bassalp
et al. provided a recombination strategy based on the coupling of the
CRISPR and λ-red systems. They co-introduced a plasmid that con-
taining Cas9 and a plasmid that containing λ-red recombinase into E.
coli, and found that Cas9 can work effectively in different genonomic
environments with λ-red recombinase. Through this method, a 10 kb
gene encoding the complete isobutanol production pathway can effi-
ciently be labeled (less than 50%) in one day [79]. This powerful re-
combination ability makes rapid metabolic transformation of micro-
organisms a reality. Using this strategy, Cho et al. used CRISPR coupled
with λ-red quickly and efficiently knock out multiple genes in
Strreptomyces glutamine [29]. Therefore, this genomic engineering system strategy is generally applicable to the development of genomic engineering systems for other microorganisms. This genomic engineering system accelerates the pace of metabolic engineering of industrial strains.

In another work, Jiang et al. co-transformed donor DNA provided in fragments and sgRNAs contained in pTargetF into E. coli cells, using the CRISPR-Cas9 system to achieve various precision genome modifications, including gene deletions and insertions, with a maximum efficiency of 100%. Multiple genes can be simultaneously edited, using this method so as to improve the efficiency of recombination while reducing the time and labor required for construction. Using this system they also successfully targeted Tatumella Citrea chromosome deletion with highest efficiency up to 100% [80]. However, Jiang et al. also found that when increasing the number of target targets or reducing the length of homologous sequences (from 300 to 400 bp to 40 bp), the recombination efficiency was significantly reduced. We suspect that dsDNA has a low transformation efficiency in E. coli, and λ-red recombinase can promote the recombination of smaller single-stranded DNA fragments, so we can further improve the recombination efficiency by using single-stranded DNA as a donor, or increasing the length of the homologous sequence.

5. Concluding remarks and future perspectives

Just as recombinant DNA technology has benefited from basic research on restriction enzymes from microorganisms, the latest generation of Cas9-based genome engineering tools is also based on components of antiphage defense system from microorganisms. In recent years, the CRISPR-Cas9 system and various technologies derived from it, such as precise editing, multi-gene editing, and precise regulation, have catalyzed innovation to provide integrated solutions, and have provided us with increased flexibility in bacterial genetic engineering. It plays an important role in the fields of single-stranded RNA editing and high-throughput gene screening. Nevertheless, the problems such as off-target rate and cytotoxicity should not be ignored. Since the DSB caused by the Cas9 protein is lethal to host cells, DNA base editor that does not introduce DSB has been developed. It is reported that an evolved Cas9 variant containing 7 mutations has relaxed the PAM requirement to NG or NNG [81]. The application of these variants is expected to significantly increase the number of targeted nucleotides in the genome. The existing base editor can only realize the base conversion between pyrimidine and between purine. Zhao et al. designed and constructed the cytosine deaminase nCas9-Ung protein complex, creating a new glycosylase base editor (GBE). They developed a single-base gene editing system that can realize the conversion between pyrimidine and purine. Based on this system, it is the first time in the world to achieve arbitrary base editing in microorganisms and specific conversion of C-G bases in mammalian cells [82]. This is important in the construction of synthetic biological systems and modification of biological traits. As microbial cell factories are increasingly used to produce biochemical products, it will be a trend to use the CRISPR-Cas9 system to optimize the genome of microbial cells in a modular, parallel, and high-throughput manner to increase the yield of target products.

Considering the striking progress of CRISPR-Cas9 system in eukaryotes, and high-throughput manner to increase the yield of target products. It plays an important role in the components of antiphage defense system from microorganisms. In recent search on restriction enzymes from microorganisms, the latest generation of Cas9-based genome engineering tools is also based on components of antiphage defense system from microorganisms. In recent years, the CRISPR-Cas9 system and various technologies derived from it, such as precise editing, multi-gene editing, and precise regulation, have catalyzed innovation to provide integrated solutions, and have provided us with increased flexibility in bacterial genetic engineering. It plays an important role in the fields of single-stranded RNA editing and high-throughput gene screening. Nevertheless, the problems such as off-target rate and cytotoxicity should not be ignored. Since the DSB caused by the Cas9 protein is lethal to host cells, DNA base editor that does not introduce DSB has been developed. It is reported that an evolved Cas9 variant containing 7 mutations has relaxed the PAM requirement to NG or NNG [81]. The application of these variants is expected to significantly increase the number of targeted nucleotides in the genome. The existing base editor can only realize the base conversion between pyrimidine and between purine. Zhao et al. designed and constructed the cytosine deaminase nCas9-Ung protein complex, creating a new glycosylase base editor (GBE). They developed a single-base gene editing system that can realize the conversion between pyrimidine and purine. Based on this system, it is the first time in the world to achieve arbitrary base editing in microorganisms and specific conversion of C-G bases in mammalian cells [82]. This is important in the construction of synthetic biological systems and modification of biological traits. As microbial cell factories are increasingly used to produce biochemical products, it will be a trend to use the CRISPR-Cas9 system to optimize the genome of microbial cells in a modular, parallel, and high-throughput manner to increase the yield of target products. Considering the striking progress of CRISPR-Cas9 system in eukaryotes, we anticipate that discoveries in the near future will expand its application in more microorganisms.

Credit author statement

I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

Declaration of competing interest

The authors indicate that they have no conflict of interest.

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