An Efficient Procedure for Marker-Free Mutagenesis of \textit{S. coelicolor} by Site-Specific Recombination for Secondary Metabolite Overproduction

Bo Zhang$^{1,*}$, Lin Zhang$^{1,2,*}$, Ruixue Dai$^1$, Meiying Yu$^1$, Guoping Zhao$^{1,2,3,4,*}$, Xiaoming Ding$^1$

$^1$Department of Microbiology and Microbial Engineering, School of Life Sciences, Fudan University, Shanghai, China, $^2$Key Laboratory of Synthetic biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China, $^3$Shanghai-MOST Key Laboratory of Health and Disease Genomics, Chinese National Human Genome Center at Shanghai, Shanghai, China, $^4$Department of Microbiology and Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong SAR, China

Abstract

Streptomyces bacteria are known for producing important natural compounds by secondary metabolism, especially antibiotics with novel biological activities. Functional studies of antibiotic-biosynthesizing gene clusters are generally through homologous genomic recombination by gene-targeting vectors. Here, we present a rapid and efficient method for construction of gene-targeting vectors. This approach is based on Streptomyces phage $\phi$BT1 integrase-mediated multistep in vitro site-specific recombination. Four ‘entry clones’ were assembled into a circular plasmid to generate the destination gene-targeting vector by a one-step reaction. The four ‘entry clones’ contained two clones of the upstream and downstream flanks of the target gene, a selectable marker and an \textit{E. coli-Streptomyces} shuttle vector. After targeted modification of the genome, the selectable markers were removed by $\phi$C31 integrase-mediated \textit{in vivo} site-specific recombination between pre-placed $attB$ and $attP$ sites. Using this method, part of the calcium-dependent antibiotic (CDA) and actinorhodin (Act) biosynthetic gene clusters were deleted, and the $rrdA$ encoding RrdA, a negative regulator of Red production, was also deleted. The final prodiginine production of the engineered strain was over five times that of the wild-type strain. This straightforward $\phi$BT1 and $\phi$C31 integrase-based strategy provides an alternative approach for rapid gene-targeting vector construction and marker removal in streptomyces.

Introduction

Members of the Gram-positive, spore-producing genus \textit{Streptomyces} play critical roles in soil ecology and are useful for synthesizing medically and industrially important secondary metabolites. Genomic sequencing of the model organism \textit{Streptomyces coelicolor} A3(2) was completed in 2002 [1], which dramatically facilitated gene function studies of these bacteria, especially those involved in antibiotic production through targeted modification [2]. Additionally, with the increased data of whole genomic actinomycetes sequences, robust and rapid methods are required to study the functions of a large number of important genes. The gene-targeting approach through homologous recombination was the first step in construction of gene-targeting vectors. The traditional method of constructing vectors is by step-by-step enzymatic digestion and ligation following PCR amplification and assembly of independent DNA segments into a plasmid. However, this method is complicated and time consuming, and often limited by lack of proper restriction enzymatic sites.

Many methodologies have been developed for gene targeting vector construction [3,4,5,6], especially those of recombination-based strategies [4,5,7,8,9,10,11,12,13,14]. Among these, a powerful method, termed REDIRECT$^\text{TM}$ technology (PCR-targeting system) [4,7,15] has been widely used in \textit{Streptomyces}. This procedure is based on phage $\lambda$-Red proteins to promote recombination, which employs Red$\alpha$ (exo), Red$\beta$ (bet) and Red$\gamma$ (gam) to mediate recombination when only tens of nucleotides are homologous to the target region [4]. In \textit{E. coli} with helper plasmids, PCR-amplified selectable markers using primers with only 39 nt homology extensions are used to amplify chromosomal sequences within a genomic cosmids library [15] to generate the desired gene-targeting vectors. Another universal cloning method, referred to as Gateway$^\text{TM}$ technology [8], is based on phage $\lambda$ site-specific integrase between $attB$ and $attP$ sites and excision between $attL$ and $attR$ sites. Using expanded properties of recombination sites with unique specificities, many segments could be cloned into a vector backbone [16], thereby expediting several simple one-week methods to construct gene-targeting vectors [5,12,14]. These two phage $\lambda$-recombination-based technologies are simple and.
efficient, and have been combined together for constructing gene targeting vectors to generate knock-out mice [17]. Furthermore, the μ transposon was developed to allow random insertion of selectable markers and other desired sequences into destination plasmids for rapid generation of gene-targeting vectors [3,18,19], which complemented existing recombination-based approaches for generation of gene-targeting constructs [3].

We previously established a highly efficient in vitro site-specific recombination system based on Streptomyces phage φBTI integrase and identified the minimal sizes of attB and attP sites (36-bp and 45-bp, respectively), which was smaller than that of λ site-specific recombination (25-bp and 200-bp, respectively) [20]. We selected 16 pairs of non-compatible recombination sites, of which the central dinucleotides were not identical, and inhibited DNA strand exchange and religation, thus no recombination could occur between site pairs containing different core sequence mutations [21]. Here, we report a simple and highly efficient system for marker-free gene targeting in S. coelicolor by combining φBTI integrase-mediated multisite recombination in vitro, homologous recombination and φC31 integrase-mediated site-specific recombination in vivo [22]. This simple strategy should be readily suitable and advantageous for poorly genetically established Streptomyces systems without an ordered cosmid library, coupled with a desire to knockout longer DNA segments, and could be easily adopted to other organisms to construct gene-targeting vectors.

Using this method, we constructed an S. coelicolor strain for overproduction of prodigine (Red), which is one of the four main antibiotics produced by S. coelicolor A3(2) with anti-fungal, anti-bacterial, anti-protozoan, anti-malarial, immunosuppressive and anti-cancer activities [23,24]. The biosynthesis of calcium-dependent antibiotic (CDA) and actinorhodin (Act), which might influence Red production by competition of common precursors, were disrupted by homologous recombination after parts of the key genes of these two biosynthetic clusters were deleted. For CDA, nonribosomal peptide synthetase (NRPS) coding genes cdaPS1, cdaPS2 and part of cdaPS3, were replaced by the apramycin resistance gene aac(3)IV, and for Act, structural genes (actII to actIX) were deleted. In addition, one of the Ter family protein genes, redA, which negatively regulates Red production by controlling the abundance of RedD mRNA [25], was also deleted using our method. The final Red production of the engineered strain was over five times that of the wild-type strain.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this work are listed in Table 1. E. coli DH5α [26] was used for plasmid propagation. Mannitol soy flour [27] agar was used to generate spores and select for S. coelicolor exconjugants. R2YE agar was used for phenotype screening. R4 liquid medium (100 mg casamino acids, 1 g yeast extract, 3 g proline, 10 g MgCl₂·6H₂O, 10 g glucose, 4 g CaCl₂·2H₂O, 5.6 g TES, and 0.2 ml trace element in 1.0 L H₂O) was used for antibiotic production. The conjugal transfer from E. coli ET12567/ppZB102 into S. coelicolor was performed as described previously [27]. Bacillus subtilis Flaggat ATCC 6462 was used as indicator strain for CDA production assay [27]. Antibiotics were added at the following final concentrations: ampicillin, 50 μg ml⁻¹; apramycin, 30 μg ml⁻¹; chloramphenicol, 34 μg ml⁻¹; kanamycin, 30 μg ml⁻¹; and thiostrepton, 20 μg ml⁻¹.

Construction of the plasmids

All plasmids used in this study are described in Table 1, and primers are listed in Table S1. To construct the 'entry vectors', three 1.0 kb cassettes, attB₁-attB₆ attB₁-attB₆ attB₁-attB₆, and attB₁-attB₆ attB₁-attB₆ attB₁-attB₆, were PCR-amplified using the PrimerTacks® PCR Kit (TaKaRa, Kyoto, Japan) and ligated into the pMD19-T vector (TaKaRa) to generate plasmids pFA0066, pTA0013 and pTA1315 (see Table 1). Primers ZB153 and ZB154 (containing attB₁-φC31), ZB155 (containing attB₁-φC31) and ZB156 were to amplify the aac(3)IV gene fragment, which was inserted into Xcm linearized pTA0006 and pTA1315 to generate pFDZ101 and pFDZ103. These two plasmids were used to carry homologous arms after Xcm digestion (see Fig. 1). Plasmid pSET152 was used as a template for the aac(3)IV gene.

Primer pairs ZB129/ZB130 and ZB131/132 were used to amplify two 2.1 kb homologous arm fragments of the CDA biosynthetic gene cluster, and the PCR products were inserted into pFDZ101 and pFDZ103 to generate pFDZ101-CDA 5’arm and pFDZ103-CDA 3’ arm, respectively. Primer pairs ZB125/ ZB126 and ZB127/128 were used to amplify the 2.1 kb upstream homologous arm and 2.2 kb downstream homologous arm fragments of the Act biosynthetic gene cluster, and the PCR products were inserted into pFDZ101 and pFDZ103 to generate the pFDZ101-Act 5’ arm and pFDZ103-Act 3’ arm, respectively. Using the same method, we obtained the pFDZ101-rnd4 5’ arm and the pFDZ103-rnd4 3’ arm, containing a 1.6 kb upstream homologous arm fragment and a 1.7 kb downstream homologous arm fragment of the rnd gene, respectively, which were amplified using primer pairs ZB180/ZB181 and ZB182/ZB183.

Primers Osj128 and Osj129 were used to amplify the aphII gene fragment from pRT802 [28], which was inserted into the Xcm linearized pTA0613 to generate pFDZ102. Fragment amplification containing the cat gene from pBC-AM [25] flanked by attB₁ and attB₁₂ were generated using the primer pair PTP00-PTB15 and the product was digested with Xhol/BamHI and inserted into the E. coli-Streptomyces shuttle vector pHZ1358 [27], which contained the plasmid origin of transfer (oriT) to generate pFDZ100. pFDZ100 is very unstable in streptomycetes and is easily lost when not maintained with antibiotic selection.

Construction of plasmid pXD34-int is described in the Supplemental Materials section. Plasmid pXD34-int was cut with EcoRV/Xhol to generate a 7.1 kb fragment, which contained the tipB promoter and φC31 integrase gene, and then inserted into the corresponding sites in vector pHZ1358, yielding plasmid pZB101. pZB101 was further digested with Stul/Eco36I and then linked with a 1.5 kb SmaI-linearized fragment containing the aac(3)IV gene from plasmid pBC-AM [25] (Table 1) to generate pZB102. pZB102 was subsequently conjugated from the donor E. coli ET12567/pUZ8002 into the null mutants to remove the resistance gene by φC31 integrase-mediated in vivo site-specific recombination (see Fig. 2B). The exconjugants were selected by growth on MS media supplemented with thioestrepton (20 μg ml⁻¹) and apramycin (30 μg ml⁻¹). The thioestrepton was used to induce the expression of φC31 integrase. As plasmids pZB101 and pZB102 were derived from pHZ1358, which contained the ori DNA region (strong incompatibility locus), they could be easily lost in non-resistance stress condition [27].

Standard in vitro recombination assays and verification of the positive assembly products

Expression and purification of φBTI integrase was performed as described previously [20]. The recombination reaction was performed in a reaction mixture (10 μl) containing 10 mM Tris-HCl, pH 8.0, 100 mM KCl, 50 mM NaCl, 5% glycerol, integrase (4 μ l 0.5 μl, ~75 nM) and ~30 ng DNA reaction substrates [21]. The reaction was incubated at 30°C overnight and treated with proteinase K at 55°C for 30 min and then heated to 80°C for
Table 1. Strains and plasmids used in this study.

| Strains or plasmids | Genotype or description | Ref. or source |
|---------------------|-------------------------|---------------|
| **S. coelicolor**   |                         |               |
| M145                | SCP1- SCP2-             | [27]          |
| ZB1                 | M145 with CDA gene cluster disrupted, containing $\text{aac}(3)\text{IV}$ gene copy in the chromosome | This work |
| ZB2                 | ZB1 with the resistance gene removed | This work |
| ZB3                 | ZB2 with Act gene cluster disrupted, containing a $\text{aphII}$ gene copy in the chromosome | This work |
| ZB4                 | ZB3 with the resistance gene removed | This work |
| ZB7                 | ZB4 with rrdA gene disrupted, containing a $\text{aphII}$ gene copy in the chromosome | This work |
| ZB8                 | ZB7 with the resistance gene removed | This work |
| ZB8/pFDZ16-rrdA     | ZB8 carrying integrative plasmid pFDZ16-rrdA | This work |
| ZB8/pFDZ16          | ZB8 carrying integrative plasmid pFDZ16 | This work |
| **E. coli**         |                         |               |
| DH5'                | F- recA lacZ ΔM15        | [26]          |
| ET12567             | *dam* *dcm* *hsdS*      | [27]          |
| **Plasmids**        |                         |               |
| pMD19-T             | 2.7-kb cloning vector; Amp\(^\delta\) | Takara |
| pBC-AM              | Donor of $\text{aac}(3)\text{IV}$; Apr\(^\delta\) Cm\(^\delta\) | [25] |
| pHZ1358             | *E. coli*-Streptomyces shuttle vector; Apr\(^\delta\) Thio\(^\delta\) | [25] |
| pXD34-int           | *E. coli*-Streptomyces shuttle vector; derivative obtained from pJ6021, containing tipA promoter and the $\delta$C31 integease gene; Apr\(^\delta\) Thio\(^\delta\) Kan\(^\delta\) | This work |
| pFDZ100             | *E. coli*-Streptomyces shuttle vector; derivative obtained from pHZ1358, containing attP\(_0\) site and attB\(_{15}\) site; Thio\(^\delta\) Amp\(^\delta\) Cm\(^\delta\) | This work |
| pTA0006             | Derivative obtained from pMD19-T, containing attB\(_8\) site and attP\(_0\) site; Apr\(^\delta\) | [32] |
| pTA0613             | Derivative obtained from pMD19-T, containing attB\(_8\) site and attP\(_{13}\) site; Apr\(^\delta\) | [32] |
| pTA1315             | Derivative obtained from pMD19-T, containing attB\(_{13}\) site and attP\(_{15}\) site; Apr\(^\delta\) | This work |
| pFDZ101             | Derivative obtained from pTA0006, containing attB\(_8\)-δC31 site | This work |
| pFDZ102             | Derivative obtained from pTA0613, replacing the aac(3)IV resistance gene with the aphII resistance gene | This work |
| pFDZ103             | Derivative obtained from pTA1315, containing attP\(_{0}\)-δC31 site | This work |
| pFDZ101-CDA-5'arm   | Derivative obtained from pFDZ101, containing upstream homologous arm of CDA biosynthetic gene cluster gene-targeting | This work |
| pFDZ103-CDA-3'arm   | Derivative obtained from pFDZ103, containing downstream homologous arm of CDA biosynthetic gene cluster gene-targeting | This work |
| pFDZ101-Act-5'arm   | Derivative obtained from pFDZ101, containing upstream homologous arm of Act biosynthetic gene cluster gene-targeting | This work |
| pFDZ103-Act-3'arm   | Derivative obtained from pFDZ103, containing downstream homologous arm of Act biosynthetic gene cluster gene-targeting | This work |
| pFDZ101-rrdA-5'arm  | Derivative obtained from pFDZ101, containing upstream homologous arm of rrdA gene | This work |
| pFDZ103-rrdA-3'arm  | Derivative obtained from pFDZ103, containing downstream homologous arm of rrdA gene | This work |
| pFDZ100-CDA-tandem  | Derivative obtained from pFDZ100, containing two homologous arms of CDA biosynthetic gene cluster gene-targeting; Apr\(^\delta\) | This work |
| pFDZ100-Act-tandem  | Derivative obtained from pFDZ100, containing two homologous arms of Act biosynthetic gene cluster gene-targeting; Kan\(^\delta\) | This work |
| pFDZ100-rrdA-tandem | Derivative obtained from pFDZ100, containing two homologous arms of rrdA gene; Kan\(^\delta\) | This work |
| pZB101              | *E. coli*-Streptomyces shuttle vector; derivative obtained from pHZ1358, containing tipA promoter and the δC31 integease gene; Kan\(^\delta\) Thio\(^\delta\) Amp\(^\delta\) | This work |
| pZB102              | *E. coli*-Streptomyces shuttle vector; derivative obtained from pZB101, replacing the aphII resistance gene with the aac(3)IV resistance gene | This work |
| pSET152             | Integrative vector for actinomycetes; containing oriT, int, and attP-δC31 site, Apr\(^\delta\) | [27] |
| pRT802              | *E. coli*-Streptomyces shuttle plasmid, encoding δBT1-int and attP, resistant to kanamycin | [28] |
| pFDZ16              | *E. coli*-Streptomyces integrative shuttle vector containing tipA promoter, Kan\(^\delta\), Thio\(^\delta\), Amp\(^\delta\). | [25] |
| pFDZ16-rrdA         | Derivative obtained from pFDZ16, containing the rrdA gene located downstream of the tipA promoter, Kan\(^\delta\), Thio\(^\delta\), Amp\(^\delta\). | [25] |

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20 min for inactivation and transformed into E. coli strain DH5α. After selection with antibiotics, plasmids of positive clones were isolated and verified by enzyme digestion.

CDA production assay

The CDA bioassay was carried out as previously described by Kieser et al. [27]. Spores were spotted onto an Oxoid nutrient agar plate and incubated for 2 days, then overlaid with soft nutrient agar containing Bacillus mycoides and calcium nitrate at a concentration to yield 12 mM throughout the plate. CDA produced by S. coelicolor killed the B. mycoides cells, resulting in a clearance zone on the plate.

Quantification of Red production

Red production was assayed according to previous descriptions [27]. A culture grown in 40 ml R4 liquid medium was filtered to separate the supernatant and pellet. The mycelium pellet was collected and dried under a vacuum, then extracted with methanol overnight at room temperature. The methanol was acidified with HCl (to a final concentration of 0.5 M). Optical density was measured at 530 nm. Measurements were always performed for triplicate samples.

Results and Discussion

Rapid construction of the gene-targeting vectors by site-specific recombination in vitro

Streptomyces phage φBT1 integrase is capable of catalyzing accurate and efficient site-specific in vitro recombination between attB and attP sites [20]. In our previous work, 16 pairs of non-compatible sites containing different core sequences were identified, which confirmed that no recombination could occur between site pairs containing different core sequence mutations [20]. Thus, these sites could be used for multisite cloning [21,29]. We established a multiple DNA fragment assembly method, termed site-specific recombination-based tandem assembly (SSRTA). SSRTA details and principles were described in our previous report [4,7,15]. As shown in Fig. 1, plasmids pFDZ101 and pFDZ103 were digested with XcmI to produce 5′-T overhangs on both ends. Homologous arms could be inserted by TA cloning to generate the pFDZ101 5′-arm and pFDZ103 3′-arm. Plasmid pFDZ102 containing the aphII gene was used for clone selection in E.coli and Streptomyces. Plasmid pFDZ101 was used for propagation of the assembled gene-targeting vector in E.coli and transformed into Streptomyces by conjugation. Fig. 2A demonstrated the one-step tandem assembly of the four DNA fragments into a circular plasmid by φBT1 integrase-mediated in vitro site-specific recombination. Each fragment was flanked by a pair of non-compatible recombination sites, and after incubation with φBT1 integrase in proper buffer conditions (see Materials and Methods), the four fragments were assembled together by four simultaneous recombination events.

Using this method, we successfully constructed three gene-targeting vectors. Vectors pFDZ100-CDA-tandem and pFDZ100-Act-tandem were used to disrupt the biosynthesis of CDA and Act, respectively, and pFDZ100-rrdA-tandem was used for rrdA gene deletion. Construction details of the three vectors are in the Supplemental Information. This tandem assembly method for gene-targeting vector construction was efficient and convenient, and the resistance genes (aph(3)IV and aphII in this study) could be easily replaced by any others in pTA0613 and pFDZ102. Plasmids pFDZ101~pFDZ103 could be XcmI-digested to produce 3′-T overhangs on both ends and the homologous PCR-amplified arm fragments can be indirectly inserted by TA cloning to generate the pFDZ101 5′-arm and the pFDZ103 3′-arm, which avoided the limitation of available restriction recognition sites and restriction recognition sites contained in the homologous arms. Thus the construction procedure was condensed to approximately one week by our method.

Markerless disruption of CDA, Act biosynthetic gene clusters and Deletion of the rrdA gene

Cross-regulation of endogenous gene clusters in S. coelicolor M145 might involve competition for common precursors [25]. Therefore, we deleted parts of two main antibiotic biosynthesis gene clusters, calcium-dependent antibiotic (CDA) and actinorhodin (Act), to disrupt their production in S. coelicolor M145 in order to increase prodigine (Red) production. After pFDZ100-CDA-tandem conjugation into S. coelicolor M145, parts of the CDA biosynthetic gene cluster containing NRPS-encoding genes cdaPS1,
cdaPS2 and part of cdaPS3 (sco3228–sco3232, ~42 kb), were replaced by the apramycin resistance gene, \( \text{aac(3)}\text{IV} \). Double-crossover colonies were obtained by screening the Aprar Thios exconjungants to generate the mutant strain ZB1 (see Table 1). We then transferred the marker-removal plasmid pZB101, which expresses the \( \text{w} \text{C31 integrase} \), into ZB1 by conjugation. As shown in Fig. 2B, the resistance gene was flanked by \( \text{attB0}-\text{wC31} \) and \( \text{attP0}-\text{wC31} \). After \textit{in vivo} site-specific recombination between these two sites catalyzed by \( \text{wC31 integrase} \), the resistance gene in the plasmid pTA0613 would be removed, leaving one \( \text{attL0}-\text{wC31} \) in the genome. Because the central dinucleotide \( \text{attP0}-\text{wC31} \) sequence was not identical to the wild-type \( \text{attB} \) site of \( \text{wC31 integrase} \), no reaction occurred between \( \text{attP0} \) and \( \text{attB} \) in the genome. Exconjungants were screened in the Thior Kanr MS media, and after the resistance gene was removed, the mutant strain was named ZB2 (see Table 1). We then constructed the CDA and Act biosynthetic double deficient strain ZB3 by further deleting part of the Act biosynthetic gene cluster (including key genes required for ACT biosynthesis, ranging from sco5087 to sco5092, ~3.6 kb).

After deletion of the resistance gene \( \text{aphII} \) by marker-removal plasmid pZB102, the strain was named ZB4 (see Table 1).

In our previous work, we identified the \( \text{rldA} \) gene as a TetR family protein gene, which regulated secondary metabolite production in \( S. \text{coelicolor} \) by negative regulation of Red biosynthesis and controlling RedD mRNA abundance [25]. Thus, we used the pFDZ100–\text{rldA}-tandem to further knock-out the \( \text{rldA} \) gene from the mutant strain ZB4 for Red overproduction. The mutated strains before and after removing the resistance gene \( \text{aphII} \) were named ZB7 and ZB8 (see Table 1).

Due to the high efficiency and accuracy of the site-specific recombination reaction, the ratio of positive exconjungants with marker removal was about 50%, and the entire process was very time-efficient. Including the construction of plasmids, the deletion of the gene cluster and removal of selectable markers, the whole process took less than a month. Furthermore, compared to other marker excision systems (Flp, Cre, Dre recombinases) [30,31], \( \text{wC31 integrase} \)-mediated recombination is highly directional in the absence of the excisionase. As no resistance marker was preserved in the chromosome, this approach avoided the
Figure 3. PCR analysis and phenotypes of mutants. (A) PCR verification of gene deletion. Lane 1, a 42,725 bp fragment was amplified using primers ZB287/ZB288. The size of the fragment was too large and could not be PCR-amplified, thus no band was seen in lane 1. Lane 2, a 1981 bp fragment was amplified using primers ZB287/ZB288. The size of the fragment was too large and could not be PCR-amplified, thus no band was seen in lane 1. Lane 2, a 1981 bp fragment was amplified using primers ZB287/ZB288. The CDA biosynthetic gene cluster was replaced with the \textit{aac(3)IV} gene. Lane 3, a 927 bp fragment was amplified using primers ZB287/ZB288. Lanes 4 and 6 showed the bands before and after removing the \textit{aphII} resistance gene. Lane 7, a 4073 bp fragment was amplified with primers ZB289/ZB290. Lanes 5 and 6 showed the bands before and after removing the \textit{aphII} resistance gene. WT, wild type strain; ZB1~ZB8, serial mutant strains. (B) PCR verification of gene deletion. Lane 1: the fragment was amplified with primers ZB145/ZB146 and primers were both within \textit{sco3229}. Lane 2: the fragment was amplified with primers ZB195/ZB196 and primer ZB195 was within \textit{sco5089} and primer ZB196 was within \textit{sco5090}. Lane 3: the fragment was amplified with primers ZB184/ZB186 and primer ZB184 was within \textit{sco1103} and primer ZB186 was within \textit{sco1104}. Lane 4: the fragment as a positive control was amplified with primers ZB147/ZB148 and primers were both within \textit{sco5888}. M: 150 bp ladder. (C) PCR verification of the resistance removing. Lane 1: M145; Lane 2: ZB1; Lane 3: ZB2. The three fragments were amplified with primers ZB189/ZB190, primers were both within the \textit{acc(3)IV} gene. Lane 4: M 145; Lane 5: ZB3; Lane 6: ZB4; Lane 7: ZB7; Lane 8: ZB8. The five fragments were amplified with primers ZB187/ZB188, primers were both within the \textit{aphII} gene. M: 150 bp ladder. (D) Bioassay of CDA extracts from the WT strain and CDA-null mutant strain ZB2. (E) Phenotypes of wild-type and three mutant strains. The spores were cultured for 2 days (left) and 5 days (right) on R2YE agar.

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Figure 4. Growth curves and prodiginine production of wild-type strain, three mutant strains and complemented strains. Growth curves (A) and prodiginine production (B) of M145, ZB2, ZB4, ZB8, ZB8/pFDZ16 and ZB8/pFDZ16-rrdA growth in 40 ml of R4 liquid medium. Incubation was carried out at 30°C. The symbols indicate the averages of three independent determinations and the error bars indicate the standard errors. OD\textsubscript{530}, optical density at 530 nm.

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limitations of selectable resistance markers and was quite convenient for the following gene replacement, especially for multiple gene knock-outs.

PCR verification of the mutants and phenotypes

Each null mutant was verified by PCR amplification using a pair of oligonucleotide primers specific for the flanking chromosomal DNA sequences of the target gene. The results are shown in Fig. 3A. In lane 1, no band was seen because the PCR fragment size was very large (~42 kb), which almost contained the entire CDA biosynthesis gene cluster, and could not be amplified. The further verification was also carried on by PCR amplification using two different pairs of oligonucleotide primers. One pair of primers was synthesized according to the internal sequences of the deleted gene clusters or gene, and the other one was based on the internal sequences of the resistance gene. The results were shown in Fig. 3B and Fig. 3C. No band was amplified with the former primers and a 500 bp-size band could be seen clearly with the later primers in the double-crossover null mutant. It turned out just the opposite result in the wild type {Streptomyces coelicolor} M145. The confirmation of removing the resistance gene was seen in Fig. 3A and Fig. 3C. The PCR products had also been sequenced to ensure excision of the antibiotic cassettes occurred precisely (Fig. S1). The aac(3)IV gene was used as a selectable marker in ZB1 while using aphII in ZB3 and ZB7, due to cross-resistance (<50 μg/ml) of the aac(3)IV gene to kanamycin that could have complicated the marker-removal process by false positives. However, almost no cross-resistance (<3 μg/ml) of the aphII gene to apramycin occurred.

Mutant phenotypes are shown in Fig. 3D and 3E. CDA production analysis was carried out in line with previous descriptions [25,27]. B. mycoides was used as an indicative strain to detect CDA activity. First, spores were spotted onto oxoid nutrient agar plates, air-dried and cultured for 36–48 h in a 30°C incubator. Then, the plate was overlaid with the soft nutrient agar containing B. mycoides and Ca(NO₃)₂ at a 12 mM final concentration and CDA activity was detected after one day. CDA production in strain ZB6 was determined three rounds in the Streptomyces genome. The utility of this method was clearly demonstrated by construction of a prodiginine (Red) overproducing strain, ZB6, which produced five times the amount of Red compared to the wild-type strain. This strategy provided an alternative approach for rapid markerless modification of the actinobacterial genome.

Supporting Information

Table S1 Primers used in this study.

(DOCX)

Text S1 Construction of plasmids pXD34-int, pFDZ100-CDA-tandem, pFDZ100-Act-tandem and pFDZ100-rrdA-tandem.

(DOCX)

Figure S1 Sequence analysis of PCR products for verification of the markerless mutants. Primers ZB469 (for ZB2), ZB472 (for ZB4) and ZB473 (for ZB8) were used for sequencing the PCR products.

(TIFF)

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Author Contributions

Conceived and designed the experiments: BZ, GPZ, XMD. Performed the experiments: BZ, RXD. Analyzed the data: BZ, LZ. Contributed reagents/materials/analysis tools: MYX. Wrote the paper: BZ, LZ.

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