Chapter 10
CRISPR ERA: Current Applications and Future Perspectives on Actinobacteria

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Abstract Genome editing technologies have been proven to be an important strategy for drug discovery in the recent years. Among them, clustered regularly interspaced short palindromic repeats (CRISPR) and associated proteins (CRISPR/Cas) is an efficient genome editing tool, which helps mainly in gene regulation and activation of biosynthetic gene clusters (BGC) for the discovery of novel bioactive natural products (NP) apart from its other applications. Amidst wide variety of organisms, CRISPR/Cas system aids extensively in activating silent BGCs in actinobacteria as they are an important source of antibiotics and other pharmaceutical drugs. Actinomycetes are the source of two third of the available antibiotics derived from microbial fermentation. Majority of these antibiotics are derived from *Streptomyces* genus, as its codes for a high number of NP BGCs. This chapter recapitulates the recent advances in CRISPR/Cas-based genome editing approaches in actinobacteria, mining of Cas3, and future perspectives in the discovery of NPs from novel species of actinomycetes of different origins.

Keywords CRISPR/Cas · Actinomycete · Streptomyces · BGC · Genome editing · Natural product

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10.1 Introduction

10.1.1 Biosynthetic Gene Clusters and Natural Products

Natural products (NPs) also known as secondary metabolites are produced in plants, microbes, and invertebrates. They help the producing organisms to tolerate stresses, to surpass nearby microbes, and play essential role at specific physiological and developmental stages of that organism (Davies 2013; Challis and Hopwood 2003). NPs derived from microorganisms are much diverse in structure and are rich sources for novel drug discovery which aid in treatment of several human diseases. Especially, a valuable source of bioactive NPs, which further developed into pharmaceutical drugs to treat cancer, infections, and diseases related to immune system, is bacteria (Sekurova et al. 2019). Though the role of NPs is important for survival of microorganisms in a particular habitat, molecules emerging from the secondary metabolism are of utmost agro-industrial and therapeutic importance (Loureiro et al. 2018; Walsh and Fischbach 2010). These bioactive NPs are produced by a kind of machinery which is encoded by a group of genes called as biosynthetic gene cluster which includes biosynthetic genes and genes for self-resistance, export, and expression control (Walsh and Fischbach 2010; Medema et al. 2015; Tenconi and Rigali 2018).

A biosynthetic gene cluster (BGC) can be defined as group of genes clustered together in a specific genome to encode a specialized secondary metabolite (Medema et al. 2015). Moreover, BGCs contain not only genes which encodes all the enzymes that are responsible for the production of a secondary metabolite, but also pathway-specific regulatory genes (Keller et al. 2005). A single BGC is capable of synthesizing several NPs which are related to each other in many aspects and whose bioactivities vary only in terms of specificity and/or strength. It has also been proved that a single BGC can be responsible for synthesis of two NPs, namely ferroverdins and bagremycins, which have different chemical compositions and structural organizations and are synthesized under different culture conditions (Martinet et al. 2019). Also, a lone BGC from a deep-sea derived microorganism accounts for four NPs, with diverse biological activities, representing two different anthracene scaffold variants (Alali et al. 2021). Polyketide synthases (PKS) and non-ribosomal peptide synthases (NRPS) are the most popular targets in genome mining for novel NPs and they are well known to synthesize diverse range of secondary metabolites which are used as antibiotics, immunosuppressants, and antifungals (Ayuso-Sacido and Genilloud 2005).

10.1.2 Actinomycetes Phyla as a Source of Bioactive Compound

Among the phylum of bacteria, Actinomycetes, apart from its role in maintaining soil microbiota, it is also a major source of pharmaceutically important natural bioactive compounds. Natural products (NPs) of microbes especially of the actinomycetes have been explored as an excellent resource in modern drugs discovery. Moreover, the genomic era has given many recent techniques to further strengthen the identification of several NP BGCs from the genomes of actinomycetes (Tao et al. 2018). Although different approaches like RNA polymerase engineering (Shima et al. 1996; Hosaka et al. 2009), one strain many compounds co-culturing technique (Bode et al. 2002), equipping with chemical elicitors (Romero et al. 2011), and transcription factor decoy approach (Wang et al. 2019; Nguyen et al. 2020) have been in use for decades, all these methods have its own draw back in activating the low expression BGCs. It is evident from earlier studies, streptomyces genome provide more than 30 BGCs and provide as a rich source of antibiotics (Culp et al. 2019). Till now only few BGCs of only few strains have been explored for its chemical importance and majority of the BGCs are overlooked due to its decreased or untraceable expression levels in the genome (Tong et al. 2015). The time-consuming homologous recombination methods with a suicide or temperature sensitive or plasmid self-replication were used earlier in editing BGCs in streptomyces. Nevertheless, for the discovery of NP drugs, streptomyces are of the utmost importance, of which the studying process depends on efficient genome editing methods. Therefore, it is necessary to have the accurate and the rapid techniques to identify the cryptic BGCs of distinct metabolic pathways in the host system to uncover the novel compounds that were hidden by the already existing NPs in actinomycetes. From the culture of marine *Streptomyces olivaceus* SCSIO T05, a single rsd BGC is identified responsible for the production of rishirilide B, rishirilide C, lupinacidin A, and galvaquinone B belonging to two different anthracene scaffolds, which have antithrombolytic activity, inhibitory effects against cancer cell invasion, epigenetic modulatory activity, and cytotoxicity against lung cancer cells (Zhang et al. 2018; Igarashi et al. 2011; Sottorff et al. 2019).

10.2 CRISPR/CAS-Based Gene Editing Strategies in Streptomyces

In 2015, the genome editing of streptomyces was first shown by cobb et al., followed by various in vivo and in vitro methods by distinct research group to identify the unidentified BGCs using CRISPR/Cas9 technique. There are four different methods that are mostly used in editing the streptomyces genome (Zhao et al. 2020a). (1) The gene deletion, point mutation, and promoter replacement can be performed, respectively, by *Homology directed repair (HDR)* editing in Streptomyces. This method

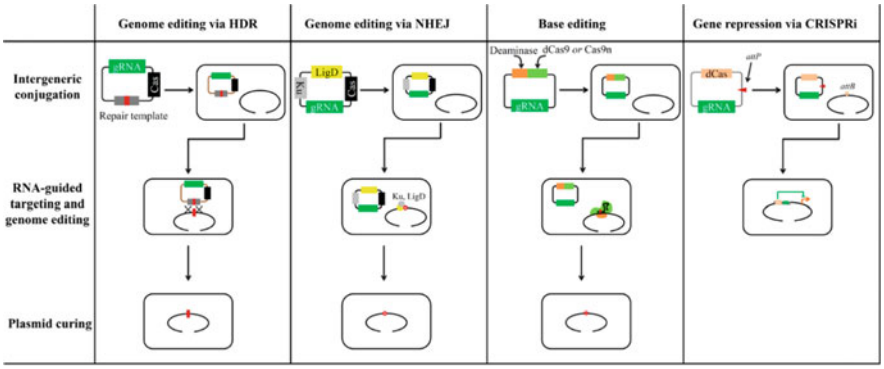

Fig. 10.1 Interpretation of the CRISPR/Cas system derived genome editing technologies in *Streptomyces* (Source: Zhao et al. 2020a)

employs the transfer of editing plasmid containing the expression cassettes of cas nuclease, a small segment of guide RNA (gRNA), and a homologous repair template into streptomyces. This would result in the cleavage of target site by the gRNA-mediated cas9 nuclease which would result in the double-stranded break (DSB) that will further be repaired by the HDR homologous repair template to provide the specific mutations in the genome. (2) *Non-homologous end joining (NHEJ)*-mediated editing in Streptomyces. This technique can be achieved by the successful transfer of editing plasmid containing the cas endonuclease segment, gRNA sequence, and the ku/ligD genes into streptomyces which would result in the DSB through intergenic conjugation. Small-sized deletion, insertion, or substitution (mostly 1–3 bp) close to the target site can be achieved by using a reconstituted NHEJ system co-expressing the scalig D. (3) *Base editor.* The target site editing can be done through intergenic conjugation while introducing the editor plasmid that would code for the gRNA and deaminase fused with cas9/cas9n to induce the base substitution in the streptomyces. In all these methods the final step of plasmid curing will be performed at the temperature of about 37 °C to obtain plasmid-free mutants and continue multiple rounds of genome editing, (4) in *CIRPSRi mediated gene editing*, gRNA is introduced into Streptomyces by conjugation for recombination at the target location for the plasmid to integrate into the genome. An inhibition of transcription of the specific genes can be done by dCas/gRNA complex by interfering with transcriptional initiation (Fig. 10.1).

On various studies, the CRISPR/Cas system has been shown as an aggressive technology for genome editing across plant, animal, and microbial kingdom. Further, these techniques employ better manipulation of genomes with many positive aspects which helps in modern drug discovery and other pharmaceutical studies (Arazoe et al. 2018). However, the toxicity and the genome instability and toxicity by the cas9 nuclease/SgRNA protein that exist in the technique has still need to be explored in depth. Multiple researches unraveled distinct approaches of using CRISPR/Cas-based gene editing technique to engineer the metabolic pathways for

its downstream applications. Recent, researches shown that the genetic engineering in actinomycetes genome solves some of the major problems that exist in the classical CRISPR techniques. A publication that appeared in 2020 by Ye et al. demonstrated that fine tuning the expression level may be useful to reduce the toxicity of Cas9 in the host strain in gene editing. High Cas9 expression level may be toxic to the cells and the level of toxicity varies between actinomycete strain. Based on their findings, the study has also introduced five set of plasmids (pCM (−cas9), pCMU, pCMUtuf, pCMU-4, and pCMU-4tuf) which have showed the distinct level of resistance for Cas9 protein in *S. coelicolor* and *S. lividans*. In addition, the plasmid pCMU 4.4 was developed by the same group to use the plasmid in various other strains of actinomycetes that are very sensitive to the Cas9 expression by using a weaker constitutive promoter.

10.2.1 Nuclease-Dependent Editing System

In the nuclease-based CRISPR editing systems, the type II-A CRISPR-Cas9 and type V-A CRISPR-Cas12a (previously referred as Cpf1) are the majorly used editing methods in prokaryotes and have showed a promising effect by their natural ability to develop immunity against the invading foreign DNA (Swarts and Jinek 2018). The defense mechanism occurs in three steps (spacer acquisition, crRNA biogenesis, and target interference) for Cas9/Cas12a (For information on the mechanism of action of Cas9/Cas12a system ref. Yao et al. 2018). Moreover, the Cas12a-based systems alleviated some of the issues present while using Cas9, like requirement of G-rich protospacer-adjacent motifs to recognize the target sequence by Cas9. Further, the Cas12a system has the RNase activity for pre-crRNA and single promoter is required for the expression of multiple guide CrRNA. Thus, these features of CRISPR/Cas12a facilitate the genome editing by targeting to multiple genomic loci through a single transcript (Abudayyeh et al. 2016).

10.2.2 CRISPRi and CRISPRa-Based Genome Editing

The very recent development in CRISPR/Cas-based genome editing is the introduction of nuclease defective cas nuclease (dcas) instead of the earlier cas9 version. The method is known as CRISPRi and the technique employs repression of genes at the transcriptional (initiation and elongation) level (Zheng et al. 2019). This simple and strong technology for repression of gene offered by dCas, which is aided by single guide RNA, can almost target any DNA sequence (Yao et al. 2018). dCas9 (D10A and H841A) of nuclease defective *S. pyogenes* are most widely used for various purposes. By blocking RNA polymerase sterically, CRISPRi inhibits transcription (Yao et al. 2018), which works like a transcriptional repressor of bacteria that have limitations such as recognizing specific DNA sequence (Xiao et al. 2016). CRISPRi

was first developed in bacteria as a method for controlling gene expression widely on a genome scale using an RNA guided dCas9 system which could also be used for gene repression in mammalian cells (Qi et al. 2013). In eukaryotes, for strong gene repression, auxiliary inhibitors are essential to bind with dCas, whereas in bacteria, dCas/sgRNA complex is single-handedly capable of repressing genes strongly (Gilbert et al. 2013; Qi et al. 2013). In multiplexed gene repression, dCas12a CRISPRi requires only a lone CRISPR array expression (Zhang et al. 2017b), whereas dCas9 needs multiple sgRNAs which express independently (Qi et al. 2013).

Three different CRISPRi gene editing tools in Streptomyces have been introduced by Tong et al., in 2015, replacing Cas9 with dCas9, which enabled efficient repression of single genes upon induction. The first method relies on the replicative plasmid pGM1190 harboring temperature-sensitive replicon pSG5, which will have the thiostrepton-inducible promoter (tipAp) driven expression of the dCas9/sgRNA complex. Later, two methods were developed in *S. coelicolor* based on dCas9 and ddCpf1 whose expression is driven by thiostrepton-inducible promoter (tipAp). Using these two CRISPRi systems, the repression of up to four genes at high efficiency was demonstrated simultaneously. The later methods have two advantages over the earlier method of using replicative plasmid (e.g., pGM1190). First, their repression effects are likely to be stable as they are integrated into the genome. Further, it is important to note that simultaneous repression of multiple targets using the dCas9-based system involves a time-consuming procedure to construct multiple sgRNA expression cassettes with independent promoters and terminators. In contrast, only a single customized CRISPR array with one promoter is required owing to the pre-crRNA processing ability of ddCpf1, which is time-saving and convenient. Therefore, the ddCpf1-based CRISPRi system has an advantage over dCas9-based systems for multiplexed gene repression (Li et al. 2018a, b, c; Zhao et al. 2018, 2020a, b). In these systems, repression of a maximum of up to four genes was achieved simultaneously at high efficiency. Also, they have some advantages such as stable repression effects and higher efficiency of transformation of pSET152 plasmid comparing to the inducible CRISPRi tool. In *S. bingchenggensis*, transcription level of the gene *sbi_04868* was down-regulated which is involved in the primary metabolism of milbemycins production by using the ddCpf-based integrative CRIPSRi system (Liu et al. 2021). Furthermore, in a recent study by Tian et al. in 2020, a novel control strategy which is pathway independent named EQCi has been developed integrating the CRISPRi tool with an endogenous quorum sensing (QS) system in the industrially important rapamycin producing strain *S. rapamycinicus*, and found that transcription repression degree in multiple targets by EQCi was equal to that of single target based on CRISPRi. Application of this EQCi genetic circuit, having advantages of both the QS system and CRISPRi, maximizes biosynthesis of the target product by aiding down-regulation of pathways which leads to deflection in metabolic fluxes.

Another recent adaptation technique in CRISPR/Cas-based genome editing for gene expression variation is CRISPR activation (CRISPRa). CRISPR-based transcriptional activation (CRISPRa), which has an activator domain, fuses RNA

polymerase from dCas9/sgRNA complex with the promoter of a target gene (Alok et al. 2020). In bacteria, especially in *E. coli*, CRISPRa is achieved by fusing dCas9 system with the ω subunit of the RNA polymerase; this complex then binds to the region upstream of the promoter, thus activating transcription (Bikard et al. 2013). In a study by Hu et al. (2018), over a 100-fold increase in transcriptional activation has been achieved by using ω-subunit ω-(I12N) bound with dCas9 system. CRISPRa is not much used in prokaryotes due to the lack of transcriptional activators suitable for the technique (Zhang et al. 2018). CRISRa needs to target a narrow region of around 90 bp upstream the transcriptional start site (TSS) for optimal activation effect. Thus, application of CRISPRa is limited to genes having no noticeable PAM sites around the 90 bp upstream the TSS (Dong et al. 2018). Therefore, study on CRISPRa genome editing in *Streptomyces* is lacking, although it has potential application in production of novel NPs through gene cluster activation.

10.2.3 CRISPR/Cas9: Knock-in Strategy

Here, knock-in of promoters is performed strategically to activate silent BGCs using CRISPR/Cas9 systems in *Streptomyces* sp. Zhang et al. 2017a used this strategy with the help of pCRISPomyces-2 plasmid (Cobb et al. 2015) to efficiently replaced the regions of native promoters with constitutive promoters in *Streptomyces* species. As a result, three uncharacterized and two known BGCs in *Streptomyces sp.* are activated using this knock-in strategy (Table 10.1). In addition to this, by knocking-in bidirectional promoters using CRISPR/Cas9 system, erythromycin production was increased by 58.3% in *Saccharopolyspora erythraea* (Liu et al. 2019).

10.2.4 CAPTURE Method

The cloning of BGCs which are larger in size and having high GC content is highly challenging in large scale. To overcome this, a new, robust, and highly efficient

Table 10.1 CRISPR/Cas9 Knock-in strategy in *Streptomyces*

BGCs	Promoter	Species	Reference
Indigoidine	*kasO*p*	*S. albus*	Zhang et al. (2017a, b)
Actinorhodin (ACT), undecylprodigiosin (RED)		*S. lividans*	
Alteramide A, Polycyclic tetramate macrolactam, FR-900098, type I polyketides		*S. roseosporus*	
Type III polyketide		*S. venezuelae*	
Pentangular type II polyketide		*S. viridochromogenes*	

direct cloning method called Cas12a-assisted precise targeted cloning using in vivo Cre-lox recombination (CAPTURE) has been developed recently by combining T4 polymerase exo + fill-in DNA assembly, Cas12a digestion and circularization of in vivo Cre-*lox* DNA (Fig. 10.2) (Enghiad et al. 2021). Regardless of their DNA sequence repetition and GC content, BGCs ranging from 10 to 113 kb can be cloned directly by this method. In this method, 43 uncharacterized NP BGCs from different *Streptomyces and Bacillus* species were cloned and heterologously expressed in *S. avermitilis*, *S. lividans,* and *B. subtilis*, resulting in identification of 7 BGC capable of producing compounds which have antibacterial activity. In addition to other direct cloning methods, CAPTURE also gives a high-level efficiency of approximately 100% and robustness.

10.2.5 CRISPR-BEST

To address the limitations of CRISPR-Cas9 system such as genome instability due to the DNA double-strand breaks (DSBs) and effects of off-target genome editing, Tong et al. (2019) have developed a novel genome editing system named CRISPR-Base Editing SysTem (CRISPR-BEST) which is a high-efficient, DSB-free, single nucleotide editing system based on pSG5 (Muth 2018) in different *Streptomyces* species. Also, CRISP-BEST reduces Cas9 toxicity and stress on chromosome by DSBs compared to the other CRISPR-based editing system. Furthermore, two variants of this system, namely CRISPR-cBEST and CRISPR-aBEST, are capable of converting the base pairs C:G to T:A and A:T to G:C, respectively (Fig. 10.3). In *Streptomyces collinus* Tü365, a non-model actinomycete, CRISPR-cBEST simultaneously inactivated two copies of a target gene upon introduction of a STOP codon. Also, Tong et al. 2020 developed a CRISPR toolkit which includes CRIPR-BEST system for Streptomycetes, where its function is inactivation of coding genes, which also can be applied in in vivo protein engineering by customizing amino acid substitution.

10.2.6 CAT-Fishing

In an attempt to simplify the process of discovery of NPs, our team developed the CRISPR/Cas12a-mediated fast direct biosynthetic gene cluster cloning (CAT-FISHING) strategy by direct cloning of large fragments of DNA from high GC *Streptomyces* genomic DNA or bacterial artificial chromosome efficiently, making it a direct and simple method. This strategy has the potential to facilitate the novel bioactive small molecules discovery from microorganisms for drug development (Liang et al. 2020).

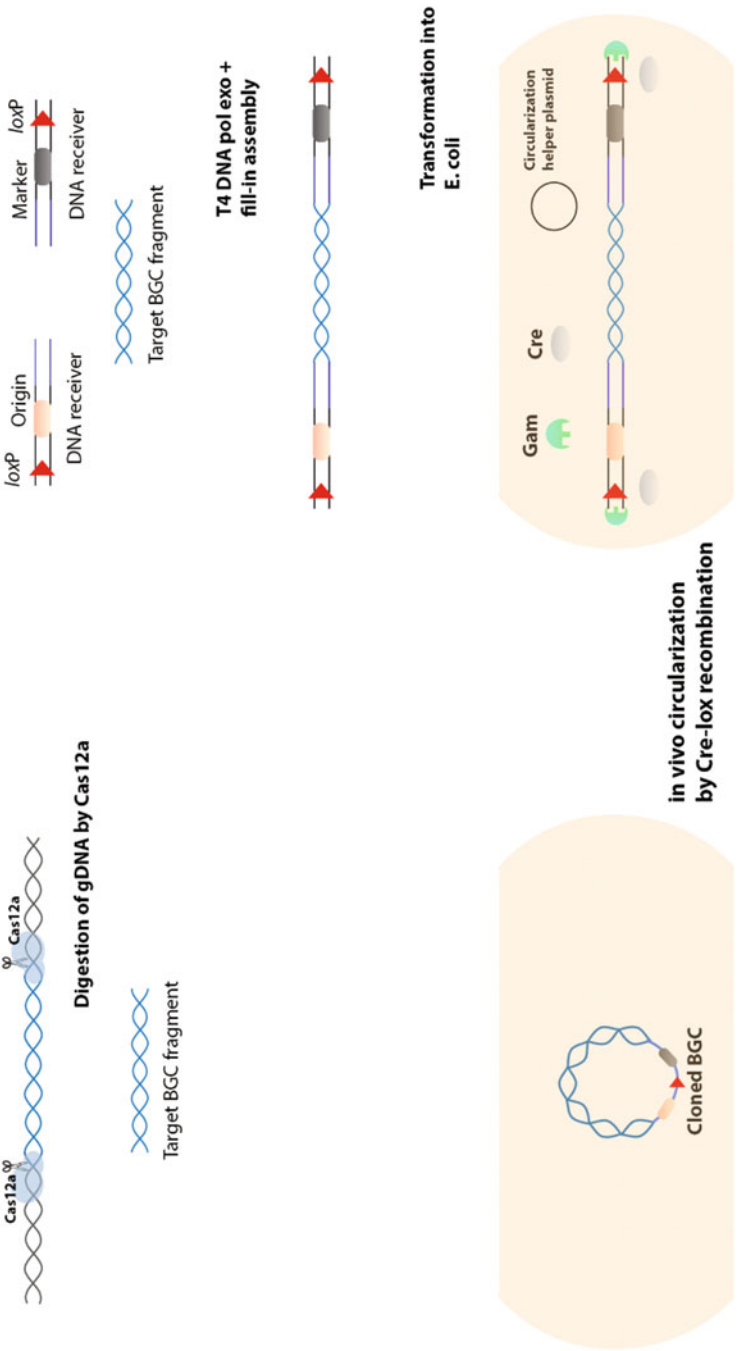

Fig. 10.2 Overview of CAPTURE method (Source: Enghiad et al. 2021)

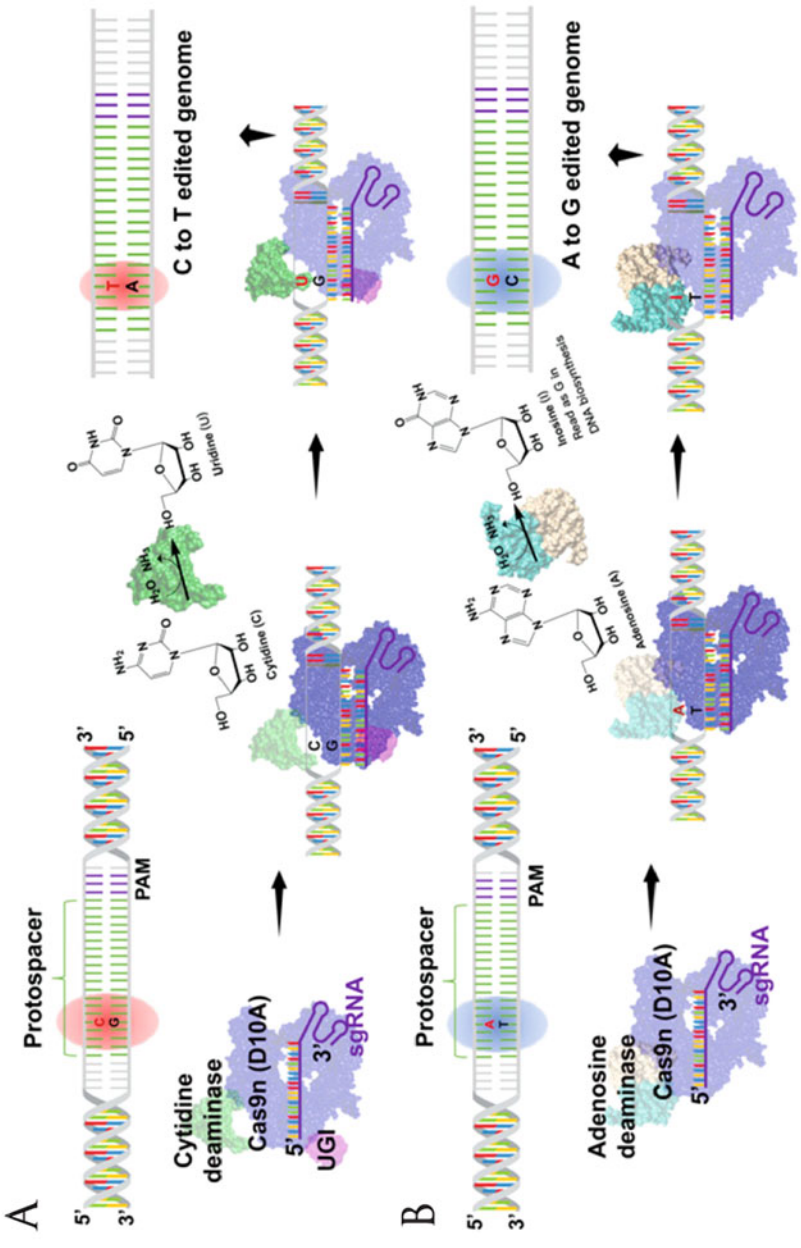

Fig. 10.3 Workflow of CRISPR-BEST. (A and B) Overview of base editing strategy for CRISPR-cBEST and CRISPR-aBEST, respectively. (Source: Tong et al. 2019)

10.3 CRISPR/CAS Systems in Other Actinomycetes

CRISPR/Cas systems and CRISPR-associated proteins are not only used as genome editing tool, but also as a defense mechanism against mobile genetic elements (MGE) in prokaryotes through an adaptive immunity by introducing short MGE sequences, called as protospacers, into the CRISPR locus (Choi and Lee 2016). In prokaryotes, CRISPRs are considered to be means of adaptive immunity against bacteriophages (Sorek et al. 2013). The variable spacers in CRISPRs have viral or plasmid DNA fragments that provide immunity upon succeeding encounters with the virus (Barrangou et al. 2007).

10.3.1 Gardnerella *spp.*

In bacterial vaginosis (BV), a most common infection which results in white gray discharge in vagina and a fishy smell among women mostly of reproductive age (Ruiz-Hernández et al. 2020), *Gardnerella* spp., another member of phylum actinobacteria, play a pivotal role in the development of the infection (Schwebke et al. 2014; Muzny et al. 2019; Morrill et al. 2020; Rosca et al. 2020). Among other bacteria which are associated with BV, *Gardnerella vaginalis* has been proved to have higher potential for virulence throughout the process of this infection (Castro et al. 2019). The presence of CRISPR/Cas systems in *G. vaginalis* strains and the possibility of genetic material transfer between the strains regulated by CRISPR/Cas system was first described by Pleckaityte et al. in 2012. An analysis of CRISPR/Cas system in *Gardnerella* spp. revealed the presence of CRISPR-mediated immunity which provides a mechanism against the effect of the phages infecting the *Gardnerella* species (Ruiz-Hernández et al. 2020).

10.3.2 Salinispora

An important emerging source for bioactive NPs is marine actinobacteria enclosing peculiar structural classes of natural compounds (Fenical and Jensen 2006). Actinobacteria are extensively distributed in the marine ecosystem, and native marine actinobacteria have also been described (Bull et al. 2005). A pan-tropical distribution of *Salinispora* genus, under phylum actinobacteria, can be found in marine sediments (Jensen and Mafnas 2006; Freel et al. 2012). In a study by Wietz et al. (2014), three previously undefined CRISPR subtypes were identified in *Salinispora* strains, and it has also been concluded that a key force in the ecology and evolution of *Salinispora* is characterized by the CRISPR-based interaction with the MGEs.

10.3.3 Nonomuraea *sp.*

Su et al. (2020) reported that for the first time, an enhanced ecumicin production was achieved through promoter engineering and CRISPR/Cas9-based genome editing of *Nonomuraea sp.*, a rare actinomycete. By combining the ribosome-binding site of *kasO*P* with that of the ecumicin gene cluster *ecuE*, increased production of active ecumicin component EcuH14 was attained. This study indicates that the *Nonomuraea* sp. might be a potential source of novel antibiotics and other pharmaceutical compounds.

10.4 Application of CRISPR/Cas Systems In Biotechnology

Industrial biotechnology is reliant on these techniques to meet the growing demands for, and expand the catalog of, chemicals, metabolites, and biomolecules that can be produced by microbial fermentation. To date, efficient genomic engineering in microbes has relied on the use of DNA donors in combination with endogenous DNA repair machinery, exogenous recombination systems, selectable markers, site-specific recombinases, group II intron retro transposition, and the use of artificial chromosomes (Esvelt and Wang 2013; David and Siewers 2014). CRISPR-Cas technologies have greatly reshaped the field of biology. In general, CRISPR-Cas systems are composed of a CRISPR RNA (crRNA) and Cas proteins. The genetic modification can then be introduced by either the error-prone NHEJ or HDR that creates precise genomic modifications. Most prokaryotes employ HDR. The initial genome engineering was done by natural CRISPR-Cas systems and later the technique was developed for gene regulation (Yao et al. 2018). These technologies have great contribution in the field of DNA imaging, bacterial immunization, virome tracking, and gene cloning (Cho et al. 2018; Donohoue et al. 2018). In 2019, Culp et al. reported the identification of various hidden antibiotics in actinomycetes by using the CRISPR/Cas9 genome engineering. Further, they have recovered distinct known variants of the antibiotics, such as thiolactomycin, amicetin, phenanthroviridin, and 5-chloro-3formalindone. The following table gives the brief overview of CRISPR/Cas-mediated plasmids that are developed by various research groups for genome editing in streptomyces (Table 10.2). Further, the industrially relevant streptomyces species is also give in Table 10.3.

In the recent years, CRISPR/Cas systems have provided a powerful tool for silent gene cluster activation, gene expression control, single or double site mutations, and in-frame gene deletion in different Streptomyces strains (Dong et al. 2017; Huang et al. 2015; Cobb et al. 2015; Zeng et al. 2015; Li et al. 2018a, b, c; Jia et al. 2017; Tong et al. 2015; Zhang et al. 2017a, b). spCas9 was used in several studies for genome editing which is codon-optimized (Huang et al. 2015; Cobb et al. 2015; Zeng et al. 2015; Tong et al. 2015; Bao et al. 2015). High efficiency in genome editing of about 66–100% has been achieved in *S. ablus* and *S. viridochromogenes*

Table 10.2 Brief list of CRISPR/Cas-based gene editing plasmids in *Streptomyces*

Editing plasmid	Endonuclease	origin	Editing efficiency (%)	Fusion sequence	Target gene	Reference
pCRISPomyces-1	Cas9	*Streptococcus pyogenes*	21–25	–	redN, *actVA-ORF5* *redN, actVA-ORF5/phpM, phpD/ redN/actVA-ORF5, shg_05713, sshg_00040/ sshg_00050*	Cobb et al. (2015)
pCRISPomyces-2	Cas9	*Streptococcus pyogenes*	67–100	–		
pKCcas9dO	Cas9	*Streptococcus pyogenes*	29–100	–	*actII-orf4*, *redD*, and *glnR and large gene clusters like,* actinorhodin (ACT), undecylprodigiosin (RED), and Ca2 + −dependent antibiotic	Huang et al. (2015)
pCRISPR-Cas9	Cas9	*Streptococcus pyogenes*	3–100	–	actIORF1, actVB actIORF1, actVB actIORF1, actVB	Tong et al. (2015)
pCRISPR-Cas9-ScaligD	Cas9	*Streptococcus pyogenes*	69–77	LigD		
pCRISPR-dCas9	Cas9	*Streptococcus pyogenes*	ND	–		
pWHU2653	Cas9	*Streptococcus pyogenes*	93–99	CodA(sm)	Actinorhodin polyketide chain length factor gene actI-ORF2	Zeng et al. (2015)
pMWCas9	Cas9	*Streptococcus pyogenes*	ND	Cod(sm)	Polyketide synthase (PKS) genes	Mo et al. (2019)
pQS-*gusA*	Cas9	*Streptococcus pyogenes*	100	GusA	actIORFI, orange-pigmented carotenoid gene cluster, abyssomicin gene cluster	Wang et al. (2019)
pQS-*idgS*	Cas9	*Streptococcus pyogenes*	100	IdgS		
pWHU2653-TRMA	Cas9	*Streptococcus pyogenes*	8.3–80	AtpD	actII-ORF4, redD	Wang et al. (2019)
pKC1139-TRMA	Cas9	*Streptococcus pyogenes*	8.3–80	AtpD		

(continued)

Table 10.2 (continued)

Editing plasmid	Endonuclease	origin	Editing efficiency (%)	Fusion sequence	Target gene	Reference
pKCCpf1	Cpf1	*Francisella novicida*	75–95	–	actI-orf1	Li et al. (2018a, b, c)
pKCCpf1-MsmE	Cpf1	*Francisella novicida*	10–56.7	LigD, Ku		
pSETddCpf1	dd Cpf1	*Francisella novicida*	11.8–95.2	–		
pCRISPomyces-Sth1Cas9	Cas9	*Streptococcus thermophilus*	100	–	?	Yeo et al. (2019)
pCRISPomyces-SaCas9	Cas9	*Staphylococcus aureus*	87–100	–		
pCRISPomyces-FnCpf1	Cpf1	*Francisella novicida*	87–100	–		
pSET-dCas9	dCas9	*Streptococcus pyogenes*	ND	–	actII4, actI1, redQ, cdaPS1 and cpkA	Zhao et al. (2018)
pSET-dCas9-actII-4-NT-S1	dCas9	*Streptococcus pyogenes*	68–99	–		
pCRISPR-cBEST	Cas9n	*Streptococcus pyogenes*	0–100	rAPOBEC1	?	Tong et al. (2019)
pCRISPR-aBEST	Cas9n	*Streptococcus pyogenes*	0–100	ecTadA		
pKC-dCas9-CDA-UL*str*	dCas9	*Streptococcus pyogenes*	15–100	PmCDA1	actVI-ORF3, actVI-ORF2 redX, redL, redX, actIII	Zhao et al. (2020b)

Table 10.3 *Streptomyces* used in CRISPR technology with industrial applications

Species	Industrial relevance	Type of modifications
Streptomyces albus	Heterologous secondary metabolites production	Recombination
Streptomyces coelicolor	Pharmacologically active and industrially relevant secondary metabolites isolation	Recombination and CRISPRi
Streptomyces lividans	Active secondary metabolites with pharmacological and industrial relevance	Recombination
Streptomyces viridochromogenes	Active secondary metabolites with pharmacological and industrial relevance	Recombination

and 70–100% in *S. coelicolor* (Cobb et al. 2015; Huang et al. 2015; Tong et al. 2015). In a study by Cobb et al. in 2015, an efficient genome deletion was reported ranging from 20 to 30 bp by the use of sgRNA CRISPR system by comparing dual tracr-crRNA expression cassette with a sgRNA expression cassette. In *S. coelicolor*, a fnCas12a assisted genome editing system has been developed so that spCas9 can be introduced into industrial Streptomyces, particularly economically important strains such as *S. pristinaespiralis* HCCB10218 and *S. hygroscopicus* SIPI-KF (Li et al. 2018a, b, c). With the help of NHEJ and HDR, gene deletion efficiency in *S. coelicolor* has reached 0–50% and 75–95%, respectively, and also CRISPRi system based on dCas12a has been developed in Streptomyces for transcriptional repression resulting in 95% repression (Li et al. 2018a, b, c). High-efficient knock-in strategies based on CRISPR/Cas9 and CRISPR/Cas12a has been reported in *S. albus*, *S. lividans*, *S. roseosporus*, *S. venezuelae*, and *S. viridochromogenes* in order to activate silent BGCs (Li et al. 2017; Zhang et al. 2017a, b). Zhang et al. (2017a, b) also reported an enhanced biosynthesis of bioactive compounds and a novel type II polyketide by replacing native promoters with the constitutive kasO*p promoter using CRISPR/Cas9 system. This strategy contributes to an enhanced technique for silent BGCs activation and to the discovery of novel uncharacterized compounds. Using CRISPR/Cas9 system, mutation in single and double site, and target gene disruption are also successfully achieved in S. rimosus, where single site mutation rate is 100% (Jia et al. 2017). Though CRISPR/Cas9 system has proven to be a powerful genome editing tool in actinomycetes, it is important to adjust the expression levels of Cas9 because in untested strains, the system was proved to be toxic by exhibiting absence or delayed growth of the organisms, but upon lowering levels of Cas9, the toxicity is reduced (Ye et al. 2020).

Furthermore, in the recent years, CRISPR/Cas systems have provided a powerful tool for silent gene cluster activation, gene expression control, single or double site mutations, and in-frame gene deletion in different Streptomyces strains (Dong et al. 2017; Huang et al. 2015; Cobb et al. 2015; Zeng et al. 2015; Li et al. 2018a, b, c; Jia et al. 2017; Tong et al. 2015; Zhang et al. 2017a, b). spCas9 was used in several studies for genome editing which is codon-optimized (Huang et al. 2015; Cobb et al. 2015; Zeng et al. 2015; Tong et al. 2015; Bao et al. 2015). High efficiency in genome editing of about 66–100% has been achieved in *S. albus* and *S. viridochromogenes* and 70–100% in *S. coelicolor* (Cobb et al. 2015; Huang et al. 2015; Tong et al.

2015). In a study by Cobb et al. in 2015, an efficient genome deletion was reported ranging from 20 to 30 bp by the use of sgRNA CRISPR system by comparing dual tracr-crRNA expression cassette with a sgRNA expression cassette. In *S. coelicolor*, a fnCas12a assisted genome editing system has been developed so that spCas9 can be introduced into industrial Streptomyces, particularly economically important strains such as *S. pristinaespiralis* HCCB10218 and *S. hygroscopicus* SIPI-KF (Li et al. 2018a, b, c). With the help of NHEJ and HDR, gene deletion efficiency in *S. coelicolor* has reached 0–50% and 75–95%, respectively, and also CRISPRi system based on dCas12a has been developed in Streptomyces for transcriptional repression resulting in 95% repression (Li et al. 2018a, b, c). High-efficient knock-in strategies based on CRISPR/Cas9 and CRISPR/Cas12a has been reported in *S. albus, S. lividans, S. roseosporus, S. venezuelae*, and S. *viridochromogenes* in order to activate silent BGCs (Li et al. 2017; Zhang et al. 2017a, b). Zhang et al. (2017a, b) also reported an enhanced biosynthesis of bioactive compounds and a novel type II polyketide by replacing native promoters with the constitutive KasO*p promoter using CRISPR/Cas9 system. This strategy contributes to an enhanced technique for silent BGCs activation and to the discovery of novel uncharacterized compounds. Using CRISPR/Cas9 system, mutation in single and double site and target gene disruption are also successfully achieved in *S. rimosus*, where single site mutation rate is 100% (Jia et al. 2017).

10.5 CRISPR Cas 3 Genes from Actinobacteria

We verified and confirmed full length sequence of CRISPR-dependent cas3 gene of *Streptomyces hygroscopicus* (A6F03_RS20145) was retrieved from NCBI gene database. The putative cas3 genes were mined from genomes of all microbes available at IMG/M database using BLASTP. The gene equivalents including the hypothetical and putative protein entries were retrieved as an amino acid FASTA file. It resulted in about 807 entries comprising cas3 genes of phylum *Actinobacteria* (Fig. 10.4). The amino acid sequences were clustered with 90% similarity using **uclust** algorithm in **USEARCH** command line tool (V- 11.0.667). Whole cas3 three genes retrieved and representative sequence from each cluster was taken and aligned using CLUSTALW algorithm in MEGA X. The phylogenetic tree data was supplied to Interactive Tree of Life webserver to visualize the phylogenetic tree. In future, if we reveal the function of these novel Cas3 genes, it can be used for actinobacteria gene editing and other applications.

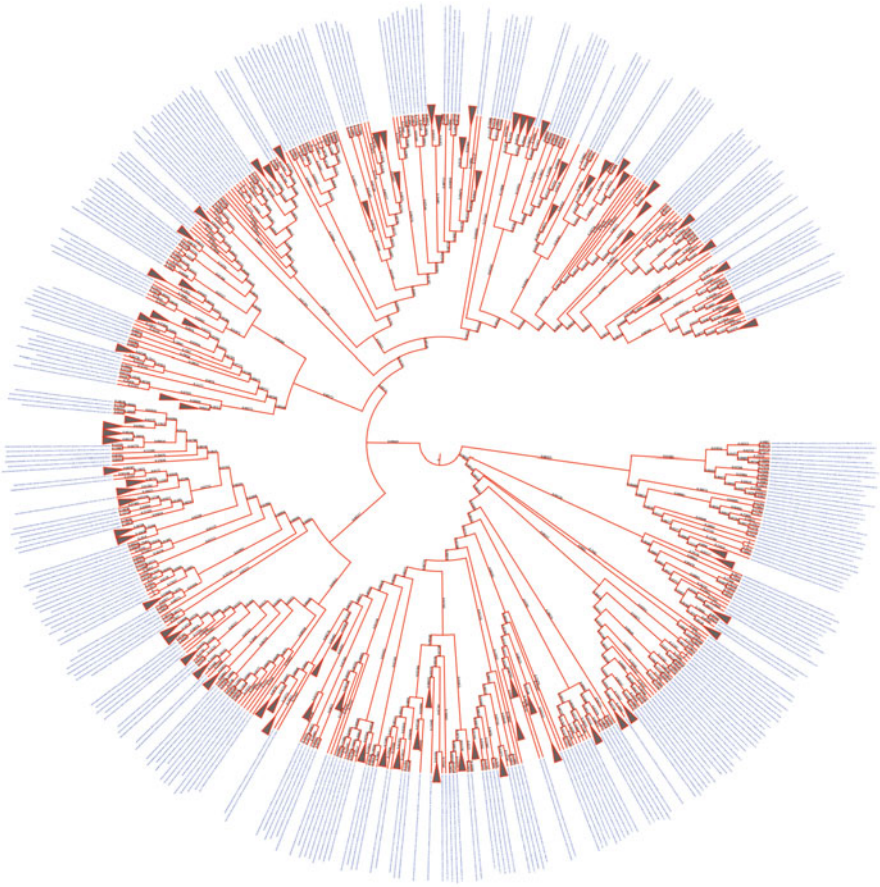

Fig. 10.4 Mining of Cas3 gene from Actinobacteria

10.6 Future Perspectives

There is no doubt that the CRISPR/Cas technology-based genome editing has revolutionized the genome editing process across organisms. The technique has been shown efficient, accurate, and less time and labor consuming. Although, in the past decade, the technique has attracted several researchers to overwhelm with CRISPR/Cas-based gene editing, use of these techniques for real application is still at the nascent stage. Number of several research group has demonstrated by using various CRISPR-based techniques to edit the single or multiple genes, small of huge BGCs in various *Streptomyces* species. However, mostly these testing level works were shown for the efficacy and the type of mutation in the selected organism. It is time and necessary to take up the approach to next level from proof of concept to application to explore the feasibility of editing the beneficial genes in metabolic

pathways. Further improvement is still needed, including the application for larger scale genome editing in rare actinomycetes. In addition, maximizing the beneficial impact of CRISPR-Cas systems on industrial biotechnology requires the expansion of this technology into strains specific to industrial applications. As all technologies come with its own setbacks, it is necessary to continue the standardization process of CRISPR/Cas technique to apply it in various organisms not just to test but also to explore the maximum possibility of real-time application especially for the discovery of the active compound for various therapies. In addition, the research should be focused to understand the major bottle necks that exist with Cas9/Cas12a nuclease toxicity in the host and the off-target effect that introduce the nonspecific DSB in the CRISPR techniques. Therefore, more tools and optimization in the CRISPR/Cas-based gene editing technique in future will shed light on several unexplored genes those were masked by the existing gene candidates in distinct streptomyces genomes. Upcoming improved CRISPR techniques may be useful in identifying more natural product compounds not only in prokaryotes but also in eukaryotic systems. In future, the following questions should be addressed such as How the microorganism will react when newer CRISPR system model introduces? And How the native microorganism controls the off-target activity?

Suggestion

Due to the higher intelligence of microorganisms and latest revolution in synthetic biology, the time has come to frame the ethical guidelines for microbial engineering research.

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