Review

CRISPR-enabled tools for engineering microbial genomes and phenotypes†

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†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/biot.201700586].

Received: December 30, 2017 / Revised: May 9, 2018 / Accepted: June 13, 2018

Keywords: CRISPR editing, CRISPR gene regulation, CRISPR tools for microbes, CRISPRa, CRISPRi.

Abbreviations: CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; **crRNA**, CRISPR RNA; **DSB**, double-strand break; **NHEJ**, non-homologous end joining.

Abstract

In recent years CRISPR-Cas technologies have revolutionized microbial engineering approaches. Genome editing and non-editing applications of various CRISPR-Cas systems have expanded the throughput and scale of engineering efforts, as well as opened up new avenues for manipulating genomes of non-model organisms. As we expand the range of organisms used for biotechnological applications, we need to develop better, more versatile tools for manipulation of these systems. Here we summarize the current advances in microbial gene editing using CRISPR-Cas based tools, and highlight state-of-the-art methods for high-throughput, efficient genome-scale engineering in model organisms *Escherichia coli* and *Saccharomyces cerevisiae*. We also review non-editing CRISPR-Cas applications available for gene expression manipulation, epigenetic remodeling, RNA editing, labeling and synthetic gene circuit design. Finally, we point out the areas of research that need further development in order to expand the range of applications and increase the utility of these new methods.

1. Introduction

CRISPR-enabled tools are rapidly replacing traditional methods as a faster, cheaper and more effective alternative for genome editing and regulation in a growing number of model and non-model organisms. Advancement of microbial biotechnology relies heavily on our ability to engineer new genotypes and phenotypes in a growing number of microbial hosts used for production of chemicals, enzymes, biofuels and materials precursors [1]. However, challenges remain as to how to improve these tools to achieve better efficiency and higher throughput gene editing, as well as more predictable and controllable multiplex gene expression without toxicity or off-target effects. In this article we review recent advances in CRISPR-mediated technologies for microorganisms, highlighting areas that still need improvement.

2. CRISPR-enabled genome editing applications

CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated) systems are an adaptive immune system found in Bacteria and Archaea. This system utilizes short RNAs (encoded by CRISPR arrays composed of short repeats and intervening protospacer sequences derived from foreign invaders) to recognize target DNA and/or RNA for degradation by Cas effector nucleases [2]. CRISPR-Cas systems can be classified as Class 1 (multisubunit effectors) or Class 2 (single protein effectors), under which there are 6 Types and numerous subtypes [3]. The Class 2 Type II CRISPR-Cas system from *Streptococcus pyogenes*, CRISPR-Cas9, has been the most extensively studied and utilized as genome editing tool in a variety of organisms [4,5]. Type II systems are composed of a single effector nuclease, Cas9, that pairs with a guide RNA (gRNA) for targeting. The gRNA is composed of a CRISPR RNA (crRNA), containing a sequence complementary to the foreign protospacer DNA and processed by the host endogenous RNase III, that hybridizes to a second RNA, a transactivating CRISPR RNA

(tracrRNA) [6]. This gRNA associates with and directs Cas9 to the targeted DNA containing protospacer adjacent motifs (PAMs), where Cas9 introduces a double-strand break (DSB) (Fig. 1A and B). The gRNA requires a 20-bp protospacer targeting sequence and the NGG PAM at the 3′-end (N(20)-NGG) for efficient target recognition [2]. To simplify this 3-component system for targeted genome editing, a fusion of the crRNA and tracrRNA (synthetic guide RNA, sgRNA) was developed. In this system, Cas9 nuclease activity can effectively be directed to any DNA sequence harboring N(20)-NGG by modifying the first 20 nt in the sgRNA [7].

For targeted genome editing, the Cas9-mediated DSB is the initiation step to induce DNA repair events. Site-specific DNA DSBs can be repaired by non-homologous end-joining (NHEJ) or homology-directed repair (HDR) [8]. While NHEJ can produce imprecise insertions/deletions at the site of the DSB, HDR (the dominant mechanism for most bacteria [9,10] and budding yeast [11]) can lead to the direct introduction of specific modifications via recombination of target sites with donor templates (Fig. 1A). CRISPR-Cas9 genome editing tools have been successfully applied in diverse organisms ranging from bacteria and yeast to plants and human cells [12].

In addition to Cas9, other Cas effectors have been developed for targeted gene editing, such as Class 2 Type V Cpf1 endonuclease [13] and various Class 1 systems [14,15], expanding the range of targets and organisms that CRISPR-based editing can be performed in. Compared to traditional homologous recombination-based methods and programmable DNA-editing tools, such as zinc-finger or TALE nucleases [16–18], CRISPR-Cas based platforms have enabled fast, efficient, marker-free and cost-effective genome engineering with precise targeting [4–6,19]. In this section, we will focus on the gene editing applications of CRISPR-Cas systems in bacteria and yeast.

2.1. CRISPR-enabled genome editing in *E. coli*

E. coli is one of the most widely used microorganisms for metabolic engineering and synthetic biology approaches, enabling rapid and efficient genome engineering (Fig. 1C). Early CRISPR-Cas9 editing by Jiang et al. in *E. coli* utilized a plasmid-based system consisting of pCas9, containing the tracrRNA and Cas9, and pCRISPR, containing a crRNA array and an editing (donor) template [20]. These plasmids were co-transformed into *E. coli* MG1655 to introduce a point mutation in the *rpsL* gene; however, the frequency of edited cells versus WT cells that had escaped Cas9 cleavage was low. This low editing efficiency was significantly enhanced by co-expression of λ-Red recombineering (recombination-mediated genetic engineering) genes *gam*, *bet*, and *exo* [21]. Further plasmid-based co-expression studies of the λ-Red machinery with Cas9 and sgRNA by Pyne and coworkers showed efficient DNA insertion of up to 3 kb and deletion of up to 19.4 kb [22], while Bassalo et al. expanded the approach for rapid integration of a 10 kb isobutanol pathway [23].

2.2. CRISPR-enabled genome editing in *S. cerevisiae*

The CRISPR-Cas9 system has also been successfully employed for genome editing in the budding yeast, *S. cerevisiae* [24–31] (Fig. 1D), which is extensively utilized for production of food, biofuels, and chemicals. Plasmid-based expression of Cas9 has been demonstrated from low (CEN/ARS) or high (2μ) copy plasmids in *S. cerevisiae*, with differing strategies for expression of the sgRNA. DiCarlo et al. used RNA polymerase III regulatory elements for gRNA expression [24], while in another study by Ryan et al., the sgRNA was fused to the 3' end of the self-cleaving hepatitis delta virus (HDV) ribozyme to enhance intracellular gRNA levels for multiplex editing [32]. Additionally, Bao et al. expressed crRNA, tracrRNA, and Cas9, relying on

RNase III (and other unknown nucleases) to process the pre-crRNA, leading to an active Cas9/gRNA complex [25].

S. cerevisiae contains a very efficient native homologous recombination system, enabling *in vivo* plasmid assembly and efficient chromosomal integration of foreign DNA [33–35]. Coupling this innately efficient HDR with the CRISPR-Cas9 system leads to greatly enhanced multiplex genome editing when multiple gRNAs are expressed from a single plasmid containing several gRNA cassettes [26,32,36] or an array of different crRNAs [25,36]. Furthermore, Cas9-facilitated multi-loci integration of *in vivo* assembled DNA parts (CasEMBLR) demonstrated that more than ten double-stranded DNA fragments with 50-bp overlap sequences could be transformed as a donor template to repair a Cas9-mediated DSB [27].

CRISPR-Cas9 has also been implemented in polyploid yeast, albeit with reduced efficiency. Zhang et al. introduced multiple gene knockouts into the industrial polyploid strain ATCC4124 with 15% to 60% efficiencies [28], while Ryan et al. showed multiplex *in vivo* assembly and integration with 70% efficiency in the same strain [32]. Together these results indicate the potential for CRISPR-Cas engineering of industrial polyploid yeast.

2.3. CRISPR-enabled genome editing in non-model microbes

S. pyogenes Cas9 has also been utilized for genome editing in a growing number of non-model microbes including *Streptomyces*, *Clostridium, Lactobacillus* and other species (Table 1) [37], which traditionally have been difficult to edit. In some cases where the use of Cas9 had not been successful, editing was improved using a "nickase" version of Cas9 where one of the two nuclease domains is inactivated, resulting in cutting of only a single DNA strand [38–41], which may lead to more efficient HDR [19]. Alternatively, some non-model organisms have been successfully edited using Type I, III and V systems (Table 1). Specifically, Type V systems have

been successfully utilized in genome editing of *Corynebacterium glutamicum* and cyanobacteria, where Cas9 had demonstrated toxicity [42–44]. Type V systems, characterized by the Cpf1 (Cas12) nuclease, have several advantages, including shorter spacer-repeat sequences (44 compared to ~100 nucleotides for Cas9 sgRNA), making it more amenable for multiplex array construction. In addition, Cpf1 cuts 17 nucleotides away from the PAM and generates DSBs with 4- to 5-nucleotide overhangs, potentially enabling more specific editing via NHEJ [13]. HDR is the dominant mechanism for DNA repair in most bacteria, but NHEJ is present in some bacteria including *Mycobacterium*, *Pseudomonas*, and *Bacillus* [9,10]. For the reasons described, alternative CRISPR-Cas systems may be of particular interest for development in non-model organisms.

2.4. CRISPR-Cas9 genome engineering tools

The versatility of the CRISPR-Cas9 system has triggered the development of an expanding array of genome engineering tools (Table 2). Iterative, multiplex genome editing facilitated by CRISPR-Cas9 has been applied in *E. coli*; self-curing of the sgRNA plasmid (via inducible expression of an sgRNA targeting the sgRNA plasmid) following initial editing allows for another round of editing with the introduction of new sgRNA plasmids [21]. Multiplex automated genome engineering (MAGE) utilized recombineering in *E. coli* for automated, iterative targeting of many locations in the genome to generate sequence diversity for the high throughput optimization of metabolic pathways [45]. Recently, Ronda et al. developed CRISPR-optimized MAGE recombineering (CRMAGE) to enable the iterative, multiplexed and automated introduction of larger modifications at higher frequency by combining CRISPR-Cas9 with ƛ-Red recombineering and a self-curing sgRNA plasmid strategy in *E. coli* [46] (Fig. 2A).

In another multiplexed method that combines recombineering with CRISPR-Cas9, Garst et al. demonstrated genome editing at multiple loci in parallel using the CRISPR-Cas9 system combined with barcode-enabled mapping of mutations that confer phenotypes of interest (Fig. 2B). In addition to plasmids for inducible expression of Cas9 and ƛ-Red genes, CRISPR-EnAbled Trackable genome Engineering (CREATE) cassettes (amplified from ~200 bp oligo arrays) containing both the targeting sgRNA and a homology arm for editing (donor template) are cloned into the sgRNA plasmid, creating a plasmid-based donor editing vectors that also acts as a "barcode" for the specific edit. Following transformation of the CREATE plasmid into *E. coli* expressing Cas9, enriched variants can be easily mapped using the plasmid barcode tracking approach after selection experiments. CREATE enabled the concurrent evaluation of up to >50,000 genome-wide mutations with parallel mapping in a single experiment and led to the identification of previously unreported mutations that are rarely found by adaptive laboratory evolution (ALE) or error-prone approaches. CREATE has also been applied to *S. cerevisiae*, showing its powerful portability as a tool for multiplex genome engineering [47].

3. Non-editing CRISPR-Cas applications

In addition to revolutionizing genome editing in microbes, the development of CRISPR-mediated technologies have expanded into a number of other molecular biology applications (Fig. 3) [48,49]. The ability of CRISPR-Cas systems to specify virtually any target offers new possibilities for investigating gene function, transcriptional regulation, visualizing genetic loci and encoding programmable logic circuits into biological systems [50–57].

3.1. Gene repression with CRISPRi

One of the first widely-adapted non-gene editing applications of CRISPR-Cas technology was CRISPR interference (CRISPRi). In this application, inactivation of the nuclease domain(s) results in a catalytically "dead" effector protein or complex that can no longer cleave DNA, but which retains the ability to bind target DNA [58,59] (Fig. 4). Directing the complex to the promoter or protein coding region of a target gene leads to targeted gene repression [60], interfering with RNA polymerase or transcription factor binding or transcriptional elongation [58]. CRISPRi is a simple and effective alternative to RNAi for targeted gene repression in bacteria and budding yeast, which lack RNAi machinery [61] and it is much easier to design and implement compared to peptide-based custom zinc-finger or TALE methods. CRISPRi also has added advantages of inducible control and reversibility [58,62], the ability to target noncoding regions [63–65] and essential genes [51,66], and simultaneous repression of multiple gene targets [52,53,67]. Therefore CRISPRi is an attractive method for investigating gene function [51,66,68], metabolic engineering [69–71], mapping enhancers, introns, and other noncoding elements [72], control over growth and cell cycle [73,74], construction of synthetic gene circuits [75,76], and interrogation of gene-to-trait relationships on a multiplex, genome-wide scale [77].

3.1.1 dCas9-based transcriptional repression

The catalytically-inactive version of the *Streptococcus pyogenes* Cas9 (dCas9) lacks nucleolytic activity due to the presence of point mutations in its RuvC-like (D10A) and HNH nuclease (H840A) domains [6]. Robust gene repression of up to 1000-fold has been demonstrated with *S. pyogenes* (dCas9) [58]. Repression can be achieved with only 12-nt homology to the 20-nt protospacer [58,78], and ChIP-seq analysis revealed that as little as 10 perfectly-matched bases in the PAM-proximal region of the sgRNA is sufficient to mediate dCas9 binding to DNA [79]. The

need for less homology makes construction of gRNA arrays easier, but it could also lead to higher probability for off-target effects [79]. Targeting the sgRNA to either strand of the RNAP-binding sites (e.g. the -35 and -10 regions of a σ70 promoter) results in steric hindrance for polymerase factor binding and inhibition of transcription initiation, while targeting to non-template DNA of the protein-coding region blocks transcription elongation [60]. CRISPRi using dCas9 has been demonstrated for sequence-specific gene repression in a variety of model and non-model microbes including *E. coli* [58,78], *Streptococcus pneumoniae* [78], *Bacillus subtilis* [80], *Corynebacterium glutamicum* [81], *Clostridium beijerinckii* [82], Cyanobacteria [83–87], and Mycobacteria [88],[66,68]. In *S. cerevisiae*, dCas9 was also fused to transcriptional repressors to enhance repression efficiency by approximately 3-fold [65].

3.1.2. Cascade-mediated transcriptional repression

The native Class 1, Type I CRISPR-Cas system in *E. coli* has also been utilized for CRISPRi. 95% of all CRISPR systems found in Bacteria and Archaea are Type I [89],[90], including industrially and medically relevant species of *E. coli*, *Thermus thermophilus, Streptococcus thermophilus*, *Clostridium autoethanogenum*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii, Salmonella typhimurium*, *Vibrio cholerae* and multiple *Bifidobacterium* species [50,91–96]. Type I systems are characterized by the signature Cas3 endonuclease and a multisubunit complex, CRISPR-ASsociated Complex for Antiviral DefensE (Cascade, encoded by *casABCDE*), that functions in gRNA procession, PAM recognition, DNA binding, and recruitment of Cas3 (Fig. 4A) [97]. *E.coli* Cascade utilizes 32-nt spacers, but spacers as short as 20-nt are sufficient for targeted interference [59,98]. The *E.coli* Cascade complex recognizes four canonical PAMs located on the 5' end of the gRNA (5'-AAG, AGG, ATG, GAG-3') [99]. However, recent high-throughput studies have shown much broader PAM sequence recognition

(at least 22 distinct PAMs), many of which show similar interference activity to canonical PAM sequences [90,100].

The *E. coli* Cascade operon is strongly repressed under normal growth conditions [59], so for CRISPRi applications, the operon (with *cas3* deactivated) must be placed under a strong promoter or expressed from a plasmid. Cascade-mediated gene repression has been shown to be as robust as that for dCas9, with highest repression levels achieved when targeting the promoter region and the template strand of the coding region [59,96]. The wide prevalence of Type I systems and their PAM promiscuity presents an attractive possibility for using native systems for manipulation of the expression of a wider range of targets in their respective host. The larger size of Cascade compared to dCas9 may also be an additional advantage when interfering with the RNA polymerase-DNA interaction [96] (Fig. 4B).

3.1.3. dCpf1-based transcriptional repression

Cpf1 is the signature effector of the Class 2 Type V CRISPR-Cas system [13]. In contrast to Cas9, which requires RNase III for crRNA processing [101], Cpf1 is capable of processing its CRISPR arrays [102]. The mature crRNAs are composed of only a short stem-loop structure in the direct repeat region and does not require an additional tracrRNA (Fig. 4C), representing the most minimalistic CRISPR-Cas system characterized to date [102]. The Cpf1 protein identified in *Francisella novicida* (FnCpf1) utilizes 42-44-bp crRNAs (19-nt repeat and 23–25-nt spacers) [13] and recognizes a YTN PAM on the 5' end of the guide RNA, similar to Type I systems [103]. Other characterized Cpf1 variants from *Acidaminococcus sp. BV3L6* (AsCpf1) and *Lachnospiraceae bacterium ND2006* (LbCpf1) recognize extended 4-nt TTTV PAMs [13].

Cpf1 crystal structure has revealed the presence of a single catalytic RuvC domain [104,105]. The inactivation of DNase activity, without inhibiting crRNA processing and DNA

binding, can be achieved via two mutations in the RuvC domain (D917A and E1006A), creating a DNase-dead Cpf1 (ddCpf1)[13,102]. The FnCpf1 inactivated variant has been used for a comprehensive screening for PAM motifs [106]. Another ddCpf1 ortholog from *Eubacterium eligens,* modified through a single deactivating mutation (D880A), was also utilized for multiplex gene repression in *E. coli* for both episomal and chromosomal targets, demonstrating robust (~330-fold) repression, similar to that of Cascade and dCas9 [107]. In contrast to the strand bias exhibited by Type I and II systems, *E. eligens* ddCpf1 exhibited bias toward the template strand when targeting the 3' UTR and the protein coding regions, but no strand bias when targeting the promoter region [107]. In addition, an AsCpf1 catalytically-dead variant (also requiring only a single point mutation) has been used alone [53] or fused to transcriptional repressors [108] for effective gene repression in *E.coli* [53] and eukaryotes [108],[109].

Although ddCpf1-mediated CRISPRi is less developed, it may prove to be the most versatile. The requirement for a single effector, no requirement for tracrRNA, and the ability to process its own crRNA makes it suitable for multiplex gene regulation in a wide array of organisms. In addition, Cpf1 T-rich PAM sequences may provide an advantage for repression of AT-rich genomes [110] and regions (e.g. origins of replication, UP elements, silencer and promoter sequences)[111–114] compared to Cas9, which requires at least one G in the PAM sequence [106]. Although the AsCpf1 and LbCpf1 variants have an even more specific PAM sequence (TTTV), mutated variants have been created that recognize more diverse PAMs [115]. Furthermore, Cpf1 has been shown to have lower toxicity than Cas9 in cyanobacteria, making it a promising candidate for CRISPRi implementation in microorganisms where dCas9 use has not been successful [44].

3.2. Gene expression activation with CRISPRa

CRISPR activation (CRISPRa) is another CRISPR-based method for controlling gene expression. Similar to CRISPRi, CRISPRa takes advantage of a catalytically-inactive Cas effector, but the inactive effector is coupled to a transcriptional activator to specifically enhance target gene expression. In bacteria, this has been accomplished by fusing dCas9 to the ω subunit of RNAP in an *E. coli* strain lacking the ω subunit (encoded by *rpoZ*) [78]. The resulting chimeric activator produced up to a 23-fold increase in gene expression when targeting ~60 bp upstream of a weak constitutive promoter. However, if the target site was within 35-70-bp of the transcription start site or more than 100-bp away, gene repression was observed, suggesting the critical role of the effector targeting position. Gene activation level varied depending on innate promoter strength, with highest activation observed for weak promoters [78].

CRISPR-mediated gene activation has also been demonstrated in eukaryotes, which possess well-characterized, modular transcriptional activation domains. In *S. cerevisiae,* a dCas9-VP64 fusion increases target gene expression by 2.5-fold, but could be increased up to 70-fold when multiple operators are added to the promoter region [116]. Using a tripartite activator (VP64-p65-Rta fusion of 3 transcriptional activator domains) or "scaffold RNAs" that recruit additional transcriptional activators/effectors can further enhance gene activation [117,118]. Deaner and Alper recently demonstrated a system where dCas9 was fused to either an activation or repression domain for metabolic engineering applications. They changed the target gRNA location to recruit the dCas9 activator/repressor to different positions in promoters, resulting in a ~40-fold range of gene expression [119]. Although most of the CRISPRa studies to date have focused on gene activation in eukaryotic systems, they demonstrate the potential for CRISPRa for the activation of gene expression in microbial systems.

3.3. Other non-editing CRISPR-enabled applications

In addition to transcriptional engineering, CRISPR-Cas systems have been modified and used for various other purposes, including imaging [54,120], mapping of regulatory elements such as enhancers, cis- and trans-acting elements [65], epigenetic remodelling [121–123], synthetic gene circuit construction [57,124,125], programmed DNA degradation [126], and localization of nucleic acids to nucleus or other cellular compartments [65,127]. Here we will focus on the two applications that are most pertinent to microbial biotechnology, namely, RNA targeting, base editing and genetic circuit design.

3.3.1. RNA targeting tools

While most genome-engineering applications focus on targeting DNA, RNA targeting is another emerging application of CRISPR-Cas technologies [128]. Cas9 can target single-stranded RNA when the PAM is presented as a DNA oligonucleotide [129], allowing targeted binding and cutting of specific RNAs *in vivo* [129–131]. Recently, certain type II-A and II-C Cas9 homologs have been shown capable of single-stranded RNA cleavage in the absence of a PAM, enabling gene repression and protection from ssRNA phages [132,133]. Additionally, Type III and Type VI CRISPR-Cas systems have both been shown capable of specifically targeting RNA. The Class 1 Type III systems, characterized by Csm and Cmr proteins, have multimeric effector complexes and possess a dual ssRNase and ssDNase activity [134–140]. Class 2 Type VI systems, on the other hand, have single protein effectors capable of targeting ssRNA and crRNA processing, making them more amenable for use in a wide array of organisms [141,142]. Two Type VI systems, Cas13a (previously known as C2c2) and Cas13b, have been extensively characterized and used for programmed knockdown of specific mRNAs in bacteria [141,143]. Unfortunately, Type VI

effectors exhibit promiscuous RNA cleavage activity [141,142]. However, catalytically-dead Cas13 variants (dCas13) can potentially be used for modulating mRNA translation, visualizing RNA localization, screening for non-coding RNAs, directing RNA localization to subcellular compartments, and capturing specific transcripts [141]. In addition, dCas13b fused to catalytic domain of the adenosine deaminase (ADAR2) gene has been used for programmable base editing of mRNA in human cells, enabling the engineering of target gene expression without permanently changing the coding DNA [143], representing another potential tool for microbial engineering.

3.3.2. Base editing

An alternative approach to gene editing using CRISPR-Cas9 technology that does not rely on introduction of double-strand breaks has recently been proposed [144]. Komor et al. have engineered the dCas9 fusion with a cytidine deaminase enzyme that enables direct conversion of cytidine to uridine for C→T conversion. This enables base editing within a 5-nucleotide window close to the PAM (nucleotides 4 to 8 of the protospacer) with average editing efficiency of 44%. The advantage of this method is that it does not require introducing double-stranded breaks, reducing the potential of indels. This method did, however, exhibit significant off-target base editing of known Cas9 off-target sites [144]. This was addressed by Rees et al. who engineered a high-fidelity base editor (HF-BE3) [145]. This method is also constrained by the PAM requirement which limits the number of accessible targets. This limitation was addressed by including five additional base editor variants with different PAM specificities to expand the number of sites that can be targeted and decreasing the editing window to 1–2 nucleotides to improve target discrimination [146]. Recently, the base editing toolbox has been expanded to include the adenine base editor that converts A•T to G•C pair [147].

3.3.3. Synthetic gene circuit design

CRISPRi and CRISPRa methods offer a new approach to genetic circuit design [57]. Modified CRISPR systems, coupled with inducible metabolite sensors, can be used as actuators to automatically regulate cellular metabolism in growth and production stages or control phenotypes such as sugar utilization, chemotaxis, biofilm formation, antimicrobial and phage resistance [74,75,125]. One of the main limitations of complex circuit design has been the limited number of orthogonal transcription factors that can work together without crosstalk [148]. CRISPR-based transcription modulators greatly expand the number of orthogonal parts that can be assembled into large, multi-layered circuits to enable complex logical operations over a wide dynamic range. Libraries of genetic circuit elements, such as NOT and NOR gates shown to work in both bacteria and yeast, have been constructed and assembled into layered structures that allow to carry out complex programming of cells [76,124,125]. These elements could be combined to build OR and AND gates, further expanding the number of logical operations that can be carried out in cells.

4. Current limitations for CRISPR-Cas Gene editing and Regulation

CRISPR-Cas technologies have overcome many of the challenges and limitations associated with previously established methods for gene editing, transcription control and other microbial biotechnology applications. However, there are still issues that need to be addressed to further improve the utility of CRISPR-based genome engineering methods. First, the editing efficiency and consistency of transcriptional repression or activation varies greatly between genes, leading to issues in reproducibility using current design rules [6,78,149]. Second, off-target effects present a challenge for editing large genomes and optimizing gene expression regulation. Third, PAM

requirement limits genomic regions that can be targeted. Below we cover the progress towards how these issues are being addressed and highlight the areas that still require research.

4.1. Editing and gene expression regulation efficiency

There are many factors that influence efficiency of CRISPR-based genome editing, including Cas enzyme kinetics [150–152], gRNA design [153], gene copy number [44], repair template and mechanism [154,155] and more. Various CRISPR-Cas systems have been extensively evaluated to determine their activity in a number of organisms [13,156,157], yet more studies are needed to understand variations in activity and potential negative effects over a wider array of organisms. As for gRNA design, an expanding suite of computational tools are now available to overcome sequence composition and secondary structure effects on gRNA stability and target binding to improve cutting and targeting efficiency [100,158–161]. In the context of transcriptional engineering, the effectiveness of repression or activation varies greatly between genes, depending on surrounding genetic context and initial gene expression strength [6,78,149]. Therefore, high-throughput analyses of gRNA functionality at different genomic loci are needed to improve our ability to understand and predict optimal gRNA design [162].

4.2. Predicting and avoiding off-target effects

Targeting specificity is determined only by a short seed region and an often non-stringent PAM sequence, which may confer off-target effects in organisms with large genomes [79,163]. In addition to carefully choosing gRNA designs to avoid high homology to other genomic sequences, several strategies have been used that reduce off-targeting. For example, off-targeting can be reduced by the use of truncated (17-nt long) gRNAs [164] or the use of Cas9 nickase with two guide RNAs [19,41,164–166]. Additionally, engineered variants such as the high-fidelity

(SpCas9-HF1), enhanced-specificity (eSpCas9), hyper-accurate (HypaCas9), and the expanded PAM specificity (xCas9) Cas9 demonstrate high targeting specificity with significantly reduced off-target activity [167–170].

For nuclease deficient applications, a standard computational search is required in order to predict potential off-target sites for a particular gRNA design [161,171–173]. A ChIP-seq analysis of sgRNA-dCas9 complex binding revealed a higher number of off-target binding sites than previously anticipated [79]. The analysis also showed the total number of mismatches at dCas9 binding sites can be as high as 10 in the PAM-distal region. This presents a potential problem for tuning of repression and activation through the incorporation of mismatches into the gRNA spacer sequence, as such designs increase the chances of off-target binding. For this type of application, a more sophisticated computational predictive models are needed to avoid off-target effects [174].

4.3. CRISPR-Cas diversity and PAM engineering

One of the strategies for increasing the range of targets, improving specificity and avoiding off-target effects is by expanding the suite of characterized CRISPR-Cas system variants [89,170,175–177]. Since the PAM requirement for a particular CRISPR-Cas system limits genomic regions that can be targeted, using different homologs or engineering PAM specificity may help overcome this limitation [100,115,156,177–180]. Flexible PAM requirements can be identified by the use of computational tools such as PAM-SCANR [100,106], and synthetic biology approaches can be utilized to alter and expand the range of PAM specificities [115,156,170]. On the other hand, longer, more stringent PAMs may be useful for avoiding off-target effects, especially in the context of flexible gRNA design such as mismatches for tuning gene repression strength.

5. Conclusions

Construction of CRISPR gRNA arrays is easy and cheap, simplifying the efforts for high-throughput metabolic engineering using CRISPR interference and activation [67,69,70,75]. Investigating the functions of essential genes is possible through the use of inducible CRISPRi systems, eliminating the need to construct thermo-sensitive or other conditional mutants [51,66]. Another advantage of CRISPR-Cas systems for gene expression manipulation is the ability to precisely control and fine-tune expression through use of titratable promoters [67,82,181] and photoactivatable systems [182,183]. The inducible and titratable nature of these systems allows to design genetic circuits that respond to environmental stimuli for engineering of industrially-relevant strains, which can undergo a metabolic shift from production to growth phase or control accumulation of metabolites (Table 3) [57,75,125]. Development of well-tested gRNA libraries targeting common regulatory elements, such as promoters (for repression) and operators (for gene activation) can streamline the use of CRISPRi in different systems and applications. The use of orthogonal CRISPR systems can expand the range of targets and organisms that it can be used in and enable customized manipulation of individual genes within a single cell and construction of complex synthetic gene circuits [62,76,150,175,184]. Recently, a combined gene deletion, activation and repression approach was demonstrated as an effective combinatorial metabolic engineering in *Saccharomyces cerevisiae* strategy using the β-carotarotene pathway as an example [185], demonstrating in practice how all these different applications can be applied together. We hope to see more of these advanced applications in the future.

Acknowledgements

This study was supported by the US Department of Energy Office of Science Biological and Environmental Research Program through a Biosystems Design project (E.O., K.T., C.E., and R.G.) and the Center for Bioenergy Innovation (C.E. and R.G.). The Center for Bioenergy Innovation is a U.S. Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science.

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Conflict of interest

The authors declare no conflict of interest

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7 Tables

Table 1. CRISPR-enabled genome editing in non-model microbes.

Microorganism	CRISPR-Cas system	Editing efficiency	Reference
Streptomyces species	Type II	60-100%	[186–189]
Clostridium species	Type II	50-100%	[15,41,190–193]
Clostridium species	Type I-B	100%	[15]
Synechococcus elongatus	Type II	57-100%	[42,194]
Lactobacillus reuteri	Type II	90%-100%	[195]

Corynebacterium glutamicum	Types II and V	38-100%	[43,196–198]
Bacillus species	Type II	10-53%	[199–201]
Pseudomonas putida	Type II	50%	[199]
Streptococcus pneumoniae	Type II	100%	[20]
Tatumella citrea	Type II	100%	[21]
Sulfolobus islandicus	Types I-A and III-B	94-100%	[14]
Cyanobacteria species	Types II and V	20-100%	[42,44,194]

Table 2. Comparison of different multiplex genome editing tools based on CRISPR-Cas9.

Method	Microorganism	Multiplex editing (no. of loci or transformation)	Efficiency	Reference
Multigene CRISPR editing	*E.coli*	3	47%	[21]
CRMAGE	*E. coli*	2	70-98%	[46]
CREATE	*E. coli*	50,000	75%	[47]

CRISPRm	*S. cerevisiae*	3	19%	[32]
HI-CRISPR	*S. cerevisiae*	3	27%-87%	[25]
Multilex CRISPR editing	*S. cerevisiae*	6	65-100%	[36]
CasEMBLR	*S. cerevisiae*	3	31%	[27]

Table 3. Examples of CRISPRi use for improving yield of industrially relevant compounds.

Class	**Products**	**Result**	**Reference**
Biofuels	n-butanol	5.4-fold increase in yield through repression of *pta, frdA, ldhA, and adhE* genes	[67]
	1,4-butanediol	100% increase in titer by repression of competing pathways (combined CRISPR editing and CRISPRi)	[71]
Terpenoids	isoprene, (-)-α-bisabolol and lycopene	Increased terpenoid yield by repression of mevalonate pathway genes	[202]
	mevalonate	41 % increase in yield by arresting cell growth	[74]
Polyphenols	(2S)-naringenin	7.4-fold increase in titer by increasing malonyl-coA flux	[70]
	pinosylvin	16-fold increase in titer by increasing malonyl-coA flux	[71]

	pinosylvin	1.9-fold increase in yield through repression of fabD	[203]
Polyhydroxy-alkanoates	poly(3-hydroxybutyrate-co-4-hydroxybutyrate)	Varying stoichiometry of 3HB/4HB from 1.4 to 18.4mol% by repression of endogenous genes	[69]

8 Figure legends

Figure 1. Cas9-based genome editing. (A) Blunt double-strand breaks in the genome are repaired by nonhomologous end-joining (NHEJ) or homology-directed repair (HDR). (B) The structure of CRISPR-Cas9 system. (C) CRISPR-Cas9 genome editing in *E. coli*. (D) CRISPR-Cas9 genome editing in *S. cerevisiae*.

Figure 2. CRISPR-Cas9-based genome engineering tools. (A) CRMAGE workflow. CRMAGE is based on the combination of MAGE recombineering and CRISPR-Cas9 system. (B) CREATE workflow. CREATE combines automated design of gRNA-editing oligo cassettes, multiplex cloning of plasmid-based donor editing vectors, and sequencing.

Figure 3: Overview of non-editing applications of CRISPR-Cas technologies.

Figure 4: Side-by-side comparison of the three modified CRISPR-Cas systems most commonly used in non-editing applications: (A) Type I Cascade complex, (B) Type II dCas9 and (C) Type V dCpf1 catalytically-dead variants are all capable of generating programmed multiplex gene repression. The target genes are specified by the spacer sequences of the CRISPR array, which is

processed into mature gRNAs that bind the effector and direct it to the target site. Each effector recognizes specific PAM sequences adjacent to the protospacer sequence in the target DNA.

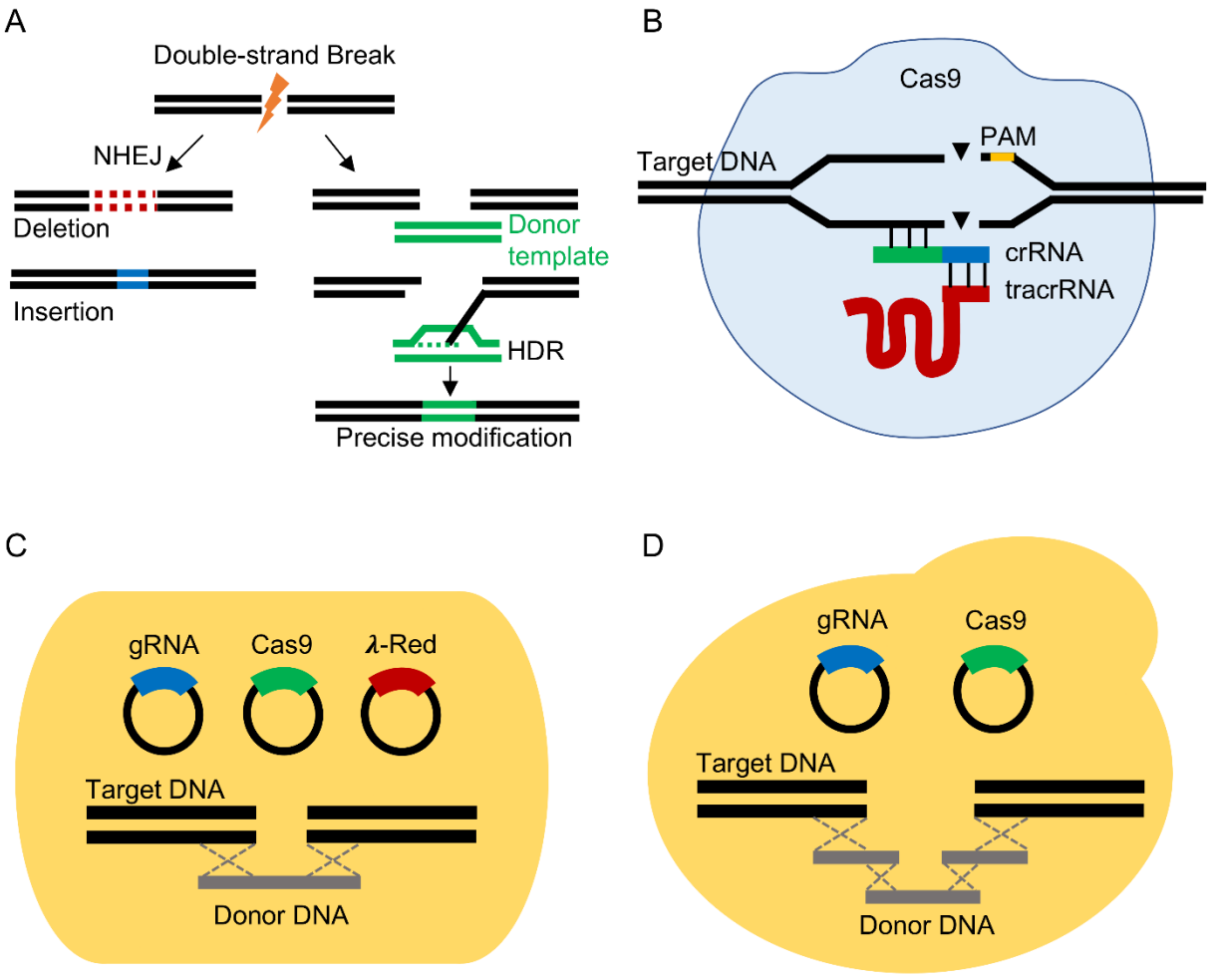

Figure 1

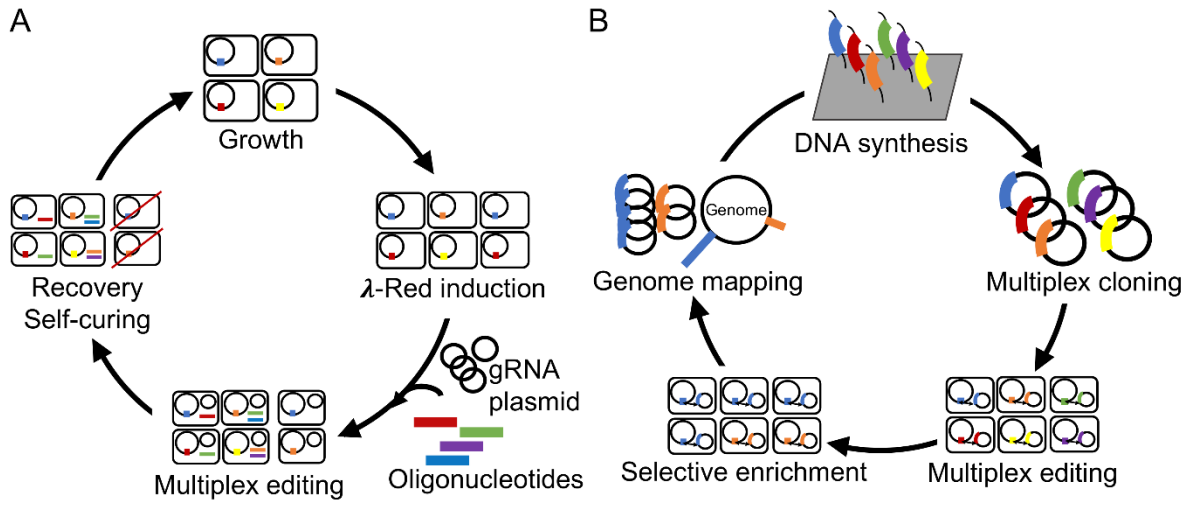

Figure 2

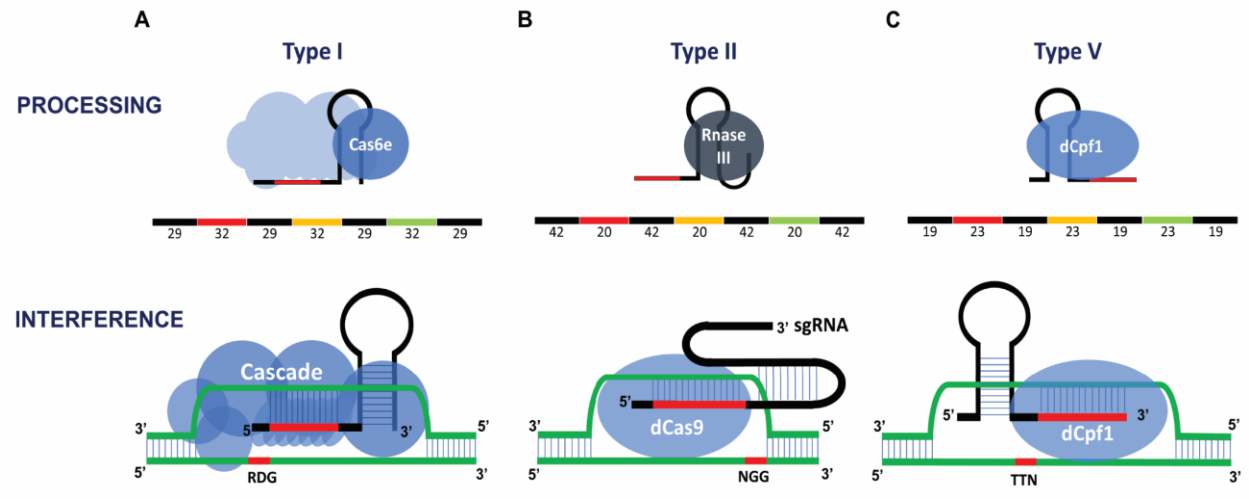

Figure 3

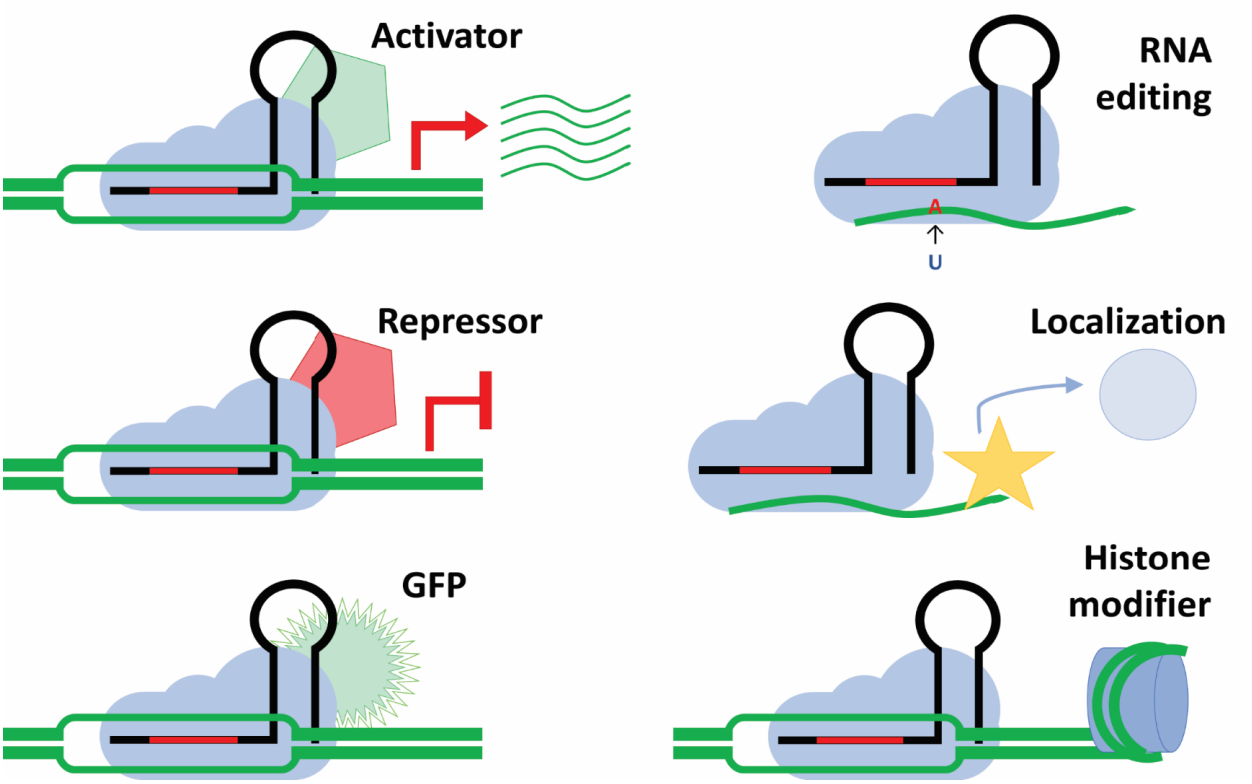

Figure 4