Appl Microbiol Biotechnol (2015) 99:10575–10585
DOI 10.1007/s00253-015-6931-4

Highly efficient editing of the actinorhodin polyketide chain length factor gene in *Streptomyces coelicolor* M145 using CRISPR/Cas9-CodA(sm) combined system

Hu Zeng[1] · Shishi Wen[1] · Wei Xu[1] · Zhaoren He[1] · Guifa Zhai[1] · Yunkun Liu[1] · Zixin Deng[1] · Yuhui Sun[1]

Received: 15 May 2015 / Revised: 4 August 2015 / Accepted: 8 August 2015 / Published online: 29 August 2015

Abstract The current diminishing returns in finding useful antibiotics and the occurrence of drug-resistant bacteria call for the need to find new antibiotics. Moreover, the whole genome sequencing revealed that the biosynthetic potential of *Streptomyces*, which has produced the highest numbers of approved and clinical-trial drugs, has been greatly underestimated. Considering the known gene editing toolkits were arduous and inefficient, novel and efficient gene editing system are desirable. Here, we developed an engineered CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat/CRISPR-associated protein) combined with the counterselection system CodA(sm), the D314A mutant of cytosine deaminase, to rapidly and effectively edit *Streptomyces* genomes. In-frame deletion of the actinorhodin polyketide chain length factor gene *actI-ORF2* was created in *Streptomyces coelicolor* M145 as an illustration. This CRISPR/Cas9-CodA(sm) combined system strikingly increased the frequency of unmarked mutants and shortened the time required to generate them. We foresee the system becoming a routine laboratory technique for genome editing to exploit the great biosynthetic potential of *Streptomyces* and perhaps for other medically and economically important actinomycetes.

Keywords CRISPR/Cas9 · CodA · Actinorhodin biosynthesis · *ActI-ORF2* · Gene editing

Electronic supplementary material The online version of this article (doi:10.1007/s00253-015-6931-4) contains supplementary material, which is available to authorized users.

✉ Yuhui Sun
yhsun@whu.edu.cn

[1] Key Laboratory of Combinatorial Biosynthesis and Drug Discovery (Wuhan University), Ministry of Education and Wuhan University School of Pharmaceutical Sciences, 185 East Lake Road, Wuhan 430071, People's Republic of China

Introduction

Actinomycetes are ubiquitous high GC Gram-positive bacteria, renowned for the production of many beneficial secondary metabolites including antibiotics, herbicides, antifungal, and anticancer agents (Hopwood 2007; Baltz 2008). *Streptomyces* is a genus of actinomyces that undergoes morphological differentiations from substrate mycelia to aerial hyphae and chains of spores (Chater 1993). Streptomycetes account for nearly 80 % of bioactive natural products derived from actinomycetes (Berdy 2005). However, the occurrence of multidrug-resistant pathogens and the diminishing returns in finding useful antibiotics create an urgent need to discover new antibiotics (Campo et al. 2002; Oliynyk et al. 2003). Whole genome sequencing of streptomycetes revealed that a large number of cryptic or orphan antibiotic biosynthetic pathways are silent under laboratory conditions. To activate silent antibiotic biosynthesis gene clusters and for the genetic manipulation of *Streptomyces* genes and gene clusters in general, novel and efficient gene editing tools are highly desired.

Compared to genetic model organisms like *Bacillus subtilis* or *Escherichia coli*, the number of biotechnological methods available in *Streptomyces* is limited. Conventional gene editing in *Streptomyces* is usually based on homologous recombination (HR) using suicide or unstable replicative vectors (Smithies 2001). The necessary amount of screening for the desired mutant depends on the frequency of HR and on the efficiency of plasmid clearance. Usually, hundreds of colonies need to be screened for the presence of the correct genetic change and the loss of the delivery plasmid, making it an arduous and time-consuming process. PCR targeting

introduced by Gust et al. (2003) was a great improvement, but it requires suitable cosmid clones, and it leaves a scar sequence in the chromosome. Site-specific recombination systems such as Cre/Dre recombinase are alternative powerful tools for high-throughput site-specific recombination resulting in efficient gene deletion. But they leave a recombinase target site in the chromosome, preventing the successive deletion of other genes (Herrmann et al. 2012). The homing endonuclease I-*Sce*I has been applied to construct unmarked mutations in *Streptomyces*: the homing endonuclease recognition site is inserted at the target locus and cleaved by I-*Sce*I. The double-strand DNA break (DSB) stimulates homologous recombination at both DNA ends. This uses a multistep process and requires screening rather than selection of the desired mutant strains (Siegl et al. 2010).

The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) system is a prokaryotic adaptive immune mechanism against invading viruses and plasmids (Bhaya et al. 2011; Deveau et al. 2010; Horvath and Barrangou 2010; Makarova et al. 2011). The type II CRISPR/Cas9 system from *Streptococcus pyogenes* showed that the Cas9 endonuclease could be guided by a single-strand guide RNA (sgRNA) to make double-strand DNA breaks at chosen target DNA sequences. The sgRNA, consists of an exchangeable 20 nt guide sequence that matches the target DNA and an invariant scaffold that binds to Cas9. The only requirement for the 20 nt guide sequence is that it must be adjacent to a PAM (Protospacer Adjacent Motif) (Jinek et al. 2012). For bacteria to multiply, the DSB must be repaired by rejoining the DNA ends. In the absence of homologous template DNA, this can be achieved by activating the non-homologous end joining (NHEJ) pathway. If homologous template DNA is available, the ends can be joined efficiently by homologous double-crossover recombination. Engineered changes in the donor template can result in precise genome modifications. To specifically target a specific DNA sequence, only the 20 nt guide part of the sgRNA needs to be changed. This cost-efficient, simple, and rapid technique has been applied for precise, targeted genome modification of mammalian (Chen et al. 2013; Cong et al. 2013; Niu et al. 2014; Qi et al. 2013), zebrafish (Hwang et al. 2013), *Plasmodium* (Ghorbal et al. 2014), *Drosophila* (Ren et al. 2013), rice (Shan et al. 2013), and *Streptomyces* (Cobb et al. (2014), Huang et al. (2015) and Tong et al. (2015)) DNA. CRISPR and *cas* homologous genes were found in several actinomycete species, but no cleavage activity was detected (Guo et al. 2011).

CodA(sm), the D314A mutant of cytosine deaminase (CodA), converts 5-fluorocytosine (5FC) supplemented in culture into 5-fluorouracil (5FU), a highly toxic compound. Dubeau et al. (2009) developed *codA*(*sm*) as a valuable counterselection marker for *Streptomyces* and other actinobacteria.

In this study, we demonstrated the efficient, precisely targeted in-frame deletion of a *Streptomyces coelicolor* M145 gene involved in the production of the polyketide antibiotic actinorhodin. A convenient *Streptomyces* delivery plasmid was constructed that expresses *S. pyogenes* Cas9, a target-specific sgRNA, and *codA*(*sm*) which confers 5FC sensitivity and thus allows selection of strains that have lost the delivery plasmid. In addition, the delivery plasmid also contains engineered sequences that are homologous to both sides of the target sequence. After conjugative transfer to *Streptomyces*, the sgRNA directed Cas9 endonuclease creates a double-strand break at the target site. The DNA ends are then rejoined by homologous recombination with the cloned homologous plasmid sequences, creating the desired unmarked chromosomal mutation. Mutant strains that have lost the delivery plasmid are then selected using 5FC.

Materials and methods

Bacterial strains, culture conditions, and reagents

Escherichia coli DH10B was used as cloning host. *E. coli* ET12567/pUZ8002 was used for intergeneric conjugation between *E. coli* and *Streptomyces*. *S. coelicolor* M145 was grown at 28 °C on SFM agar medium (20 g soy flour, 20 g mannitol, 20 g agar per liter) for sporulation or in tryptone soya broth with yeast extract (TSBY) liquid medium (30 g tryptone soya broth powder, 10 g yeast extract, 103 g sucrose per liter) for growth of mycelium and isolation of total DNA. SFM with addition of 10 mM $MgCl_2$ was used for intergeneric conjugation between *E. coli* and *Streptomyces*. *E. coli* strains were maintained in 2× YT medium (16 g typtone, 10 g yeast extract, 5 g sodium chloride per liter) at 37 °C with the appropriate antibiotic selection (25 μg/mL apramycin, 100 μg/mL ampicillin, 25 μg/mL chloramphenicol, and 25 μg/mL kanamycin). For *Streptomyces*, nalidixic acid (25 μg/mL) and apramycin (25 μg/mL) were used in SFM or TSBY. R5 agar plates (103 g sucrose, 0.25 g K_2SO_4, 10.12 g $MgCl_2{\cdot}6H_2O$, 10 g glucose, 0.1 g casamino acids, 2 mL trace element solution, 5 g yeast extract, 5.73 g TES buffer, 22 g agar per liter) supplemented by 10 mL KH_2PO_4 (0.5 %), 4 mL $CaCl_2{\cdot}2H_2O$ (5 M), 15 mL L-proline (20 %), and 7 mL NaOH (1 N) were used for actinorhodin production.

Restriction endonucleases, T4 polynucleotide kinase, and Phusion High-Fidelity Master Mix with GC-buffer were purchased from NEB. *Aar*I, FastAP, and T4 DNA ligase were purchased from Fermentas. DIG DNA labeling and detection kits were purchased from Roche. RNAprep pure bacteria kit was purchased from TIANGEN, RNase-free DNase I and RevertAid First Strand cDNA Synthesis Kit were purchased from Thermo Scientific. 5-fluorocytosine (5FC) was purchased from Adamas. Oligonucleotide primer synthesis and

DNA sequencing of PCR products were performed by either GenScript or Tsingke.

Plasmids constructions

scas9, the *Streptomyces* codon-optimized version of *cas9* flanked by *Nhe*I and *Sac*I restriction sites, was synthesized by GenScript and provided on pUC57-Simple. The 4107 bp *Nhe*I-*Sac*I fragment was inserted into pYH7 (Sun et al. 2009; Sun et al. 2006), replacing most *aac*(*3*)*IV* coding sequence between *Nhe*I and *Sac*I, under the control of the promoter of *aac*(*3*)*IV* using the same restriction sites. Then, the thiostrepton resistance gene *tsr* on pYH7 was replaced with the apramycin resistance gene *aac*(*3*)*IV*, yielding the plasmid pWHU2650 which can be selected both in *E. coli* and *Streptomyces* using apramycin. Two basic templates of sgRNA(+48) and sgRNA(+85) cloning cassette (*Aar*I) (Fig. S1), flanked by *Xba*I and *Spe*I restriction sites, were synthesized by GenScript and again provided on pUC57-Simple. The two sgRNA cloning cassettes were inserted at *Xba*I of pWHU2650 to give pWHU2651 and pWHU2652, respectively. The 20 nt *S. coelicolor* M145 *actI-ORF2* target sequence was identified using CasOT, an online genome-wide searching tool for potential Cas9/sgRNA off-target (Xiao et al. 2014). The 20 nt guide sequence was synthesized as overlapping oligonucleotides for insertion between the two *Aar*I cut sites on the basic template of the two sgRNA cloning cassettes to give pWHU2654 (sgRNA2(+48)) and pWHU2655 (sgRNA2(+85)), respectively. The c. 2 kb UHA and DHA sequences were amplified from genomic DNA of *S. coelicolor* M145, cloned into pUC18, then cut out and cloned together into the above plasmids containing the *scas9* and sgRNA expression cassettes to obtain the *actI-ORF2*-specific deletion plasmids pWHU2656, pWHU2657, and pWHU2658. *codA*(*sm*) and P*neo* were amplified from pMG303M (Dubeau et al. 2009) and inserted at *Xba*I into pWHU2658, yielding pWHU2659. As *Aar*I site exists in *codA*(*sm*), for future convenient plasmid construction during target gene deletion, *codA*(*sm*) with P*neo* was cloned into backbone by *Xba*I after the two *Aar*I sites on pWHU2652 being replaced by *Bae*I recognition site which is used for embedding the gene-specific 20 nt guide sequence, yielding pWHU2653. All oligonucleotide primers and plasmids are in Supplementary materials, Tables S1 and S2, respectively.

CRISPR/Cas9 cleavage activity

To test the efficacy of CRISPR/Cas9 expressed by the synthetic gene *scas9* and sgRNA, pWHU2650, pWHU2655, pWHU2656, and pWHU2658 were introduced into *S. coelicolor* M145 by conjugation (Kieser et al. 2000). *E. coli* ET12567/pUZ8002 containing the above plasmids were incubated to OD_{600} of 0.4–0.6. For each plasmid, the same volume of *E. coli* ET12567/pUZ8002 and *S. coelicolor* M145 spores were used for conjugation. Exconjugants grown after overlaying with apramycin and nalidixic acid were counted.

Screening for in-frame deletion mutant using CRISPR/Cas9 system

Independent apramycin-resistant exconjugants were patched on SFM agar, again containing apramycin and nalidixic acid, and grown at 28 °C for 2 or 3 days. Genomic DNA was extracted from mycelium grown on the plate and amplified by PCR using primers *actI-ORF2*-P1/*actI-ORF2*-P3 and *actI-ORF2*-P2/*actI-ORF2*-P4, respectively. To obtain plasmid-free progeny, mycelium from the patches was streaked on non-selective SFM agar plate, followed by replica plating to SFM agar plate with and without apramycin. Genomic DNA of single apramycin-sensitive colonies was extracted and amplified by PCR using primers *actI-ORF2*-CK1/*actI-ORF2*-CK2.

Screening for in-frame deletion mutants using CRISPR/Cas9-CodA(sm) combined system

Following conjugation, single exconjugants were picked and streaked on SFM agar containing 800 μg/mL 5FC and grown in the dark at 28 °C for 3 or 4 days. The $5FC^R$ colonies were then replicated to SFM with and without apramycin to confirm plasmid loss. Genomic DNA of apramycin-sensitive single colonies was extracted, followed by PCR amplification using primers *actI-ORF2*-CK1/*actI-ORF2*-CK2. Mutants verified by PCR amplification were inoculated into TSBY medium for high-quality genomic DNA isolation, followed by Southern blot analysis.

Selection of the sgRNA guide sequence and assessment of potential off-target activity

Identification of potential sgRNA 20 nt guide sequences in the *Streptomyces* chromosome was carried out using CasOT (Xiao et al. 2014). The program outputs all the available target sequences in the genomic region that is to be deleted and possible secondary target sequences in the entire genome related to each of the available target sequences. One of the available target sequences was then selected on the basis that it had the lowest chance of targeting undesirable secondary target sequences. The eight most likely secondary target sites were PCR amplified from ΔactI-ORF2 mutants as 550–850 bp fragments and sequenced using the amplification primers. Sequence alignment with the wild-type genome was performed using the Basic Local Alignment Search

Tool in the National Center for Biotechnology Information (NCBI-BLAST).

Analysis of sgRNA and *scas9* transcription by RT-PCR

Mycelia of *S. coelicolor* M145 grown in TSBY medium were collected. Total RNA was extracted using the RNAprep pure bacteria kit according to the manufacturer's protocol. To eliminate DNA contamination, the RNA preparations were treated with RNase-free DNase I. RNA concentration and purity were determined by measuring OD260/280, and an equal amount of RNA from each studied strain was used for the RT reaction. cDNA first-strand synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit according to the manufacturer's instructions. Primers *scas9*-RTP1/*scas9*-RTP2 and sgRNA2-RTP1/sgRNA2-RTP2 were used to analyze *scas9* and sgRNA2 transcription in *S. coelicolor* M145/pWHU2659 and wild-type strains. RT-PCR reaction parameters were as follows: 95 °C for 3 min, followed by 30 amplification cycles: denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s. A final extension at 72 °C for 7 min finished the amplification. 16S rRNA was used as positive internal control for the RT-PCR assays. Negative controls to rule out the possibility of DNA contamination were conducted using PCR amplification without prior RT. Samples of PCR products were separated by 2 % agarose gel electrophoresis and visualized by staining with ethidium bromide. The RT-PCR experiments were done in duplicate using RNA samples from two independent cultures.

Production, isolation, and analysis of actinorhodin

To assess actinorhodin production, each plate containing 20 mL R5 medium was inoculated with plasmid-free progeny from SFM (800 μg/mL 5FC) and grown at 28 °C for 7 days. The whole plate culture was treated with 20 mL 1 M KOH and centrifuged (3000×*g* for 5 min) (Kieser et al. 2000). The supernatants were then subjected to liquid chromatography-electrospray ionization-high-resolution mass spectrometry (LC-ESI-HRMS) analysis. Online LC-ESI-HRMS analysis was carried out on a Thermo Fisher LTQ Orbitrap XL using negative-mode electrospray ionization. The LTQ Orbitrap XL was coupled to a Thermo Accela 600 fitted with a Phenomenex Luna C18 column (250 × 4.6 mm 5 μ) at a flow rate at 1.0 mL/min. The gradient for separation of actinorhodin from crude sample was as follows: 0–5 min 95 % H_2O with 0.1 % ammonium hydroxide (A) and 5 % methanol with 0.1 % ammonium hydroxide (B), 5–20 min 5 % B to 95 % B, 20–23 min 95 % B, 23–25 min 95 % B to 5 % B, 25–30 min 5 % B. The mass spectrometer was set to full scan (from 200 to 2000 *m/z*).

Nucleotide sequence accession number

The nucleotide sequence of *Streptomyces* codon-optimized *scas9* was deposited into the GeneBank under the accession number KR154349.

Results

Transcription of modified CRISPR/Cas9 and sgRNA genes in *Streptomyces*

To ensure expression in high GC *Streptomyces*, the low GC content *S. pyogenes cas9* was codon-optimized to give *scas9* (Fig. S2) and put under the control of the *aac(3)IV* promoter (Kaster et al. 1983). The strong, constitutive *ermE** promoter (P*ermE**) was used to initiate transcription of the sgRNA, and the transcript was terminated by the artificial B1006 terminator (http://parts.igem.org) (Fig. 1a). The *Streptomyces* optimized genes on pWHU2659 were introduced into *S. coelicolor* M145. RT-PCR using two pairs of specific primers detected *scas9* and sgRNA2 transcripts in *S. coelicolor* M145/pWHU2659. The results showed that both *scas9* and sgRNA2 in pWHU2659 were transcribed constitutively in *Streptomyces* (Fig. 1d).

The modified CRISPR/Cas9 are probably cleaving *Streptomyces* DNA

Cas9 and sgRNA should combine to form a nuclease that cleaves both strands of the *Streptomyces* genome at the target site specified by the 20 nt guide sequence at the 5′ end of the sgRNA. Chromosome breaks are lethal unless repaired. In the absence of a homologous sequence spanning the DNA break, efficient repair by homologous recombination was impossible. Only the inefficient non-homologous end joining pathway in *Streptomyces* was expected to repair a small proportion of the broken chromosomes (Siegl et al. 2010). Therefore, introducing pWHU2655 expressing *scas9* and sgRNA2(+85) (sgRNA2 contains the 20 nt guide sequence from *actI-ORF2*; (+85) identifies the longer, more efficient scaffold (Fig. S1)) was expected to produce fewer apramycin-resistant *Streptomyces* colonies than pWHU2650 which contained *scas9* without the sgRNA gene. This was indeed the case: pWHU2650 without sgRNA produced about 1000 exconjugants compared to seven exconjugants from pWHU2655 expressing *scas9* and sgRNA2(+85) together (Table 1). PCR and sequence analysis of the seven exconjugants revealed no changes at or near the sgRNA2 target site, indicating that the target sequence may not have been cut in these exconjugants because NHEJ would probably have introduced insertion and/or deletion mutations of variable length at the site of the DSB (Gong et al. 2005).

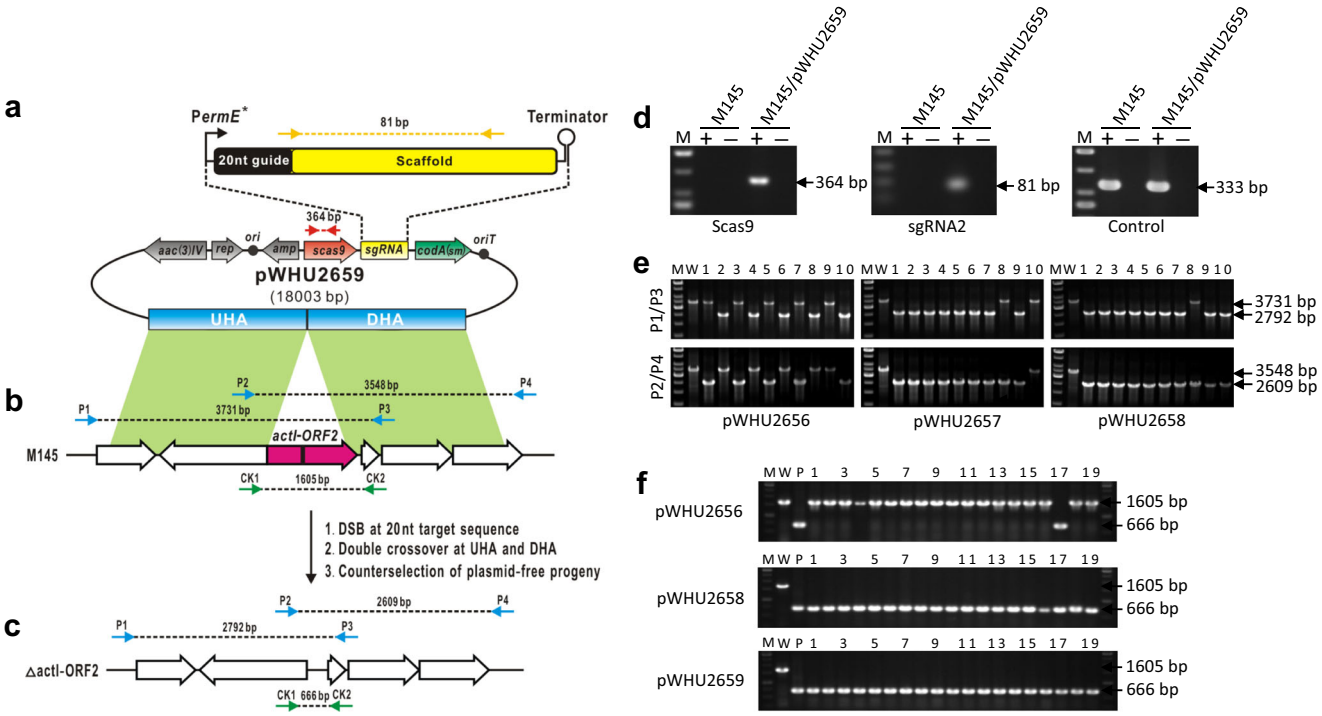

Fig. 1 Targeted in-frame deletion of *actI-ORF2* in *S. coelicolor* M145 using the CRISPR/Cas9-CodA(sm) combined system. **a** Schematic representation of the ultimate delivery plasmid pWHU2659 featuring the *Streptomyces* codon-optimized *scas9* encoding nuclease Cas9, *actI-ORF2*-specific sgRNA (single-guide RNA), the counterselectable cytosine deaminase gene *codA*(*sm*), and UHA and DHA homologous arms flanking *actI-ORF2*. The sgRNA consists of the 20 nt target gene-specific guide sequence (*black*) and the invariant scaffold RNA (*yellow*). The sgRNA binds to the Cas9 nuclease for target-specific double-strand DNA cleavage. The *angled arrow* indicates the P*ermE** transcriptional promoter and the stem-loop indicates the artificial B1006 terminator; the *red* and *orange horizontal arrows* indicate the locations of primer pairs used for detecting *scas9* or sgRNA2 transcripts. *aac*(*3*)*IV* apramycin resistance gene, *rep* multicopy replicon of *Streptomyces* plasmid pIJ101, *ori* origin of replication of *E. coli* plasmid *ColE1*, *amp* ampicillin resistance gene, *oriT* origin of transfer of plasmid RK2. **b** *actI-ORF2* and flanking ORFs in the genome of wild-type *S. coelicolor* M145. The *black bar* in *actI-ORF2* indicates the 20 nt guide sequence at the 5′ end of the sgRNA in pWHU2659. *Olive green parallelograms* connect the identical UHA and DHA sequences on pWHU2659 and the M145 chromosome where recombination can take place. **c** Targeted mutant after in-frame deletion of *actI-ORF2*. *Horizontal blue and green arrows* indicate PCR primers used to distinguish wild-type, single crossover, and double crossover progeny. **d** Transcripts *scas9* and sgRNA in *Streptomyces* detected using RT-PCR. PCR amplification of the cDNA after reverse transcription (+) and PCR amplification of the RNA without reverse transcription (−) are indicated. Endogenous 16S rRNA served as an internal control. *M* 50 bp DNA ladder. **e** Agarose gels showing PCR amplification products from independent progeny before plasmid elimination. The larger fragments correspond to the wild-type and the smaller ones to the *actI-ORF2*-deleted strains. Note, that most progeny obtained using pWHU2656 contain the plasmid integrated into the chromosome by a single crossover. *M* 1 kb DNA ladder, *W* wild-type. **f** PCR screening of progeny obtained using the plasmids pWHU2656 (*scas9*, UHA+DHA), pWHU2658 (*scas9*, sgRNA2, UHA+DHA), and pWHU2659 (*scas9*, *codA*(*sm*), UHA+DHA) after plasmid elimination. *M* 1 kb DNA ladder, *W* wild-type, *P* plasmid

If the dramatic reduction of the number of exconjugants from 1000 to seven was caused by DNA cleavage, and NHEJ was, as expected, very inefficient, providing DNA homologous to the sequences left and right of the target sequence (UHA and DHA) might result in efficient rejoining of the DNA ends by homologous recombination (HR). This homologous DNA should, of course, not itself contain the sequence matching the 20 bp guide sequence.

To test whether adding homologous DNA could give rise to HR end joining, we constructed pWHU2656 and pWHU2658. Both plasmids contained two homologous arms (2120 and 2035 bp) flanking the target *actI-ORF2* gene from the actinorhodin (*act*) biosynthesis gene cluster. The 939 bp DNA of *actI-ORF2*, which contains the 20 nt target sequence of sgRNA2, has been deleted in-frame. End joining by homologous recombination should therefore generate an *act* gene cluster lacking *actI-ORF2* (see Fig. 1b for locations of the genes and PCR primers; Table 1 gives the genes which are present on each plasmid). pWHU2658, which contains *scas9*, sgRNA2(+85), and *act* homologous DNA (DHA+UHA) produced proportionally about tenfold more (6 % instead of 0.7 %) exconjugants than pWHU655 which lacked DHA+UHA (Table 1).

Further analysis in the next section demonstrated that the complete system in pWHU2658 (*scas9*, sgRNA, DHA+UHA) produced a high proportion of double crossover gene replacements which are necessary to re-join DNA ends.

Table 1 Repair by HR of CRISPR/Cas9 induced double crossover chromosome breaks in *S. coelicolor* M145 and promotes double crossover HR

Delivery plasmids	pWHU2650	pWHU2655	pWHU2656	pWHU2657	pWHU2658	pWHU2659
Features of delivery plasmids						
Base vector pYH7	*Ori*(*ColE1*), *rep*(*pIJ101*), *oriT*(*RK2*), *aac*(*3*)*IV*					
scas9	*scas9*	*scas9*	*scas9*	*scas9*	*scas9*	*scas9*
sgRNA2(+85) or (+48)	–	sgRNA2(+85)	–	sgRNA2(+48)	sgRNA2(+85)	sgRNA2(+85)
actI-ORF2 flanking regions	–	–	UHA+DAH	UHA+DAH	UHA+DAH	UHA+DAH
codA(*sm*)	–	–	–	–	–	*codA*(*sm*)
Statistics of $Apra^R$ exconjugants						
Number of $Apra^R$ *S. coelicolor* M145 exconjugants	1000	7 (7/1000 = 0.7 %)	600	nd	33 (33/600 = 6 %)	nd
Interpretation	Normal conjugative plasmid transfer	Cas9-sgRNA DSB kills most recipients (NHEJ is inefficient)	Normal conjugative plasmid transfer	nd	HR rescues some of the broken recipient chromosomes	nd
PCR analysis before elimination of the delivery plasmids ($Apra^R$)						
Double crossover/total exconjugants tested (%)	nd	nd	3/80 (4 %)	71/76 (93 %)	80/81 (99 %)	nd
Single crossover/total exconjugants tested (%)	nd	nd	75/80 (94 %)	2/76 (3 %)	1/81 (1 %)	nd
Wild-type/total exconjugants tested (%)	nd	nd	2/80 (3 %)	3/76 (4 %)	0/81 (0 %)	nd
PCR analysis after elimination of the delivery plasmids						
Number of $Apra^S$ progeny/total progeny tested (%)	nd	nd	24/160 (15 %)	nd	63/160 (39 %)	128/135 (95 %)
Double crossover/total progeny tested (%)	nd	nd	1/160 (0.6 %)	nd	63/160 (39 %)	127/135 (94 %)

nd not done, *ori*(*ColE1*) origin of replication of *E. coli* plasmid *ColE1*, *rep*(*pIJ101*) multicopy replicon of *Streptomyces* plasmid pIJ101, *oriT*(*RK2*) origin of transfer of plasmid RK2, *scas9 Streptomyces* codon-optimized *cas9*, *codA*(*sm*) *Streptomyces* codon-optimized cytosine deaminase coding gene conferring 5FU sensitivity, *sgRNA* single-guide RNA, *UHA* upstream homologous arm, *DHA* downstream homologous arm, $Apra^R$ apramycin resistance phenotype, $Apra^S$ apramycin-sensitive phenotype

The modified CRISPR/Cas9 increased the level of homologous recombination in *Streptomyces*

To investigate whether CRISPR/Cas9 can be used to increase the frequency of double crossover HR in *Streptomyces*, the c. 2 kb sequences UHA and DHA were incorporated in the plasmid as shown in Fig. 1a. HR between the UHA and DHA of the plasmid and the *Streptomyces* chromosome should result in the precise 939 bp in-frame deletion of *actI-ORF2*, which was not required for the survival of the host strain. To reduce the influence of spontaneous homologous recombination during propagation, apramycin-resistant transconjugants were examined in the primary apramycin-resistant exconjugants using PCR (Fig. 1b, c, e). Three plasmids were compared: pWHU2656 (*scas9*, UHA+DHA, no sgRNA) produced mostly progeny with single crossover plasmid integration into the *Streptomyces* chromosome either at UHA or DHA (Fig. 1e). This was expected because pYH7-derived plasmids are segregationally unstable, and integrated copies are replicated stably as part of the host chromosome (Sun et al. 2009; Sun et al. 2006). pWHU2657 (*scas9*, sgRNA2(+48), UHA+DHA) and pWHU2658 (*scas9*, sgRNA2(+85), UHA+DHA) produced predominantly double crossover replacements of the chromosomal *actI-ORF2*. pWHU2658 with the longer sgRNA2(+85) gave a slightly higher percentage of double crossover progeny than pWHU2657 containing the shorter sgRNA2(+48) (Table 1). Note, that the two primer pairs could not amplify sequences from autonomous plasmids which were probably still present. These data suggested that CRISPR/Cas9 significantly increased the level of double crossover homologous recombination in *Streptomyces*.

CRISPR/Cas9 combined with CodA(sm) accelerates the screening process for isolating unmarked progeny containing the targeted deletion

The process of plasmid removal for obtaining unmarked progeny is inevitably time-consuming with segregationally unstable plasmid like the pYH7 derivatives used here, or even non-replicating (suicide) plasmids, because these vectors readily recombine with the *Streptomyces* chromosome via their homologous sequences. The integrated plasmids are then replicated stably as part of the host chromosome.

To test the rate of plasmid clearance, four exconjugants of *S. coelicolor* M145/pWHU2658 (*scas9*, sgRNA2(+85), UHA+DHA) were randomly picked and plated for single colonies on non-selective medium. Replica plating onto selective and non-selective medium identified 63 (39 %) apramycin-sensitive progeny; all were confirmed by PCR to be double crossover mutants (Fig. 1f, lanes 1–19; Table 1). pWHU2656 (*scas9*, UHA+DHA, without sgRNA) gave 24 out of 160 (15 %) apramycin-sensitive progeny, of which only one was a double crossover mutant (Table 1; Fig. 1f, lane 17). This demonstrated the huge advantage of using the CRISPR/Cas9-targeted DNA cleavage in *Streptomyces*. Even though pWHU2658 produced a much higher proportion of the desired double crossover progeny than pWHU2656, the time required to isolate such mutants (growing single colonies, replica plating, growing) remained almost the same. To make double crossover mutants available more quickly, we constructed pWHU2659 featuring the 5FC counterselectable *codA*(*sm*) (Table 1, Fig. 1a). Forty apramycin-resistant exconjugants of *S. coelicolor* M145/pWHU2659 were picked and streaked onto SFM plates containing 800 μg/mL 5FC (Fig. 2). Of the 135 surviving, independent single colonies, 128 (95 %) were apramycin sensitive, 127 were verified to be double crossover mutants (Fig. 1f, lanes 1–19) Table 1), and actinorhodin production was lost (Fig. 3c, d). This showed that 5FC was effectively selected for progeny that had lost pWHU2659, making the tedious screening for apramycin-resistant colonies unnecessary.

To test for possible DNA rearrangements (especially deletions that were expected from NHEJ), genomic DNA from seven *actI-ORF2* deletion mutants was randomly selected, digested with *Age*I, and the Southern transfer was probed with labeled 666 bp probe fragment (Fig. 3a). All seven strains produced the expected 4229 bp positive band, typical for the precise excision of the *actI-ORF2* sequence without detectable rearrangements (Fig. 3b). Using the CRISPR/Cas9-CodA(sm) combined system, both the plasmid clearance rate and the ultimate yield of gene deletion mutant were remarkably increased and, most usefully, 5FC counterselection of *codA*(*sm*) saved several days of the screening process.

Evaluation of potential off-target activity

One concern using CRISPR/Cas9 gene editing systems is the occurrence of non-specific indels that have been observed in eukaryotic cells at sites that resemble the 20 nt guide sequence adjacent to a NGG (PAM) sequence. CRISPR/Cas9 can induce mutations at sites that differ by as many as five nucleotides from the intended target (Fu et al. 2013; Hsu et al. 2013). To evaluate potential off-target activity of CRISPR/Cas9 in *S. coelicolor* M145, seven *actI-ORF2* deletion mutants were picked and analyzed for mutations at the eight most likely secondary target sites identified using the program CasOT (Table S3). No mutation was found in these potential target regions, suggesting that off-target mutations were not a problem for our particular gene replacement. Furthermore, PCR products using primers *actI-ORF2*-CK1/CK2 of the seven selected mutants and one wild-type descendant from the pWHU2659 experiment were sequenced, confirming that no mutations had occurred at the target site. These results indicated that the CRISPR/Cas9-CodA(sm) system was highly efficient, specific, and precise.

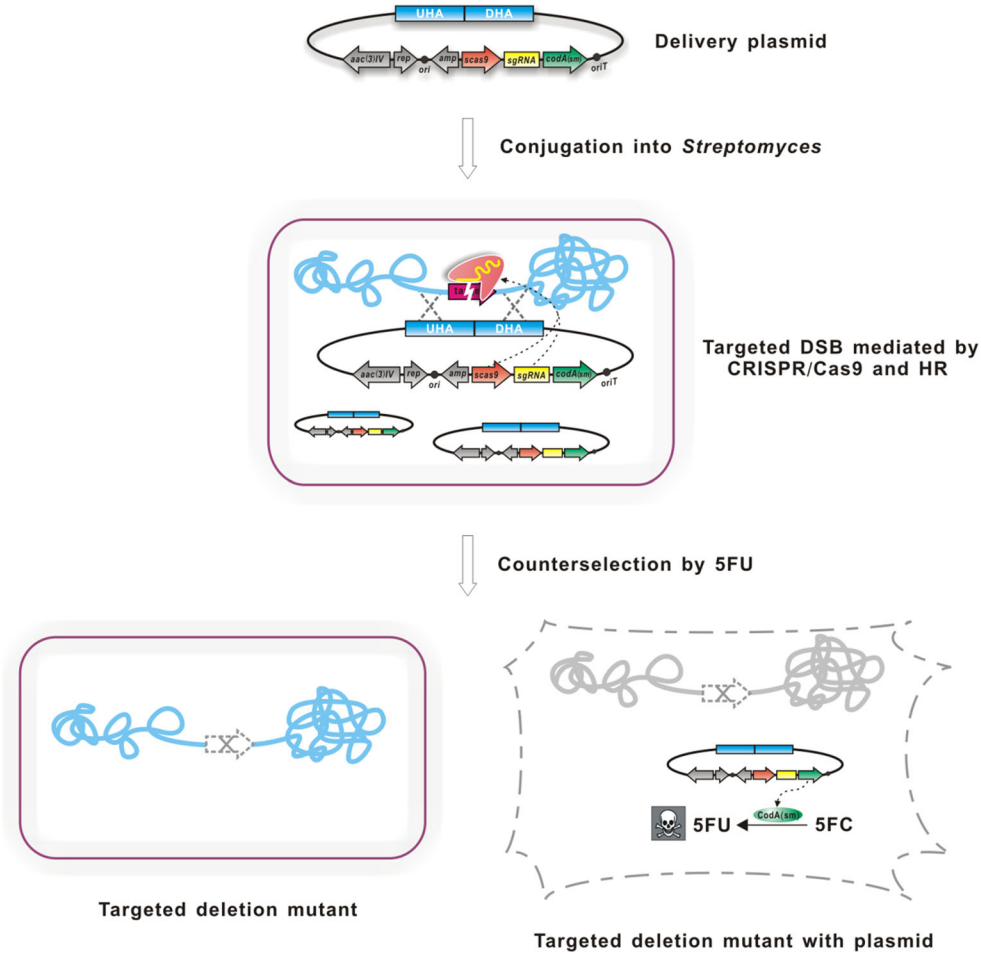

Fig. 2 Flowchart of targeted gene deletion in *S. coelicolor* M145 using the CRISPR/Cas9-CodA(sm) combined system. The multicopy delivery plasmid featuring *scas9*, *actI-ORF2*-specific single guide RNA (sgRNA), *codA*(*sm*), and two homologous arms (UHA and DHA) flanking *actI-ORF2* is transferred from *E. coli* to *S. coelicolor* M145 by intergeneric conjugation, followed by apramycin selection. In the exconjugant, Cas9 guided by the sgRNA causes a double-strand break (DSB) in the target sequence. Then, homologous recombination crossovers at UHA and DHA efficiently rejoins the chromosome ends and creates the desired chromosomal deletion. After removing apramycin, the host delivery plasmids are spontaneously lost from the host, and the desired plasmid-free progeny is selected using 5FC which is converted to cytotoxic 5FU, thus killing bacteria expressing CodA(sm) from the plasmid. The surviving colonies contain the desired chromosomal deletion (*grey dashed arrow* with internal X)

Discussion

In this study, a CRISPR/Cas9-CodA(sm) combined gene editing system was developed and successfully applied to create an in-frame deletion of *actI-ORF2* in the *Streptomyces* model strain *S. coelicolor* M145. Target-specific DSB introduced by CRISPR/Cas9 stimulate homologous recombination, and two crossovers are required for rejoining the DNA ends by HR (Choulika et al. 1995; Rouet et al. 1994). Initially, all the progeny still contained the multicopy delivery plasmid which is segregationally unstable in *Streptomyces* even at normal growth temperature. The delivery plasmid carried the c. 2 kb UHA and DHA chromosomal sequences. These sequences readily recombine (single crossover) with their chromosomal counterparts, resulting in plasmid integration and stable replication as part of the host chromosome. It was therefore very useful to be able to select, rather than screen, for progeny which has become spontaneously plasmid free. This was achieved by integrating cytosine deaminase gene *codA*(*sm*) into the delivery plasmid. CodA(sm) converts 5FC to toxic 5FU. In our experiments, 95 % of the 5FC-resistant cells had also lost apramycin resistance, the other plasmid marker, and 99 % of those plasmid-free strains were tested to be double crossover mutants, indicating that the combined system was indeed not only useful for selecting plasmid-free strains but also highly efficient for gene deletion.

During the development of the CRISPR/Cas9 technique, the fused sgRNA composed of crRNA- and tracrRNA-derived sequences connected by an artificial tetraloop was extended from sgRNA(+48) (48 nt tracrRNA tail) to sgRNA(+85) (85 nt tracrRNA) tail (Jinek et al. 2012). Previous studies in mammalian cells showed that sgRNAs

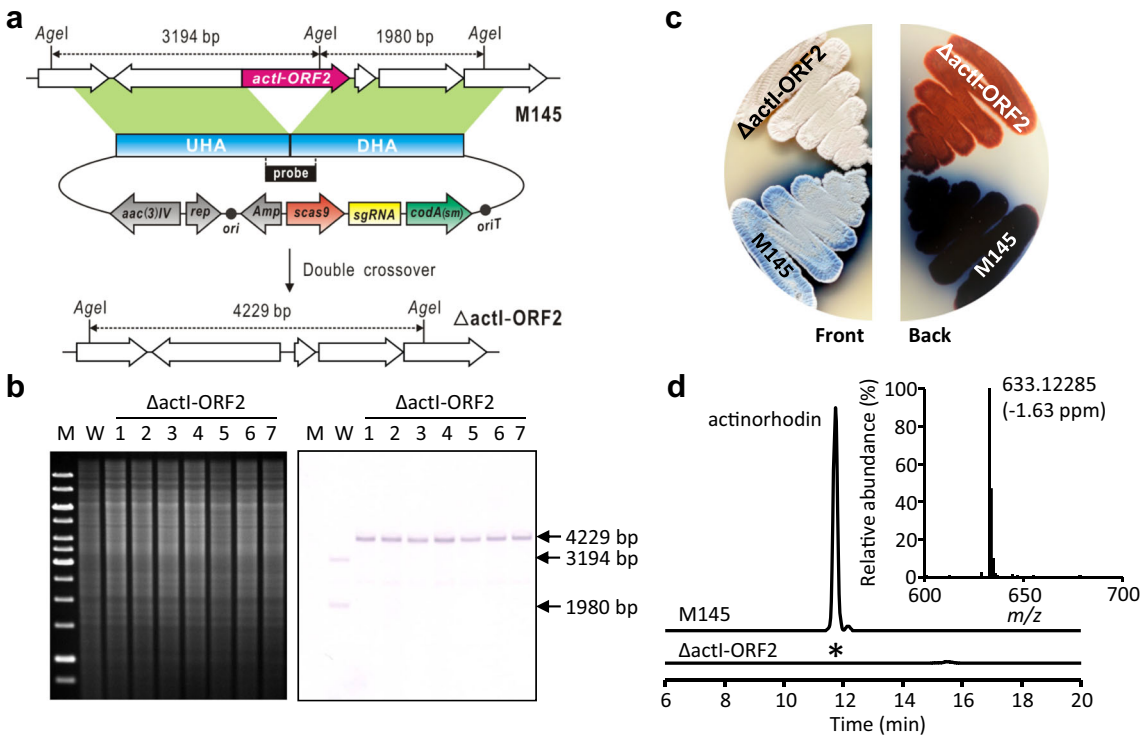

Fig. 3 Southern blot confirmation and antibiotic production by an *actI-ORF2* in-frame deletion mutant. **a** Schematic representation of in-frame deletion of *actI-ORF2*. The *horizontal dotted arrows* indicate the expected sizes of wild-type and mutant genomic DNA after digestion with *Age*I. The *black box labeled probe* indicates 666 bp fragment amplified by primers *actI-ORF2*-CK1/*actI-ORF2*-CK2 (Fig. 1c). **b** *Age*I-digested genomic DNA of seven independent isolates. Ethidium bromide-stained agarose gel and Southern hybridization using the labeled 666 bp probe. *M* 1 kb DNA ladder. **c** Actinorhodin production in wild-type and *actI-ORF2* deletion mutant on R5 medium. The *blue color* of actinorhodin is visible on the reverse side of the wild-type. The *orange color* of mutant is from undecylprodiginin in the absence of actinorhodin production. **d** Actinorhodin detected by LC-ESI-HRMS. Selective ion monitoring was carried out on $[M-H]^-$ (calculated: *m/z* 633.12389; observed: *m/z* 633.12285). No actinorhodin was detected in the ΔactI-ORF2 mutant strain

with extended tracrRNA tails dramatically improved the Cas9 cleavage activity in vivo (Hsu et al. 2013). The crystal structure of Cas9 indicated that the longer tracrRNA tail of the sgRNA improved the in vivo activity by stabilizing both the sgRNA and the functional Cas9-sgRNA complex (Hsu et al. 2013; Nishimasu et al. 2014). In this study, pWHU2657 containing the shorter sgRNA2(+48) was already very efficient, but pWHU2658 with the longer sgRNA2(+85) showed a slightly higher percentage of double crossover progeny. Therefore, sgRNA2(+85) was incorporated into the final delivery vector pWHU2569.

Homologs of the *ku* gene, which plays a key role in mutagenic NHEJ were reported in *Streptomyces*, but the NHEJ system of *Streptomyces* is thought to serve as a major DSB repair mechanism only during spore germination (Moeller et al. 2007; Weller et al. 2002; Zhang et al. 2012). In actively growing culture, HR was the major pathway for DSB repair in *Streptomyces* with NHEJ playing a minor role (Siegl et al. 2010). In this study, no mutation indicative of NHEJ action was observed at potential off-target sites identified by CasOT. Probably no off-target double-strand cleavage occurred in *S. coelicolor* M145. We have shown that the CRISPR/Cas9-CodA(sm) system can be used to introduce gene knock-outs in the *Streptomyces* genome with high efficiency. It is expected that it can be applied equally effectively for generating gene knock-ins, gene replacements, large fragment deletions, and other genome editing (Smithies 2001). Our delivery vector (pWHU2653 with gene-specific UHA+DHA and 20 nt guide sequences) can probably be used in most *Streptomyces* strains and even in some related actinomyces genera because all its elements, including promoters and terminators of CRISPR/Cas9, and the pIJ101 replicon have been used successfully in many different strains (Kieser et al. 1982; Schmitt-John and Engels 1992). The convenient restriction sites on the "precursor delivery vector" pWHU2653 make it very easy to build target-specific delivery vectors containing 20 nt guide sequence, UHA and DHA (Fig. S1). Recently, gene editing in *Streptomyces* using an engineered CRISPR/Cas system has been reported by Cobb et al. (2014), Huang et al. (2015), and Tong et al. (2015). In all three reports, temperature-sensitive delivery plasmids derived from pSG5 (copy number 20–50, Muth et al. 1988) were used. The procedures to edit single genes using HR were efficient (70 to 100 % by Cobb et al. (2014), 60 to 100 % by Huang et al. (2015), and nearly 100 % by Tong et al. (2015)), but the elimination of the temperature-sensitive plasmid is time-consuming and it

critically depends on the precise temperature control and incubation time (Muth et al. 1988; Muth et al. 1989). By contrast, we used a segregationally unstable *sti*⁻ pIJ101-derived *E. coil-Streptomyces* shuttle vector with high copy number of about 50 per chromosome (Kieser et al. 2000) producing large amounts of single-strand plasmid DNA (Deng et al. 1988), thus providing ample template DNA for HR. The proportion of double crossover recombinants immediately after conjugation was nearly 100 %. 5FC selection against CodA(sm) eliminated all progeny still containing the delivery plasmid independent of whether it was replicating autonomously or integrated by single crossover into the *Streptomyces* chromosome.

To sum up, in addition to the dramatically high efficiency of double crossover recombination, this combined system gets rid of the tedious multistep process to obtain plasmid-free mutants. The end product is a totally unmarked (no resistance gene or scar sequence) mutant strain, which allows the generation of successive gene replacements, always using the same delivery vector. The deployment of this system will greatly improve our ability to understand gene function and explore the huge potential of streptomycetes and probably also some related actinomycetes.

Acknowledgments This research was supported by grants from National Basic Research Program of China (973 Program), Research Fund for the Doctoral Program of Higher Education of China, and Program for Collaborative Innovative in Wuhan University School of Medicine. We thank Dr. Ryszard Brzezinski at Universite´ de Sherbrooke in Canada for the gift of pMG303M containing *codA*(*sm*). We are grateful to associate Prof. Changming Zhao for his help in LC-ESI-HRMS detection of actinorhodin and Dr. Tobias Kieser for his critical reading of the manuscript.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Baltz RH (2008) Renaissance in antibacterial discovery from actinomycetes. Curr Opin Pharmacol 8:557–563

Berdy J (2005) Bioactive microbial metabolites. J Antibiot (Tokyo) 58:1–26

Bhaya D, Davison M, Barrangou R (2011) CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. Annu Rev Genet 45:273–297

Campo N, Daveran-Mingot ML, Leenhouts K, Ritzenthaler P, Le Bourgeois P (2002) Cre-loxP recombination system for large genome rearrangements in *Lactococcus lactis*. Appl Environ Microbiol 68:2359–2367

Chater KF (1993) Genetics of differentiation in *Streptomyces*. Annu Rev Microbiol 47:685–713

Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, Li GW, Park J, Blackburn EH, Weissman JS, Qi LS, Huang B (2013) Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. Cell 155:1479–1491

Choulika A, Perrin A, Dujon B, Nicolas JF (1995) Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of *Saccharomyces cerevisiae*. Mol Cell Biol 15:1968–1973

Cobb RE, Wang Y, Zhao H (2014) High-efficiency multiplex genome editing of *Streptomyces* species using an engineered CRISPR/Cas system. ACS Synth Biol [Epub ahead of print]

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339:819–823

Deng ZX, Kieser T, Hopwood DA (1988) "Strong incompatibility" between derivatives of the *Streptomyces* multi-copy plasmid pIJ101. Mol Gen Genet 214:286–294

Deveau H, Garneau JE, Moineau S (2010) CRISPR/Cas system and its role in phage-bacteria interactions. Annu Rev Microbiol 64:475–493

Dubeau MP, Ghinet MG, Jacques PE, Clermont N, Beaulieu C, Brzezinski R (2009) Cytosine deaminase as a negative selection marker for gene disruption and replacement in the genus *Streptomyces* and other actinobacteria. Appl Environ Microbiol 75:1211–1214

Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, Sander JD (2013) High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat Biotechnol 31:822–826

Ghorbal M, Gorman M, Macpherson CR, Martins RM, Scherf A, Lopez-Rubio JJ (2014) Genome editing in the human malaria parasite *Plasmodium falciparum* using the CRISPR-Cas9 system. Nat Biotechnol 32:819–821

Gong C, Bongiorno P, Martins A, Stephanou NC, Zhu H, Shuman S, Glickman MS (2005) Mechanism of nonhomologous end-joining in mycobacteria: a low-fidelity repair system driven by Ku, ligase D and ligase C. Nat Struct Mol Biol 12:304–312

Guo P, Cheng Q, Xie P, Fan Y, Jiang W, Qin Z (2011) Characterization of the multiple CRISPR loci on *Streptomyces* linear plasmid pSHK1. Acta Biochim Biophys Sin (Shanghai) 43:630–639

Gust B, Challis GL, Fowler K, Kieser T, Chater KF (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci U S A 100:1541–1546

Herrmann S, Siegl T, Luzhetska M, Petzke L, Jilg C, Welle E, Erb A, Leadlay PF, Bechthold A, Luzhetskyy A (2012) Site-specific recombination strategies for engineering actinomycete genomes. Appl Environ Microbiol 78:1804–1812

Hopwood DA (2007) *Streptomyces* in nature and medicine: the antibiotic makers. Oxford University Press, New York

Horvath P, Barrangou R (2010) CRISPR/Cas, the immune system of bacteria and archaea. Science 327:167–170

Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, Cradick TJ, Marraffini LA, Bao G, Zhang F (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol 31:827–832

Huang H, Zheng G, Jiang W, Hu H, Lu Y (2015) One-step high-efficiency CRISPR/Cas9-mediated genome editing in *Streptomyces*. Acta Biochim Biophys Sin (Shanghai) 47:231–243

Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR, Joung JK (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol 31:227–229

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337:816–821

Kaster KR, Burgett SG, Rao RN,.Ingolia TD (1983) Analysis of a bacterial hygromycin B resistance gene by transcriptional and translational fusions and by DNA sequencing. Nucleic Acids Res 11: 6895–6911

Kieser T, Bibb M, Buttner M, Chater K, Hopwood D (2000) Practical *Streptomyces* genetics. The John Innes Foundation, Norwich

Kieser T, Hopwood DA, Wright HM, Thompson CJ (1982) pIJ101, a multi-copy broad host-range Streptomyces plasmid: functional

analysis and development of DNA cloning vectors. Mol Gen Genet 185:223–228

Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, Moineau S, Mojica FJ, Wolf YI, Yakunin AF, van der Oost J, Koonin EV (2011) Evolution and classification of the CRISPR-Cas systems. Nat Rev Microbiol 9:467–477

Moeller R, Stackebrandt E, Reitz G, Berger T, Rettberg P, Doherty AJ, Horneck G, Nicholson WL (2007) Role of DNA repair by nonhomologous-end joining in *Bacillus subtilis* spore resistance to extreme dryness, mono- and polychromatic UV, and ionizing radiation. J Bacteriol 189:3306–3311

Muth G, Nußbaumer B, Wohlleben W, Pühler A (1989) A vector system with temperature-sensitive replication for gene disruption and mutational cloning in streptomycetes. Mol Gen Genet 219:341–348

Muth G, Wohlleben W, Puhler A (1988) The minimal replicon of the *Streptomyces ghanaensis* plasmid pSG5 identified by subcloning and Tn5 mutagenesis. Mol Gen Genet 211:424–429

Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, Ishitani R, Zhang F, Nureki O (2014) Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell 156:935–949

Niu Y, Shen B, Cui Y, Chen Y, Wang J, Wang L, Kang Y, Zhao X, Si W, Li W, Xiang AP, Zhou J, Guo X, Bi Y, Si C, Hu B, Dong G, Wang H, Zhou Z, Li T, Tan T, Pu X, Wang F, Ji S, Zhou Q, Huang X, Ji W, Sha J (2014) Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell 156: 836–843

Oliynyk M, Stark CB, Bhatt A, Jones MA, Hughes-Thomas ZA, Wilkinson C, Oliynyk Z, Demydchuk Y, Staunton J, Leadlay PF (2003) Analysis of the biosynthetic gene cluster for the polyether antibiotic monensin in *Streptomyces cinnamonensis* and evidence for the role of monB and monC genes in oxidative cyclization. Mol Microbiology 49:1179–1190

Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152:1173–1183

Ren X, Sun J, Housden BE, Hu Y, Roesel C, Lin S, Liu L-P, Yang Z, Mao D, Sun L, Wu Q, Ji J-Y, Xi J, Mohr SE, Xu J, Perrimon N, Ni J-Q (2013) Optimized gene editing technology for *Drosophila melanogaster* using germ line-specific Cas9. Proc Natl Acad Sci U S A 110:19012–19017

Rouet P, Smih F, Jasin M (1994) Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. Mol Cell Biol 14:8096–8106

Schmitt-John T, Engels JW (1992) Promoter constructions for efficient secretion expression in *Streptomyces lividans*. Appl Microbiol Biotechnol 36:493–498

Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu JL, Gao C (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. Nat Biotechnol 31:686–688

Siegl T, Petzke L, Welle E, Luzhetskyy A (2010) I-SceI endonuclease: a new tool for DNA repair studies and genetic manipulations in *Streptomyces*. Appl Microbiol Biotechnol 87:1525–1532

Smithies O (2001) Forty years with homologous recombination. Nat Med 7:1083–1086

Sun Y, He X, Liang J, Zhou X, Deng Z (2009) Analysis of functions in plasmid pHZ1358 influencing its genetic and structural stability in *Streptomyces lividans* 1326. Appl Microbiol Biotechnol 82:303–310

Sun Y, Hong H, Samborskyy M, Mironenko T, Leadlay PF, Haydock SF (2006) Organization of the biosynthetic gene cluster in *Streptomyces* sp. DSM 4137 for the novel neuroprotectant polyketide meridamycin. Microbiology 152:3507–3515

Tong Y, Charusanti P, Zhang L, Weber T, Lee SY (2015) CRISPR-Cas9 based engineering of actinomycetal genomes. ACS Synth Biol [Epub ahead of print]

Weller GR, Kysela B, Roy R, Tonkin LM, Scanlan E, Della M, Devine SK, Day JP, Wilkinson A, d'Adda di Fagagna F, Devine KM, Bowater RP, Jeggo PA, Jackson SP, Doherty AJ (2002) Identification of a DNA nonhomologous end-joining complex in bacteria. Science 297:1686–1689

Xiao A, Cheng Z, Kong L, Zhu Z, Lin S, Gao G, Zhang B (2014) CasOT: a genome-wide Cas9/gRNA off-target searching tool. Bioinformatics 30:1180–1182

Zhang X, Chen W, Zhang Y, Jiang L, Chen Z, Wen Y, Li J (2012) Deletion of ku homologs increases gene targeting frequency in *Streptomyces avermitilis*. J Ind Microbiol Biotechnol 39:917–925