Development of a CRISPR/Cas9-mediated gene-editing tool in Streptomyces rimosus

Haiyan Jia, Longmei Zhang, Tongtong Wang, Jin Han, Hui Tang and Liping Zhang*

Abstract
Clustered regularly interspaced short palindromic repeats, associated proteins (CRISPR/Cas), has been developed into a powerful, targeted genome-editing tool in a wide variety of species. Here, we report an extensive investigation of the type II CRISPR/Cas9 system for targeted gene editing in Streptomyces rimosus. S. rimosus is used in the production of the antibiotic oxytetracycline, and its genome differs greatly from other species of the genus Streptomyces in the conserved chromosome terminal and core regions, which is of major production and scientific research value. The genes zwf2 and devB were chosen as target genes, and were edited separately via single-site mutations, double-site mutations and gene fragment disruptions. The single-site mutation guided by sgRNA-1 or sgRNA-2, respectively, involved GG changing to CA, GC changing to AT, and GG changing to CC. The double-site mutations guided by sgRNA-1 and sgRNA-2 included deletions and/or point mutations. Consistently, all mutations occurred in the gRNA sequence regions. Deletion mutations were characterized by the absence of eight bases, including three bases upstream of the PAM (protospacer adjacent motif) sequence, the PAM sequence itself and two bases downstream of the PAM sequence. A mutant (zwf2 devB) with a high yield of oxytetracycline was successfully obtained, whose oxytetracycline level was increased by 36.8 % compared to the original strain. These results confirm that CRISPR/Cas9 can successfully serve as a useful targeted genome editing system in S. rimosus.

INTRODUCTION
The ability to biologically edit genes has tremendous potential for the development of products that benefit society and further our understanding of the working mechanisms of many organisms. In general, there are two methods for gene editing: sequence-dependent homologous recombination, and non-homologous end joining [1]. Recently, a number of gene-editing technologies have emerged. These technologies include engineered meganucleases [2], zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALEN) [3] and the RNA-guided CRISPR/Cas nuclease system. Distinct from the protein-guided DNA cleavage utilized by ZFNs and TALENs, CRISPR/Cas9 uses a bacterially derived DNA endonuclease and depends on a small RNA for sequence-specific cleavage [4]. CRISPR/Cas9 is the most recently characterized and rapidly developing genome-editing technology. Since the first descriptions of genome editing using CRISPR/Cas9 technology were published, it has been applied to many model organisms, including humans [5], mice [6], zebrafish [7], plants [8, 9] and yeasts [10]. CRISPR/Cas9 allows efficient and precise gene editing, and provides new insights into gene regulation and genotype identification.

Actinomycetes are a special group of prokaryotes with great commercial value. Among the currently available antibiotics, 67 % are products of microbial fermentation and approximately two-thirds of these are secondary metabolites produced by actinomycetes [11]. Most of these antibiotics are produced by species of the genus Streptomyces, including several with important applications in human medicine and agriculture. Streptomyces rimosus is one such species and is used in the production of the antibiotic oxytetracycline. The S. rimosus genome is about 8 Mb in size and is similar to that of Streptomyces coelicolor. In the S. rimosus genome, there are conserved genes, tapA, tpgA and ttrA, that ensure the entire chromosome is linear [12]. However, the genome of S. rimosus differs greatly from other species of the genus Streptomyces. Kirby et al. [13] analysed the differences between the S. rimosus genome and that of other species of the genus Streptomyces using DNA chip analysis.
and found that the conserved regions of *S. rimosus*, including chromosomal end regions and core regions, were not the same as in other species of the genus *Streptomyces* species. In addition, the *S. rimosus* genome contains some rare genes, such as HTR2L, HTR2T and HRT2V, which are currently only otherwise found in the horizontal transfer region (HTR) of *S. coelicolor*, not in other species of the genus *Streptomyces* [12]. Therefore, *S. rimosus* is of high productivity and scientific research value.

The use of CRISPR/Cas9 system to edit *Streptomyces* has been reported. Cobb et al. used the CRISPR/Cas9 system to design pCRISPomycoses, and subsequently conducted multiplex gene editing of several species of the genus *Streptomyces* [14]. Huang et al. delivered a high-efficiency CRISPR/Cas9 genome editing plasmid, pKCcas9dO, into *S. coelicolor* M145 [15]. Wang et al. designed and engineered a type II CRISPR/Cas9 system and targeted multiplex gene deletions in *Streptomyces lividans*, *Streptomyces albus* and *Streptomyces viridochromogenes* [16]. Zhang et al. reported an efficient CRISPR/Cas9 knock-in strategy to activate silent biosynthetic gene clusters (BGCs) in streptomycetes [17]. However, this system remains deficient, particularly in *S. rimosus*, whose genome differs from other species of the genus *Streptomyces*. There is currently no detailed description of the efficiency of the different editing results. Therefore, in the present study the genes zwf2 and devB of *S. rimosus* were chosen as target genes to be edited through single-site mutations, double-site mutations or gene fragment disruptions using pCRISPomycoses. Deletions were made in zwf2 encoding isozymes of glucose 6-phosphate dehydrogenase, the first enzyme in the oxidative pentose phosphate pathway (PPP) [18]. The gene devB encodes the only protein known to be catalytically involved in the hydrolysis of 6-phosphogluconolactone. It is changes in flux through the PPP that are largely responsible for the effects on antibiotic production [19]. Based on our experimental results, some regulations of base mutation were summarized in this situation. In addition, multiple locus editing and fragment deletion methods were creatively designed. Our work provides a convenient and accurate molecular tool for genetic manipulation. This tool can be used to efficiently study the function of *Streptomyces* genes and to explore unknown genes and new genetic metabolic pathways.

**METHODS**

**Strains and plasmids**

*Escherichia coli* JM110 was grown in Luria–Bertani (LB) broth. The *S. rimosus* 1139 strain was grown in mycelium growth medium CRM (per litre: 10 g glucose, 103 g sucrose, 10.12 g MgCl₂·6H₂O, 15 g tryptone and 5 g yeast extract). Putative transformants were purified in CRM supplemented with appropriate antibiotics. pCRISPomycoses-2 was purchased from Addgene.

**Construction of recombinants**

According to genome sequences and parameters obtained from the sgRNACas9 software for designing CRISPR sgRNA and evaluating off-target sites (http://sourceforge.net/projects/sgrnacas9/) [20], three types of CRISPR array were designed: an array that contains one spacer (single crRNA), an array that contains two spacers (two independent crRNAs), and an array that contains one spacer with homologous arms (single crRNA with homologous arms).

To construct the single crRNA, primers zwfspacer1F/zwfspacer1R, zwfspacer2F/zwfspacer2R, devBspacer1F/devBspacer1R and devBspacer2F/devBspacer2R were synthesized (Table 1), and the annealed products were inserted into the *Bbs* I sites of pCRISPomycoses-2. This mode of mutation was called single-site mutation.

To construct the two independent crRNAs, two sgRNA cassettes targeting two different positions were included in a synthetic DNA fragment containing (from 5′ to 3′) the first gRNA sequence (spacer1), sgRNAtracr, a terminator (fd), a promoter [gapdh(EL)] and the second gRNA sequence.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′–3′)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>zwf F</td>
<td>GGGCATCTGCACCCGCACA</td>
<td>Amplification of zwf</td>
</tr>
<tr>
<td>zwf R</td>
<td>AGTCGAGACGGTGGTTC</td>
<td>Amplification of zwf</td>
</tr>
<tr>
<td>devB F</td>
<td>AGGGTCAAAGGTATCCG</td>
<td>Amplification of devB</td>
</tr>
<tr>
<td>devB R</td>
<td>CGGATAGGGCAGATTCTG</td>
<td>Amplification of devB</td>
</tr>
<tr>
<td>zwf spacer1F</td>
<td>ACGGCGAGGCCAGCAGTCACGACC</td>
<td>Spacer1 for pCRISPomycoses-zwf guide RNA</td>
</tr>
<tr>
<td>zwf spacer1R</td>
<td>AACGCGTGCTCGAGTGGCTGGCGTTCG</td>
<td>Spacer1 for pCRISPomycoses-zwf guide RNA</td>
</tr>
<tr>
<td>zwf spacer2F</td>
<td>ACGCCGGGCGCTGGTACCTCTTT</td>
<td>Spacer2 for pCRISPomycoses-zwf guide RNA</td>
</tr>
<tr>
<td>zwf spacer2R</td>
<td>AAACAGGTGGCATCCCGAGGCCC</td>
<td>Spacer2 for pCRISPomycoses-zwf guide RNA</td>
</tr>
<tr>
<td>devB spacer1F</td>
<td>ACGGGTGACCCACCCACCATCGACCC</td>
<td>Spacer1 for pCRISPomycoses-devB guide RNA</td>
</tr>
<tr>
<td>devB spacer1R</td>
<td>AAACGAGTGGCAGGGTGGCTGC</td>
<td>Spacer1 for pCRISPomycoses-devB guide RNA</td>
</tr>
<tr>
<td>devB spacer2F</td>
<td>AACGCGTGACCCFGCTCGAGTGGCTGC</td>
<td>Spacer2 for pCRISPomycoses-devB guide RNA</td>
</tr>
<tr>
<td>devB spacer2R</td>
<td>AAAACAGGTGGCATCCCGAGGCCC</td>
<td>Spacer2 for pCRISPomycoses-devB guide RNA</td>
</tr>
<tr>
<td>pCRISPomycoses F</td>
<td>TCCCTGCAAGGCTCAGC</td>
<td>Detection of pCRISPomycoses-2</td>
</tr>
<tr>
<td>pCRISPomycoses R</td>
<td>GGGAAGAAGCGGACAG</td>
<td>Detection of pCRISPomycoses-2</td>
</tr>
</tbody>
</table>
Table 2. zwf2 gene fragment deletion mutation sequence

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′–3′)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>zwf2 sgRNA-1 donors</td>
<td>gaagacGATCTTCAGAAGGCATCTGCAGGCGACGGCTACGAGCCAGCCGAGCTACCAGCGAAGGCCATCTCGAGGCAGCCAGCTACCAGGACCGCAAGGTCGAGGAAGCCATCCGGATGATCACGACGTCGCGACCGCGGCCAGCTCACCAGACC gaaagac</td>
<td>zwf2 sgRNA-1 100 bp HR disruption donors</td>
</tr>
<tr>
<td>devB sgRNA-1 donors</td>
<td>gaagacGATCTTCAGAAGGCATCTGCAGGCGACGGCTACGAGCCAGCCGAGCTACCAGCGAAGGCCATCTCGAGGCAGCCAGCTACCAGGACCGCAAGGTCGAGGAAGCCATCCGGATGATCACGACGTCGCGACCGCGGCCAGCTCACCAGACC gaaagac</td>
<td>devB sgRNA-1 100 bp HR disruption donors</td>
</tr>
</tbody>
</table>

(spacer 2), all flanked by Bbs I sites (Table 2) and inserted into the Bbs I sites of pCRISPomyces-2 (Fig. 1). This mode of mutation was called double-site mutation.

To construct single crRNA with homologous arms, sgRNA-1 was selected as the target. A synthetic DNA fragment containing (from 5′ to 3′) the 100-bp HR donor and the gRNA-1 sequence (spacer 1), all flanked by Bbs I sites, was inserted into the Bbs I sites of pCRISPomyces-2. The 100-bp HR donor harbouring an 8-bp deletion includes the PAM (protospacer adjacent motif) sequence and the last 3 bp of the guide sequence (Fig. 2). This mode of mutation was called gene fragment disruption.

Electrotransformation of intact cells

Cells of strain 1139 from 50 ml of a late exponential phase culture were harvested by centrifugation (1006 g, 10 min, 4°C) and washed three times with ice-cold distilled water and twice with cold electroporation medium (0.5 mol l⁻¹ sucrose and 1 mmol l⁻¹ MgCl₂). Finally, cells were concentrated 20-fold in the same medium. Competent cells were either used directly for electroporation or stored at –80°C. Electroporation was done using a Bio-Rad Gene Pulser with pulse controller. Before electroporation, 50 µl competent cells were mixed with 1 µl DNA in a 1 mm cuvette and preincubated at 50°C for 5 min. The mixture was subjected to an electric pulse (voltage 1.5 kV, resistance 800 Ω, capacitance 25 µF) for various time periods [21] and CRM was added immediately. Pulsed cells were incubated for 4–6 h at 30°C, then plated on CRM agar supplemented with the appropriate antibiotic and incubated at 30°C for 3–5 days.

Screening of mutants

Individual exconjugants were randomly picked and separated on CRM agar plates supplemented with apramycin, grown at 30°C for 2–3 days and subcultured twice. Single colonies were then picked into liquid CRM for genomic DNA isolation. To eliminate false positives, positive transformants were confirmed by PCR with pCRISPomyces-2 specific primers pCRISPomyces F/pCRISPomyces R.

![Fig. 1. Construction of pCRISPomyces-zwf2 for single-site mutations and double-point mutations. pCRISPomyces figure from Cobb et al. and wang et al. [14] [16].](image-url)
The zwf2 or devB locus was PCR-amplified and sequenced using zwf F/zwf R or devB F/devB R primers, respectively.

**Fermentation and oxytetracycline detection**

A spore suspension (1.0 × 10⁸) was inoculated into 40 ml seed medium and grown for 24 h. Then, a 4 ml seed culture was inoculated into 40 ml optimum FM medium [5% corn starch, 2% soya bean, 1.4% calcium carbonate, 1.4% (NH₄)₂SO₄, 0.4% NaCl, 0.01% KH₂PO₄, 0.4% corn steep liquor, 0.001% CoCl₂ and 0.1–0.2% amylase]. All fermentation cultures were grown at 30°C for 10 days.

To quantify the production of oxytetracycline, cultures were adjusted to pH 1.5–2.0 with 9 M HCl, and 1 ml of each culture was centrifuged at 12 000 g for 10 min. Samples were then subjected to HPLC analysis on a Shimadzu Prominence HPLC system with dual UV detector and YMC polymer C18 column (4.6 × 250 mm). Separation was performed under the following conditions: 60% H₂O, 10% methanol, 20% acetonitrile and 10% phosphoric acid (2 mM), with a constant flow rate of 1 ml min⁻¹. Corresponding peak areas detected at 350 nm were used to calculate the concentration of oxytetracycline.

**RESULTS**

**Single-site mutation and sequence analysis of zwf2**

Ten positive transformants were selected and cultured in the presence of apramycin. The mutation rate of mutants edited by zwf2sgRNA-1 or zwf2sgRNA-2 was 100% in both cases. The mutation sites were mostly distributed within the 15 and 16 bases upstream of the PAM sequence. It included two base mutations, GG changed to CA (zwf2sgRNA-1), and CG changed to TA (zwf2sgRNA-2). Sequence analysis of the mutants is shown in Fig. 3.

**Double-site mutation and sequence analysis of zwf2**

The mutation site guided by zwf2sgRNA-1 was located within the first to fourth bases upstream of the PAM sequence. The mutation site guided by zwf2sgRNA-2 was located within the third to fifth bases upstream of the PAM sequence. The mutation rate of the double-site mutation simultaneously guided by both zwf2sgRNA-1 and zwf2sgRNA-2 was 33.3%, including deletions and/or point mutations (Fig. 4).

**Gene fragment disruption and sequence analysis of zwf2**

Based on traditional CRISPR components, we used a 50 bp homology arm to introduce gene disruptions. The gene disruption rate was 100%. The sequence alignment of the mutants is shown in Fig. 5.

**Editing of devB**

The single-site mutations guided by devBsgRNA-1 or devBsgRNA-2 were largely distributed within the 12–13 bases and 8–9 bases upstream of the PAM sequence, respectively, which included two base mutations, GC changed to AT (devBsgRNA-1), and GG changed to CC (devBsgRNA-2). In the double-site mutation, the mutation guided by devBsgRNA-1 occurred at the second to ninth bases upstream of the PAM sequence, and the mutation guided by devBsgRNA-2 occurred at the ninth to fourteenth bases upstream of the PAM sequence. The mutation rate simultaneously guided by both devBsgRNA-1 and devBsgRNA-2 was 40%, including deletions and/or point mutations. Sequence analysis of the single-site mutations and double-site mutation is shown in Fig. 6(a, b). Sequence alignment of the disruption mutants is shown in Fig. 6(c).

**DISCUSSION**

Gene editing using a variety of methods, including the CRISPR system, has been implemented in several species of...
the genus Streptomyces, including *S. coelicolor*, *S. albus*, *S. lividans* and *S. iranensis*. However, most of these experiments have focused on deleting genes or gene clusters, implementing gene replacement, achieving functional verification of genes of interest, increasing yields of target products or forming new metabolites [22–27]. Detailed procedures for multiple modes of gene editing have not yet been reported.

In the current study, the CRISPR/Cas9 gene-editing system was applied to *S. rimosus*, and successfully produced single-site mutations, double-site mutations and disruptions in the target genes. While developing this strategy, we discovered several regulations. First, there is high probability of introducing a point mutation, as observed here as well as in rice [28, 29] and bacteria [30]. In our experiments, the rate of single site mutation was 100%, probably because we used subcultures so that the CRISPR/Cas9 system was able to continue working. Second, we show that the mutation sites

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**Fig. 3.** zwf2 gene single-site mutation (sgRNA-1/sgRNA-2) sequence analysis. gRNA target sequences are within the black rectangle; PAM sequences are within the red rectangle; and mutated sequences are shown in purple. ‘p’ indicates the single-site mutation.

**Fig. 4.** zwf2 gene double-site mutation (sgRNA-1 and sgRNA-2) sequence analysis. gRNA target sequences are within the black rectangle; PAM sequences are within the red rectangle; mutated sequences are represented by different colours; deletion sequences are indicated by dots. ‘d’ indicates the double-site mutation.

**Fig. 5.** zwf2 gene disruption mutations sequence analysis. gRNA target sequences are within the black rectangle; PAM sequences are within the red rectangle; deletion sequences are indicated by dots. ‘h’ represents the gene disruption mutation.
Fig. 6. (a) devB gene single-site mutation (sgRNA-1/sgRNA-2) sequence analysis results. gRNA target sequences are within the black rectangle; PAM sequences are within the red rectangle; and mutated sequences are shown in purple. (b) devB gene two-site mutation (sgRNA-1 and sgRNA-2) sequence analysis. gRNA target sequences are within the black rectangle; PAM sequences are within the red rectangle; mutated sequences are represented by different colours; deletion sequences are indicated by dots. (c) devB gene disruption mutations sequence analysis. gRNA target sequences are within the black rectangle; PAM sequences are within the red rectangle; deletion sequences are indicated by dots.

Fig. 7. Oxytetracycline yield of zwf2 gene mutants.

Fig. 8. Oxytetracycline yield of devB gene mutants.
are predictably and regularly distributed within the 14 bases upstream of the PAM sequence (included in the sgRNA sequence), and these included two base mutations. It is also interesting that the sgRNA-1 and sgRNA-2 mutations are exactly the same type. The Cas9 protein used in this study was derived from the Streptococcus pyogenes cas9 gene [16]. The location of the mutation site and the number of mutated bases in this study may differ from those in other studies due to differences in the type and source of the Cas9 protein.

Compared with single-site mutation, the double-site mutation strategy induces several mutation types that are different from single-site mutation, and it shows less regularity and predictability. We identified the following mutations in the gRNA-gene disruptions (10 %), two-base gene disruptions (10 %) and four-base gene disruptions (40 %). We identified the following mutations in the gRNA-2 region: C–T point mutations (10 %), T–C point mutations (20 %), single-base gene disruptions (20 %) and three-base gene disruptions (40 %). Consistently, all mutations occurred in the gRNA sequence region. The rate of double-site mutation was much lower than that of the single-site mutation. In contrast, similar experiments in rice resulted in similar mutation rates, regardless of whether paired gRNAs or a single gRNA was used [29]. The position of the double-site mutation was also different from the location of the two single-site mutations, and the proportion of gene disruptions in the double-site mutations was relatively large, which may be due to interactions between the two gRNAs. This could in turn change the location of the mutation site, and lead to multiple cutting sites, which would likely cause a gene disruption. The physiological effect of a double-site mutation, as determined by oxytetracycline levels, is similar to that of a fragment deletion; however, the experimental procedure is more convenient. The mutation rate obtained through a deletion strategy is also very high (100 %) due to addition of the homologous arms [17].

The oxytetracycline yield of zwf2− mutants was shown to be increased by 24.8 % in the disruption mutant (Fig. 7). On the basis of the zwf2− mutants, we edited the devB gene and the mutants (zwf2− devB−) increased the yield of oxytetracycline by 9.9 % more than the zwf2− mutants (Fig. 8), which is 36.8 % higher than the original strain used in zwf2− gene mutation.

Glucose 6-phosphate dehydrogenase (G6PDH) encoded by zwf2 and 6-phosphogluconolactonase encoded by devB are both key enzymes in the pentose phosphate pathway. Besides acting as the rate-limiting enzyme in this pathway, G6PDH is also a key enzyme in the formation of NADPH. The final two steps of oxytetracycline biosynthesis require large amounts of oxygen and NADPH. When the activity of G6PDH and 6-phosphogluconolactonase is reduced, more NADPH and oxygen enter the oxytetracycline biosynthesis pathway, causing an increase in oxytetracycline synthesis.

In the present study, editing systems of single-site mutations, double-site mutations and gene fragment disruptions were established, providing a new strategy for the efficient implementation of gene editing in S. rimosus. This has potential general applications in prokaryotes, and has both theoretical and practical significance.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
There are no human or animal experiments in this study.

References


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