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CRISPR-Cpf1 assisted multiplex genome editing and transcriptional repression in *Streptomyces*

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Abstract

Streptomyces has strong capability to produce a large number of bioactive natural products and remains invaluable sources for the discovery of novel drug leads. Although the *Streptococcus pyogenes* (*Sp*) CRISPR-Cas9-assisted genome editing tool has been developed for rapid genetic engineering in *Streptomyces*, it has a number of limitations, including the toxicity of *Sp*Cas9 expression in some important industrial *Streptomyces* strains and the need for complex expression constructs when targeting multiple genomic loci. To address these problems, in this study, we develop a high-efficiency CRISPR-Cpf1 system (from *Francisella novicida*) for multiplex genome editing and transcriptional repression in *Streptomyces*. Using an all-in-one editing plasmid with homology-directed repair (HDR), our CRISPR-Cpf1 system precisely deletes single or double genes at efficiencies of 75-95% in *Streptomyces coelicolor*. When no templates for HDR are present, random sized DNA deletions are achieved by *Fn*Cpf1-induced double-strand breaks (DSBs) repair by a reconstituted non-homologous end joining (NHEJ) pathway. Furthermore, a DNase-deactivated Cpf1 (ddCpf1)-based integrative CRISPRi system is developed for robust, multiplex gene repression using a single customized crRNA array. Finally, we demonstrate that *Fn*Cpf1 and *Sp*Cas9 exhibit different suitability in tested industrial *Streptomyces* species, and show that *Fn*Cpf1 can efficiently promote HDR-mediated gene deletion in the 5-oxomilbemycin-producing strain *Streptomyces hygroscopicus* SIPI-KF, in which *Sp*Cas9 does not work well. Collectively, *Fn*Cpf1 is a powerful and indispensable addition to *Streptomyces* CRISPR toolbox.

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Importance

Rapid, efficient genetic engineering of *Streptomyces* strains is critical for genome mining of novel natural products (NPs) as well as strain improvement. Here, a novel and high-efficiency *Streptomyces* genome editing tool is established based on the *Fn* CRISPR-Cpf1 system, which is an attractive and powerful alternative to the *Sp* CRISPR-Cas9 system due to its unique features. When combined with HDR or NHEJ, *Fn*Cpf1 enables the creation of gene(s) deletion with high efficiency. Furthermore, a ddCpf1-based integrative CRISPRi platform is established for simple, multiplex transcriptional repression. Of importance, *Fn*Cpf1-based genome editing proves to be a highly efficient tool for genetic modification of some important industrial *Streptomyces* strains (e.g. *S. hygroscopicus* SIPI-KF) that cannot utilize the *Sp* CRISPR-Cas9 system. We expect the CRISPR-Cpf1 assisted genome editing tool to accelerate discovery and development of pharmaceutically active NPs in *Streptomyces* as well as other actinomycetes.

Keywords

Streptomyces, CRISPR, *Fn*Cpf1, genome editing, multiplex repression

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Introduction

Streptomyces species, as Gram-positive and high G+C% soil actinomycetes, have evolved to produce a large number of important bioactive natural products, such as insecticides, immunosuppressors and antitumor agents (1-3). Recent large-scale bacterial genome sequencing analysis has revealed that ~90% of secondary metabolite biosynthetic gene clusters (BGCs) in streptomycetes are silent or cryptic under laboratory conditions and are thus regarded as a treasure for the discovery of novel drug leads (4-6). Convenient and efficient *Streptomyces* genetic engineering will significantly advance functional genomic research, genome mining of bioactive compounds as well as strain improvement (7-10).

CRISPR-Cas system is a widely distributed adaptive immunity against invading mobile genetic elements in archaea and bacteria (11-14). Class 2 CRISPR-Cas system, consisting of a single multi-domain effector paired with guide RNA, has been developed as a powerful genome editing tool for a broad spectrum of organisms due to its simplicity and versatility (15-17). As a kind of fast and high-throughput genome editing tools, CRSIPR-based systems have been widely employed for fundamental research and development of advanced cell factories in the microbial systems, such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Streptomyces* (18-21). Previous studies have demonstrated that *Streptococcus pyogenes* (*Sp*) CRISPR-Cas9 system can be utilized for high-efficiency multiplex genome editing by homologous recombination (HR) or non-homologous end joining (NHEJ)-mediated double-strand breaks (DSBs) repair and reversible transcriptional gene expression in multiple model

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and industrial *Streptomyces* species (22-27). However, *Sp*Cas9-based DNA editing has some shortcomings to be addressed. For instance, a G-rich protospacer-adjacent motif (PAM) sequence (5′-NGG-3′) is required for *Sp*Cas9 to recognize a target site (28, 29). Although this PAM is rather frequently distributed across the *Streptomyces* genomes (GC content >70%, ca. 260 unique genomic targets per 1000 bp, which is the average size of *S. coelicolor* genes, Table S1), it is not always available in the desired loci, particularly in AT-rich regions. Furthermore, complex plasmid construction for independent expression of multiple single guide RNAs (sgRNA) in the *Sp*Cas9-mediated editing system is not convenient for multiplex genome editing and transcriptional regulation. Notably, our earlier study indicated that the *Sp*Cas9-based editing plasmid did not work in several industrial *Streptomyces*, such as the 5-oxomilbemycin-producing strain *Streptomyces hygroscopicus* SIPI-KF. Similar phenomenon has also been observed in *Corynebacterium glutamicum*, where that the native *Sp*Cas9 cannot be constitutively expressed due to its toxicity in the recipient cells (30, 31). These limitations of *Sp*Cas9-based editing tool propel us to explore a novel, alternative CRISPR-Cas system for high-efficiency genetic engineering of *Streptomyces* species.

Cpf1 (also known as Cas12a), a new class 2 type V CRISPR-Cas RNA-guided endonuclease, was recently identified to possess efficient genomic DNA cleavage activity in bacteria, plant and mammalian cells (32-35). Three Cpf1-family proteins, including *Fn*Cpf1 from *Francisella novicida*, *As*Cpf1 from *Acidaminococcus sp*. BV3L6 and *Lb*Cpf1 from *Lachnospiraceae bacterium*, have been studied most

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107 intensively (34-36). Over the past two years, Cpf1 has been rapidly developed as a
108 powerful genome editing tool in many organisms due to its unique features distinct
109 from *Sp*Cas9:

110 i. Cpf1 recognizes T-rich PAM sequences, i.e., 5′-TTTV-3′ for AsCpf1/LbCpf1
111 and 5′-TTV-3′ for *Fn*Cpf1 (V=A/G/C) (34). This feature can expand the genomic
112 DNA scope that can be targeted. In addition, studies have demonstrated that Cpf1
113 could provide higher levels of target specificity than *Sp*Cas9 in mammalian cells (37,
114 38). These results suggest that Cpf1 may be an attractive alternative tool for genetic
115 modification of actinomycetal genomes (particularly AT-rich regions).

116 ii. Cpf1 is guided by a single CRISPR RNA (crRNA) without trans-activating
117 crRNA (tracrRNA) and harbors a distinct RNase activity for pre-crRNA processing
118 (35, 39, 40). Additionally, the Cpf1 crRNA (19 nt direct repeat and 23-25 nt spacer) is
119 shorter than that of *Sp*Cas9 chimeric sgRNA by ~60 nt (40). These advantages of
120 Cpf1 make it easy to perform multiplex genome editing and transcriptional regulation
121 by only using a single, customized crRNA array. Recently, the CRISPR-Cpf1 system
122 was used to simultaneously edit up to four genes by NHEJ repair in rice and
123 mammalian cells (35, 41). Furthermore, a DNase-deactivated Cpf1 (ddCpf1) paired
124 with a single crRNA array was also employed for inducible transcriptional activation
125 of up to three genes in human cells and multiplex gene repression of up to four genes
126 in *E. coli* (42, 43).

127 Thus far, Cpf1-mediated genome editing has been demonstrated in a few bacteria,
128 including *E. coli*, *C. glutamicum*, *Cyanobacteria*, *Yersinia pestis*, and *Mycobacterium*

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129 *smegmatis* (31, 33, 44). The goal of the present study was to develop a high-efficiency
130 CRISPR-Cpf1 system for genome editing as well as multiplex gene repression in the
131 industrial important microbe, *Streptomyces*. First, we established an all-in-one
132 *Fn*Cpf1-based plasmid for targeted genomic DNA cleavage (Fig. 1A). It should be
133 noted that *As*Cpf1/*Lb*Cpf1 were not chosen for genome editing of *Streptomyces* due to
134 their excessively low PAM occurrence frequencies compared to *Fn*Cpf1 (Table S1).
135 Second, *Fn*Cpf1-assisted editing system was developed for precise single or double
136 genes deletions by homology-directed repair (HDR) and random sized deletions of
137 target genes by the reconstituting NHEJ system (Fig. 1B). Third, a ddCpf1-based
138 integrative CRISPR interference (CRISPRi) system was also designed for robust and
139 multiplex gene expression control (Fig. 1B). Finally, we demonstrated that
140 *Fn*Cpf1-mediated editing system is an alternative and powerful tool for industrial
141 *Streptomyces* strains, in which the *Sp* CRISPR-Cas9 system does not work well.
142

143 ## Materials and Methods

144 ### Strains, plasmids and growth conditions

145 The strains and plasmids used in this study are listed in Table S2. *S. coelicolor* M145
146 and its derivatives were grown on MS medium (g/L, soybean flour 20, mannitol 20,
147 and agar 20) for spore preparation and intergeneric conjugal transfer (45). Medium
148 MS was also used for spore preparations of *Streptomyces albus* J1074, *Streptomyces*
149 *venezuelae* ATCC10712 and *Streptomyces avermitilis* NRRL8165. Medium RP (g/L,
150 tryptone 5, yeast extract 5, valine 0.5, NaCl 2, KH_2PO_4 0.5, $MgSO_4 \cdot 7H_2O$ 1 and agar

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20, pH 6.4), YMD (g/L, malt extract 10, yeast extract 4, glucose 4 and agar 20), MB (g/L, sucrose 4, skim milk 1, yeast extract 2, malt extract 5 and agar 20, pH 7.0-7.2) and Isp4 (g/L, starch 10, tryptone 1, yeast extract 0.5, NaCl 1, $(NH_4)_2SO_4$ 2, K_2HPO_4 1, $CaCO_3$ 2, agar 20, and trace element solution 1 mL, pH 7.0) were used for spore preparations of *Streptomyces pristinaespiralis* HCCB10218, *Streptomyces roseosporus* SIPI-DT51, *Streptomyces hygroscopicus* SIPI-KF and *Streptomyces verticillus* SIPI-BL, respectively. When the CRISPR-Cas9 or CRISPR-Cpf1 genome editing tool was employed, M-Isp4 medium (g/L, soybean flour 5, mannitol 5, starch 5, tryptone 2, yeast extract 1, NaCl 1, $(NH_4)_2SO_4$ 2, K_2HPO_4 1, $CaCO_3$ 2, agar 20, and trace element solution 1 mL, pH 7.0-7.2) with 10 mM or 60 mM $MgCl_2$ was used for conjugal transfer between *E. coli* and *Streptomyces*. *S. coelicolor* and S. *hygroscopicus* cultures grown in liquid MB and YEME medium (g/L, yeast extract 3, peptone, 5, malt extract 3, glucose 10, sucrose 340 and $MgSO_4 \cdot 7H_2O$ 1.24), respectively, were harvested for total DNA isolation (45). All *Streptomyces* strains were cultivated at 30°C.

Escherichia coli DH5α was used for DNA cloning. *E. coli* ET12567/pUZ8002 or S17-1 was used for conjugal transfer from *E. coli* to *Streptomyces*. The detailed conditions of conjugal transfer were listed in Table S3. *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) medium or on LB agar plates. Antibiotics (50 μg/mL of ampicillin, apramycin and kanamycin) were added when necessary.

Construction of Cpf1- and ddCpf1-based editing plasmids

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173 The *cpf1* gene originating from *Francisella novicida* U112 was codon-optimized
174 based on the bias of *S. coelicolor* M145 (the optimized sequence was listed in
175 Supplementary material Data 1 and named as *scocpf1*) and synthesized. Then, the
176 *scocpf1* gene was cloned into the plasmid pKCCas9(*tipAp*) between the *Eco*R I and
177 *Nde* I sites to replace the *scocas9* gene (26), which is under the control of the
178 inducible promoter *tipAp*, generating the plasmid pKCCpf1(*tipAp*)-E. Using the
179 plasmid pAH91*kasO*p*-*cmlR* as the template (46), the crRNA scaffold, containing the
180 strong promoter *kasOp** and 19 nt direct repeat, was obtained by PCR amplification
181 with the primers *kasOp**-crRNA-fw/rev. The resulting DNA fragment was
182 double-digested by *Spe* I and *Xba* I, and ligated into pKCCpf1(*tipAp*)-E to generate
183 pKCCpf1(*tipAp*).

184 Based on pKCCpf1(*tipAp*), the plasmid pKCCpf1 containing the *scocpf1* gene
185 driven by the constitutive promoter *ermEp** was constructed as follows. The *ermEp**
186 promoter was amplified from pIB139 using the primers *ermEp**-fw/rev. The resulting
187 DNA fragment was recombined into pKCCpf1(*tipAp*) that was digested by *Bam*H I
188 and *Nde* I, generating the plasmid pKCCpf1.

189 The plasmid pSETddCpf1 containing the DNase-dead Cpf1 (ddCpf1) encoding
190 gene was constructed on the basis of the integrative plasmid pSET152 as follows.
191 Using pKCCpf1 as the template, the upstream and downstream regions (884 and 3356
192 bp) of the CRISRP/Cpf1 expression cassette were obtained by PCR amplification
193 using the primer pairs ddcpf1-up-fw/ddcpf1-up-E1006A-rev and ddcpf1-down-fw/rev
194 (the primer ddcpf1-up-E1006A-rev contains the mutational DNA sequence for

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195 generating the point mutation E1006A in *Fn*Cpf1), and then ligated together by
196 overlapping PCR with the primers ddcpf1-up-fw and ddcpf1-down-rev. The resulting
197 DNA fragment was cloned into the *Eco*R I/*Xba* I-digested pSET152 by recombination,
198 generating the plasmid pSETddCpf1. The E1006A mutation in pSETddCpf1 was
199 confirmed by DNA sequencing.

200 On the basis of pKCCpf1, the plasmid pKCddCpf1 containing the ddCpf1 encoding
201 gene was constructed as follows. The *ddcpf1* gene was amplified from pSETddCpf1
202 by PCR using the primers ddcpf1-fw/rev, followed by double-digestion with *Eco*R I
203 and *Nde* I. Then, the resulting DNA fragment was cloned into pKCCpf1 to yield
204 pKCddCpf1. Similarly, based on pKCCas9(*tipAp*), the plasmid pKCdCas9(*tipAp*)
205 containing the DNase-deficient *cas9* (*dcas9*) encoding gene was constructed. The
206 *dcas9* gene originating from *S. pyogenes* was codon-optimized based on the bias of *S.*
207 *coelicolor* M145 and synthesized. Then, the *dcas9* gene was cloned into
208 pKCCas9(*tipAp*) between the *Eco*R I and *Nde* I sites to replace the *scocas9* gene (26),
209 thus generating pKCdCas9(*tipAp*).

210 Cpf1-based gene editing plasmids and ddCpf1-based CRISPRi plasmids were
211 constructed by introducing 23 nt gene-specific spacer sequence into the crRNA
212 scaffold of pKCCpf1 and pSETddCpf1, respectively. Briefly, using pKCCpf1 as the
213 template, the crRNA expression cassettes were amplified with the forward primer
214 5′-CACTAGTN$_{23}$ATCTACAACAGTAGAAATTTGG-3′ (N$_{23}$ represents the 23 nt
215 gene-specific spacer sequence) and the reverse primer (crRNA-rev). The PCR product
216 was digested with *Nde* I and *Spe* I, and ligated to *Nde* I/*Spe* I-digested pKCCpf1 and

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pSETddCpf1, respectively. Different Cpf1 or ddCpf1-based editing plasmids could be generated simply by just changing the N_{23} region of the forward primer. The primers used are listed in Table S4 in the supplementary materials.

Construction of pKCCpf1-based NHEJ plasmids

Based on pKCCpf1, a series of recombinant plasmids containing different NHEJ pathways driven by the strong promoter *gadphp* or *ermEp** were constructed as follows. The *Sda-ligD* and *Sda-ku* genes from *Streptomyces daghestanicus* under the control of *gadphp* were synthesized and cloned into pKCCpf1 between *Hin*d III and *Spe* I, thus generating pKCCpf1-SdaP. The promoter *ermEp** was amplified from pIB139 using the primers *ermEp**-fw (Spe I) and *ermEp**-rev (*Eco*R V) and then ligated into pKCCpf1-SdaP to yield the plasmid pKCCpf1-SdaE. The *Msm-ligD* and *Msm-ku* genes from *Mycobacterium smegmatis* were amplified from the plasmid pZX09 using the primer pairs *Msm-ligD*-fw/rev and *Msm-ku*-fw/rev, respectively (47). The *Msm-ligD* and *Msm-ku* genes were double-digested by *Eco*R V/*Xba* I and *Xba* I/*Hin*d III, respectively, and ligated simultaneously into pKCCpf1-SdaP or pKCCpf1-SdaE between *Eco*R V and *Hin*d III to generate pKCCpf1-MsmP or pKCCpf1-MsmE. Using the *Pseudomonas putida* genome as the template, the *Ppu-ligD* and *Ppu-ku* genes were amplified using the primer pairs *Ppu-ligD*-fw/rev and *Ppu-ku*-fw/rev. The *Ppu-ligD* and *Ppu-ku* genes were double-digested by *Spe* I/*Xba* I and *Xba* I/*Hin*d III, respectively, and cloned simultaneously into pKCCpf1 between *Spe* I and *Hin*d III to yield pKCCpf1-Ppu. The promoter *gadphp* was

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amplified from pKCCpf1-SdaP using the primers *gadphp*-fw/rev, and then ligated into pKCCpf1-Ppu between by *Spe* I and *Pme* I to yield pKCCpf1-PpuP. Similarly, the promoter *ermEp** was amplified from pIB139 using the primers *ermEp**-fw (*Spe* I) and *ermEp**-rev (*Pme* I) and ligated into pKCCpf1-Ppu between *Spe* I and *Pme* I, generating pKCCpf1-PpuE.

To utilize *Fn*Cpf1-assisted NHEJ systems for random deletions of *redX*, *actIorfI* or RED biosynthetic gene cluster (BGC), the editing plasmids pKCCpf1-*redX*, pKCCpf1-*actIorf1* and pKCCpf1-RED BGC was first constructed as above by introducing the gene(s)-specific spacer sequence into the crRNA scaffold of pKCCpf1. This design was to check whether crRNAs was capable of efficiently mediating DSBs at the target loci in *Streptomyces*. Then, NHEJ-mediated editing plasmids were constructed as follows. Here, we took pKCCpf1-MsmE-*redX* as an example to describe the construction process. In brief, the MsmE expression cassette containing the *Msm-ligD* and *Msm-ku* genes under the control of *ermEp** were obtained by double-digestion of pKCCpf1-MsmE with *Hin*d III and *Spe* I, and then ligated into pKCCpf1-*redX* to yield pKCCpf1-MsmE-*redX*. The other five NHEJ-mediated editing palsmids, including pKCCpf1-SdaE-*redX*, pKCCpf1-PpuE-*redX*, pKCCpf1-MsmE-*actIorf1* pKCCpf1-SdaE-*actIorf1* and pKCCpf1-PpuE-*actIorf1* were constructed similarly. The primers used are listed in Table S4 in the supplementary materials.

Construction of the *S. coelicolor* and *S. hygroscopicus* mutants

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261 The Δ*actIorf1*, Δ*redX and* Δ*actIorf1-redX* mutants with in-frame deletion of *actIorf1*,
262 *redX* and both *actIorf1* and *redX* were constructed, respectively, using the
263 CRISPR-Cpf1-assisted HDR strategy. Here, we took Δ*actIorf1* as an example to
264 introduce the mutant construction process. Briefly, the upstream and downstream
265 regions (1019 and 1033 bp) of *actIorf1* were obtained by PCR using the primer pairs
266 Del-*actIorf1*-up-fw/rev and Del-*actIorf1*-down-fw/rev, respectively. Using the
267 plasmid pKCCpf1 as the template, the crRNA expression cassette containing the
268 strong promoter *kasOp**, 19 nt repeat sequence and 23 nt protospacer sequence
269 targeting *actIorf1*, was obtained by PCR amplification with primers
270 *actIorf1*-crRNA-fw/rev. Then, three DNA fragments were overlapped together by
271 PCR using the primers Del-*actIorf1*-up-fw and *actIorf1*-crRNA-rev, followed by
272 double-digestion with *Nde* I and *Spe* I. The resulting DNA fragment was ligated into
273 pKCCpf1 to yield pKCCpf1-*actIorf1*-HR, which was introduced into the wild-type *S.*
274 *coelicolor* M145 by conjugal transfer. The resulting exoconjugants were subsequently
275 grown on solid MS medium without apramycin at 37°C for two rounds to remove the
276 plasmid pKCCpf1-*actIorf1*-HR. The correct double cross-overs were verified by
277 colony PCR with the primers ID-*actIorf1*-fw/rev, yielding the Δ*actIorf1* mutant. The
278 Δ*redX* and Δ*actIorf1-redX* mutants were constructed similarly.

279 The ΔRED-BGC mutant with deletion of the ~28 kb RED BGC region between
280 *redX* and *redG*, was constructed by using CRISPR-Cpf1-assisted NHEJ strategy.
281 Briefly, using the plasmid pKCCpf1 as the template, the crRNA array expression
282 cassette, containing the strong promoter *kasOp** and two crRNAs targeting *redX*

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283 and *redG*, respectively, was obtained by PCR amplification with primers
284 crRNA-RED-BGC-fw and crRNA-rev, followed by double-digestion with *Nde* I
285 and *Spe* I. The resulting DNA fragment was cloned into pKCCpf1 to generate
286 pKCCpf1-RED-BGC. Then, the *ligD* and *ku* expression cassette under the control of
287 *ermEp** from pKCCpf1-MsmE by double-digestion with *Hin*d III and *Spe* I, was
288 ligated into pKCCpf1-RED-BGC to generate pKCCpf1-MsmE-RED-BGC, which
289 was introduced into the *S. coelicolor* wild-type strain M145. The resulting strains
290 were subsequently grown on solid R2YE medium for phenotypic analysis. These
291 strains, which only produced blue-pigmented actinorhodin (ACT), were cultivated on
292 MS medium without apramycin at 37°C for two rounds to remove the plasmid
293 pKCCpf1-MsmE-RED-BGC. The correct strains were verified by colony PCR with
294 the primers ID-NHEJ-RED-BGC-fw/rev and DNA sequencing, yielding the
295 ΔRED-BGC mutant. The primers used are listed in Table S4.

296 The Δ*SBI00792* mutant with an in-frame deletion of *SBI00792* was constructed on
297 the basis of the 5-oxomilbemycin-producing strain *S. hygroscopicus* SIPI-KF, using
298 the CRISPR-Cpf1-assisted HDR strategy. Briefly, the crRNA expression cassette was
299 amplified from pKCCpf1 using the primers *SBI00792*-crRNA-fw/rev, and
300 recombined into pKCCpf1 to generate pKCCpf1-*SBI00792*. Then, the upstream
301 and downstream regions (2409 and 2470 bp) of *SBI00792* were obtained by PCR
302 amplification using the primer pairs Del-*SBI00792*-up-2.5-fw/Del-*SBI00792*-up-rev
303 and Del-*SBI00792*-down-fw/Del-*SBI00792*-down-2.5-rev, respectively. Two DNA
304 fragments were ligated together by overlapping PCR using the primers

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305 Del-*SBI00792*-up-2.5-fw and Del-*SBI00792*-down-2.5-rev. The resulting DNA
306 fragment was ligated into pKCCpf1-*SBI00792* to yield pKCCpf1-*SBI00792*-HR2.5
307 by Gibson assembly (48), which was transferred into *S. hygroscopicus* SIPI-KF. The
308 resulting strains were subsequently grown on solid MB medium without apramycin at
309 37°C for two rounds to remove the plasmid pKCCpf1-*SBI00792*-HR2.5. The correct
310 double cross-overs were verified by colony PCR with the primers
311 ID-*SBI00792*-fw/rev, yielding the Δ*SBI00792* mutant.

312 To confirm the role of *SBI00792*, the complemented strain Δ*SBI00792*/pIB-00792
313 was constructed. Briefly, using the *S. hygroscopicus* SIPI-KF genome as the DNA
314 template, the gene *SBI00792* was obtained by PCR amplification using the primers
315 Com-SBI00792-fw/rev and double-digested with *Eco*R I and *Nde* I. The resulting
316 DNA fragment was cloned into the *Eco*R I/*Nde* I-digested pIB139, thus generating the
317 plasmid pIB-00792. Then, the plasmid pIB-00792 was introduced into the Δ*SBI00792*
318 mutant, yielding the complemented strain Δ*SBI00792*/pIB-00792. As controls, two
319 strains, SIPI-KF/pIB139 and Δ*SBI00792*/pIB139 were constructed by introducing the
320 empty vector pIB139 into *S. hygroscopicus* SIPI-KF and the Δ*SBI00792* mutant,
321 respectively. The primers used are listed in Table S4 in the supplementary materials.

322

323 **Fermentation of *S. hygroscopicus* and HPLC analysis of 5-oxomilbemycin A3/A4**
324 **production**

325 For 5-oxomilbemycin A3/A4 production, *S. hygroscopicus* strains were first grown on
326 solid MB medium at 30 °C for 6-7 days and then inoculated into 25 mL seed medium

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(g/L, sucrose 20, meat peptone 2.5, yeast extract 5 and K_2HPO_4 0.1, pH 7.2) in 250 mL Erlenmeyer flasks on an orbital shaker (240 rpm). After incubation at 28 °C for 24-28 h, 1 mL of pre-culture was inoculated into 25 mL of fermentation medium (g/L, sucrose 120, cotton seed meal 5, soybean flour 5, K_2HPO_4 0.5, $FeSO_4 \cdot 7H_2O$ 0.1 $ZnSO_4 \cdot 7H_2O$ 0.01, $CuSO_4 \cdot 7H_2O$ 0.05 and $CaCO_3$ 3) in 250 mL Erlenmeyer flasks. The pH of fermentation medium was adjusted to 7.2 prior to $CaCO_3$ addition.

1 mL fermentation samples were collected at five time points (4, 6, 10, 14 and 16 days) and extracted with the same volume of ethanol for 1-2 h. Subsequently, the mixtures were centrifuged at 12,000 rpm for 10 min, and the supernatants (ethanol extracts) were directly analyzed by HPLC (1100 series, Agilent) using a 4.6×150 mm Hypersil C18 column (Agilent). For HPLC detection, a mixture of acetonitrile/H_2O (73:27, v/v) was used as the mobile phase, with a flow rate of 1.2 mL/min and retention time of 14 min of 5-oximilbemycin A3 and 20 min of 5-oximilbemycin A4. The eluates were monitored at 240 nm, and the column temperature was 30 °C.

RNA preparation and quantitative real-time RT-PCR (RT-qPCR)

RNA preparation and RT-qPCR analysis were performed as previously described (49). The primers used are listed in Table S4. *S. coelicolor* strains were grown on R2YE covered with sterilized plastic cellophane. Cultures were harvested for RNA preparation at different time points (24, 48 and 72 h). RT-qPCR analysis was performed in the MyiQ2 two-color real-time PCR detection system (Bio-Rad, USA) by using iQ™ SYBR Green Supermix (Bio-Rad, USA). The reactions were

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conducted in triplicate for each transcript and repeated with three independent samples. The *hrdB* gene (*SCO5820*, encoding the principal sigma factor) was used as an internal control. The relative expression levels of tested genes were normalized to those of *hrdB*. The relative fold-changes in the transcription of each gene were determined using the $2^{-\Delta\Delta Ct}$ method (50). Error bars indicate the standard deviations from three independent biological replicates.

Results

Design and functionality of an all-in-one *Fn*Cpf1-editing system in *Streptomyces*

To develop a *Fn*Cpf1-mediated genome editing tool in *Streptomyces*, an all-in-one CRISPR-Cpf1 editing plasmid, pKCCpf1(*tipAp*), in which the expression of the codon-optimized *scocpf1* gene and target-specific crRNA were driven by the inducible promoter *tipAp* and the strong promoter *kasOp** (51), respectively, was constructed. The editing plasmid pKCCpf1(*tipAp*) harboring the temperature-sensitive replicon pSG5, could be quickly eliminated when the edited strains were cultivated at 37°C (45). This feature would significantly facilitate *Fn*Cpf1-assisted iterative genome editing. To confirm that *Fn*Cpf1 was functional, we constructed the editing plasmid pKCCpf1(*tipAp*)-*actIorf1*, which specifically targeted the *actIorf1* gene from the biosynthetic pathway of the blue-colored antibiotic actinorhobin (ACT) in the model strain *S. coelicolor*. It should be noted that a 5′-TTC-3′ PAM sequence in *actIorf1* was chosen to test *Fn*Cpf1-mediated genome cleavage activity. As a positive control, *Sp*Cas9-derived editing plasmid pKCCas9(*tipAp*)-*actIorf1* was also

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371 constructed. Then, five plasmids, namely, the control plasmid pKC1139,
372 pKCCas9(*tipAp*) (without target-specific sgRNA expression cassette),
373 pKCCas9(*tipAp*)-*actIorf1*, pKCCpf1(*tipAp*) (without target-specific crRNA
374 expression cassette) and pKCCpf1(*tipAp*)-*actIorf1* were introduced individually into *S.*
375 *coelicolor* by conjugal transfer. It was observed that introduction of
376 pKCCas9(*tipAp*)-*actIorf1* into M145 caused a 99.6% decrease in exoconjugant
377 numbers compared to pKCCas9(*tipAp*). However, only a 41.5% decrease in
378 exoconjugant numbers was caused when pKCCpf1(*tipAp*)-*actIorf1* was introduced
379 into M145, indicating that the activity of CRISPR-Cpf1 system was not enough for
380 targeted genome cleavage (Fig. 2A). To address this problem, the strong constitutive
381 promoter *ermEp** was used to replace *tipAp* for *Fn*Cpf1 expression, thus generating
382 plasmids pKCCpf1 and pKCCpf1-*actIorf1*. Compared with pKCCpf1, introduction of
383 pKCCpf1-*actIorf1* into M145 only generated very few exoconjugants (12±3 per
384 10^8 recipient cells), with a nearly 100% killing frequency (Fig. 2A). The results
385 clearly indicate that *Fn*Cpf1 expression in *S. coelicolor* is not toxic and the all-in-one
386 CRISPR-Cpf1 system can efficiently mediate targeted DNA cleavage, which is a
387 prerequisite for site-directed genetic modification by HDR or NHEJ.

388 It has been previously reported that *Fn*Cpf1 could cleave target DNA specifically at
389 YTV (Y=T/C and V=A/G/C) PAM located on the 5′-end of the protospacer (40, 52).
390 Here, PAM sequence preference of *Fn*Cpf1 was further checked in *S. coelicolor*. We
391 designed six crRNAs targeting 5′-TTV-3′ or 5′-CTV-3′ PAM-proceded sequences
392 located in two genes, *actIorf1* and *actVB*. It was observed that all 5′-TTV-3′

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393 PAM-targeted crRNAs could result in significantly reduced exoconjugant formation
394 with several orders of magnitude, while 5′-CTV-3′ PAM-targeted crRNAs only caused
395 41.1-88.6% decreases in exoconjugant numbers compared with the control plasmid
396 (Fig. 2B). Furthermore, we found that 86.7-100% of the obtained exoconjugants
397 produced blue-pigmented ACT (Fig. S1), indicating that most of colonies have not
398 undergone targeted Cpf1-mediated DNA cleavage. These results revealed that the
399 5′-CTV-3′ PAM is capable of mediating DNA interference but not suitable for
400 high-efficiency genome editing, and 5′-TTV-3′ PAM would be the preferable choice
401 for genetic engineering in *Streptomyces*.

402 The mature crRNA of CRISPR-Cpf1 system composes of a 19 nt direct repeat and
403 23-25 nt of spacer sequence (34). It has been demonstrated that to achieve efficient
404 DNA cleavage *in vitro*, a minimum of 17 nt of guide sequence is required (34, 53). To
405 determine the minimum spacer length of crRNA for efficient genomic DNA cleavage
406 in *S. coelicolor*, we designed a series of crRNAs with different spacer lengths (16-23
407 nt), which targeted two different sequences located in *actIorf1*. The results showed
408 that *Fn*Cpf1 required at least 19 nt of spacer sequence to maintain efficient
409 RNA-guided DNA cleavage activity in *S. coelicolor* (Fig. 2C and D).

410

411 **_Fn_Cpf1-assisted single gene and double genes deletions by HDR**

412 We firstly validated the CRISPR-Cpf1 system in *S. coelicolor* by in-frame deleting
413 single or double genes based on homology-directed DSB repair (HDR). Two genes,
414 *actIorf1* and *redX* from the biosynthetic pathways of the blue-colored antibiotic ACT

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415 and the red-pigmented antibiotic prodiginines (RED), respectively, were chosen for
416 deletion trials. Three editing plasmids, including pKCCpf1-*actIorf1*-HR (targeting
417 *actIorf1* only), pKCCpf1-*redX*-HR (targeting *redX* only) and
418 pKCCpf1-*actIorf1*-*redX*-HR (targeting both *actIorf1* and *redX*), were constructed and
419 introduced individually into M145 by conjugal transfer. The lengths of homolog arms
420 (HA) used for *actIorf1* and *redX* deletion were 1017/1033 bp and 1019/1033 bp
421 (up/downstream), respectively. The experiments were repeated twice. The
422 exoconjugants for *actIorf1*, *redX* and *actIorf1*-*redX* deletions were 54±5, 41±6 and
423 23±4, respectively. Then, 10 colonies were randomly picked to determine the editing
424 efficiency by PCR analysis for each trial, and the experiments were repeated twice. As
425 shown in Fig. 3A and B, the deletion rates of *actIorf1* and *redX* were 95%
426 (10/10+9/10) and 90% (10/10+8/10), respectively. The efficiency of simultaneous
427 deletion of both genes also reached up to 75% (8/10+7/10). As expected, visual
428 analysis on agar plates showed that disruption of *actIorf1* resulted in complete loss of
429 blue pigment (ACT) formation, while deletion of *redX* led to loss of red color (RED)
430 production. The mutant with simultaneous deletion of both genes does not produce
431 ACT and RED any more (Fig. 3C). It could be therefore concluded that similar to the
432 *Sp* CRISPR-Cas9 system, the *Fn* CRISPR-Cpf1 system is capable of HDR-dependent
433 multiplex genome engineering in *S. coelicolor*.
434
435 **_Fn_Cpf1-assisted random sized deletion by the reconstituted NHEJ system**
436 The repair of endonuclease-induced DSBs by the NHEJ pathway can give rise to

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small insertions and deletions (indels) in genomes, which thus lead to inactivation of target genes (54-56). Compared to HDR-dependent genome editing that requires two homologous arms as the template for DSBs repair, template-free NHEJ repair is imprecise but more convenient. Unfortunately, most streptomycetes lack complete NHEJ pathway, which composes of a DNA ligase D (LigD) and its cognate Ku protein. Here, three different NHEJ systems from *Mycobacterium smegmatis*, *Streptomyces daghestanicus* and *Pseudomonas putida*, named as Msm-LK, Sda-LK and Ppu-LK, respectively, were selected to promote *Fn*Cpf1-induced DSBs repair in *S. coelicolor*. The reasons why we selected the three NHEJ pathways were as follows. First, Msm-LK exhibited higher DSBs repair activity than other two NHEJ systems from *M. tuberculosis* and *Bacillus subtilis* in *E. coli* (47). Second, Sda-LK is the only known NHEJ system of *Streptomyces* origin (NCBI GenPept ID of *ligD* and *ku* are WP_086839147.1 and WP_086839677.1, respectively). Finally, Ppu-LK possesses different NHEJ repair mechanism from Msm-LK and Sda-LK, and the genome of *P. putida* has a high G+C content (61.9%) similar to *Streptomyces* speices (55). These features promote Ppu-LK as a considerable alternative for reconstituting complete NHEJ system in *S. coelicolor*.

To develop an all-in-one CRISPR-Cpf1 editing plasmid containing the reconstituted NHEJ system, *ligD* and the cognate *ku* gene were directly ligated to pKCCpf1. Two different strong promoters, *ermEp** and *gadphp* (57), were used, respectively, to drive co-expression of these two genes. As such, six plasmids, including pKCCpf1-MsmP, pKCCpf1-MsmE, pKCCpf1-SdaP, pKCCpf1-SdaE,

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459 pKCCpf1-PpuP and pKCCpf1-PpuE, were constructed. As the first step, the toxicity
460 of foreign NHEJ pathways was tested. It was observed that overexpression of these
461 three different NHEJ systems under the control of *gadphp* all resulted in dramatically
462 decreased formation of exoconjugants. The exoconjugant numbers were reduced by
463 90.2% to 98.9%, compared with the control (pKCCpf1). In contrast, expression of the
464 NHEJ systems drived by *ermEp** led to a decrease in exoconjugant numbers by
465 43.8% to 85.9%, which was acceptable for *S. coelicolor* genome editing. Additionally,
466 we noticed that Msm-LK showed the lowest cell toxicity among three tested NHEJ
467 systems.

468 Then, *redX* and *actIorf1* were chosen for template-free deletion trials. Totally, six
469 editing plasmids harboring both the NHEJ systems driven by *ermEp** and the crRNA
470 expression cassettes targeting *redX* or *actIorf1*, including pKCCpf1-MsmE-*redX*,
471 pKCCpf1-SdaE-*redX*, pKCCpf1-PpuE-*redX*, pKCCpf1-MsmE-*actIorf1*,
472 pKCCpf1-SdaE-*actIorf1*, and pKCCpf1-PpuE-*actIorf1* were constructed. As controls,
473 pKCCpf1-*redX* and pKCCpf1-*actIorf1* with the *redX*- and *actIorf1*-targeted crRNA
474 expression cassettes cloned in pKCCpf1, were also constructed, respectively. We
475 observed that compared with the controls (pKCCpf1-*redX* and pKCCpf1-*actIorf1*)
476 that no exoconjugants grew out, introduction of these editing plasmids harboring
477 different NHEJ systems significantly increased exoconjugant numbers (Fig. 4B).
478 More precisely, the numbers of exoconjugants were increased from 0 to 168 ± 13
479 (MsmE-LK), 195 ± 12 (SdaE-LK) and 180 ± 9 (PpuE-LK) for editing *redX*.
480 Meanwhile, the exoconjugant numbers for editing *actIorf1* were increased from 0 to

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481 185 ± 11 (MsmE-LK), 51 ± 4 (SdaE-LK) and 16 ± 2 (PpuE-LK). These results
482 indicated that the three foreign NHEJ systems were functional in *S. coelicolor*. 15
483 colonies were randomly picked to determine the editing efficiency by visual analysis
484 on the R2YE plate for each experiment, and the tests were repeated twice. The results
485 showed that, the editing efficiencies for disruption of *redX* (the blue clones, producing
486 no red-pigmented RED) were 0% (0/15+0/15, PpuE-*redX*), 33.3% (5/15+5/15,
487 SdaE-*redX*) and 50% (7/15+8/15, MsmE-*redX*), respectively. In contrast, the
488 efficiencies for inactivation of *actIorf1* (the red clones, producing no blue-pigmented
489 ACT) were 20% (3/15+3/15, PpuE-*actIorf1*), 43.3% (7/15+6/15, SdaE-*actIorf1*) and
490 56.7% (9/15+8/15, MsmE-*actIorf1*) (Fig. 5C and D). To further validate these results,
491 PCR analysis combined with DNA sequencing was performed and primers were
492 designed to amplify the ~2800 bp DNA fragments covering the cleavage site of the
493 MsmE-*redX*-crRNA. Totally, 15 exoconjugants were selected and tested. As shown in
494 Fig. 4E, the deletions were precisely located at the target sites, but varied in size (from
495 409 to 1624 bp). These results clearly demonstrated that Msm-LK, the NHEJ pathway
496 from *M. smegmatis*, exhibited the lowest cell toxicity and the highest NHEJ activity
497 (~50% DSBs repair effciency) in *S. coelicolor*.

498 Next, to check whether the combination of CRISPR-Cpf1 system and the
499 reconstituted NHEJ pathway (Msm-LK) could be used to delete large DNA fragments
500 (eg. the antibiotic biosynthetic pathway) guided by a pair of crRNAs, the RED
501 biosynthetic gene cluster (BGC) was selected and the editing plasmid
502 pKCCpf1-MsmE-RED-BGC harboring two crRNA expression cassettes targeting

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503 *redX* and *redG* simultaneously were constructed (Fig. S2A). The plasmid was
504 introduced into the wild-type strain M145 and then the exoconjugants were selected
505 on MS agar plate. Visual analysis on R2YE plate showed that 30% (4/15+5/15) of
506 tested colonies failed to produce the red-pigmented RED (Fig. S2B), indicating the
507 existence of mutations within the RED BGC. However, further analysis by PCR
508 amplification and DNA sequencing revealed that only 10% (1/15+2/15) colonies
509 showed different sizes of deletions in the RED BGC (Fig. S2C). Collectively, these
510 data presented here demonstrated that *Fn*Cpf1-assisted NHEJ system from *M.*
511 *smegmatis* can efficiently mediate random sized deletions of single genes in *S.*
512 *coelicolor*; meanwhile, this system could also be employed for large-size deletions of
513 secondary metabolites BGCs, but with a relatively low editing efficiency.
514
515 **ddCpf1-mediated multiplex gene repression using a single crRNA array**
516 A reversible dCas9-based CRISPRi system was previously developed for efficient
517 gene expression regulation in *S. coelicolor* (25). However, this system exist two
518 limitations to be improved. First, the replicative plasmid pKC1139, which was used to
519 express CRISPR-dCas9 system, may lead to fluctuant transcriptional repression of
520 target genes due to the plasmid structural and segregational instability (45, 58, 59).
521 Second, simultaneous repression of multiple targets with dCas9 is limited by the
522 time-consuming procedure of the construction of multiple independent-expressed,
523 large single guide RNAs (sgRNAs) expression cassettes. By contrast, a
524 DNase-deactivated Cpf1 (ddCpf1) harboring the E1006A mutation in the RuvC

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525 domain of *Fn*Cpf1 remains pre-crRNA processing but not DNA cleavage activity, and
526 can process a single customized CRISPR array for simplified, multiplex gene
527 expression control (42, 43, 60). Additionally, whole-transcriptomic analysis revealed
528 that ddCpf1-based gene repression showed no significant off-target effects in *E.coli*
529 (42). In the present study, we developed a robust, multiplex CRISPRi system with
530 chromosomal integration expression of ddCpf1 and crRNA(s) using the integrative
531 plasmid pSET152. Again, the *redX* gene was chosen as a test case. Four crRNAs
532 targeting the template DNA strand (T strand) and four targeting the non-template
533 DNA strand (NT strand) in the *ORF* of *redX* were designed. Phenotypic and
534 transcriptional analysis showed that three of four crRNAs targeting the T strand
535 enabled effective gene repression from 48.2% to 88.3%, and crRNA targeting the site
536 nearest to the start codon exhibited the highest repression activity (Fig. 5A and B).
537 The crRNAs targeting the end of T strand or the NT strand showed less effective or
538 ineffective at all for transcriptional repression (Fig. 5A and B). Overall, these results
539 indicate that ddCpf1-based CRISPRi system can efficiently mediate repression of
540 transcription elongation and the repression is more effective when the crRNAs
541 specifically target the T strand of the target gene, which is just opposite to the data
542 obtained from the dCas9-based CRISPRi system in *S. coelicolor* (25).

543 Subsequently, three genes, including *redX*, *actIorf1* and *cpkA* (from the BGC of
544 the yellow-pigmented cryptic polyketone (CPK)), were chosen to test
545 ddCpf1-mediated multiplex gene repression. In prior to multiplex repression, single
546 gene repression for these three genes was first investigated. Three crRNAs were

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547 designed for targeting the T strands of the ORFs of *redX*, *actIorf1* and *cpkA*,
548 respectively, and three corresponding plasmids pSETddCpf1-*redX*
549 pSETddCpf1-*actIorf1* and pSETddCpf1-*cpkA* were constructed. Phenotypic analysis
550 showed that individual introduction of three CRISPRi editing plasmids could
551 significantly reduce the production of the corresponding antibiotics (Fig. 5C).
552 Furthermore, the transcriptional levels of three target genes in strains with the
553 corresponding CRISPRi plasmid were repressed by the efficiencies of 82.1-95.2%
554 (Fig. 5D). Then, two crRNA arrays expressing pre-crRNAs in the orders of
555 *actIorf1-cpkA-redX* (array 1) and *cpkA-redX-actIorf1* (array 2) were constructed using
556 19 nt direct repeats and 23 nt spacers for multiplex gene repression. It was observed
557 that production of three pigmented antibiotics (CPK, RED and ACT) and mRNA
558 levels of their respective biosynthetic genes were significantly decreased in the
559 engineered strain with the co-expression of array 1 and ddCpf1 compared to the
560 wild-type strain only expressing ddCpf1, with the repression efficiencies of 69.9% for
561 *cpkA*, 70.6% for *redX* and 67.4% for *actIorf1* (Fig. 5C and D). Interestingly, when
562 array 2 was used to mediate multiplex gene regulation, the last crRNA unit targeting
563 *actIorf1* only resulted in a slight reduction of ACT biosynthesis and it just gave a
564 repression efficiency of 11.8% at the transcriptional level, indicating an obvious order
565 effect of *actIorf1*-targeted crRNA on repression efficiency.

566 Totally, there are six combination of arrays for three crRNA in different orders. To
567 systematically explore the effect of the crRNA orders on repression efficiency, the
568 other four arrays (array 3-6) were also constructed and tested (Fig. S3A). The results

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revealed that, as shown in Fig. S3A and B, similarly as the results obtained from array 1 and 2, the effect of different crRNA orders on repression efficiency was only observed for the crRNA targeting *actIorf1*, but not for other two tested crRNAs targeting *cpkA* and *redX*. Transcriptional analysis showed that the highest *actIorf1* repression efficiency (76.6%) was observed when its crRNA unit was put immediately downstream of *kasOp** (array 5). However, when the *actIorf1*-targeted crRNA unit was put at the middle and last orders in the arrays, they resulted in much lower repression efficiencies, 37.7% for array 4 (middle) or 51.6% for array 6 (middle) and 17.9% for array 3 (last) (Fig. S3B). Overall, the results presented here clearly indicated that the crRNA order in arrays might only influence the repression efficiency of specific genes. Additionally, we noticed that the repression strength of gene transcription using a crRNA array was lower than those using individual crRNAs for all of the three tested genes (Fig. 5D and S3B), which might be ascribed to the insufficient expression quantity of ddCpf1. In our multiplex repression test, ddCpf1 was not only used to process pre-crRNA to the mature crRNA but also simultaneously target three loci, which was significantly different from the role of ddCpf1 in the single repression test. This reduced repression activity might be addressed by replacing *ermEp** with the stronger promoter *kasOp** or its derivatives (51, 61). Together, these results establish that ddCpf1-based CRISPRi system is a convenient and powerful tool for efficient repression of multiple genes in *S. coelicolor*.

Applicability of the *Fn* CRISPR-Cpf1 system in other *Streptomyces* species

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591 To determine whether *Fn*Cpf1-assisted genome editing tool is applicable to other
592 *Streptomyces* species other than *S. coelicolo*r, as the first step, pKCCpf1 was
593 introduced into seven model or industrial *Streptomyces* strains, including *S. albus*
594 J1074, *S. venezuelae* ATCC10712, *S. avermitilis* NRRL8165, *S. roseosporus*
595 SIPI-DT51, *S. pristinaespiralis* HCCB10218, *S. hygroscopicus* SIPI-KF and *S.*
596 *verticillus* SIPI-BL, for toxicity test of *Fn*Cpf1 expression. Plasmids pKC1139 and
597 pKCCas9(*tipAp*) were used as the controls. The results showed that for four
598 *Streptomyces* strains tested, including *S. albus* J1074, *S. venezuelae* ATCC10712, *S.*
599 *avermitilis* NRRL8165 and *S. roseosporus* SIPI-DT51, introduction of both *Fn*Cpf1-
600 and *Sp*Cas9-expressing plasmids reduced the numbers of exoconjugants in varying
601 degrees from 14.4% to 91.7% compared to pKC1139, but there was no obvious
602 difference in transformation efficiencies between two different CRISPR-Cas systems
603 (Fig. 6A). However, introduction of pKCCas9(*tipAp*) could generate 196 ± 5
604 exoconjugants, but very few colonies (5 ± 1) harboring the plasmid pKCCpf1 could
605 be obtained in *S. pristinaespiralis* HCCB10218. By contrast, introduction of
606 pKCCpf1 into *S. hygroscopicus* SIPI-KF could generate 204 ± 9 exoconjugants with
607 over 100-fold transformation efficiency as that of pKCCas9(*tipAp*) (Fig. 6A). Finally,
608 no colonies could be obtained when introducing pKC1139, pKCCas9(*tipAp*) or
609 pKCCpf1 into *S. verticillus* SIPI-BL, which should be ascribed to the incompatibility
610 of temperature-sensitive replicon pSG5 in the recipient cells (Fig. 6A). Notably,
611 similar results were previously observed in *S. verticillus* ATCC15003 that
612 introduction of pKC1139 failed to yield any true exoconjugants (62). Together, these

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613 results clearly indicated that the two well-known Cas proteins, *Sp*Cas9 and *Fn*Cpf1,
614 exhibited different suitability in the tested *Streptomyces* species.

615 Subsequently, as an example, the *Fn* CRISPR-Cpf1 system was employed for
616 HDR-mediated precise genome editing in the 5-oxomilbemycin-producing strain *S.*
617 *hygroscopicus* SIPI-KF, in which *Sp* CRISPR-Cas9 system did not work well as
618 described above. Here, we attempted to knock out a TetR-family regulatory gene
619 *SBI00792*, which is adjacent to the 5-oxomilbemycin BGC and may therefore be
620 involved in 5-oxomilbemycin biosynthesis. 1-kb homologous arms (HAs) was first
621 designed for *SBI00792* deletion, but no exoconjugants could be obtained (Fig. 6B). So,
622 lengths of HAs were extended to 1.5-, 2- and 2.5-kb for optimizing DSBs repair
623 efficiency. As shown in Fig. 6B, along with the extension of HAs length, the
624 exoconjugant numbers were gradually increased. Notably, the repeated transformation
625 trials demonstrated that introduction of the plasmid pKCCpf1-*SBI00792*-HA2.5 with
626 two 2.5-kb HAs could stably generate more than 20 exoconjugants, which were
627 enough for screening of positive mutants (Fig. 6B). These results indicated that HA
628 lengths should be carefully considered for HDR-mediated gene deletion in some
629 specific *Streptomyces* species.

630 Then, 10 colonies were randomly picked for PCR and DNA sequencing analysis,
631 and this experiment was repeated twice. It was observed that the positive rate for
632 in-frame deletion of *SBI00792* was 85% (8/10+9/10), which was comparable to the
633 data obtained from *S. coelicolor* (Fig. 6C). Phenotypic analysis showed that there was
634 no growth difference between the parental strain SIPI-KF and the *SBI00792* deletion

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635 mutant (Fig. S4). Fermentation analysis showed that inactivation of *SBI00792* resulted
636 in decreased production of 5-oxomilbemycin A3/A4 (the precursor of milbemycin
637 oxime) by 99% compared with the parental strain (from 2226 mg/L to 1120 mg/L)
638 and the complemented strain Δ*SBI00792*/pIB-00792 could successfully restore
639 5-oxomilbemycin production (Fig. 6D). These results suggested that SBI00792 was a
640 positive regulator for 5-oxomilbemycin biosynthesis, whose detailed regulatory
641 mechanism required to be further explored. Collectively, we demonstrated that
642 *Fn*Cpf1 is capable of HDR-mediated targeted gene disruption in *S. hygroscopicus*
643 SIPI-KF and can serve as a useful alternative for genome editing in other
644 *Streptomyces* strains that cannot utilize the *Sp* CRISPR-Cas9 system.
645

646 **Discussion**

647 In this study, the *Fn* CRISPR-Cpf1-based genome editing tool (cloned in an all-in-one
648 plasmid) was successfully established to efficiently cleave *Streptomyces* genome,
649 there by promoting high-efficiency HDR- and NHEJ-dependent genetic engineering.
650 Interestingly, we found that without the addition of inducer, the expression of *Sp*Cas9
651 driven by the inducible promoter *tipAp* but not of *Fn*Cpf1 is enough for efficient
652 genomic DNA cleavage in *S. coelicolor*, suggesting that high expression levels of
653 *Fn*Cpf1 are required for genome editing (26). Guided by previous studies performed
654 in mammalian cell, crRNA used in this work was designed to contain a 19 nt direct
655 repeat and a 23 nt spacer proceeded by 5′-TTV-3′ PAM (34, 35). Here, we
656 demonstrated that the PAM sequence could be relaxed from 5′-TTV-3′ to 5′-YTV-3′

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(Y=T/C and V=A/G/C) for DNA cleavage in *S. coelicolor*. However, 5′-CTV-3′ PAM cannot mediate efficient genome editing due to its halfway DNA cleavage activity compared to 5′-TTV-3′ PAM. On the other hand, differing from a previous report that *Fn*Cpf1 enables complete DSB activity *in vitro* when paired with 17-25 nt spacer crRNA (53), our *in vivo* study revealed that the shortest length of spacers must be 19 nt for efficient genome cleavage. Therefore, the PAM sequence and crRNA spacer lengths require to be precisely determined in a specific organism before large scale genome editing are conducted. Additionally, compared to the original crRNA spacer length (23-25 nt), the truncated spacer could reduce the price of primer synthesis for expression of a long crRNA array used in multiplex gene editing or expression regulation.

*Fn*Cpf1 can induce a site-specific DSB in the genome, which relies on the repair by homologous recombination or NHEJ. In the present study, high effciencies (75-95%) of *Fn*Cpf1-based system for precise single and double genomic loci editing in the presence of HDR templates were achieved, which is comparable to *Sp*Cas9-assisted editing system in *Streptomyces* (26, 27). Therefore, it is reasonable to believe that *Fn*Cpf1-mediated HDR could also be expanded to mediate deletion of large-size secondary metabolites BGCs (10-100 kb) or precise point mutations, as previously reported for *Sp*Cas9 (26). On the other hand, NHEJ-mediated DSBs repair results in stochastic indels at the target loci, but this strategy is very simple and convenient due to no need for homology templates. Here, we demonstrated that, in *S. coelicolor*, reconstituted NHEJ systems from actinomycetes, including Msm-LK and Sda-LK,

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679 exhibited higher DSBs repair activities than that of Ppu-LK from the Gram-negative
680 bacteria *P. putida*. *Fn*Cpf1 combined with Msm-LK could generate large, random
681 sized deletions in target genes from 0.4 to 1.6 kb. Interestingly, previous studies have
682 showed that only very short indels (1-3 bp) was introduced by *Sp* CRISPR-Cas9
683 combined with *N. carenus* NHEJ system in *S. coelicolor* (25). This difference migh be
684 due to the distinct cleavage mechanisms of these two different class 2 CRISPR-Cas
685 systems, i.e. *Sp*Cas9 generates a PAM-proximal DSB with a blunt end, whereas
686 *Fn*Cpf1 creates a PAM-distal DSB with a 5′-overhang (15, 63). Additionally, the
687 efficiency for the deletion of large-size RED BGC (~28 kb) by *Fn*Cpf1-based
688 Msm-LK system was low (only 10%) in *S. coelicolor*. However, in *E. coli*, the
689 effciencies for deleting 46 kb and 123 kb DNA fragements reached 46% and 36%,
690 respectively, by combining CRISPR-Cas9 with Msm-LK system (47). Therefore, the
691 developed *Fn*Cpf1-based Msm-LK system in *Streptomyes* still required to be further
692 optimized by the approaches, such as fine-tuning the expression of Msm-LK pathway
693 and introducing other more efficient NHEJ systems.

694 In addition to *Fn*Cpf1-mediated *Streptomyces* genome editing tool, in this study, a
695 ddCpf1-based CRISPRi system was also developed for multiplex gene repression.
696 Using this novel CRISPRi system, herein we achieved simulteneous repression of
697 three antibiotic biosynthetic genes at efficiencies of ~70%. Herein, the integrative
698 plasmid pSET152 instead of the replicative plasmid pKC1139 was employed as the
699 backbone for the expression of the CRISPRi system, which would theoretically
700 enhance the stability of ddCpf1-based gene expression control. Additionally, the

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701 *Φ*C31 integration system in pSET152 has a broad host range for *Streptomyces* species
702 (64), ensuring that our CRISPRi system can be widely used in the genus *Streptomyces*.
703 Similar as the data obtained in *E. coli*, crRNA recognizing the T strand but not the NT
704 strand of target genes could achieve efficient ddCpf1-based gene repression in
705 *Streptomyces* (42, 65). However, we found that the crRNA order in a single array
706 obviously influenced repression efficiency for *actIorf1* involved in ACT biosynthesis
707 at 72 h after growth, which was not identical to the previously reported in *E. coli* (42).
708 We speculated that the effect of different crRNA orders on repression efficiency of
709 *actIorf1* transcription might be ascribed to the below three reasons. First, microarray
710 analysis showed that *actIorf1* is strongly transcribed at 72 h after growth, but the other
711 two tested genes *redX* and *cpkA* are basically not transcribed at that time (66). Second,
712 ddCpf1 and crRNA array were under the control of the promoters *ermEp** and
713 *kasOp**, respectively. Previous studies showed that the mRNA levels of these two
714 promoters significantly decreased in the later growth stages (60-96 h) of *S. coelicolor*
715 (51). Therefore, the expressional levels of both ddCpf1 and crRNA array would be
716 very low at 72 h, which might be the reason for the strong leakiness in *actIorf1*
717 expression. Finally, the low expressional level of ddCpf1 might also be insufficient
718 for completely processing crRNA array, which thus led to the lower level of the
719 crRNA for targeting *actIorf1* in the middle or last unit of array compared to that in the
720 first unit of array. Nevertheless, we believe that ddCpf1-based CRPISRi system can
721 be broadly applicable to the study of essential genes and complex regulatory network,
722 as well as quick screening of functional genes in *Streptomyces* (67).

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723 Previously, it has been repeatedly reported that no transformant could be obtained
724 when the constitutive or leaky expression of a native *Sp*Cas9 in *C. glutamicum* (30, 31,
725 68). The toxic effect of *Sp*Cas9 expression could be overcome by three different
726 strategies. First, the plasmid harboring a codon-optimized *cas9* gene based on the bias
727 of *Streptomyces* could be effiectively transformed into *C. glutamicum* due to some
728 unknown reasons (30). Second, two inducible promoters P*tac* and P*prpD2* were
729 utilized to strictly control the expression of the native *cas9* gene (68). The strategy
730 was also employed to overcome the toxicity of constitutive expression of the
731 nulcease-deactivated *Sp*Cas9 (dCas9) in *C. glutamicum* (69). Finally, Jiang and
732 coworkers successfully uitilized the *Fn* CRISPR-Cpf1 system to achieve highly
733 efficient genome editing in *C. glutamicum*, which skillfully bypassed the fine-tuning
734 of *Sp*Cas9 and sgRNA expression (31). In the present study, we found that no
735 exoconjugant could be obtained when pKCCas9(*tipAp*) was introduced into *S.*
736 *hygroscopicus* SIPI-KF possibly due to the leaky expression of the codon-optimized
737 *cas9* under the control of the inducible promoter *tipAp*. Similarly, the toxic effect was
738 effectively addressed by replacing *Sp*Cas9 with *Fn*Cpf1. Alternatively, other inducible
739 promoters, including tetracycline-, glycerol- and cumate-inducible systems, may be
740 used to strictly control *Sp*Cas9 expression in *S. hygroscopicus* SIPI-KF (70). In
741 contrast, the constitutive expression of *Fn*Cpf1 without a customized crRNA cassette,
742 resulted in serious cell toxicity in another industrial strain *S. pristinaespiralis*
743 HCCB10218 compared to *Sp*Cas9. These results suggested that the two well-known
744 Cas proteins exhibited distinct toxicity effects in several specific *Streptomyces* strains.

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745 Notably, bioinformatical analysis showed that there was no any typical *Sp*Cas9 or
746 *Fn*Cpf1 crRNA sequence in the above two industrial *Streptomyces*. Therefore, *Sp*Cas9
747 or *Fn*Cpf1 toxicity is unlikely to be explained due to the Cas endonuclease-mediated
748 target or off-target DSBs. On the other side, introduction of both dCas9- and
749 ddCpf1-expressing plasmids reduced the numbers of exoconjugants compared to
750 pKC1139 in both *S. pristinaespiralis* HCCB10218 and *S. hygroscopicus* SIPI-KF, (Fig.
751 S5). Therefore, we speculated that *Sp*Cas9 or *Fn*Cpf1 may bind tightly to PAMs in the
752 absence of crRNA, which thus results in different global transcriptional expression
753 and influences bacterial growth at varying degrees. The general toxic effects of Cas
754 proteins should be further explored in the future. Nevertheless, from a practical
755 perspective, *Fn*Cpf1 provides a useful complement to *Sp*Cas9 for high-efficiency
756 genome editing and multiplex transcriptional regulation in *Streptomyces*. Diverse
757 CRISPR toolbox will significantly advance metabolic engineering and natural
758 products discovery in different kinds of industrial *Streptomyces* strains as well as
759 other actinomycetes (9, 22, 71).

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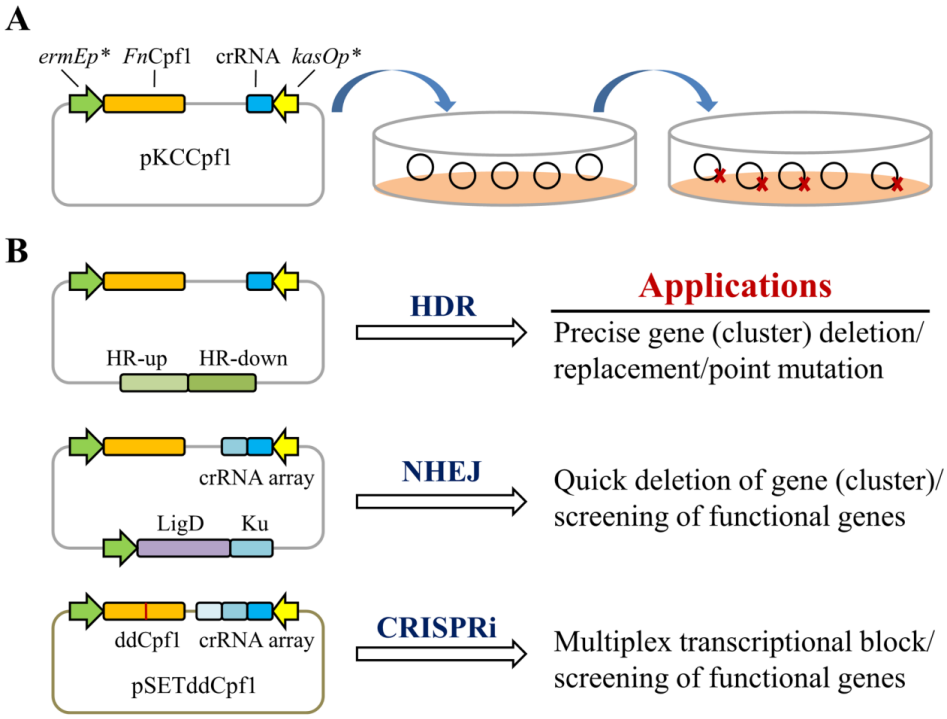


Fig. 1. Overview of *Fn*Cpf1-assisted multiplex genome editing and transcriptional repression in *Streptomyces*. (A) Design of *Fn*Cpf1-mediated genome editing plasmid. Two strong promoters *ermEp** and *kasOp** were used to drive the expression of *Fn*Cpf1 and crRNA, respectively. (B) Broad applications of *Fn*Cpf1-assisted genome editing and ddCpf1-based gene repression in *Streptomyces*. HR, homology arm; HDR, homology-directed repair; NHEJ, non-homologous end joining; CRISPRi, CRISPR interference.

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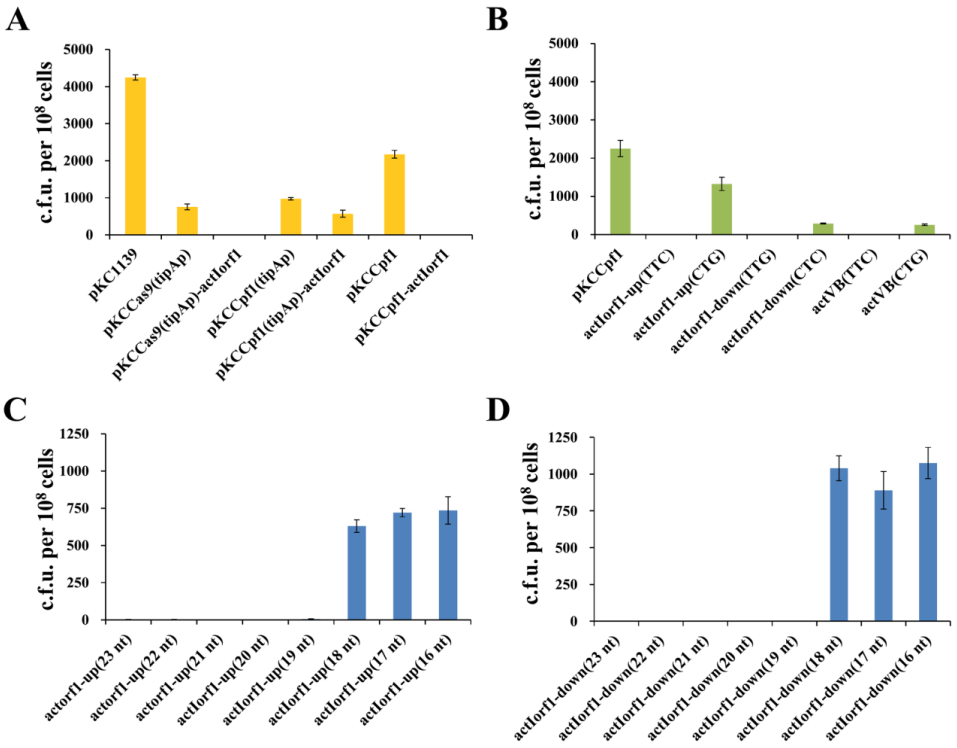


Fig. 2. Optimization of *Fn*Cpf1-mediated genome editing system for targeted DNA cleavage in *S. coelicolor*. (A) Growth of *S. coelicolor* expressing *Sp*Cas9 or *Fn*Cpf1, with or without the combined expression of crRNA targeting *actIorf1*. The co-expression of *Fn*Cpf1 under the control of *ermEp** but not *tipAp* and target-specific crRNA (pKCCpf1-*actIorf1*) can efficiently induce DSBs and thus leads to significantly reduced exoconjugant survival. The plasmids pKC1139 and pKCCas9(*tipAp*) were used as the negative controls and pKCCas9(*tipAp*)-*actIorf1* as the positive control. c.f.u. represents colony-forming unit. (B) Effects of different PAM sequences on *Fn*Cpf1 cleavage activity. The plasmid pKCCpf1 was used as the control. The bases in bracket represent the PAM sequences. Six PAMs located in two genes, *actIorf1* and *actVB*, were tested. (C, D) Effects of spacer lengths on *Fn*Cpf1 cleavage activity. The numbers in bracket represent the spacer lengths.

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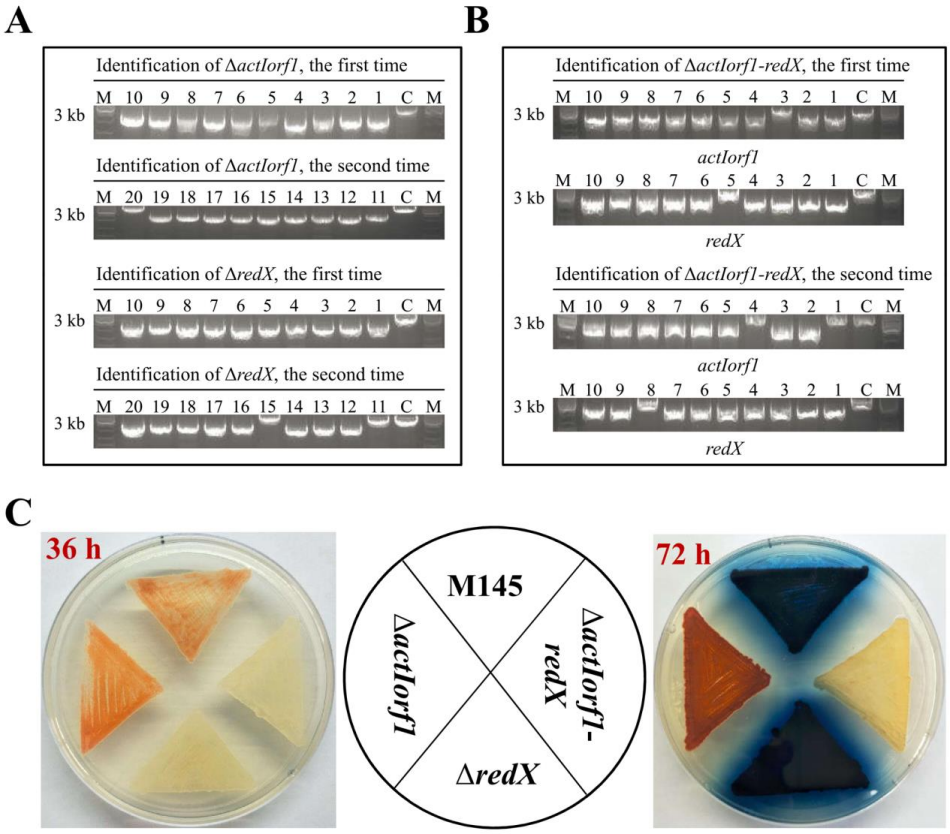


Fig. 3. *Fn*Cpf1-assisted in-frame deletions of single gene and simultaneous double genes. (A) Identification of *actIorf1* and *redX* deletion mutants (Δ*actIorf1* and *ΔredX*). Ten exoconjugants were selected and verified by PCR. The experiments were repeated twice. C and M represent the wild-type *S. coelicolor* M145 and 1 kb DNA ladder, respectively. (B) Identification of *actIorf1* and *redX* double deletion mutants (Δ*actIorf1-redX*). (C) Effects of single or double deletion of *actIorf1* and *redX* on ACT and RED biosynthesis. The mutants were grown on R2YE plates and the images were photographed from the back side at 36 and 72 h.

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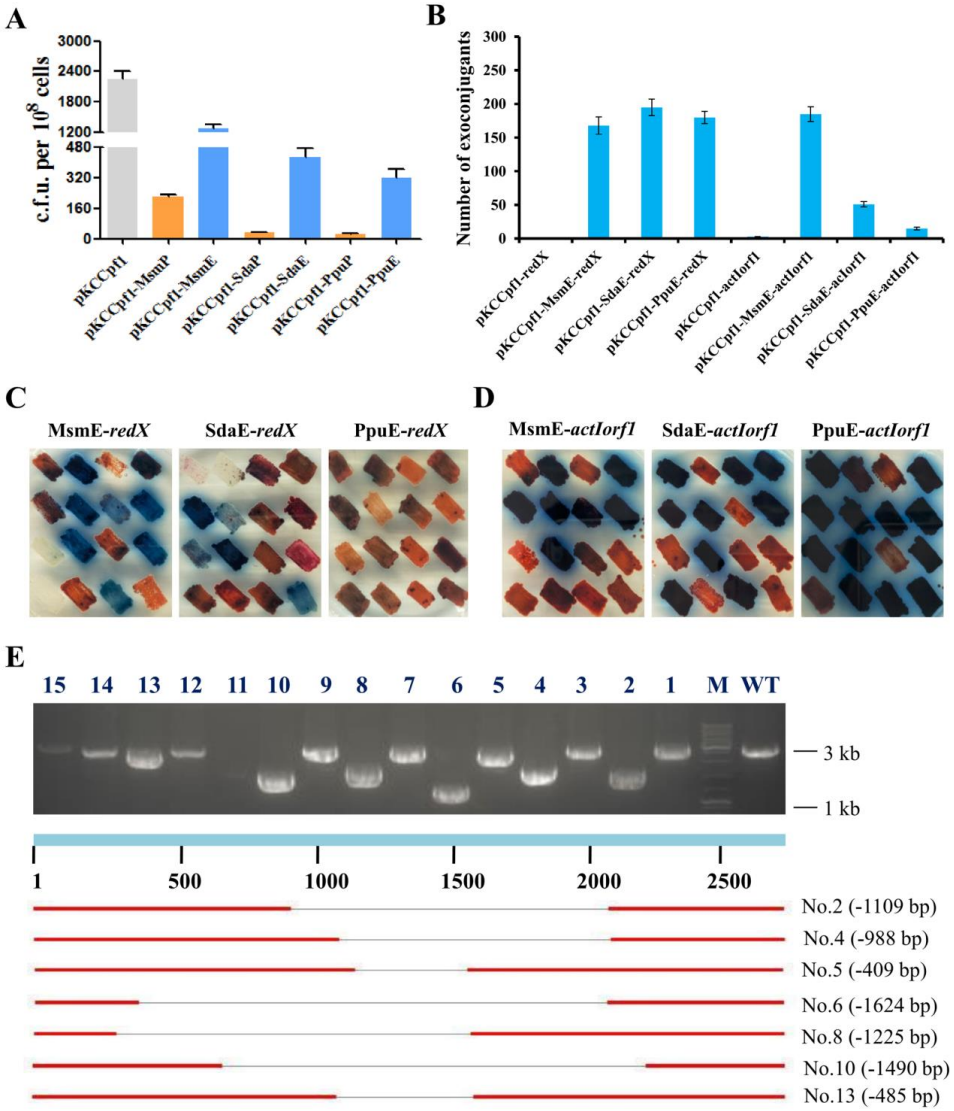


Fig. 4. Rapid gene inactivation by the CRISPR/Cpf1 system combined with reconstituted NHEJ pathways in *S. coelicolor*. (A) Growth of *S. coelicolor* expressing *Fn*Cpf1 with three different reconstituted NHEJ pathways. Based on pKCCpf1, six editing plasmids containing the reconstituted NHEJ pathways, namely pKCCpf1-MsmP, pKCCpf1-MsmE, pKCCpf1-SdaP, pKCCpf1-SdaE, pKCCpf1-PpuP, pKCCpf1-PpuE, were constructed, in which the co-expression of *ligD* and *ku* genes

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1012 from *M. smegmatis*, *S. daghestanicus* or *P. putida* was drived by the strong promoter
1013 *gadphp* or *ermEp**. c.f.u. represents colony-forming unit. (B) Effects of
1014 *Fn*Cpf1-induced DSBs repair by three different NHEJ pathways on two target loci,
1015 *redX* and *actIorf1*. (C, D) Phenotypic analysis of *redX* or *actIorf1* inactivation by
1016 *Fn*Cpf1 combined with the NHEJ systems. The strains with *redX* inactivation only
1017 produced blue-pigmented ACT on R2YE plates (imaged at 48 h). In contrast, these
1018 strains with *actIorf1* inactivation only produced red-pigmented RED on R2YE plates
1019 (imaged at 72 h). 15 random exoconjugants were randomly picked for phenotypic
1020 observations and the strain in the top right corner represented the wild-type in each
1021 group. Due to the good data repeatability, we only showed the results of the first
1022 phenotypic analysis. (E) Large random sized deletions in the *redX* gene caused by
1023 *Fn*Cpf1 combined with the Msm-NHEJ system.

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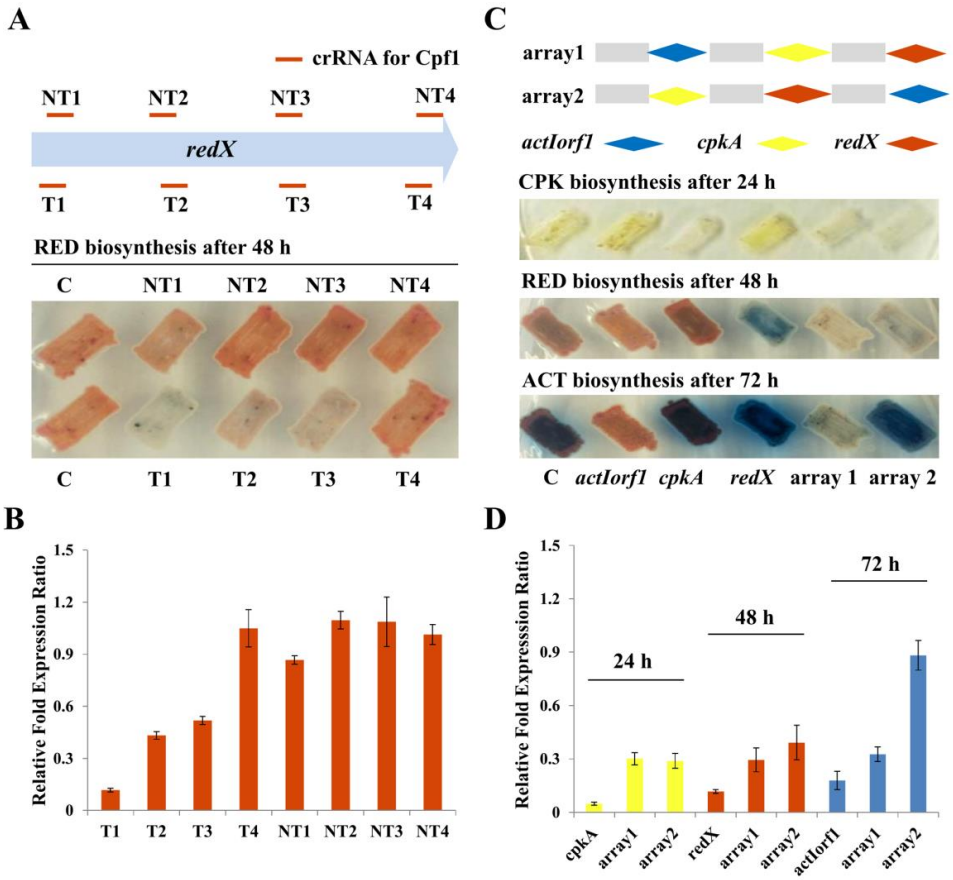


Fig. 5. ddCpf1-mediated multiplex gene repression in *S. coelicolor*. (A, B) ddCpf1-mediated efficient block of *redX* transcriptional elongation. crRNAs targeting the T or NT strand of the *redX* coding region are illustrated. The phenotypic (A) and transcriptional analysis (B) were carried out after growth on R2YE agar for 48 h. Error bars indicate the standard deviations from three independent biological replicates. (C, D) Multiplex gene repression with ddCpf1 and a single customized crRNA array. Three target genes (*cpkA*, *actIorf1* and *redX*) were selected for simultaneous repression. The order of crRNAs targeting three genes is designed as array 1 or array 2. The transcriptional levels of each gene were analyzed in the engineered strains expressing ddCpf1 with individual crRNA or crRNA arrays, and

1035 the strain only expressing ddCpf1 was used as the control. The phenotypic and
1036 transcriptional analysis was carried out for *cpkA*, *redX* and *actIorf1* at 24, 48 and 72 h,
1037 respectively.

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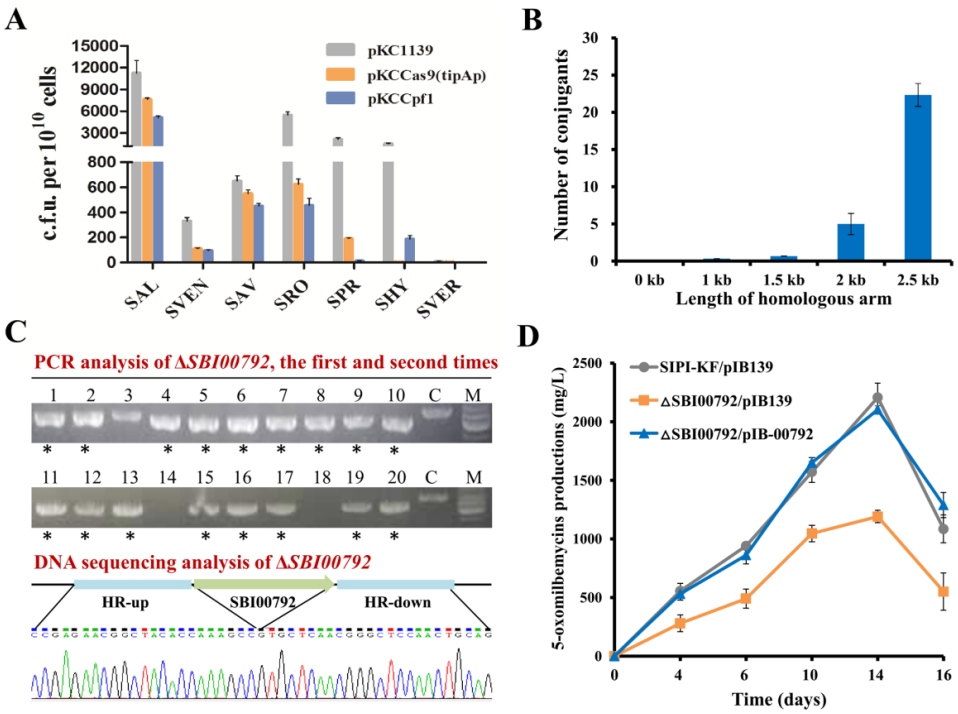


Fig. 6. Applicability of the CRISPR-Cpf1 system in multiple model and industrial *Streptomyces*. (A) Growth of seven different *Streptomyces* species only expressing *Sp*Cas9 or *Fn*Cpf1. pKC1139 was used as the control. c. f. u. represents colony-forming unit. SAL: *S. albus* J1074, SVEN: *S. venezuelae* ATCC10712, SAV: *S. avermitilis* NRRL8165, SRO: *S. roseosporus* SIPI-DT51, SPR: *S. pristinaespiralis* HCCB10218, SHY: *S. hygroscopicus* SIPI-KF, SVER: *S. verticillus* SIPI-BL. (B) Effects of homologous arm lengths on the exoconjugant numbers when deleting *SBI00792* in *S. hygroscopicus* SIPI-KF. (C) PCR and DNA sequencing analysis of the *SBI00792* deletion mutant (Δ*SBI00792*). C and M represent the parental strain KF and 1 kb DNA ladder, respectively. (D) 5-oxomilbemycin A3/A4 production by the Δ*SBI00792* mutant. Fermentation samples from the three *S. hygroscopicus* strains, including SIPI-KF/pIB139, the Δ*SBJ00792* mutant with pIB139 (Δ*SBJ00792*/

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1051 pIB139), and the complemented strain Δ*SBJ00792*/pIB-00792, were collected at 4, 6,

1052 10, 14 and 16 days.

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