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CRISPR/dCas9-Mediated Multiplex Gene Repression in *Streptomyces*

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Streptomycetes are Gram-positive bacteria with the capacity to produce copious bioactive secondary metabolites, which are the main source of medically and industrially relevant drugs. However, genetic manipulation of *Streptomyces* strains is much more difficult than other model microorganisms like *Escherichia coli* and *Saccharomyces cerevisiae*. Recently, CRISPR/Cas9 or dCas9-mediated genetic manipulation tools have been developed and facilitated *Streptomyces* genome editing. However, till now, CRISPR/dCas9-based interference system (CRISPRi) is only designed to repress single gene expression. Herein, the authors developed a novel CRISPRi system for multiplex gene repression in the model strain *Streptomyces coelicolor*. In this system, the integrative plasmid pSET152 is used as the backbone for the expression of the dCas9/sgRNA complex and both dCas9 and sgRNAs are designed to be under the control of constitutive promoters. Using the integrative CRISPRi system, the authors achieved efficient repression of multiple genes simultaneously; the mRNA levels of four targets are reduced to 2–32% of the control. Furthermore, it is successfully employed for functional gene screening, and an orphan response regulator (RR) (encoded by *SCO2013*) containing an RNA-binding ANTAR domain is identified being involved in bacterial growth. Collectively, this integrative CRISPRi system is very effective for multiplex gene repression in *S. coelicolor*, which could be extended to other *Streptomyces* strains for functional gene screening as well as for metabolic engineering.

1. Introduction

The CRISPR/Cas system (Clustered regularly interspaced short palindromic repeats/CRISPR associated nuclease), which is the adaptive immune system widely distributed among bacteria and archaea,[1] has been extensively developed as a very powerful technology for genome editing in both eukaryotes and prokaryotes.[2,3] A simplified version of the type II CRISPR/Cas9 system from *Streptococcus pyogenes*, which only consists of the Cas9 nuclease and a chimeric single guide RNA (sgRNA), is currently the most popular genome editing tool.[4,5] The sgRNA, which harbors a 20 nt protospacer that specifically recognizes and binds to the target sequence, and a 42 nt structural Cas9-binding handle, directs Cas9 to the host genome, and creates a DNA double-strand break (DSB). Deriving from CRISPR/Cas9, the CRISPR interference (CRISPRi) system containing a nuclease-deficient Cas9 (dCas9) coexpressed with a sgRNA, has also been established for efficient gene repression.[6] This CRISPRi platform has now been widely used for identifying functional genes as well as for metabolic engineering in microbes, such as *Escherichia coli*,[7] *Corynebacterium glutamicum*,[8] *Saccharomyces cerevisiae*,[9,10] *Clostridium*,[11] *Synechocystis*,[12,13] and *Mycobacteria*.[14]

Streptomycetes are soil-dwelling, Gram-positive bacteria with high GC content genomes, belonging to the family of Streptomycetaceae and the order of Actinomycetales. They have strong capability to produce copious medically and industrially important bioactive secondary metabolites, such as antibiotics, immunosuppressants, anti-tumor drugs, and herbicides.[15] However, in the past decades, it becomes more and more difficult to find novel secondary metabolites by traditional tools. With the progress of microbial genome projects, a great number of *Streptomyces* genome sequences have been sequenced and numerous silent secondary metabolite biosynthetic gene clusters (BGCs) are being discovered.[16] These BGCs are likely to encode secondary metabolites with novel structures, which are a valuable source for drug discovery. However, in comparison with other model organisms, such as

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S. cerevisiae and *E. coli*, few genetic manipulation tools have been established in *Streptomyces*,[17–19] which hampered the functional genome research of this kind of industrially important bacteria.

With the rapid development of the emerging CRISPR/Cas9-based technologies, several efficient CRISPR/Cas9-assisted genetic manipulation tools have been recently developed in *Streptomyces* as well, which will be very helpful to address the above challenges. Until now, CRISPR/Cas9-based *Streptomyces* genome editing tools have been established by four different labs worldwide, enabling efficient genome editing in several *Streptomyces* strains, including point mutations, deletions of single or double genes, and deletions of single or double BGCs.[20–23] Furthermore, a dCas9-mediated CRISPRi system has also been developed in the model strain *S. coelicolor*, allowing for efficient repression of single genes in a reversible manner.[22] In this system, the backbone for expression of the dCas9/sgRNA complex is a temperature sensitive pSG5 replicon and the *dCas9* gene is under the control of the thiostrepton inducible promoter (*tipA*p). However, repression of multiple genes simultaneously in *Streptomyces* has not been reported yet.

In this study, we report the development of a novel dCas9-based CRISPRi system in *S. coelicolor*, which was cloned in the chromosomal integrative vector pSET152 and the *dCas9* gene and sgRNAs were designed to be under the control of strong constitutive promoters, *ermE**p, and *j23119*, respectively. Using this CRISPRi system, we achieved efficient repression of four genes simultaneously, and a two-component system (TCS) response regulator (RR) involved in regulation of bacterial growth was functionally identified. dCas9, which is the catalytically dead *S. pyogenes*-derived Cas9 with point mutations in the HNH and RuvC domains (D10A and H840A),[6] was codon-optimized for *Streptomyces* and synthesized. To facilitate the construction of sgRNA expression arrays for multiplex gene repression, the Golden Gate Assembly method was adopted.[24,25] Collectively, we developed a new CRISPRi system for efficient multiplex gene repression in *S. coelicolor*, which will surely accelerate functional gene screening. This system may be further extended to other streptomycetes for genetic manipulation.

2. Experimental Section

2.1. Strains, Plasmids, Media, and Culture Conditions

The strains and plasmids used in this study are listed in Table S1, Supporting Information. *Escherichia coli* strains were cultivated on LB agar medium or in LB liquid medium.[26] *Streptomyces coelicolor* M145 and its derivatives were grown on MS, R2YE or minimal medium (MM) supplemented with 75 mM glutamate (Glu) for phenotypic analysis of antibiotics production.[27,28] MS medium supplemented with 10 mM $MgCl_2$ was used for intergeneric conjugation between *E. coli* and *S. coelicolor*.[28] MS medium was also used for preparing spore suspensions of *S. coelicolor*. Apramycin (50 $\mu g\,mL^{-1}$), kanamycin (50 $\mu g\,mL^{-1}$), chloramphenicol (25 $\mu g\,mL^{-1}$), ampicillin (100 $\mu g\,mL^{-1}$), and nalidixic acid (25 $\mu g\,mL^{-1}$), were added when necessary.

2.2. Construction of the CRISPRi Plasmids

The plasmid pSET-dCas9, in which the codon optimized *dCas9* gene was cloned in the integrative vector pSET152 and its expression was under the control of the strong constitutive promoter *ermE**p, was constructed as follows. Firstly, the *dCas9* gene optimized for expression in *S. coelicolor* was synthesized and cloned into the plasmid pGH (Generay, Shanghai, China), generating the plasmid pGH-dCas9. Using the primers dCas9-F and dCas9-R, the *dCas9* gene was amplified from pGH-dCas9. After digestion with NdeI and EcoRI, the PCR product was cloned into the plasmid pIB139 to yield pIB-dCas9. Secondly, the *ermE**p promoter and the *dCas9* gene was amplified from pIB-dCas9 with the primer pair ermEp-dCas9-F and ermEp-dCas9-R and then inserted into pSET152 between XbaI and EcoRV to generate pSET-*ermE**p-dCas9. Thirdly, the *fd* terminator was amplified with the primers fd-F and fd-R from the plasmid pIJ6902 and ligated into pSET-*ermE**p-dCas9 by the Gibson assembly method,[29] generating pSET-*ermE**p-dCas9-fd (abbreviated to pSET-dCas9) (Figure S1, Supporting Information). The primers are listed in Table S2, Supporting Information.

Based on pSET-dCas9, the CRISPRi plasmids harboring single or multiple sgRNA expression cassettes were constructed according to the method described in supplementary materials. The construction workflows of these CRISPRi plasmids are presented in Figure S2 and S3, Supporting Information.

2.3. Determination of CDA Production

CDA production was determined by a bioassay using *Bacillus subtilis* as an indicator strain according to the method described by Kieser *et al.*,[28] with some modifications. In brief, after the germination of *S. coelicolor* spores, the same amounts of spores were spotted on R2YE agar medium without $CaCl_2$. The agar plates were grown at 30 °C for 48 h, followed by being overlaid with soft LB agar medium containing $CaCl_2$ at a concentration of 12 mM and 1% (v/v) of the indicator strain, *B. subtilis* (cultured overnight in LB liquid medium). Soft LB agar medium containing only *B. subtilis* was overlaid as a control. After incubation for another 12 h, a clearance zone of inhibition around *S. coelicolor* strains indicates CDA production. The inhibition activity of CDA is dependent on Ca^{2+}. So, when no $CaCl_2$ was added in the soft LB agar medium, no clearance zone of inhibition would be observed.

2.4. Quantification of mRNA

Real-time RT-PCR (qRT-PCR) analysis was conducted as reported previously.[27] Briefly, spores of *S. coelicolor* strains were plated on agar plates of R2YE or on MM supplemented with 75 mM Glu. Cultures were harvested at different time points (mainly at 36 and 48 h) and frozen and then ground into powder in liquid nitrogen. RNA samples were extracted with the Ultrapure RNA Kit (Cwbio, Shanghai, China), followed by digestion with DNase I (Takara, Dalian, China) to remove residual chromosomal DNA. The primers used are listed in Table S2, Supporting Information in the supplemental

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materials. qRT-PCR was carried out in a MyiQ2 thermal cycler (Bio-Rad), using a SYBR Green PCR premix kit (Bio-Rad). PCR analysis was carried out in triplicate (technical replicates). The mRNA levels of tested genes were normalized to *hrdB* (an internal control) and then determined by the $2^{-\Delta\Delta CT}$ method.[30] The transcription of each gene in the control strain (containing pSET-dCas9) at 30 h was arbitrarily assigned a value of 1. The experiments were conducted with three independent RNA samples (biological replicates). Error bars indicate the standard deviations (SD).

2.5. Construction of the Δ*SCO2013* Mutant Strain

The Δ*SCO2013* mutant with an in-frame deletion of *SC02013* was constructed by traditional homologous recombination.[28] Briefly, the upstream and downstream regions (1447 and 1435 bp, respectively) of *SCO2013* were amplified from the genome of *S. coelicolor* M145 using primers SCO2013-up-F/R and SCO2013-down-F/R. After digestion with HindIII/BamHI and BamHI/EcoRI, two DNA fragments were cloned into the replication temperature sensitive plasmid pKC1139 between HindIII and EcoRI to yield pKC-*SCO2013*. The obtained plasmid was transformed into *E. coli* ET12567/pUZ8002 and subsequently introduced into *S. coelicolor* M145 by conjugal transfer. Exoconjugants were passaged on MS agar plates containing apramycin at 37 °C to obtain single crossover strains. Then, they were passaged in YEME liquid medium at 30 °C to obtain double crossover strains. The apramycin-sensitive strains were selected by replica plating and the Δ*SCO2013* mutant was identified by PCR with primers SCO2013-ID-F and SCO2013-ID-R, followed by DNA sequencing.

2.6. Complementation of the Δ*SCO2013* Mutant

The open reading frame (ORF) of *SCO2013* was amplified with primers SCO2013-ex-F and SCO2013-ex-R. After digestion with NdeI and EcoRI, the PCR product was ligated into the plasmid pIB139 between NdeI and EcoRI to yield the complemented plasmid pIB-*SCO2013*. The constructed plasmid was transferred into the Δ*SCO2013* mutant by conjugal transfer to obtain the complemented strain Δ*SCO2013*/pIB-*SCO2013*. The empty plasmid pIB139 was also transferred into the Δ*SCO2013* mutant and the parental strain M145 to obtain two control strains, M145/pIB139 and Δ*SCO2013*/pIB139, respectively.

3. Results

3.1. Construction of an Integrative dCas9-Based CRISPRi System for *Streptomyces*

Herein, the integrative plasmid pSET152 was used as the backbone for coexpression of the dCas9/sgRNA complex. In this CRISPRi system for *Streptomyces*, the expression of the *dCas9* gene is driven by the strong constitutive promoter *ermE**p and terminated by the *fd* terminator. The *dCas9* gene was codon optimized for expression in the model strain *S. coelicolor*, which is derived from the Cas9 nuclease of *S. pyogenes* with point mutations in the HNH and RuvC domains (D10A and H840A).[6] Each sgRNA was designed to be transcribed by the promoter *j23119* from *E. coli* and terminated by the *T0* terminator. The activity of *j23119* has been verified previously as a strong promoter in *S. coelicolor*.[21]

Many studies showed that sgRNAs targeting the promoter regions are very effective for gene repression irrespective of whether they are targeted to the template (T) or nontemplate strand (NT); however, when sgRNAs are targeted to the coding region, effective repressive effects are only observed on the NT strand.[6,22,31,32] Considering that the promoter regions of most genes in *Streptomyces* are unknown, in this study, all sgRNAs were designed to target the NT strand of gene coding regions, which were conducted by the software CRISPRy to minimize off-target effects.[33] To facilitate the construction of the CRISPRi plasmids harboring single sgRNAs with different specific 20 nt target sequences, two restriction sites (SpeI and EcoRI) were inserted after the *j23119* promoter and *T0* terminator, respectively. The schematic map of the CRISPRi system is illustrated in Figure S4A, Supporting Information.

3.2. Feasibility Study of the Developed CRISPRi System in *S. coelicolor*

To test whether our CRISPRi system could efficiently repress gene transcription, the *actII4* gene (*SCO5085*), which encodes a pathway-specific activator for the biosynthesis of the blue-colored antibiotic actinorhodin (ACT) from *S. coelicolor*,[34] was selected as a reporter gene. The transcriptional level of *actII4* correlates well with ACT production,[35] which facilitates the detection of repression effects of *actII4* by only observing the changes of blue color (ACT). Three different sgRNAs were designed for *actII4* repression: NT-S1 and NT-S2 targeting the coding region of *actII4* (+30 and +61 bp from the start codon ATG, respectively), and Out-S0 targeting outside of the gene (Figure S4B, Supporting Information). These three sgRNAs were inserted into the plasmid pSET-dCas9 carrying the dCas9 expression cassette (Figure S1, Supporting Information), resulting in pSET-dCas9-*actII4*-NT-S1, pSET-dCas9-*actII4*-NT-S2, and pSET-dCas9-*actII4*-Out-S0, respectively. The plasmid pSET-dCas9 (containing the *dCas9* gene only) and the empty plasmid pSET152 were used as controls. Five plasmids were individually introduced into the wild-type *S. coelicolor* strain M145 by conjugal transfer. Exoconjugants were streaked on R2YE agar medium to observe ACT production. As shown in Figure S4C, Supporting Information, in strains harboring the CRISPRi plasmid, pSET-dCas9-*actII4*-S1 or pSET-dCas9-*actII4*-NT-S2, targeting the 5′-end of *actII4* coding region, almost no ACT production was observed by visual inspection. However, no obvious reduction in ACT production was observed in clones containing pSET-dCas9-*actII4*-Out-S0 targeting outside of the target gene. As controls, clones with pSET-dCas9 (expressing dCas9 only) or the empty plasmid pSET152 still produced a high level of ACT and little difference of growth was observed between these two strains, indicating dCas9 has no effect on bacterial growth and ACT biosynthesis.

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We next checked *actII4* repression at the transcriptional level by qRT-PCR. *S. coelicolor* strains were grown on R2YE agar medium and cultures were harvested at two time points (36 and 48 h) for RNA isolation. As shown in Figure S4D, Supporting Information, consistent with the phenotypic changes in ACT production, both pSET-dCas9-*actII4*-NT-S1 and pSET-dCas9-*actII4*-NT-S2 resulted in dramatically reduced transcription of *actII4*. Compared with the control (pSET-dCas9), the mRNA levels of *actII4* in the strain with pSET-dCas9-*actII4*-NT-S1 were reduced to 15% (36 h) and 3% (48 h); and were downregulated to 18% and 5% in the strain with pSET-dCas9-*actII4*-NT-S2 at 36 and 48 h, respectively. As expected, only slight downregulation of *actII4* (≈70% of the control) was observed in the strain with pSET-dCas9-*actII4*-Out-S0 (targeting outside of the gene) at 48 h, but the repression extent was much lower than the strains with sgRNAs targeting the coding region. The results presented above clearly demonstrated that our CRISPRi system could be used for efficient gene repression in *S. coelicolor*, and further confirmed that sgRNAs targeting the coding regions of target genes on the NT strand are very effective for gene repression as reported in other bacteria.[6,22,31,32]

3.3. Multiplex Gene Repression by the Integrative CRISPRi System in *S. coelicolor*

To investigate simultaneous multiple gene repression using this CRISPRi system in *S. coelicolor*, four genes, namely, *actI1* (*SCO5087*), *redQ* (*SCO5887*), *cdaPS1* (*SCO3230*), and *cpkA* (*SCO6275*), were selected. They encode a polyketide beta-ketoacyl synthase alpha subunit, an acyl carrier protein, a non-ribosomal peptide synthetase (NPRS), and a type I polyketide synthase essential for the biosynthesis of four known antibiotics, including the blue-colored ACT, red-pigmented undecylprodigiosin (RED), calcium-dependent antibiotic (CDA), and yellow pigmented type I polyketide (yCPK), respectively. These four antibiotics are very easy to be detected, allowing for convenient detection of repression effects of target genes by visual inspection. ACT and RED could be observed on R2YE agar medium according to the characteristic color; CDA can be determined by a bioassay experiment; yCPK could be observed on minimal medium (MM) supplemented with 75 mM Glu. Thus, the repression of *actI1*, *redQ*, *cdaPS1*, and *cpkA*, could be easily checked by observing the alterations in the production of these four antibiotics on agar media described above.

In prior to multiplex repression, single gene repression for these four genes was studied firstly. Four sgRNAs were designed to target the 5′-ends of the coding regions of these four genes on the NT strand and cloned in pSET-dCas9, respectively, generating pSET-dCas9-*actI1*, pSET-dCas9-*redQ*, pSET-dCas9-*cdaPS1*, and pSET-dCas9-*cpkA*. The plasmid pSET-dCas9 was used as a control. After transferring each plasmid into *S. coelicolor* M145 by conjugation, excoconjugants were streaked on different agar medium for phenotypic analysis of antibiotics production. Compared with the control plasmid pSET-dCas9, the CRISPRi plasmids pSET-dCas9-*actI1*, pSET-dCas9-*redQ*, pSET-dCas9-*cdaPS1*, and pSET-dCas9-*cpkA* strongly repressed the production of ACT, RED, CDA, and yCPK, respectively. Nearly no production of these four antibiotics could be observed in strains with the corresponding CRISPRi system by visual inspection, as shown in **Figure 1**A. Further qRT-PCR confirmed the dramatically repression of respective target genes by the CRISPRi plasmids, particularly at 48 h; the mRNA levels of *actI1*, *redQ*, *cdaPS1*, and *cpkA* were reduced to 1%, 9%, 7%, and 0.7%, respectively, compared with the control pSET-dCas9 (Figure 1B). It should be noted that *cpkA* transcription was significantly reduced on both R2YE and Glu-supplemented MM medium (Figure S5, Supporting Information). As such, the alteration in the mRNA level of *cpkA* was only analyzed on R2YE medium in the following study. The different repression ratio of target genes may be mainly ascribed to the difference in repression efficiency of sgRNAs. Collectively, this novel CRISPRi system could be employed to efficiently repress single genes in *S. coelicolor*. It is worth to note that all of four target genes tested here (*actI1*, *redQ*, *cdaPS1*, and *cpkA*) are likely to cotranscribed with downstream genes (Figure S6, Supporting Information), so in theory, repression of these four genes would possibly affect the transcription of downstream genes. Therefore, the phenotypic alterations in antibiotics production of the strains harboring the CRISPRi systems should be the consequence of repression effects of the entire operon where four tested genes are located.

To check whether the CRISPRi system could efficiently repress multiple genes simultaneously, CRISPRi plasmids targeting two, three, and four genes were constructed, respectively. Totally, three CRISPRi plasmids, namely, pSET-dCas9-*actI1-cdaPS1* (targeting *actI1* and *cdaPS1* simultaneously), pSET-dCas9-*actI1-cdaPS1-cpkA* (targeting *actI1*, *cdaPS1*, and *cpkA* simultaneously), and pSET-dCas9-*actI1-cdaPS1-cpkA-redQ* (targeting *actI1*, *cdaPS1*, *cpkA*, and *redQ* simultaneously) were constructed, in which each sgRNA cassette that has been verified to efficiently repress the corresponding gene was assembled together by the Golden Gate Assembly method.[24,25] These four plasmids were introduced individually into the wild-type strain M145 and antibiotics production of exoconjugants was analyzed. We found that, as presented in Figure, 3A, and 4A , antibiotics production were greatly repressed in strains with these CRISPRi systems. However, it could be obviously observed that strains with the CRISPRi plasmids containing an increasing number of sgRNAs (particularly from three to four) produced more and more ACT levels, indicating the repression effect was gradually weakened when more targets were included to be repressed.

Further qRT-PCR analysis revealed that these CRISPRi plasmids could repress two, three, and four genes simultaneously with high efficiencies. When two or three sgRNAs were included in the CRISPRi system, the repression efficiencies were comparable to those with single sgRNAs. However, we observed that similar as the phenotypic changes in ACT production, when four genes were repressed simultaneously, the repression efficiencies of target genes become weaker. Compared with those with single CRISPRi plasmids, in which the mRNA levels of *actI1*, *cdaPS1*, *cpkA*, and *redQ* were reduced to 16%/1%, 16%/9%, 16%/7%, and 3%/0.7% at the tested time points (36/48 h), the degrees of reduction in the mRNA levels of four corresponding target genes were narrowed down to 16%/32%, 30%/19%, 18%/16%, and 6%/2%, respectively (**Figure 2**B, 3B, and 4B). Nevertheless, the integrative CRISPRi system could be used to efficiently repress multiple genes simultaneously in *S. coelicolor*.

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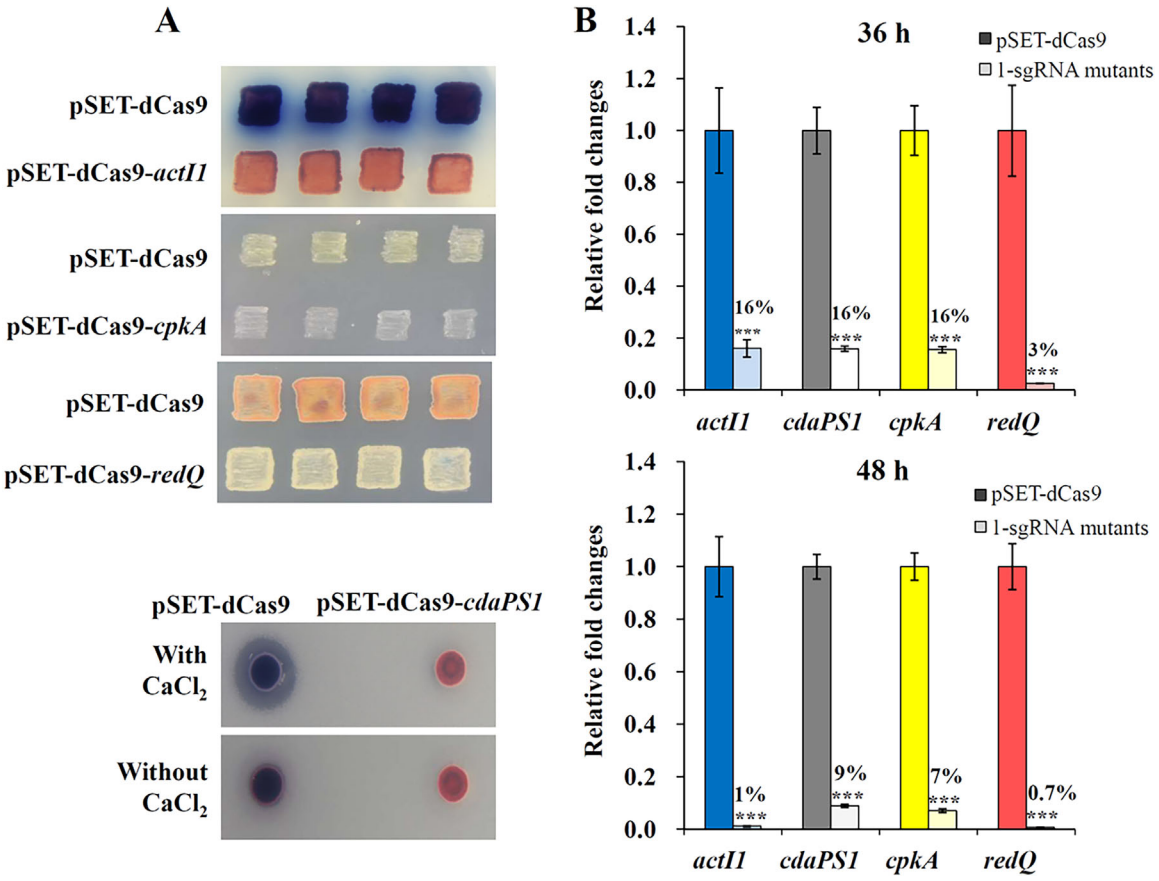


Figure 1. CRISPRi-mediated single gene repression in *S. coelicolor*. A) Visual inspection of antibiotics production of *S. coelicolor* strains harboring the CRISPRi systems targeting four genes, including *actI1*, *cdaPS1*, *cpkA*, and *redQ*, respectively. The strains containing the CRISPRi plasmid pSET-dCas9-*actI1* or pSET-dCas9-*redQ* were grown on R2YE agar for 72 h before imaging. The strains harboring pSET-dCas9-*cpkA* were cultivated on MM supplemented with 75 mM Glu for 24 h before imaging. The clones containing pSET-dCas9-*cdaPS1* were analyzed by a bioassay experiment using *B. subtilis* as an indicator strain. The strains harboring pSET-dCas9 (with the expression of dCas9 only) were used as controls. Four colonies were randomly taken for visual inspection per engineered strain. For the bioassay of CDA production, the inhibition zone of one clone was imaged. B) Transcriptional analysis of *actI1*, *cdaPS1*, *cpkA*, and *redQ* in strains (1-sgRNA mutants) harboring the corresponding CRISPRi plasmid by qRT-PCR. RNA samples were isolated from *S. coelicolor* strains grown on R2YE for 36 and 48 h, respectively. Error bars represent the standard deviations (SD) from three biological replicates. $^{***}p < 0.001$.

3.4. Application of the CRISPRi System to Screen Functional Genes in *S. coelicolor*

The ability of this CRISPRi system to repress multiple genes simultaneously enable us to screen functional genes quickly. In this study, as an example, we sought to repress the genes encoding an important family of proteins with RNA-binding activity that contains the AmiR and NasR Transcriptional Antiterminator Regulator domain (ANTAR) in *S. coelicolor*. This class of proteins are widespread bacterial regulatory proteins,[36–39] in which the ANTAR domain always occurs in combination with a variety of signal-sensing domains (nearly 50% containing a two-component signal transduction receiver domain).[38] The ANTAR domain specifically recognizes a specific RNA motif comprising two tandem stem-loops in the 5′-UTR region of mRNA and utilizes antitermination (by forming an antiterminator step-loop) to regulate target gene expression at the post-transcriptional level.[38] The function and regulatory mechanism of ANTAR-mediated transcription antitermination have been intensively studied in *Enterococcus faecalis*, which are involved in regulation of the expression of the ethanolamine-utilization genes (*eut*).[37,38] In *S. coelicolor*, there are 10 genes encoding ANTAR proteins, and so far, none of them have been investigated.[39] The genetic positions of these genes on the genome are presented in Figure S7, Supporting Information. Interestingly, among these 10 genes, the nucleotide sequences of *SCO0018* and *SCO7829* are completely the same. The biological significance for the duplication of this ANTAR protein-coding gene is unknown yet.

Nine sgRNAs were designed to repress the tested 10 genes, with sgRNA-*SCO0018* targeting *SCO0018*, and *SCO7829* simultaneously. We divided nine sgRNAs into three arrays: array 1 contained three sgRNAs targeting *SCO0018*, *SCO0049*, and *SCO0058*; array 2 targeting *SCO2013*, *SCO4028*, and *SCO4029*; array 3 targeting *SCO7326*, *SCO7340*, and *SCO7423*. After transferring these three multiplex repression plasmids into M145 individually, exoconjugants were streaked on MS medium.

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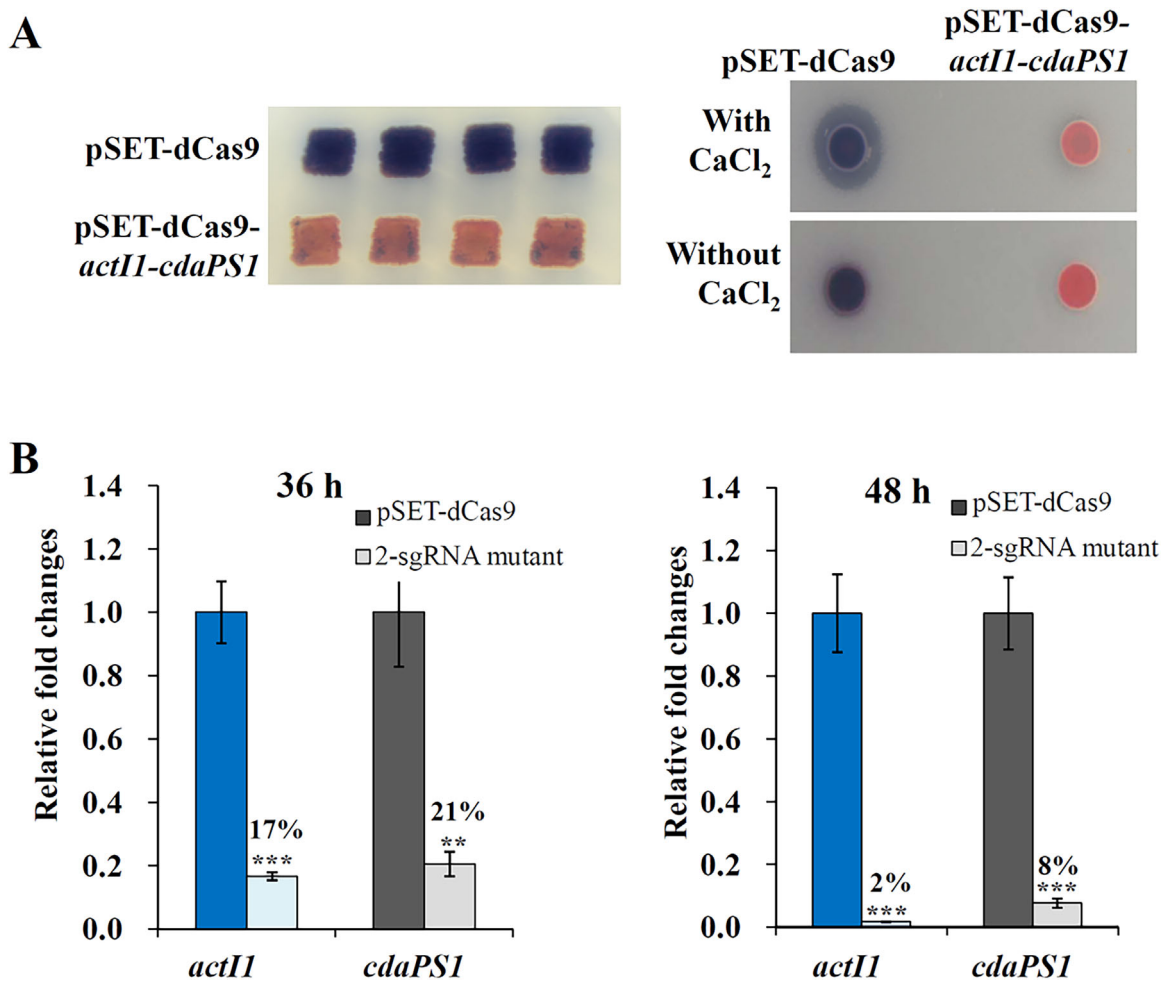


Figure 2. CRISPRi-mediated double gene repression in *S. coelicolor*. A) Visual inspection of antibiotics production of *S. coelicolor* strains harboring the CRISPRi system targeting two genes (*actI1* and *cdaPS1*) simultaneously. The strains containing the pSET-dCas9-*actI1-cdaPS1* were grown on R2YE agar for 72 h before imaging (for the analysis of ACT production) and analyzed by a bioassay experiment using *B. subtilis* as an indicator strain (for the analysis of CDA production). The strains harboring pSET-dCas9 (with the expression of dCas9 only) were used as controls. Four colonies per engineered strain were randomly taken for visual inspection. For the bioassay of CDA production, the inhibition zone of one clone was imaged. B) Transcriptional analysis of *actI1* and *cdaPS1* in the strain (2-sgRNA mutant) harboring the CRISPRi plasmid targeting both genes by qRT-PCR. RNA samples were isolated from *S. coelicolor* strains grown on R2YE for 36 and 48 h, respectively. Error bars represent the standard deviations (SD) from three biological replicates. $^{**}p < 0.01$; $^{***}p < 0.001$.

We found that strains with array 2 grow worse than the control strains (expressing dCas9 only), while strains with array 1 and 3 grew as well as the control. The results implied that one gene or more in array 2 play(s) an important role in *S. coelicolor* growth (**Figure 5**A). Subsequently, three genes in array 2 were repressed independently by CRISPRi. We found that only repression of *SCO2013*, which encodes a response regulator (RR) with an ANTAR domain, resulted in an impaired bacterial growth, while repression of the other two genes had no effect on growth (Figure 5A).

To further prove the role of *SCO2013* in bacterial growth, the mutant Δ*SCO2013* with an in-frame deletion of *SCO2013* was constructed. The obtained mutant exhibited an identical phenotypic alteration in bacterial growth as that of M145 with the CRISPRi plasmid targeting *SCO2013*. Furthermore, introduction of the complementation plasmid could easily rescue the impaired growth of Δ*SCO2013* (Figure 5B). These data clearly indicated that the ANTAR protein encoded by *SCO2013* is important for *S. coelicolor* growth. Therefore, the integrative CRISPRi system developed here is effective for multiplex gene repression and will surely facilitate the screening of functional genes in *S. coelicolor*, which could be also extended to functional genomic research in other *Streptomyces* strains.

4. Discussion

In summary, we constructed a novel integrative dCas9-mediated CRISPRi system, enabling efficient repression of single genes or multiple genes simultaneously in *S. coelicolor*. A step-by-step protocol for this CRISPRi system is provided in the supplementary materials. This technology will be valuable for screening

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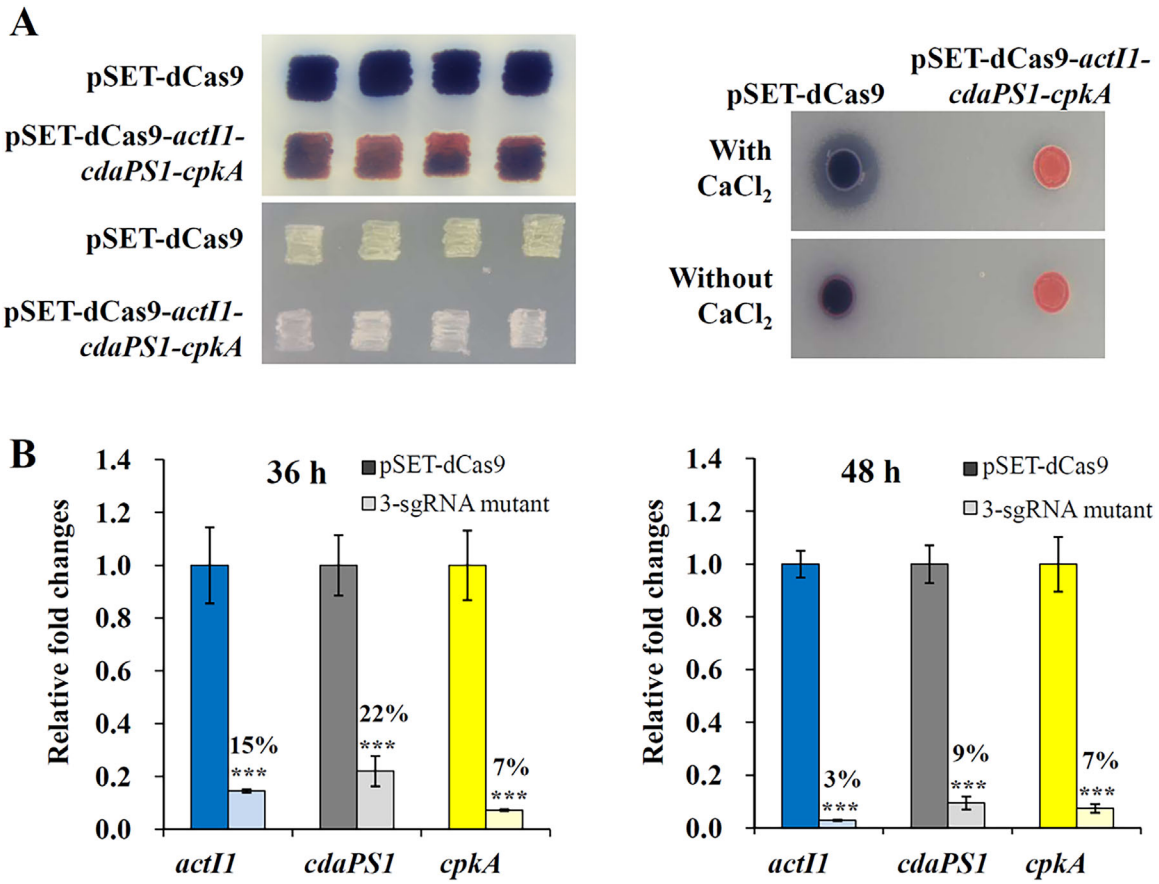


Figure 3. CRISPRi-mediated simultaneous repression of three genes in *S. coelicolor*. A) Visual inspection of antibiotics production of *S. coelicolor* strains harboring the CRISPRi system targeting three genes (*actI1*, *cdaPS1*, and *cpkA*) simultaneously. For the analysis of ACT production, the strains containing the CRISPRi plasmid pSET-dCas9-*actI1-cdaPS1-cpkA* were grown on R2YE agar for 72 h before imaging. For analysis of CDA production, the strains were analyzed by a bioassay experiment using *B. subtilis* as an indicator strain. For yCPK production analysis, the strains were cultured on MM supplemented with 75 mM Glu for 24 h before imaging. The strains harboring pSET-dCas9 (with the expression of dCas9 only) were used as controls. Four colonies per engineered strain were randomly taken for visual inspection. For the bioassay of CDA production, the inhibition zone of one clone was imaged. B) Transcriptional analysis of *actI1*, *cdaPS1*, and *cpkA* in the strain (3-sgRNA mutant) harboring the CRISPRi plasmid targeting three genes simultaneously by qRT-PCR. RNA samples were isolated from *S. coelicolor* strains grown on R2YE for 36 and 48 h, respectively. Error bars represent the standard deviations (SD) from three biological replicates. $^{***}p < 0.001$.

functional genes, thereby accelerating functional genome research of *Streptomycs* strains. Compared with the CRISPRi system developed in *Streptomyces* previously,[22] in which the backbone of expression of the dCas9/sgRNA complex is a temperature sensitive pSG5 replicon, there are two main advantages of our CRISPRi system. Firstly, the pSET152 backbone is chromosomal integrative mediated by site-specific recombination system, so the CRISPRi system constructed here may be more stable than the pSG5-based one. Secondly, the plasmid harboring the pSG5 replicon can not be transferred into many *Streptomyces* strains with high efficiency, which would somehow hamper the application of the previously established pSG5-based CRISPRi system. However, our CRISPRi system is based on the integrative vector and its integration into the genome is via the ϕ31 integrase. Considering that the ϕ31 *attB* site is present on the genome of all *Streptomyces* strains (whose genome sequences are available so far) and the efficiency of ϕ31 integrase-mediated DNA transformation is much higher than that of the pSG5-based plasmids, it could be concluded that the integrative CRISPRi system will be more widely used for multiplex gene repression in *Streptomyces*. Nevertheless, both CRISPRi methods established so far are potentially adaptable for efficient *Streptomyces* gene repression in functional genomic research.

CRISPRi-mediated multiplex gene repression has been successfully employed for metabolic engineering for strain improvement in many bacteria, such as *E. coli*,[7,40,41] *Synechcocystis* sp. PCC6803,[12] *Halomonas* sp. TD01,[42] and *C. glutamicum*,[8] etc. As for the application of the CRISPRi constructed here is concerned, besides for functional gene screening, it could also be employed for *Streptomyces* metabolic engineering to improve the production level of desired secondary metabolites. In streptomycetes, primary metabolism provides various precursors for secondary metabolism. Genes encoding enzymes in primary metabolism are difficult to be inactivated as they are always essential for strain growth, so

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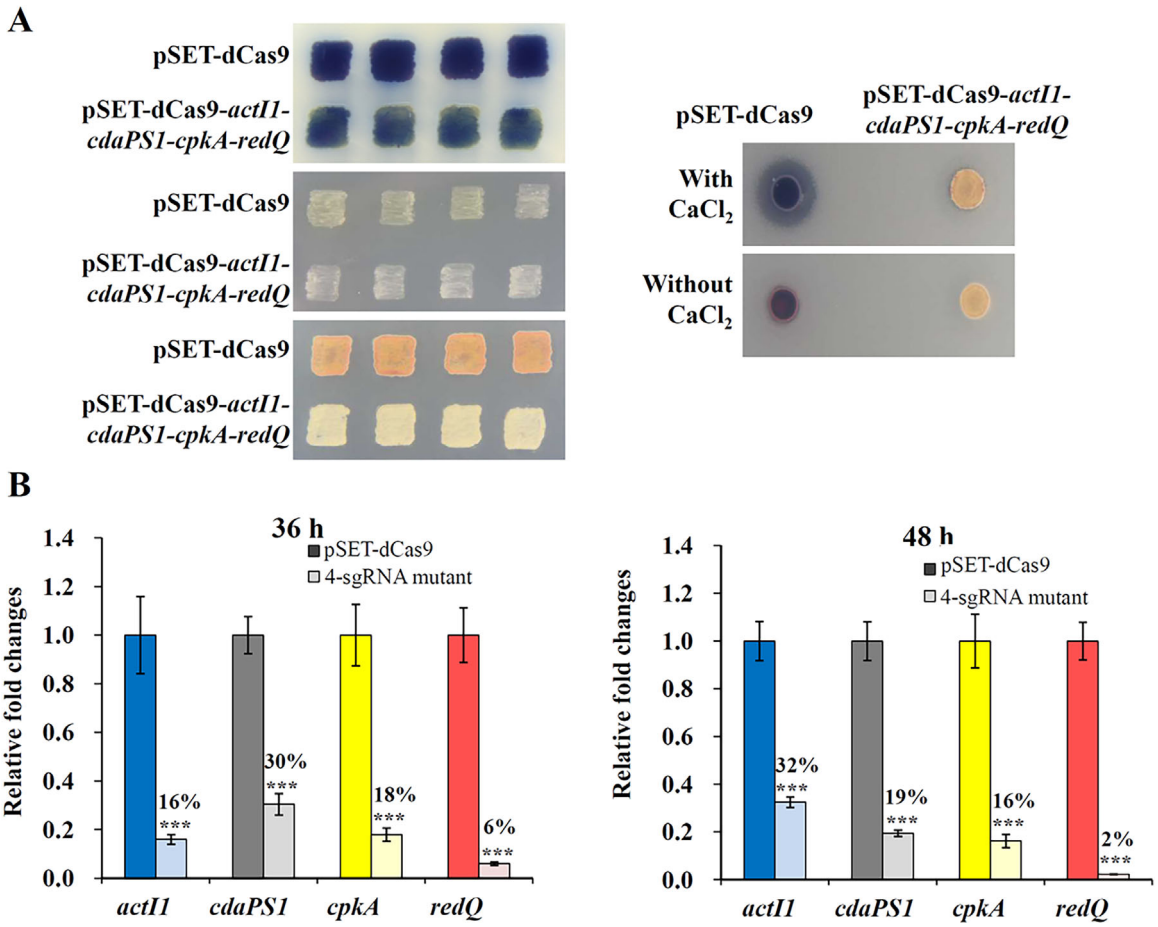


Figure 4. CRISPRi-mediated simultaneous repression of four genes in *S. coelicolor*. A) Visual inspection of antibiotics production of *S. coelicolor* strains harboring the CRISPRi system targeting four genes (*actI1*, *cdaPS1*, *cpkA*, and *redQ*) simultaneously. For the analysis of ACT and RED production, the strains with the CRISPRi plasmid pSET-dCas9-*actI1-cdaPS1-cpkA* were grown on R2YE agar for 72 and 36 h, respectively, before imaging. For CDA production analysis, the strains were analyzed by a bioassay experiment using *B. subtilis* as an indicator strain. For yCPK analysis, the strains were cultured on MM supplemented with 75 mM Glu for 24 h before imaging. The strains harboring pSET-dCas9 (with the expression of dCas9 only) were used as controls. Four colonies per engineered strain were randomly taken for visual inspection. For the bioassay of CDA production, the inhibition zone of one clone was imaged. B) Transcriptional analysis of *actI1*, *cdaPS1*, *cpkA*, and *redQ* in the strain (4-sgRNA mutant) harboring the CRISPRi plasmid targeting four genes simultaneously by qRT-PCR. RNA samples were isolated from *S. coelicolor* strains grown on R2YE for 36 and 48 h, respectively. Error bars represent the standard deviations (SD) from three biological replicates. $^{***}p < 0.001$.

gene repression will be a good choice as suggested previously.[43] Therefore, we anticipate that our CRISPRi system could be used for metabolic engineering of primary metabolism to improve secondary metabolite production in the industrial *Streptomyces* strains. It should be noted that, when primary metabolism of *Streptomyces* is engineered, the high repression efficiencies (≈85% for singles genes and 70% for four genes simultaneously) of this CRISPRi system will greatly affect bacterial growth, thereby resulting in much lower biomass as well as reduced levels of target secondary metabolites. To address this problem, inducible or relatively weak promoters may be adopted to drive the expression of dCas9.

In this study, we observed from both the phenotypic changes in ACT production (blue color) as well as the transcription of four target genes, including *actI1*, *cdaPS1*, *cpkA*, and *redQ*, when four genes were repressed simultaneously, the repression efficiency of target genes become weaker. This phenomenon has also been reported previously that increase in the number of repression targets generally decreases the repression efficiency.[3] In our case, it may be mainly ascribed to the promoter strength of *ermE**p driving dCas9 expression, which might be not strong enough to supply sufficient dCas9 nuclease for transcriptional repression of four target genes simultaneously. This point could be addressed by using stronger promoters, e.g., *kasO**p and its derivatives developed previously.[44] As for the sudden drop in repression efficiency of *actI1*, we reasoned that it may be dependent on the timing of *actI1* transcription coupled with lower expression of dCas9. DNA microarray showed that among four tested genes, *actI1* is transcribed the latest.[45] Therefore, when the dCas9 nuclease is sufficient, its remaining activity at later growth may still repress *actI1* transcription efficiently. However, if dCas9 is insufficient, its activity may be very low at the stage when *actI1* being

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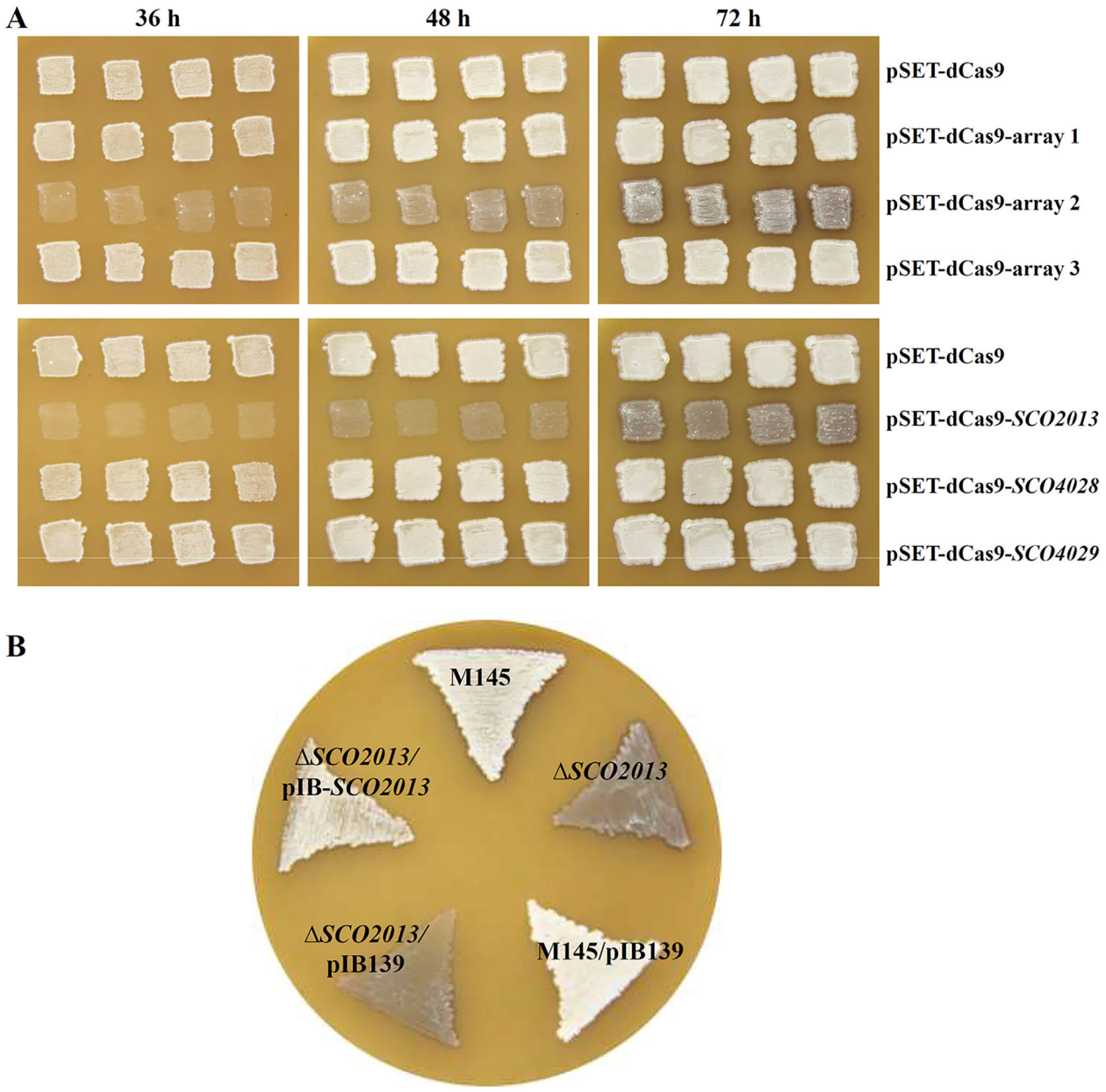


Figure 5. CRISPRi-mediated simultaneous repression of multiple genes encoding proteins with an RNA-binding ANTAR domain in *S. coelicolor*. A) CRISPRi-mediated multiplex repression of genes encoding the ANTAR RNA-binding proteins. Nine sgRNAs were designed to repress 10 genes encoding proteins with the ANTAR domain in *S. coelicolor*, with sgRNA-*SCO0018* targeting *SCO0018* and *SCO7829* simultaneously. The sgRNAs were divided into three arrays. Array 1 included three sgRNAs targeting *SCO0018, SCO0049, SCO0058*; array 2 included three sgRNAs targeting *SCO2013, SCO4028, SCO4029*; array 3 included three sgRNAs targeting *SCO7326, SCO7340, SCO7423*. Bacterial growth of the exoconjugants was analyzed on MS agar medium. Four colonies per engineered strain were randomly taken for visual inspection. B) Effect of an in-frame deletion of *SCO2013* on *S. coelicolor* growth. For the complementation assay, three strains, including the wild-type strain M145 with the empty plasmid pIB139 (M145/pIB139), the Δ*SCO2013* mutant with pIB139 (Δ*SCO2013*/pIB139), and the complemented strain (Δ*SCO2013*/pIB-*SCO2013*) were generated. *S. coelicolor* strains were cultivated on MS agar medium for visual inspection.

strongly transcribed, thus leading to the strong leakiness in *actI1* expression. Herein, we also observed a big difference in repression efficiency at 36 and 48 h, which might be due to the different timing of expression of dCas9 and target genes. We speculated that target genes may be strongly transcribed prior to dCas9, thus resulting in the difference in repression efficiency at two tested time points.

Abbreviations

ANTAR, AmiR and NasR transcription antitermination regulators; Cas, CRISPR-associated protein; CDA, calicium-dependent antibiotic; CRISPR, the clustered regularly interspaced short palindromic repeats; dCas9, catalytically dead Cas9; PCR, polymerase chain reaction; qRT-PCR, real-time reverse-transcription PCR; sgRNA, single guide RNA; UTR, untranslated region.

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

Keywords

CRISPRi, dCas9, multiplex gene repression, *Streptomyces*

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