Volume 36 | Number 9 | September 2019 | Pages 1225–1372

Natural Product Reports

rsc.li/npr

Themed issue: Engineering of Cell Factories for the Production of Natural Products
Guest Editor: Tilmann Weber

ISSN 0265-0568

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REVIEW ARTICLE
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CRISPR/Cas-based genome engineering in natural product discovery

new area. Back in 1987, Ishino and colleagues had discovered the sequence of a gene called *iap* belonging to the gut microbe *Escherichia coli*.[9] However, at that time, they did not determine the function of those sequences. In 2002, those sequences were first named “Clustered Regularly Interspaced Short Palindromic Repeats” (CRISPR).[10]

Fig. 1 The working model of CRISPR immunology.

identify alien DNA. These tags are named "protospacer-adjacent motifs" (PAMs). For example, Cas9 from *Streptococcus pyogenes* recognizes 5′-NGG-3′ as its PAM on the nontarget DNA strand[22] (Fig. 2 left panel).

1.3 CRISPR/Cas as genome editing tool

Among the large CRISPR/Cas families, Class 2 CRISPR systems have been in main focus due to their simplicity and modularity. They require only two components: a single Cas effector (endonuclease) and a CRISPR RNA (crRNA, also known as guide RNA (gRNA)), which functions as a homing device (Table 1). The best studied example is the CRISPR/Cas9 system of *Streptococcus pyogenes*. In this system, the effector Cas9 (referred to below as SpCas9) contains two nuclease domains, an HNH domain cleaving the targeting strand and a RuvC-like domain cutting the nontargeting strand.[34] The effector associates with two RNA molecules coined crRNA and trans-activating CRISPR RNA (tracrRNA). For SpCas9, these two small RNAs can be artificially linked to an 82 nt single RNA molecule known as single guide RNA (sgRNA) while retaining full functionality.[24] These components are the only two needed for the cleavage of target double-strand DNA.[12,23,24] Based on these observations, the CRISPR/Cas9 system was initially used as a genome editing tool for editing bacterial genomes and mouse and human cells.[25,26] Since then, the genome editing platform CRISPR/Cas9 has reached almost every corner of the genetic manipulation field, providing a basis for tools to edit the genomes of phages,[27] bacteria,[25] yeasts,[28] algae,[29] plants,[30–32] fly,[33] mouse,[26] and human[26] (these references are the first reports in each case).

The general CRISPR/Cas9-based genome editing process can be described as follows: (1) sgRNA and Cas9 molecules are generated; (2) a Cas9-sgRNA complex is formed; (3) this complex searches throughout the genome for PAMs by random collisions; (4) when a PAM is found, the complex starts to bind to this PAM and interrogates the flanking DNA for spacer complementarity; (5) when a complementary target sequence is found, the complex binds to the target DNA, and then Cas9 undergoes a series of conformational changes that triggers the nuclease activity of its HNH and RuvC-like domains, which results in a DNA double-strand break (DSB); (6) the DSB is repaired.[34,35] Multiple DSB repair pathways are available intracellularly, which can lead to diverse genome editing events such as indels, in-frame deletions/insertions, random-sized deletions, open reading frame (ORF) inactivations, and nucleotide substitutions (Fig. 2). In addition to DSB-based genome editing, engineering of CRISPR/Cas9 can lead to other applications: a SpCas9 nickase (SpCas9n) was engineered by mutating one of the nuclease domains (D10A or H840A) and was reported to enhance genome editing specificity in some cases.[36] Furthermore, a catalytically dead Cas9 variant (dCas9) was engineered by mutating the active sites of both nuclease domains (D10A and H840A); it has no endonuclease activity but preserves the ability of sgRNA-guided DNA binding. Therefore, dCas9 can serve as a roadblock to inhibit gene transcription initiation and/or elongation, which is a process named CRISPRi (CRISPR

Fig. 2 An overview of the Class 2 CRISPR/Cas-based genome editing mechanism.

Table 1 Known properties of experimentally evaluated Class 2 CRISPR/Cas systems

Type	Cas endonucleases	Target	Nuclease domain	crRNA processing	PAM/PFS	Cleavage pattern	Guide RNA type
II	Cas9	dsDNA	RuvC and HNH	No	G-rich	Blunt	tracrRNA:crRNA (engineered as sgRNA)
V-A	Cas12a (Cpf1)	dsDNA	RuvC	Yes	T-rich	Staggered	crRNA
V-B	Cas12b (C2c1)	dsDNA	RuvC	ND	T-rich	Staggered	tracrRNA:crRNA
VI-A	Cas13a (C2c2)	ssRNA	HEPN	Yes	H (A, U, C)[a]	ssRNA target and collateral	crRNA
VI-B	Cas13b	ssRNA	HEPN	Yes	5′ D (A, U, G), 3′ NAN or NNA	ssRNA target and collateral	crRNA

[a] PFS is short for protospacer flanking site; the Cas13a from *Leptotrichia shahii* needs an H (A, U, C) PFS, while the Cas13a from *Leptotrichia wadei* does not need a significant PFS.[45]

nterference)[37] (Fig. 3A). It was reported by different indepen-
lent groups[37–39] that the transcription repression was strand-
specific, if targeting coding region of a gene, only those
gRNAs target on non-template strand can inhibit the gene
ranscription. However, Howe and colleagues reported that in
east, rather than acting simply as a roadblock, sgRNA/dCas9
inding creates an environment that is permissive for tran-
cription initiation/termination, thus generating novel sense
nd antisense transcripts. Thus, by targeting *HMS2* in *Saccha-
omyces cerevisiae*, they observed that CRISPRi is not strand-
specific at all loci.[40] In addition, dCas9 and Cas9n could be
used as vehicles for the delivery of other functional proteins to
lefined genome loci. For example, by fusion with transcription
ctivators, dCas9 can mediate the transcription activation of
arget genes, becoming thus known as CRISPRa (CRISPR acti-
vator, see Fig. 3B).[41,42] By fusion with cytidine deaminase or
denine deaminase, dCas9/Cas9n can assist a single base-pair
exchange in the target locus without a DSB (from a C/G pair
to a T/A pair by a cytidine deaminase and from a A/T pair to a G/C pair by an adenine deaminase).[43,44]

In addition to Cas9 from *S. pyogenes*, more than 3000 predicted Cas9 orthologs were identified in the database.[46] Several of these Cas9 orthologs have been developed into genome editing tools as well (see Table 2 for details).

To increase the specificity and fidelity of CRISPR/SpCas9, many SpCas9 variants were obtained by structure-guided protein engineering. For example, PAM specificity was altered by introducing mutations into the PAM-interacting domains of wild-type SpCas9, creating proteins such as the SpCas9 variant VRER (D1135V/G1218R/R1335E/T1337R), which recognizes NGCG, and the variants VQR (D1135V/R1335Q/T1337R) and EQR (D1135E/R1335Q/T1337R), which recognize NGAG.[47,48] The SpCas9 fidelity was increased by introducing mutations into the DNA-binding domains of wild-type SpCas9 to create such proteins as the variants spCas9-HF1 (N497A/R661A/Q695A/Q926A), spCas9-K855A, and espCas9 [1.1] (K848A/K1003A/R1060A), which can dramatically reduce genome-wide off-target effects.[49–51]

In addition to the successful applications of Cas9 as a genome editing tool, several other Class 2 CRISPR systems were also engineered as genome editing platforms. For example, CRISPR/Cas12a (Cpf1), belonging to Type V CRISPR systems, the mode-of-action is conceptually similar to the CRISPR/Cas9 system, and the single Cas effector Cas12a can be guided by a crRNA to cut the target DNA[52,53] (Fig. 2 right panel). CRISPR/Cas13a (C2c2), belonging to Type V CRISPR systems, is an RNA-guided RNA targeting system.[54] It has been used in RNA detection methods, such as SHERLOCK, which can detect specific DNA or RNA molecules at an attomolar level.[55] The CRISPR/Cas13a system was also applied to 7(r432chang.0175Tc06.908-1.34Td[(CRI318.8didesi(er7877(ems,(n)1.78-

2 CRISPR/Cas as genome editing tools in natural product research

2.1 CRISPR/Cas applications in microbes relevant to natural products

For many model organisms, well established "traditional genetic manipulation tools" exist, such as PCR targeting[58] or MAGE.[59] However, often considerable efforts are required to transfer these methods to non model organisms, which includes most natural product producers, for example, actinomycetes, filamentous fungi and plants. For many of these organisms, the availability of CRISPR/Cas-based genome editing tools has opened new possibilities. In this section, we are going to summarize CRISPR/Cas applications in *Streptomyces*, *Myxobacteria*, *Bacillus*, *Pseudomonas*, and *Cyanobacteria*, which are famous bacterial producers of natural products.

2.1.1 CRISPR/Cas applications in ***Streptomyces*** and other actinomycetes. As one of the most important resources for natural products, actinomycetes have been drawing much attention for many decades. The history of actinomycete genetics can basically be divided into three phases based on the approach to genetic manipulation that was taken. In Phase I (until-1978), the random mutagenesis era, the only direct way to manipulate a gene was random mutagenesis. Phase II, the pre-CRISPR era (1978–2014), started with the successful establishment of DNA transformation protocols for *Streptomyces*;[81] in this period, directed genetic manipulation was possible for the first time, one of the widely used approaches is PCR-targeting method (a simple workflow was shown in Fig. 4), but this method was relatively time consuming (it takes more than one month to successfully manipulate a gene), and the protocols did not allow a high degree of parallelization/throughput; with the

Fig. 4 A simple workflow of PCR-targeting genetic manipulation approach in *Streptom ces*.

advent of CRISPR, Phase III (2014–), the CRISPR era, began. New protocols that allow genetic manipulations with considerably higher efficiencies were developed (Table 3). In addition to their use in *Streptomycetes*, CRISPR/Cas-based genome editing techniques had been successfully applied in some other actinomycetes such as *Corynebacteria*,[82–87] *Actinoplanes*,[88] and *Mycobacteria*.[89,90]

At the time of writing this review, four CRISPR/Cas9 systems used for actinomycetal genome editing have been published, including pCRISPomyces-2 (another version is pCRISPomyces-1, which uses not sgRNA but crRNA:tracrRNA), published by Cobb *et al.*;[74] pCRISPR-Cas9, published by Tong *et al.*;[39] pKCcas9dO, published by Huang *et al.*;[76] and pWHU2059 (also known as CRISPR/Cas9-CodA(sm)), published by Zeng *et al.*[75] All of these four systems share some common designs and applications: they use Sp(d)Cas9 in a one-vector strategy that combines the *Sp(d)Cas9* gene, the sgRNA(s), and, if applicable, the editing template on a single plasmid. With the exception of the pCRISPomyces-1 system,[74] sgRNA is used as the homing device. Furthermore, the CRISPR plasmids are based on the temperature-sensitive pSG5 replicon in order to facilitate multiple rounds of editing. Most of the applications described so far are DSB-mediated DNA fragment knockout or promoter knockin (technically, taking advantage of the host homologous recombination (HR) machinery, all DSB-based knockout system can be used for knockin by putting the gene-of-interest between the two HR templates). Only Tong *et al.* used nonhomologous end-joining (NHEJ) repair to introduce deletions or frameshifts.[39] Furthermore, successful CRISPRi applications have been reported as well by Tong and colleagues.[39] For more detailed information, please see Table 3.

The sgRNA plays a crucial role as a homing device in successful CRISPR/Cas9 applications. Unfortunately, not many sgRNA identification tools are available for nonmodel organisms. One general tool is sgRNAcas9,[91] a script-based sgRNA finder, which can identify sgRNAs of the sequences uploaded by users. An alternative is CRISPy-web (https://crispy.secondarymetabolites.org),[92] a web-based tool that allows users to upload their own genomes or directly uses antiSMASH-job id for sgRNA identification.

2.1.2 CRISPR/Cas9 applications in ***Myxobacteria***. The Gram-negative *Myxobacteria* are also prolific producers of diverse secondary metabolites. Like for actinomycetes, the genetic tools to engineer *Myxobacteria* are also limited. As a model strain, *Myxococcus xanthus* is widely used for proof-of-concept of new techniques. Indeed, a two-plasmid based CRISPR/Cas9 system was established in *Myxococcus xanthus* for gene deletion.[93] The SpCas9 and its sgRNA were cloned into the single *attB*-site-specifically integrating plasmid pSWU30; while the editing templates (homologous recombination templates) were carried by the second suicide plasmid, pBJ113. At first, pBJ113 integrated either up- or downstream of the target gene by a single crossover event. In a second step, the pSWU30 plasmid was site-specifically integrated into the genome. As a third step, the Cas9:sgRNA complex introduced a DSB when the Cas9 was induced. Finally, the DSB was repaired by HR, resulting in a double crossover event to achieve in-frame deletion with high efficiency. A 92 kb DNA fragment was successfully deleted with an efficiency of 14.3%. Authors also observed that *Myxococcus xanthus* cannot tolerate high levels of Cas9 expression.

2.1.3 CRISPR/Cas9 applications in ***Bacillus***. The soil-dwelling Gram-positive bacterium *Bacillus* is an important producer of enzymes and valuable small molecules. A CRISPR/SpCas9 toolkit that generates mutations with up to 100% efficiency was developed for genome editing in *Bacillus subtilis*.[94] It was successfully applied for gene knockout, knockin, and knockdown. Multiplexing sgRNAs made simultaneous multigene editing possible. With the counterselectable marker gene *mazF*, this toolkit allowed continuous editing. A simple workflow of this system could be described as follows: firstly, the *SpCas9* gene was integrated into the *lacA* locus with a constitutive promoter. Secondly, this *SpCas9* gene-harboring strain was then transformed with a linearized sgRNA delivery vector and editing template(s). As a result, the sgRNA transcription cassette(s) was/were integrated into the *thrC* locus, and then with HR, the desired genome editing occurred. Thirdly (optionally), the successfully edited strain could be transformed with the wild-type *thrC* editing templates to eliminate the sgRNA transcription cassette, and the *thrC* locus was then restored for a second round of editing. Moreover, a CRISPRi system was also successfully constructed with a xylose-inducible dCas9 for gene transcription modulation.[94] Another genome-integrated CRISPR/SpCas9 nickase-based genome editing system was developed for *Bacillus licheniformis*.[95] With an editing template 1 kb in length, the efficiency of gene knockout reached nearly 100%. In addition, this system could multiplex genome editing. The reported efficiency of knocking out two genes simultaneously was 11.6%. Authors also demonstrated that this system was capable of large DNA fragment deletion and DNA fragment knockin. A 42.7 kb fragment was successfully deleted with the efficiency of 79.0%, while the knockin efficiency reached 76.5%.[95]

2.1.4 CRISPR/Cas9 applications in ***Pseudomonas***. *Pseudomonas* is a diverse genus of Gammaproteobacteria. Many *Pseudomonas* species are capable of producing different types of natural products.[96] For example, mupirocin (pseudomonic acid)[97] is a topical antibiotic useful against superficial skin infections. In addition to being producers of natural products, some pseudomonads such as *Pseudomonas putida* have received much attention as a cell factory due to their unique features such as their high tolerance to many organic solvents and their wide metabolic diversity. Though the single-stranded DNA (ssDNA) recombineering method enables genetic manipulations of *P. putida*, its efficiency was limiting. A method that combines ssDNA recombineering and CRISPR/Cas9 dramatically boosted the editing efficiency. In this system, CRISPR/SpCas9 is used for counterselection.[98] Use of this system resulted in a single nucleotide substitution; 315 bp; 693 bp; a large (69 kb) DNA fragment deletion; and the simultaneous deletion of two genes with efficiencies of 97%, 93.2%, 54.2%, 0.8%, and 3%, respectively. Using the *Streptococcus pasteurianus* dCas9, a CRISPRi system was established for gene transcription modulation. A Ptac promoter-controlled *Mycobacteria* codon-optimized *S. pasteurianus* dCas9 (NNGCGA was identified as

Table 3 A summary of CRISPR/Cas genome editing applications in *Streptom cetes* (up to February, 2018)

Species	Cas type[f]	sgRNA	DSB repair	Multiplex	Editing size (bp)	Editing efficiency[g] (%)	Backbone plasmid	Editing type	Related compound	Ref.
S. lividans	SpCas9	Yes[a]	HR	Yes	20–31 415	100/21.4–25	pSG5	Knockout	Undecylprodigiosin, actinorhodin	74
S. viridochromogenes	SpCas9	Yes	HR	Yes	20–23	66.7–100	pSG5	Knockout	Phosphinothricin tripeptide	
S. albus	SpCas9	Yes	HR	Yes	67–13 214	66.7–100	pSG5	Knockout	Polycyclic tetramic acid, macrolactam	
S. coelicolor	SpCas9	Yes	HR	No	666	94	pWHU	Knockout	Actinorhodin	75
S. coelicolor	SpCas9	Yes	Defective NHEJ	No	1–37 173	3–54	pGM1190	Knockout	Actinorhodin	39
			NHEJ	No	Indel[b]	69–77	(pSG5 replicon)			
			HR	No	1795–1952	97–100				
S. coelicolor	Dead SpCas9	Yes	N.R.[e]	No	NR[e]	100	pGM1190 (pSG5 replicon)	Knockdown	Actinorhodin	39
S. coelicolor	SpCas9	Yes	HR	No	768–82 867	29–100	pKC1139 (pSG5 replicon)	Knockout	Undecylprodigiosin, actinorhodin	76
S. formicae	SpCas9	Yes	HR	No	~43 000[c]	ND[d]	pSG5	Knockout	Formicamycins	77
S. rimosus	SpCas9	Yes	HR	Yes	1–8	100 33.3–40	pSG5	Knockout	Oxytetracycline	78
S. sp. SD85	SpCas9	Yes	HR	Yes	14–833	ND[d]	pGM1190 (pSG5 replicon)	Knockout	Sceliphrolactam	79
S. albus	SpCas9	Yes	HR	No	94–1097	60–100	pSG5	Knockin	Indigoidine	80
S. lividans	SpCas9	Yes	HR	No	97	66–100	pSG5	Knockin	Undecylprodigiosin, actinorhodin	
S. roseosporus	SpCas9	Yes	HR	No	97–774	50–100	pSG5	Knockin	Macrolactam photocyclized alteramide A, FR-900098	
S. venezuelae	SpCas9	Yes	HR	No	774	38–64	pSG5	Knockin	*m/z* 425[f]	
S. viridochromogenes	SpCas9	Yes	HR	No	97	100	pSG5	Knockin	*m/z* 405[f], *m/z* 780[f]	

[a] crRNA:tracrRNA was also tested. [b] Error-prone repair results in small deletion, insertion, and substitution. [c] The exact size was not indicated. [d] ND, not detected. [e] NR, not relevant. [f] Cas protein coding sequence in this table was codon-optimized to be capable of expression in *Streptomyces*; normally, *S. coelicolor* codon usage frequency effects this expression. [g] The efficiency of knockdown means that all tested sgRNA had repression effects.

the PAM) was integrated into the attTn7 locus, while the sgRNA transcription was controlled by a Ptet promoter.[99] Single and multigene repression in *P. putida* and *P. aeruginosa* were successfully tested.

2.1.5 CRISPR/Cas9 applications in ***Cyanobacteria***. As autotrophic bacteria, *Cyanobacteria* can directly convert sunlight and carbon dioxide into industrially important products, which makes them attractive cell factories. They have been recognized as important sources of a variety of bioactive natural products as well.[100] Pakrasi and colleagues established a CRISPR/Cpf1-based genome editing toolkit that was used for markerless knockouts, knockins and point mutations in three model cyanobacteria, *Synechococcus*, *Synechocystis* and *Anabaena*, in a fast and efficient manner.[101] Hu and colleagues reported a CRISPR/Cas9-based highly efficient genome editing tool for *Synechococcus elongatus* PCC 7942.[102] This tool is a two-plasmid system: a SpCas9 and crRNA:tracrRNA transcript cassette were cloned in one plasmid, and a second plasmid was used to provide HR templates. However, Pakrasi and colleagues found that SpCas9 is toxic in cyanobacteria, and they derived a CRISPR/SpCas9 system from pCRISPomyces-2 (ref. 74) that worked only when the codon-optimized *SpCas9* was transiently expressed.[103] This system performed in *Synechococcus elongatus* UTEX 2973 with 100% efficiency and no markers left in the genome. Moreover, Pfleger and colleagues established a CRISPRi system in *Synechococcus* sp. strain PCC 7002 for gene transcription modulation. They used an integrative strategy: dCas9 was introduced at the *acsA* locus, while the sgRNA was introduced at the NS1 site.[104] A CRISPRi system that can nicely tune gene expression in *Anabaena* sp. PCC 7120 was also established,[105] and the difference is a replicative plasmid carrying both dCas9 and sgRNA.

2.2 CRISPR/Cas applications in **fi**lamentous fungi important for natural productsiTcalegerNS15J/T1spais oneainNS1pigTf0s11Tf-06480Td93803

Table 4 A summary of CRISPR/Cas genome editing applications in filamentous fungi relevant to natural products

Species	Cas type	sgRNA[k]	DSB repair	Multiplex	Editing efficiency[c] (%)	Editing type[a]	Related compound/ molecule	Ref.
A. aculeatus	*A. niger* optimized SpCas9-SV40, in an AMA1 plasmid	HH-HDV embedded, in the same AMA1 plasmid with Cas9	HR[d] NHEJ	No	High[b]	Knockout/in indel	Pigment	108
A. brasiliensis			NHEJ			Indel		
A. carbonarius			NHEJ			Indel		
A. luchuensis			NHEJ			Indel		
A. nidulans			HR[d] NHEJ			Knockout/in indel Indel		
A. niger			NHEJ					
A. nidulans	Same as above	Same as above[g]	HNEJ	Yes	13–100[f]	Indel	Pigment	112
A. niger			HR			Knockout		
A. oryzae			HR[e]			Point mutation		
A. carbonarius	Same as above	Same as above	HR	No	1	Knockout/in	Pigment	113
A. fumigatus	*A. niger* optimized SpCas9-SV40, integrated	HH-HDV embedded	HR	No	80	Knockout (1 bp)	Trypacidin	109
A. fumigatus	Human optimized SpCas9-SV40, integrated	SNR52	NHEJ	No	25–53	Indel	Pigment	114
A. fumigatus	Human optimized SpCas9-SV40, integrated	U6/*in vitro* transcribed	NHEJ MMEJ	YES	~43 95–100	Indel[h] Knockin	Pigment	115
A. niger	*A. niger* optimized SpCas9-SV40, in an AMA1 plasmid	*In vitro* transcribed	HR	No	27.5–100	Knockout/in	Galactaric acid	116
A. oryzae	*Aspergillus* optimized SV40-SpCas9-SV40, integrated	U6	NHEJ	No	10–100	Indel	Pigment	117
Alternaria alternata	*A. niger* optimized SpCas9-SV40, in an AMA1 plasmid	HH-HDV embedded, in the same AMA1 plasmid with Cas9	NHEJ	No	10–25	Indel	Melanin	118
Beauveria bassiana	*B. bassiana* optimized SpCas9-SV40, in an AMA1 plasmid	*In vitro* transcribed	NHEJ HR	Yes	100 100, 39, 5[j]	Indel Knockout/in	Uridine	119
G. lingzhi *G. lucidum*	*G. lucidum* optimized SpCas9-SV40, integrated	*In vitro* transcribed	NHEJ	No	88.9 66.7–100	Indel	Uridine	120
M. thermophila	*Myceliophthora* optimized hac-1-SpCas9-hac1, in a plasmid	U6	NHEJ HR	Yes	100 95, 61–70, 30, 22[i]	Indel Knockout/in	Fluoroacetamide, cellulase	121
M. heterothallica			HR	No	90	Knockout/in		
Mucor circinelloides	*In vitro* expressed	*In vitro* transcribed	NHEJ HR	No	100	Indel knockout/in	Terpene, β-carotene	122
N. crassa	Human optimized SpCas9-SV40, transiently expressed in a nonreplicative plasmid	SNR52	HR	No	20–30	Knockin	Cellulase	123
Nodulisporium sp.	*T. reesei* optimized SpCas9-SV40, integrated	*In vitro* transcribed	NHEJ HR	No	75 4.5–68.3	Indel Knockin	P450 Pigment	110
A. oryzae *Sporormiella minima*			NHEJ		72.7 91.7	Knockin	Histone deacetylase A	

without editing templates in a highly efficient manner. This system was also successfully used in *A. fumigatus* to edit a single nucleotide insertion in the polyketide synthase of the trypacidin biosynthetic pathway and reconstitute its production in a nonproducing strain.[109] Notably, when the *yA* gene in *A. nidulans* was targeted, 70–80% of the transformants retained a wild-type phenotype (green) on the primary transformation plates; the authors re-inoculated 12 green transformants on the selective plates, and all transformants showed the genome-edited phenotype (yellow). Their explanation is that the CRISPR/Cas9-mediated mutagenesis of *yA* is efficient in a growth-dependent manner.[108] In *Aspergillus*, circular editing templates were found to be more efficient for gene targeting than the corresponding linear ones.[108] In contrast, in a *Nodulisporium* sp. (no. 65-12-7-1), the efficiency of linear editing templates was much higher than that of a circular plasmid editing template (68.4 *vs.* 4.5).[110] A linear template was also used in *T. atroroseus* to successfully identify a new gene that is responsible for production of polyketide-nonribosomal peptide hybrid products.[111] Mortensen and colleagues extended their

A similar approach was adopted in the development of a CRISPR/Cas9 genome editing system in *Aspergillus niger*, one of the most important model species. This system was successfully evaluated by manipulating related genes to increase the galactaric acid titer.[116] Additionally, Lu and colleagues established another toolkit for *A. fumigatus*. The human codon-optimized SpCas9-SV40 was integrated into the chromosome, while the sgRNA was either *in vivo*-transcribed by a U6 promoter or *in vitro*-transcribed by a T7 system in *E. coli*.[115] Instead of using NHEJ or HR to repair DSBs, the authors proposed using microhomology-mediated end joining (MMEJ) for DSB repair. Their method required only a very short editing template (approximately 35 bp) and yielded >95% editing efficiency.[115] In the higher fungus *Ganoderma*, such genome editing tools were set up as well.[120]

In addition to many applications of the strategies of "produce both Cas9 and sgRNA *in vivo*" and "produce Cas9 *in vivo* while transcribe sgRNA *in vitro*," only two reported cases before this review used the "produce both Cas9 and sgRNA first and then preassemble them into the Cas9:sgRNA complex *in vitro*" strategy. With this strategy, Cas9 protein and *in vitro*-generated sgRNA were mixed in a certain buffer to form the active Cas9:sgRNA complex, then this preassembled CRISPR/Cas9 ribonucleoproteins (RNPs) was directly delivered into the target strains. One case was reported by Nygård and colleagues in *Penicillium chrysogenum*;[124] and the other case was reported by Papp and colleagues in *Mucor circinelloides*.[122] For more information, please see Table 4. Interestingly, in the timeframe of this review, no toxicity of Cas9 had yet been reported in filamentous fungi. All reported systems and applications were SpCas9 based, and neither CRISPRi nor CRISPRa was reported in filamentous fungi.

2.3 CRISPR/Cas applications in plants

Plants play extremely important roles in the whole ecosphere and food chain. They provide us with oxygen, food, and bioactive natural products. Due to this importance, plants were one of the very first testing fields of CRISPR/Cas9 genome editing technology. Three different groups reported their successful CRISPR/Cas9 genome editing systems for model plants in the same journal back-to-back-to-back.[30–32] CRISPR/Cas has been widely used in plants for many purposes. In this section, we will mainly focus on reviewing the CRISPR/Cas genome editing applications in plants that have already been revealed as having potential as native producers or heterologous hosts of natural products. We also discuss single-celled algae, mainly the green alga *Chlamydomonas reinhardtii*, with the Kingdom plantae in this section.

2.3.1 CRISPR/Cas9 applications in chlorophytes. Microalgae, as photosynthetic organisms, use sunlight to produce chemicals from CO_2 and H_2O. They have drawn our attention as promising sustainable producers of vitamins, carotenoids, fatty acids, and many other valuable compounds. They have also shown great potential to produce bioactive secondary metabolites such as antioxidant, antiviral, antibacterial, antifungal, anti-inflammatory, antitumor, and antimalarial compounds[127,128] and to function as cell factories for producing fuel-like molecules, biomass, and synthetic chemistry feedstock.[129] The green unicellular alga *C. reinhardtii* is a model organism for both basic studies and applied sciences. As was the case for many bacterial and fungal nonmodel systems, the lack of efficient genetic manipulation approaches hampered the use of this organism. However, CRISPR/Cas technology opened a door for microalgal genome editing as well. Weeks and colleagues set up a NHEJ-based CRISPR/Cas9 genome editing system in *C. reinhardtii* with a single plasmid carrying the *Chlamydomonas* codon-optimized SpCas9 and an U6 promoter-controlled sgRNA, and they found that Cas9 and even dCas9 are very toxic to *C. reinhardtii*. Therefore, the Cas9 needed to be transiently expressed, which allowed a low level of intracellular Cas9 to be produced. Using this system, they successfully mutated targeted sequences with up to approximately 50% editing efficiency ($\sim 1/10^9$ – 46.7%).[130] To reduce the Cas9 toxicity and improve the editing efficiency, Shin and colleagues established a direct delivery system to deliver the Cas9 RNPs (*in vitro*-preassembled Cas9:sgRNA complex). This strategy was successfully tested by knocking out genes in *Chlamydomonas*. The editing efficiency was indeed dramatically increased, up to 100-fold.[131] Hegemann and colleagues established an HR-based two-plasmid CRISPR-saCas9 system: one plasmid carried a codon-optimized Cas9 from *Staphylococcus aureus*, and the other carried sgRNA and editing templates in *C. reinhardtii*. This system achieved editing efficiencies up to 9% in preselected colonies. It seemed that saCas9 was less toxic to *Chlamydomonas* than SpCas9. The authors also modified the *in vitro*-preassembled SpCas9:sgRNA RNPs system by testing different kinds of editing templates. They found that with the single-stranded oligodeoxynucleotide (ssODN) editing templates, the system could create low amounts of errorless knockin mutants, whereas with the short double-stranded editing templates (90 bp), it created high numbers of unpredictable gene disruptions/modifications.[132] A similar *in vitro*-preassembled Cas9 RNP system with editing templates was also established by Bae and colleagues in *C. reinhardtii*.[133] This Cas9 RNP-based genome editing tool was successfully applied in *C. reinhardtii* to improve the macular pigment titer.[134] Notably, a well-studied CRISPR system other than CRISPR/Cas9, CRISPR/Cpf1, was also implanted into *C. reinhardtii*. Instead of using a plasmid-based system, Molnar and colleagues developed a single-step codelivery of CRISPR/Cpf1 RNPs with a ssDNA repair template genome editing system for *C. reinhardtii*. The precise gene replacement efficiency was approximately 10%.[135] In summary, though CRISPR/Cas systems were successfully applied for genome editing in *Chlamydomonas*, at the time of this review, the editing efficiency requires further optimization.

2.3.2 CRISPR/Cas9 applications in plants important for natural products. Natural products from plants are widely used as pharmaceuticals, nutraceuticals, seasonings, pigments, flavors, *etc.* for a very long time. We have never stopped studying and exploiting plants for natural products, which has also now accelerated because of the revolutionary CRISPR/Cas technology. Most of the investigations of CRISPR/Cas applications in plants that have been reported were proof-of-concept studies. Generally, CRISPR/Cas-based genome editing in plants was accomplished during NHEJ-mediated DSB repair. For most studies, a human codon-optimized or specific plant

codon-optimized SpCas9 with an NLS was used. The sgRNA transcripts were mainly controlled by a U6 promoter. The CRISPR/Cas9 components were delivered by *Agrobacterium*-mediated transformation.

Papaver somniferum, known as opium poppy, biosynthesises morphine and is considered a very important medical plant. Unver and colleagues successfully established a CRISPR/Cas9-based gene knockout system in *P. somniferum*. This system is a gene inactivation system that causes small indels during NHEJ repair of the DSB introduced by Cas9. Authors used *Agrobacterium*-mediated transformation of leaves with TRV-based synthetic plasmids expressing sgRNA and a human codon-optimized SpCas9-encoding synthetic vector to inactivate 4′OMT2, a regulator of benzylisoquinoline alkaloid (BIA) biosynthesis in *P. somniferum*.[136]

As the second largest family of flowering plants, Orchidaceae is used not only for decoration but also for medicinal purposes. For instance, *Dendrobium officinale* produces many useful secondary metabolites.[137] Cai and colleagues established a similar CRISPR/Cas9 gene inactivation system for *Dendrobium officinale* to facilitate its genetic manipulation. This system achieved 10–100% editing efficiency using *Agrobacterium*-mediated transformation.[138]

Camelina sativa, a member of the Brassicaceae family, has received tremendous attention because of its unique oil profile, with the majority of its fatty acids being linolenic (C18:3), oleic (C18:1), linoleic (C18:2), and eicosenoic (C20:1) acids. Some of these acids can be used for industrial purposes, human nutrition, and pharmaceuticals.[139] Two back-to-back reports, one from Weeks and colleagues and one from Faure and colleagues, showed the successful application of CRISPR/Cas9-based genome editing technology to successfully increase the oleic acid content by knockout of fatty acid desaturase 2 (FAD2) genes.[140,141] Moreover, Durrett and colleagues simultaneously targeted three conserved homologous genes by the same sgRNA to alter the oil content in *Camelina sativa*.[142]

Nicotiana tabacum, a perennial herbaceous plant, is known as the source of tobacco; however, it has great potential to produce secondary metabolites, including terpenoids, alkaloids, flavonoids, phenylpropanoids, *etc.* Moreover, it is used as a molecular farm to produce special recombinant proteins such as biotherapeutic glycoproteins. Boutry and colleagues established a CRISPR/Cas9 genome editing system in suspended *Nicotiana tabacum* cells for gene deletion.[143] Later, this system was extended for multiplexing knockout genes Involved in glycan biosynthesis.[144]

Salvia miltiorrhiza, an old and well-recognized herb, produces many diterpene compounds.[145] Qi and colleagues reported successful applications of CRISPR/Cas9 to delete the committed diterpene synthase gene (*SmCPS1*) involved in tanshinone biosynthesis in *Salvia miltiorrhiza* by *Agrobacterium rhizogenes*-mediated transformation.[146] Zhang and colleagues also applied CRISPR/Cas9 in *Salvia miltiorrhiza* to knockout the rosmarinic acid synthase gene (SmRAS) in the water-soluble phenolic acid biosynthetic pathway.[147]

3 The remaining challenges in using CRISPR/Cas for natural product discovery

3.1 Common challenges and limitations

3.1.1 **Off**-target **eff**ects. When a gene is targeted using a CRISPR toolkit, some of the most detrimental effects are off-target effects. There are fewer reports of off-target effects in bacteria than in other organisms, which may be due to the lower occurrence of sequences homologous to a given spacer-PAM combination in smaller genomes.[148] Moreover, in microbes that have not had their whole genome sequenced, many of these off-target effects that do not result in obvious phenotypes are overlooked. Therefore, similar to the use of these systems in higher eukaryotes, great efforts are undertaken to minimize the off-target effects. Effective solutions comprise controlling the intracellular Cas9 amount, as low Cas9 abundance reduces off-target effects; designing "better" sgRNAs, which minimizes mismatches and secondary structures of sgRNAs that promote off-target binding; optimizing Cas9 for higher specificity by protein engineering; and using paired Cas9 nickases.

3.1.2 E**ffi**cient delivery. In general, the delivery of Cas9/sgRNA into cells, such us bacteria, fungi and plants, seems to be a crucial event in determining whether CRISPR application is possible in a specific organism. Therefore, successful CRISPR/Cas9 application requires robust Cas9 and sgRNA delivery, either by plasmids, which mediate the expression of Cas9 and sgRNA intracellularly, or by *in vitro*-preassembled Cas9:sgRNA complex. Unfortunately, many organisms discussed in this review lack efficient transformation methods and somewhat even lack plasmid vector systems, which hamper the use of the CRISPR systems.

3.1.3 Precise editing. In eukaryotes, such as filamentous fungi and plants, NHEJ is the dominant pathway for DSB repair, while NHEJ acts in an error-prone manner that will randomly generate some small indels around the DSB site. Generally, the sizes of an indel cannot be precisely predicted. They often lead to frameshift mutations. Even if an editing template is provided for HDR, the native NHEJ will still be dominant and heavily affect the efficiency of HDR. To overcome this limitation, several strategies have been successfully employed to suppress the native NHEJ pathway such as using the small molecule Scr7 to inhibit the activity of DNA ligase IV, thus suppressing the native NHEJ activity.[149] Strategies can be employed in parallel to specifically improve the HDR repair pathways.[150]

3.1.4 Link phenotype to genotype. CRISPR/Cas generally significantly reduces the time and cost for editing a gene. The rate-limiting step of the whole gene editing cycle is now identifying the correct edits. High-throughput screening using biosensors that link the genotype to the desired phenotype is an excellent tool for this purpose. However, such screening systems are still missing for most applications.

3.1.5 Lack of basic support. In comparison with model organisms such as *E. coli* or yeast, the basic genetic

manipulation toolbox for most of the proficient natural product producers is very limited with respect to vectors, characterized promoters, selection markers and general transformation rates. All these aspects clearly also restrict CRISPR/Cas application and development.

3.2 Special challenges

3.2.1 Cas9 toxicity. Cas9 toxicity has not been reported in filamentous fungi or plants. However, in most bacteria and chlorophytes, strong Cas9 expression often results in toxicity that severely impacts growth. Multiple studies have shown that these toxic effects can be overcome by reducing the intracellular Cas9 content,[151,152] which can be done by using weak promoters or by transient expression of Cas9. A new approach is to integrate anti-Cas9 components that modulate the activity of Cas9 into the different toolkits in order to reduce the toxicity and off-targets.[153]

3.2.2 Genome instability. The DSBs are considered one of the most dangerous cellular events; they lead to instability of the genome and, when left unrepaired, cause cell death.[154,155] Bridging the broken DNA ends by NHEJ contributes to the evolution and stability of eukaryote genomes.[154] However, NHEJ does not widely occur in bacteria. Bacterial DSB repair mainly relies on HDR pathways. The introduction of a DSB by CRISPR/Cas puts the cells under severe stress. In *Streptomycetes*, which have linear chromosomes, such stress could result in large-scale genomic deletions and facilitate genome rearrangements,[156,157] which may explain some of the toxic effects caused by strong Cas9 expression. For all Cas9 applications that involve the generation of DSBs, it therefore is advisable to check the integrity of the genomes. Alternative strategies that do not rely on DSBs, such as CRISPRi or deaminase-based single base editor,[43,44,158,159] may also be considered to inactivate a gene by introducing mutations within the coding region or scrambling the promoter/RBS region to inhibit transcription/translation.

3.2.3 Influence from the native CRISPR system. One effect that has so far been largely neglected is how the CRISPR/Cas system used for engineering natural product cell factories interferes with CRISPR systems that are already present in the cells.[160–162] For example, an active type I-E CRISPR/Cas system was identified in *Streptomyces avermitilis* by Qiu and colleagues in 2016.[162] This kind of native CRISPR/Cas system could have crosstalks with the externally introduced CRISPR/Cas systems, however, no such studies have been published yet.

3.2.4 Unique genome characteristics that impede CRISPR engineering. The genomes of most *Streptomycetes* and related genera, which are proficient natural product producers, have a remarkably high GC-content (over 70%), which generally makes genetic manipulation difficult. In the case of SpCas9 genome editing, this characteristic results in the required PAMs (which is NGG in the case of SpCas9) being very abundant throughout the genomes. However, this characteristic also has a negative impact, as the chance for nonspecific binding of the sgRNA and thus off-target effects also increase. Furthermore, it limits the use of Cas nucleases such as Cas12a proteins, which have AT-rich PAMs such as the TTTV PAM for fnCas12a.

4 The prospects of CRISPR/Cas in natural product discovery

Although having attracted much interest during the past several years, the use of CRISPR/Cas technologies in the natural product community is still in its infancy. Only a handful of "real applications" have been reported, and most of the work has been largely confined to proof-of-concept studies such as evaluating the feasibility of CRISPR/Cas systems in actinomycetes, in filamentous fungi, and in plants. It is remarkable that CRISPR/Cas9 engineering succeeded in almost all tested organisms; in other words, as long as the Cas protein and its crRNA are correctly expressed and transcribed in the target cell or Cas9/sgRNA complexes can be directly delivered, CRISPR/Cas system are very likely to work. After so many proof-of-concept studies, it is now approximately time to move from the testing ground to application in the field. CRISPR/Cas-based genome editing technology has great potential in system metabolic engineering, cell factory construction, biosynthetic pathway elucidation, and large DNA fragment cloning, which will be extremely useful in gene cluster cloning.[163–165] For those organisms that cannot be transformed with external DNA and RNPs or that have a long life cycle, especially woody plants, directly editing the genome to increase the yield of some desired products may not be the preferred method. However, in those cases, easy-to-handle microbial cell factories may certainly be used to heterologously express the key enzyme or/and the whole pathway. Some successful cases had already been reported during the pre-CRISPR era, such as expressing a taxol precursor in *E. coli*,[166] expressing triterpenoids in yeast,[167] expressing an artemisinin precursor in yeast,[168] and expressing opioids in yeast.[169] Among the CRISPR/Cas applications discussed in this review, wild-type SpCas9-based gene knockout is currently the main field of application. However, these knockout systems can be engineered for reversible knockdown and activation of target gene expression, known as CRISPRi[37,170] and CRISPRa,[171,172] respectively, which might be a better option than direct gene knockout for many metabolic pathway studies. In addition to SpCas9, there are Cas proteins such as Cas12a (Cpf1),[52,53] other Cas9 orthologs with different PAMs,[66] and engineered Cas9s with higher specificity and broader PAM recognition[47–51,173] that can now be tested. Moreover, thanks to the CRISPR community, an increasing number of CRISPR systems with unique features and potential that may open completely new application fields have been described and studied. For example, Cas13a (C2c2) is an RNA-guided RNA targeting system that can be reprogrammed to target mRNA for knockdown.[56]

CRISPR systems are the results of the endless coevolution between bacteria and phages. It therefore is not surprising that another product from this coevolution was anti-CRISPR proteins. Anti-CRISPR proteins are originally utilized by phages to neutralize host CRISPR immunology.[174–177] However, they can be artificially engineered for tuning the genome editing activities of CRISPR/Cas systems.[153]

Undoubtedly, the enormous potential of CRISPR/Cas related technologies and applications will have a severe impact on

natural products discovery and engineering of production strains in the future. However, as no technology is prefect, it will be necessary to extend the general scope of applications, and keep optimizing, developing, and innovating the current technologies. For example, as mentioned above, off-target effects of SpCas9/sgRNA that introduce DSBs (and with that mutations) in undesired regions of the genome are a major challenge for many CRISPR applications, which may be overcome by using optimized Cas9 nucleases or alternatives with higher target specificity. But it also has to be considered that DSB as introduced by most Cas nucleases may have different effects and impacts to the target organisms. As one example, we and others observed enhanced genome instability that can lead to the loss of huge portions of the genome in some *Streptomycetes* (unpublished results) after engineering attempts with CRISPR/SpCas9. This instability is likely caused due to duplicated target regions that lead to simultaneous DSBs of the linear genomes. In such cases, it therefore may not be a wise choice to use DSB-based genome editing tools, like CRISPR/SpCas9 for gene manipulation. We therefore need to extend the currently available CRISPR/Cas9 based toolkits and also include CRISPR-based engineering strategies that don't rely on DSB.

5 Conflicts of interest

Y. T., T. W., and S. Y. L. are co-inventors on a patent application on actinomycete CRIRSPR application (WO2016150855) filed by Technical University of Denmark.

6 Acknowledgements

The work of the authors was supported by grants from the Novo Nordisk Foundation (NNF10CC1016517, NNF15OC0016226, NNF16OC0021746). The work of SYL was also supported by the Technology Development Program to Solve Climate Changes on

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