CRISPR-mediated Genome Engineering and its Application in Industry

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Abstract

The CRISPR (clustered regularly interspaced short palindromic repeat)/Cas9 (CRISPR-associated nuclease 9) method has been dramatically changing the field of genome engineering. It is a rapid, highly efficient and versatile tool for precise modification of genome that uses a guide RNA (gRNA) to target Cas9 to a specific sequence. This novel RNA-guided genome-editing technique has become a revolutionary tool in biomedical science and has many innovative applications in different fields. In this review, we briefly introduce the Cas9-mediated genome-editing tool, summarize the recent advances in CRISPR/Cas9 technology to engineer the genomes of a wide variety of organisms, and discuss their applications to treatment of fungal and viral disease. We also discuss advantageous of CRISPR/Cas9 technology to drug design, creation of animal model, and to food, agricultural and energy sciences. Adoption of the CRISPR/Cas9 technology in biomedical and biotechnological researches would create innovative applications of it not only for breeding of strains exhibiting desired traits for specific industrial and medical applications, but also for investigation of genome function.

Introduction

Although in 1987 a group of scientists led by Atsuo Nakata (Osaka University) reported an unusual pattern of non-coding DNA in *Escherichia coli* (Ishino *et al.*, 1987), biological function of CRISPR arrays was not understood until 2005, when for the first time three studies suggested a role of it in adaptive immunity (Bolotin *et al.*, 2005; Mojica *et al.*, 2005; Pourcel *et al.*, 2005). Then, in 2007, Barrangou *et al.* (2007) provided evidence of adaptive immunity in bacteria by monitoring clustered regularly interspaced short palindromic repeats (CRISPR) loci in phage-challenged cultures of *Streptococcus thermophilus* (one type of bacteria used to make yogurt and cheese). In addition, Horvath's research group reported that bacteria harbouring a particular viral sequence as a CRISPR spacer were resistant to that virus, and that the CRISPR arrays were certified to provide protection against invading viruses when combined with Cas genes (Doudna and Charpentier, 2014). The mechanism of this immune system based on RNA-mediated DNA targeting was illustrated shortly thereafter (Brouns *et al.*, 2008; Deltcheva *et al.*, 2011; Garneau *et al.*, 2010; Marraffini and Sontheimer, 2008). In 2012, a research team led by Emmanuelle Charpentier and Jennifer Doudna devised the Type II CRISPR system from *Streptococcus pyogenes* for genome editing (Jinek *et al.*, 2012). Consequently, researchers can now utilize this mechanism to break the genome of most organisms, prokaryotic or eukaryotic, at almost any site (Fig. 7.1). The system requires only two components: the Cas9 DNA endonuclease and a single guide RNA (sgRNA) encoding the reverse complement to the sequence in the DNA to be targeted.

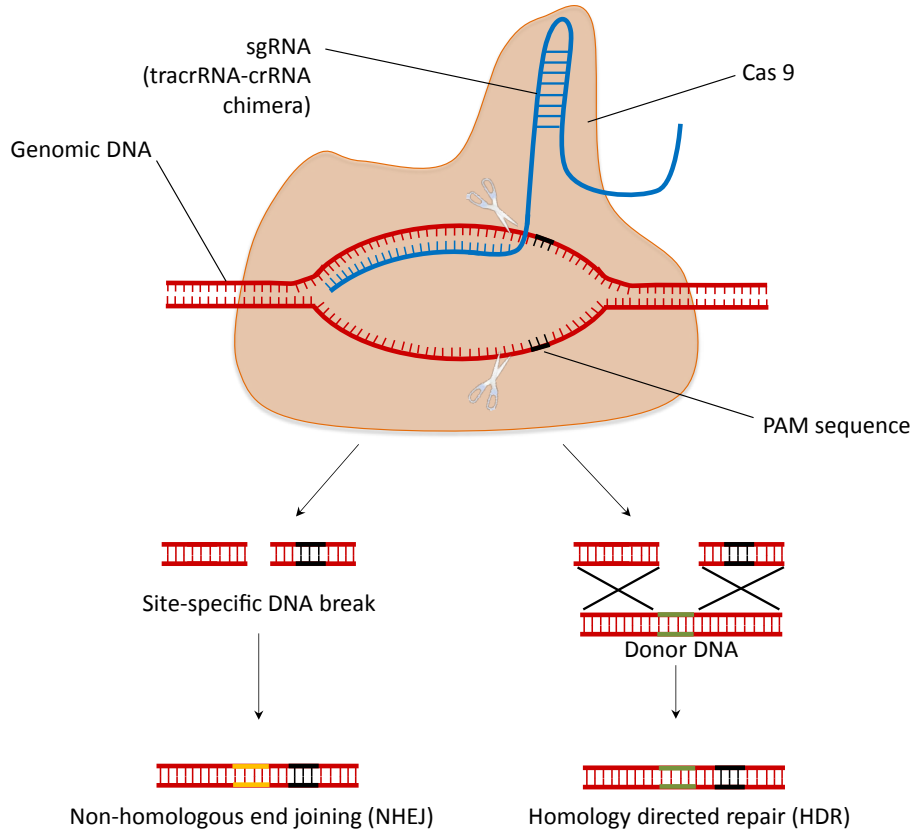

Figure 7.1 The CRISPR/Cas system. The CRISPR-associated endonuclease Cas9 could target specific DNA loci and make double-strand breaks (DSBs) under the guidance of the sgRNA. The presence of the Protospacer Adjacent Motif (PAM) at the target sequence is mandatory for successful Cas9 binding and catalysis – a protection mechanism to avoid self-cutting in CRISPR-containing organisms. DNA double strand breaks can be repaired by homologous recombination (HR) or non-homologous end joining (NHEJ). NHEJ re-ligates DSBs in an error-prone manner allowing for insertion or deletion of several bases at the DSB site. HR, on the hand, is a high-fidelity repair mechanism that uses an identical (or very similar) copy of the DSB region as a template for repair.

The only restriction for designing a CRISPR/Cas9 guiding sequence is a need for a protospacer adjacent motif (PAM) close to genomic target site (Jinek *et al.*, 2012; Cong *et al.*, 2013). Unlike ZFNs and TALENs, which achieve sequence recognition via protein–DNA interactions, Cas9 can be targeted to specific genomic loci with a guide RNA (gRNA). In addition, ZFNs and TALENs are costly, less reliable and time-consuming techniques than CRISPR/Cas9 for research. In eukaryotes, once the Cas9–sgRNA complex introduces a Double-Strand Break (DSB) at the target site, it is immediately repaired through the evolutionarily conserved pathways of error-prone non-homologous end joining (NHEJ) (which consists of re-ligation of the DSB resulting in loss or addition of a few nucleotides) or homology-directed repair (HDR) (when cells harbour a second copy of chromosomes) (Fig. 7.1) (Barnes, 2001; Van den Bosch *et al.*, 2002). With the utilization of these repair processes, researchers have been able to disrupt specific genes, add exogenous DNA elements into intended genomic sites, introduce single-nucleotide substitutions, and perform many other applications.

Despite slight improvement by using traditional molecular biology tools, most organisms relevant to biomedical and biotechnological sciences still show resistance to genetic engineering which could be problematic from the viewpoint of breeding new energy producing industrial organisms. To date,

Table 7.1 Application of CRISPR/Cas9 to industrially important organisms

Organism	Cell type	Industry	Reference
Fungi			
Saccharomyces cerevisiae	CEN.PK	Energy	Ronda *et al.*, 2015
Candida albicans	SC5314	Health care	Vyas *et al.*, 2015
	SC5314, BWP17, SN152	Health care	Min *et al.*, 2016
Trichoderma reesei	Qm6a	Chemical	Liu *et al.*, 2015
Aspergillus oryzae		Brewing	Katayama *et al.*, 2016
Yarrowia lipolytica	NS18 and NS432	Energy	Friedlander *et al.*, 2016
Streptomyces coelicolor	A3(2)	Energy	Tong *et al.*, 2015
Bacteria			
Escherichia coli	BL21 and BW25113-T7	Health care	Ahmed *et al*., 2014
	IYB5670 and IYB5671	Health care	Yosef *et al.*, 2015
	DH5α	Health care	Stovicek *et al.*, 2015
Clostridium autoethanogenum	DSM10061	Energy	Nagaraju *et al.*, 2016
Virus			
HIV-1	T cells	Health care	Ebina *et al.*, 2013
	Microglia, promonocytes, T cells	Health care	Hu *et al.*, 2014a
HPV18	HeLa	Health care	Kennedy *et al.*, 2014
HPV16	SiHa	Health care	Kennedy *et al.*, 2014
HPV16	SiHa and CaSki	Health care	Hu *et al.*, 2014b
HBV	HepAD38	Health care	Kennedy *et al.*, 2015
HBV	HepG2.2.15	Health care	Zhen *et al.*, 2015
EBV	Raji	Health care	Wang and Quake, 2014

CRISPR/Cas9 has been used to overcome the challenges with these organisms through its application for treatment of microbial disease (bacterial, fungal and viral), for generation of animal disease models and drug discovery, and for improvement of food, agricultural and energy sciences. However, one outstanding limitation to the technique is off-target mutations, in which Cas9-directed genome editing occurs at unintended DNA sites (Jinek *et al.*, 2012; Cong *et al.*, 2013; Fu *et al.*, 2013). Although current works represent that these objections are not a major difficulty, precise qualification of Cas9 is needed before direct usage of the technology in humans. Ethical concerns are a second leading issue (Webber, 2014). There are concerns about the risk of errors in heritable modifications and (unintended) consequences. Moreover, *S. pyogenes* Cas9 is the most widely used orthologue for genome editing and requires an NGG (PAM) (Jiang *et al.*, 2013). Each of the GG dinucleotides is present on average in 1 of every 8 base pairs; therefore, extended genomic regions without PAM are very rare in most organisms (Cong *et al.*, 2013).

Application of CRISPR technology to treatment of microbial infection

Application of CRISPR technology to the treatment of fungal infection

Mycosis is a fungal infection of animals and humans (Kirk *et al.*, 2008). Mycoses are common and a variety of environmental and physiological conditions can contribute to the development of fungal diseases. Fighting mycosis is an increasingly important global health concern and involves dominating both emerging infectious agents and

newly drug-resistant strains. Among microbial infection, mycosis accounts for more deaths annually than even either tuberculosis or malaria (Denning and Bromley, 2015). Therefore, new biological leads for pathogen identification and therapeutics are required, and researchers have developed new genome engineering tools for the study of pathogenic fungi such as those that are recombination-based techniques (Krappmann *et al.*, 2006; Takahashi *et al.*, 2008). By using these techniques, a major limitation for functional analysis of genome in fungal pathogens has been the low rates of homologous recombination between exogenous DNA sequences and recipient genomes. On the other hand, because there are a limited number of validated selectable markers for transformation and making knockouts of multiple genes, it is impractical for large numbers of genes to be analysed in a single-strain background. To circumvent these problems several site-specific recombinase systems have been developed (Hartmann *et al.*, 2010; Shahana *et al.*, 2014; Khrunyk *et al.*, 2010). However, among all genome editing tools the CRISPR/Cas9 system has been exhibiting to be a rapid and facile genome manipulation for both yeasts (Ronda *et al.*, 2015; Laughery *et al.*, 2015; DiCarlo *et al.*, 2013; Vyas *et al.*, 2015; Min *et al.*, 2016) and filamentous fungi (Liu *et al.*, 2015; Arazoe *et al.*, 2015; Nødvig *et al.*, 2015; Fuller *et al.*, 2015; Zhang *et al.*, 2016; Katayama *et al.*, 2015; Matsu-ura *et al.*, 2015). For example, *Candida albicans* is pathogenic yeast that causes mucosal and systematic infections with high mortality. Gerald R. Fink's group used the CRISPR system to create homozygous gene knockouts, mutations in multiple genes and genes that encode essential function. The ability to analyse essential genes provides an opportunity to explore potential antifungal targets in *C. albicans* (Vyas *et al.*, 2015). Their finding that CRISPR works effectively in a recent antifungal resistant clinical isolate suggests a new route to characterize clinical isolates of drug-resistant strains of *Candida*.

Application of CRISPR technology to the treatment of viral infection

Viral infections are of therapeutic challenge since viral life cycles occur within the host cells. Recent years have seen the rapid development of novel application for genetic-engineering technologies including those for the treatment of viral infections. Studies have shown that the CRISPR/Cas9 system can clear the HIV-1 genome and prevent new HIV infection (Ebina *et al.*, 2013; Hu *et al.*, 2014a). In fact, sgRNA expression vector targeting the long terminal repeats (LTR) of HIV-1 efficiently cleaves and mutates LTR target sites and suppresses LTR-driven viral gene expression. In addition, Ebina group showed that this system can delete viral genes from the host cell chromosome (Ebina *et al.*, 2013). The high specificity of Cas9/sgRNAs in editing the HIV-1 target genome has also been recently demonstrated (Hu *et al.*, 2014a). Cas9/sgRNAs efficiently inactivate HIV gene expression and replication in latently infected cells, including microglial, promonocytic and T cells. Significantly, Cas9/sgRNA mediated genome editing has been shown to immunize cells to prevent HIV-1 infection (Hu *et al.*, 2014a). These results indicate that the CRISPR/Cas9 technology can serve as a potential tool for clinical applications to cure viral-based infectious diseases. CRISPR system has also been exhibited the potential to be developed as an effective therapy for human papillomavirus (HPV)-associated tumours (Kennedy *et al.*, 2014; Hu *et al.*, 2014b), hepatitis B virus (HBV)-associated diseases (Kennedy *et al.*, 2015; Zhen *et al.*, 2015) and Epstein–Barr virus (EBV) (Wang and Quake, 2014) in the clinic.

Application of CRISPR technology to drug design

So far, drug design investments have not been returned as medicines and budgets are being cut (Paul *et al.*, 2010), partly because some clinical failures happen due to molecule quality and in some cases therapeutic hypothesis is without foundation (Arrowsmith, 2011; Prinz *et al.*, 2011). However, the abilities to apply biomedical knowledge in drug discovery have been improving; availability of knock-out and knock-in mice has helped model generation (Heck, 2004) and RNA interference (RNAi) has aided target validation (Bartz and Jackson, 2005). However, RNAi has its drawbacks, including partial knock-down that can fail to produce a measurable phenotype (Sachse *et al.*, 2005) and off-target effects (Marine *et al.*, 2012). Despite these drawbacks, both short hairpin RNA (shRNA) and small interfering RNA (siRNA) genome-wide screens have been used to look for new therapeutic targets in a number of diseases.

The discovery of the Cas9 nuclease removed many of the technical and financial barriers to high-throughput knock-out screens (Malina *et al.*, 2013; Wang *et al.*, 2014; Shalem *et al.*, 2014; Koike-Yusa *et al.*, 2014; Zhou *et al.*, 2014; Sanjana *et al.*, 2014; Doench *et al.*, 2014): specific endonucleases can be assembled in cells simply by co-expressing Cas9 and an sgRNA, which is short enough to be encoded by oligonucleotides that can be synthesized in large-scale arrays. Antibiotic overuse in medicine and abuse in animal agriculture has led to the rise of multidrug-resistant pathogens that are increasingly tolerant to the current antibiotic arsenal. Accordingly, there is a need for novel antimicrobials that can bypass common modes of multidrug resistance while being selective for individual strains. Because CRISPR/Cas9 is a more precise and a sequence-specific technique than previous similar ones, it is possible to use its specificity to design it to target a single bacterial species. It will only cut up essential genes from that one species, even when the target species is mixed up with others. Therefore, antimicrobial CRISPR/Cas systems may be better weapons against bacteria than antibiotics. In this regard, the Marraffini group in their work suggested that CRISPR/Cas systems could be used for the sequence-specific killing of bacteria (Bikard *et al.*, 2012). Later studies by Bikard *et al.* (2014), Citorik *et al.* (2014) and Gomaa *et al.* (2014) offered a promising solution to the problem of antibiotic resistance by using CRISPR/Cas9 system. Yosef *et al.* (2015) also designed a CRISPR-based system that could selectively kill antibiotic-resistant bacteria. They used temperate phages to deliver a functional CRISPR/Cas system into the genome of antibiotic-resistant bacteria. The delivered CRISPR/Cas system destroyed both antibiotic resistance-conferring plasmids and genetically modified lytic phages. With further development, CRISPR has the potential to treat multidrug-resistant infections without impacting beneficial microbes, to remove contaminating microbes from industrial fermentations (Stovicek *et al.*, 2015) and to provide further insights into microbial communities.

Application of CRISPR technology to generation of animal models

Cas9-mediated genome editing has enabled accelerated generation of transgenic models and expands biological research beyond traditional, genetically tractable animal model organisms (Sander and Joung, 2014). For generation of cellular models, Cas9 can be easily introduced into the target cells

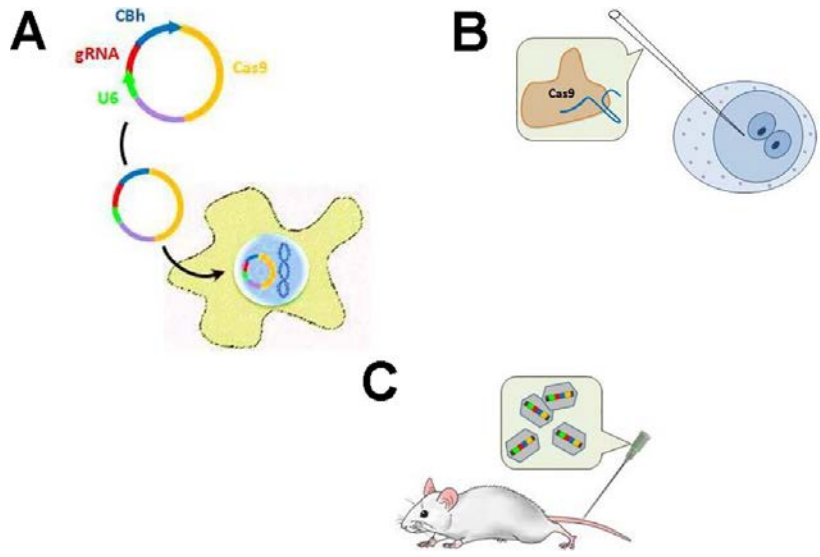

Figure 7.2 Applications of Cas9. (A) Expression plasmids encoding both the Cas9 gene and a short sgRNA cassette driven by the U6 RNA polymerase III promoter can be directly transfected into any cell line of interest. (B) Purified Cas9 protein and *in vitro* transcribed sgRNA can be microinjected into fertilized zygotes for rapid generation of transgenic animal models. (C) In order to perform somatic genetic modification, high-titre viral vectors encoding CRISPR reagents can be transduced into tissues or cells of interest.

using transient transfection of plasmids carrying Cas9 and the appropriately designed sgRNA (Fig. 7.2A). Additionally, the multiplexing capabilities of Cas9 offer a promising approach for studying common human diseases – such as diabetes, heart disease, schizophrenia, and autism – that are typically polygenic. For generation of transgenic animal models, Cas9 protein and transcribed sgRNA can be directly injected into fertilized zygotes to achieve heritable gene modification at one or multiple alleles in models such as rodents and monkeys (Wang *et al.*, 2013; Li *et al.*, 2013; Yang *et al.*, 2013; Niu *et al.*, 2014) (Fig. 7.2B). Successful multiplex targeting in cynomolgus monkey models was also recently reported (Niu *et al.*, 2014), suggesting the potential for establishing more accurate modelling of complex human diseases such as neuropsychiatric disorders using primate models. Additionally, Cas9 could be harnessed for direct modification of somatic tissue, obviating the need for embryonic manipulation (Fig. 7.2C) as well as enabling therapeutic use for gene therapy.

Studies to date have typically relied on the injection of Cas9 mRNA into zygotes (fertilized embryos at the single-cell stage). However, because transcription and translation activity is suppressed in the mouse zygote, Cas9 mRNA translation into active enzymatic form is likely delayed until after the first cell division (Oh *et al.*, 2000). Because NHEJ-mediated repair is thought to introduce indels of random length, this translation delay likely plays a major role in contributing to genetic mosaicism in CRISPR-modified mice. To overcome this limitation, Cas9 protein and sgRNA could be directly injected into single-cell fertilized embryos. The high rate of non-mutagenic repair by the NHEJ process may additionally contribute to undesired mosaicism because introducing indels that mutate the Cas9 recognition site would then have to compete with zygotic division rates. To increase the mutagenic activity of NHEJ, a pair of sgRNAs flanking a small fragment of the target gene may be used to increase the probability of gene disruption. Tan and colleagues (Tan *et al.*, 2013) used CRISPR system to produce biomedical model pigs to enhance productivity in the livestock industry. Xue *et al.* (2014) used hydrodynamic injection to deliver plasmids encoding sgRNA and Cas9 to the murine liver where simultaneous targeting of the Pten and Trp53 led to the development of liver tumours in all five mice treated within 3 months. Platt *et al.* (2014) generated a Cre-dependent Cas9 knock-in mouse, which could be crossed with a variety of Cre-driver strains to enable the expression of Cas9 either constitutively or in various tissues. Multiple reports describe the use of custom nucleases to engineer cultured human stem cells. Cas9-sgRNA and a plasmid donor were employed to correct the cystic fibrosis transmembrane conductance receptor by homologous recombination in cultured intestinal stem cells from cystic fibrosis patients: organoids derived from these cells restored the normal cAMP-induced swelling phenotype (Schwank *et al.*, 2013).

Application of CRISPR technology to food and agricultural sciences

One of the major goals in the food and agricultural sciences is to get technologies to develop health-promoting products for a growing world population. In this regard, the use of recombinant genetic technologies has profoundly impacted molecular biology research and applications in fields such as biosynthesis of vitamins, enzymes, pharmaceuticals, antibiotics, and bioactive peptides. The CRISPR/Cas9 technology is a modern, fashionable method in plant research. Immediately after its early use to edit the genomes of animals and bacteria (Hwang *et al.*, 2013; Jiang *et al.*, 2013; Mali *et al.*, 2013), in August 2013, five reports were published discussing the first application of CRISPR/Cas9-based genome editing in plants (Feng *et al.*, 2013; Li *et al.*, 2013; Nekrasov *et al.*, 2013; Shan *et al.*, 2013; Xie and Yang, 2013). Subsequent work focused on additional crop species such as sorghum (Jiang *et al.*, 2013), wheat (Upadhyay *et al.*, 2013; Wang *et al.*, 2014) and maize (Liang *et al.*, 2014). Two research groups simultaneously proposed a possible strategy to make plants virus resistant using CRISPR/Cas9 technology (Baltes *et al.*, 2015; Ji *et al.*, 2015). In addition, the application of CRISPR/Cas9 could extend to a direct knock-out strategy in the disease susceptible genes, often termed 'S-genes', of a host genome; the result is the development of durable disease-resistant crops. The S-gene knock-out strategy was employed successfully in hexaploid bread wheat using the TALEN and CRISPR/Cas9

system, and the fungi-resistant wheat was generated (Wang *et al.*, 2014). Thus, novel and valuable plants generated by CRISPR/Cas9 can regain useful traits overlooked during domestication; these traits help plants survive unpredictable global environmental changes.

Application of CRISPR technology to energy sciences

Because CRISPR/Cas9 has been shown to lead to precise and affordable genome edition in bioenergetics systems, then it can be very powerful tool in energy sciences. *Yarrowia lipolytica* is valuable oleaginous microbial host for chemical production known for converting sugars to lipids and hydrocarbons as energy source that are difficult to make synthetically (Friedlander *et al.*, 2016). Genome of the *Y. lipolytica* has been hard to manipulate at the genetic level by old genome engineering tools. In a recent work, however, a research team could adapt CRISPR/Cas9 system for *Y. lipolytica*, showing that the system could be used for markerless gene knock-out and introduction of new genes (Schwartz *et al.*, 2016). This approach, based on the team's claim, is the first step of a project to create long chain hydrocarbons – used to make specialty polymers, adhesives, coatings and fragrances – from yeast rather than synthetically.

Bacteria are almost unlimited source of enzymes, and they are extensively used in industry in various ways for the manufacture of dairy products, and the production of biological substances such as enzymes, vaccines, antibiotics and biofuels. In this regard, *Clostridium autoethanogenum* is a model acetogen that is being pursued for fuel (ethanol) and chemical (2,3-butanediol) production at commercial scale (Liew *et al.*, 2016; Daniell *et al.*, 2016). Recently, Nagaraju *et al.* (2016) constructed and screened a small library of tetracycline-inducible promoters in CRISPR/Cas9 system and reported that they could improve the efficiency of CRISPR/Cas9-mediated desired gene deletion to over 50%, making a viable tool for engineering *C. autoethanogenum*.

Sustainable and cost-effective biofuels are attractive sources for renewable energy. This could be achieved by either creating of efficient metabolic pathways for ethanol production in organisms such as algae or by engineering of yeast, mould and bacteria which have shown the potential to generate biofuel from plant biomasses like maize biomass. Despite some improvement, most of these organisms show resistant to engineer by traditional molecular biology tools which could be problematic from the viewpoint of breeding new energy producing industrial strains. CRISPR/Cas9 has been used to overcome those genome editing drawbacks and limitations with organisms (DiCarlo *et al.*, 2013; Min *et al.*, 2016; Nødvig *et al.*, 2015; Matsu-ura *et al.*, 2015; Stovicek *et al.*, 2015; Shan *et al.*, 2013; Mans *et al.*, 2015; Jacobs *et al.*, 2014; Ninomiya *et al.*, 2004; Huang *et al.*, 2015; Tong *et al.*, 2015; Kim *et al.*, 2015; Feng *et al.*, 2016).

Future direction

Cas9-based technology is becoming an ultimate molecular powerful tool for studies in basic, biomedical and biotechnological sciences. This technology has been successfully applied for genetic manipulation in numerous bacterial, fungal, viral and other species. Adapting CRISPR/Cas to industrially and medically important organisms is highly desirable because they are the central core in energy, pharmaceutical and health care industries. Therefore, for those host organisms a better characterization of genetics and subsequently a more validated Cas9-based engineering package are essential (Crook and Alper, 2012). In this way, one major obstacle might be off-target effects and their consequences. In the future, the use of high-throughput methods that enable comprehensive profiling of off–target cleavage sites (Jinek *et al.*, 2012; Cong *et al.*, 2013; Fu *et al.*, 2013) should provide low-cost and high-speed engineering in each host system. In case of ethical issues as one of the increasingly important concerns, the possibility of modifying the germline – in particular the human germline – should be considered carefully.

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Conflict of interest statement

There is no conflict.

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