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High-Efficiency Multiplex Genome Editing of *Streptomyces* Species Using an Engineered CRISPR/Cas System

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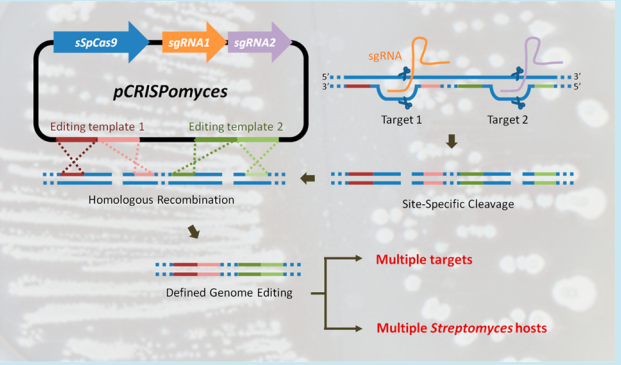
Supporting Information

ABSTRACT: Actinobacteria, particularly those of genus *Streptomyces*, remain invaluable hosts for the discovery and engineering of natural products and their cognate biosynthetic pathways. However, genetic manipulation of these bacteria is often labor and time intensive. Here, we present an engineered CRISPR/Cas system for rapid multiplex genome editing of *Streptomyces* strains, demonstrating targeted chromosomal deletions in three different *Streptomyces* species and of various sizes (ranging from 20 bp to 30 kb) with efficiency ranging from 70 to 100%. The designed pCRISPomyces plasmids are amenable to assembly of spacers and editing templates via Golden Gate assembly and isothermal assembly (or traditional digestion/ligation), respectively, allowing rapid plasmid construction to target any genomic locus of interest. As such, the pCRISPomyces system represents a powerful new tool for genome editing in *Streptomyces*.



KEYWORDS: *genome engineering, CRISPR, Cas9, synthetic guide RNA, Streptomyces*

Actinobacteria of the genus *Streptomyces* are among the most prolific and well-studied producers of diverse secondary metabolites.[1,2] Over the past several decades, *Streptomyces* strains have been found to produce a number of important bioactive natural products, such as the anticancer compound daunorubicin from *Streptomyces peucetius*,[3] the herbicide phosphinothricin from *Streptomyces hygroscopicus*[4] and *Streptomyces viridochromogenes*,[5] and the antibacterial daptomycin from *Streptomyces roseosporus*.[6] While these decades of study might suggest that the supply of *Streptomyces* natural products is nearing exhaustion, in fact, genome sequencing efforts have revealed that the well is far from dry, even in the most comprehensively studied strains.[7]

Access to this "silent" majority of uncharacterized natural product gene clusters would benefit greatly from the development of new genetic manipulation tools that leverage genomic information to aid natural product discovery, characterization, engineering, and production. In the context of a *Streptomyces* strain of interest, for example, facile genome manipulation would aid both discovery and validation of new natural products from uncharacterized gene clusters, as well as ensuing biochemical and mechanistic studies. In heterologous production strains, such techniques would further enable genomic remodeling to direct metabolic flux toward a pathway of interest and eliminate competing pathways, as well as pathway engineering for product diversification. Nevertheless, the current *Streptomyces* genetic toolkit, though well-developed and widely employed, often mandates a significant investment of time and effort.

Typically, for gene disruption in *Streptomyces*, single crossover integration of a suicide plasmid can be employed, resulting in disruption of the gene of interest with a selectable marker.[8] However, the limited number of selectable markers limits the reusability of this approach. Further, disruption via single-crossover can revert in the absence of selective pressure, resulting in undesired restoration of the wild type allele. Inclusion of flanking recombinase target sites and expression of the corresponding recombinase can enable recycling of markers and improve mutation stability, but this mandates additional steps and leaves a scar sequence at the target site.

Alternatively, clean genomic deletions can be made via double-crossover integration.[8] However, this multistep process is often labor and time intensive. First, integration of the disruption vector at the target locus (the first single-crossover event) is identified with a selectable marker. Next, loss of the disruption vector and its selectable marker (the second single-crossover event) is identified via nonselective culture. Finally, colonies exhibiting loss of the selectable marker (upon replica plating) must be further screened to separate those that have lost the vector via the desired second crossover event from those that have reverted the first crossover event to restore the

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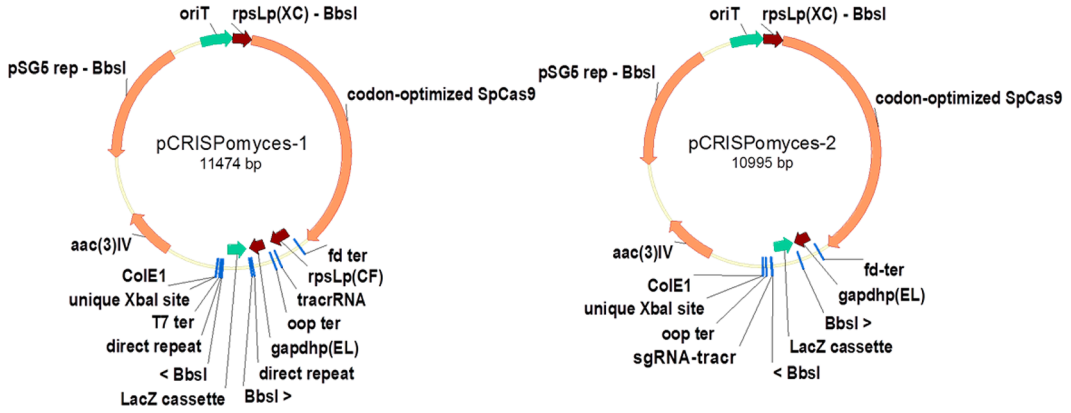

Figure 1. pCRISPomyces plasmids for targeted genome editing in *Streptomyces* species. Notable features include a codon-optimized *cas9* from *Streptococcus pyogenes*, a *Bbs*I-flanked lacZ cassette for Golden Gate assembly of spacer sequences, an *Xba*I site for addition of editing templates, and a temperature-sensitive pSG5 origin.

original genotype. Counterselectable markers such as *rpsL* and *glkA* can facilitate identification of the second crossover event but are limited to use only in particular mutant hosts.[8] To facilitate identification of double-crossover integrants in one step, a double-strand break (DSB) can be introduced at the genomic locus of interest, as has been demonstrated using a homing endonuclease.[9] However, this method is dependent upon prior integration of the homing endonuclease recognition site at the target locus, as homing endonucleases are minimally amenable to specificity alteration.[10]

Recently, DSB-mediated genome editing has been achieved via the type II Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated proteins (Cas) system of *Streptococcus pyogenes*.[11] Functioning similarly to a bacterial immune system, CRISPR/Cas requires three components to realize targeted cleavage of foreign DNA: Cas9, the nuclease and scaffold for the recognition elements; CRISPR RNA (crRNA), short RNAs conferring target site specificity, encoded as spacers in a CRISPR array; and trans-activating crRNA (tracrRNA), a short RNA that facilitates crRNA processing and recruitment to Cas9.[12] The Cas9/crRNA/tracrRNA complex can target any DNA sequence, known as a protospacer, provided that the requisite protospacer-adjacent motif (PAM) is present at the 3′ end (NGG in the case of *S. pyogenes*, where N represents any nucleotide).[13]

To repurpose this system for genome engineering, spacer sequences matching genomic loci of interest can be directly programmed into a heterologously expressed CRISPR array. To further simplify the system, fusion of the crRNA and tracrRNA into a single synthetic guide RNA (sgRNA) transcript has been demonstrated, obviating the need for processing of the transcribed CRISPR array (pre-crRNA) into individual crRNA components.[14] The *S. pyogenes* CRISPR/Cas system has been successfully reconstituted in a variety of hosts across all domains of life, including (but not limited to) *Escherichia coli*,[15] *Saccharomyces cerevisiae*,[16] and human cell lines.[17] Compared with other tools for site-specific genome engineering, such as zinc-finger nucleases and transcription activator-like effector (TALE) nucleases, the engineered CRISPR/Cas system offers unprecedented modularity. Targeting any site of interest requires only the insertion of a short spacer into a CRISPR array/sgRNA construct, which can be achieved rapidly and with high throughput using modern DNA assembly techniques. A suitable target site needs only to have an adjacent NGG sequence, which are notably abundant in GC-rich *Streptomyces* genomes.

To harness the CRISPR/Cas system for genome editing in *Streptomyces* species, the pCRISPomyces expression system was designed (Figure 1). Two versions of the pCRISPomyces plasmid were constructed: pCRISPomyces-1, which includes both tracrRNA and CRISPR array expression cassettes along with *cas9*, and pCRISPomyces-2, which includes a sgRNA expression cassette and *cas9*. In both cases, previously characterized strong promoters[18] were selected to drive expression of the CRISPR/Cas elements, along with widely used terminators from phages fd, *λ*, and T7. Analysis of the *Streptococcus pyogenes cas9* gene revealed the presence of several rare codons. Among them were many *bldA* codons, translation-level regulators of secondary metabolism in *Streptomyces* species.[19] As a result, a refactored *cas9* gene codon-optimized for *Streptomyces* expression was designed.

To facilitate seamless, one-step Golden Gate assembly of custom-designed spacers into the CRISPR array of pCRISPomyces-1, a *lacZ* cassette flanked by unique *Bbs*I restriction sites was incorporated between two direct repeat sequences in the empty CRISPR array. A *Bbs*I-flanked *lacZ* cassette was included in the sgRNA cassette of pCRISPomyces-2 for the same purpose. In both plasmids, a unique *Xba*I restriction site allows linearization of the backbone for insertion of additional elements, such as editing template sequences for recombination-driven repair, via Gibson assembly or traditional ligation. Inclusion of *aac(3)-IV* allows selection in both *Escherichia coli* and *Streptomyces* hosts, the colE1 origin enables replication in *E. coli*, and the RP4 origin of transfer *oriT* enables conjugative transfer of pCRISPomyces plasmids from *E. coli* to *Streptomyces* hosts.[20] Finally, the temperature-sensitive *rep* region from pSG5[21] allows rapid clearance of the pCRISPomyces plasmid following the desired genome editing.

To assess the functionality of the pCRISPomyces system, initial experiments were carried out in the well-studied strain *Streptomyces lividans* 66.[22] Two genomic protospacer sequences were selected: one in *redN*, from the undecylprodigiosin gene cluster,[23] and one in *actVA-ORF5*, from the actinorhodin gene cluster.[24] In both cases, a 20 bp sequence with the requisite NGG PAM sequence was chosen, with preference given to sites on the noncoding strand and those with multiple purine bases at the 3′ end.[25] To minimize off-target effects, sites were chosen in which the last 12 bp of the protospacer plus the PAM (15 bp

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Table 1. Summary of Genome Editing Results in *Streptomyces* Species

strain	plasmid	target(s)	deletion size	result[a]
S. lividans	pCRISPomyces-1	*redN*	20 bp	3/14
S. lividans	pCRISPomyces-1	*actVA-ORF5*	34 bp	2/8
S. lividans	pCRISPomyces-2	*redN*	20 bp	6/6
S. lividans	pCRISPomyces-2	*actVA-ORF5*	34 bp	8/8
S. lividans	pCRISPomyces-2	*redN*/*actVA-ORF5*	20 bp and 34 bp	4/4
S. lividans	pCRISPomyces-2	*redD*/*redF*	31 415 bp	4/4
S. viridochromogenes	pCRISPomyces-2	*phpD*	23 bp	7/7
S. viridochromogenes	pCRISPomyces-2	*phpM*	20 bp	4/6
S. albus	pCRISPomyces-2	*sshg_05713*	67 bp	6/6
S. albus	pCRISPomyces-2	*sshg_00040*/ *sshg_00050*	13 214 bp	2/3

[a]Number of correctly edited exconjugants/total number of exconjugants screened.

in total) were unique,[17] as confirmed by BLAST analysis against the published genome sequence. To enable defined editing via homologous recombination, a 2 kb editing template was supplied on the pCRISPomyces plasmid. The editing template consisted of two 1 kb arms homologous to the corresponding sequences upstream and downstream of the protospacer, designed to introduce a short deletion (20−34 bp) to partially or fully eliminate the protospacer sequence and create a frame shift.

The pCRISPomyces plasmids were transferred to *S. lividans* via conjugation. Exconjugants displaying apramycin resistance were confirmed by restreaking on selective ISP2 plates. To screen for the desired editing event, genotyping of multiple exconjugants was carried out by first isolating the genomic DNA and then PCR amplifying the target locus. To ensure that the PCR product was amplified from the chromosome rather than the pCRISPomyces plasmid, primers were designed to anneal slightly upstream and downstream of the editing template sequence. Each PCR product was sequenced with internal primers to determine if the intended deletion had been introduced. Of note, conjugation efficiency was found to be reduced by 5- to 10-fold for any plasmid bearing the *cas9* gene, suggesting an inherent toxicity associated with overexpression of the heterologous endonuclease.

Using the pCRISPomyces-1 system with separate tracrRNA and CRISPR array elements, only modest editing efficiency was observed (Table 1). For the *redN* target, three out of 14 exconjugants possessed the desired deletion, while the remaining 11 strains were unedited at the *redN* locus. As a negative control, a pCRISPomyces-1 derivative plasmid bearing all CRISPR elements (tracrRNA, *redN* spacer, and *redN* editing template) except for the *cas9* gene was constructed and conjugated into *S. lividans*. In this case, 12 exconjugants were screened, and each possessed the unedited genotype. For the *actVA-ORF5* target, a similar efficiency was observed as the *redN* target, with two out of eight exconjugants displaying the edited genotype.

To demonstrate clearance of the pCRISPomyces-1 plasmid, one of the identified *S. lividans* strains carrying the desired *redN* deletion was cultured nonselectively at high temperature (37−39 °C). After growth to stationary phase, a fraction of the culture was plated to isolate individual colonies. Multiple colonies were obtained that had regained apramycin sensitivity and did not produce the red undecylprodigiosin pigment (Supporting Information Figure S1), indicating successful clearance of the temperature-sensitive plasmid. This sequence allows the apramycin selection marker to be reused in this strain for future applications, such as further genome editing or introduction of heterologous genes.

Since the three-component CRISPR/Cas9 system of the pCRISPomyces-1 plasmid did not exhibit high editing efficiency, it was speculated that employing the simpler two-component system could yield improvement. To test this hypothesis, the same protospacers in the *redN* and *actVA-ORF5* target genes were evaluated with the sgRNA-expressing pCRISPomyces-2 plasmid. Editing efficiency was again evaluated by genotyping multiple isolated exconjugants. With the pCRISPomyces-2 *redN*-targeting construct, significantly higher editing efficiency was observed, as six out of six randomly selected exconjugants were revealed to possess the edited phenotype (Table 1; Supporting Information Figure S2). Similar results were observed for the *actVA-ORF5* target, where eight out of eight randomly selected exconjugants were found to be correctly edited. Taken together, these results show that sgRNA targeting affords higher efficiency than tracr/crRNA targeting. A possible explanation could be that pre-crRNA processing by native RNase enzymes is inefficient when applying CRISPR/Cas in *Streptomyces*, in contrast to results in other hosts where native RNase enzymes appear sufficient.[17]

One of the key advantages of the CRISPR/Cas system compared with other targeted nucleases is the relative ease with which multiple sequences can be simultaneously targeted using the same Cas9 endonuclease. Given the observed superiority of sgRNA over the tracrRNA/CRISPR array configuration for single-target genome editing, additional constructs were designed with two sgRNA cassettes to evaluate the potential for multiplex targeting. In these constructs, tandem sgRNA cassettes were driven by two copies of the same strong promoter. Insertion of both cassettes into the same pCRISPomyces-2 backbone was carried out in one step by Golden Gate assembly with a synthetic DNA fragment containing the first sgRNA sequence, a terminator, a promoter, and the second sgRNA sequence, all flanked by *Bbs*I sites.

The first dual-targeting construct was designed to introduce simultaneous short deletions in both the *redN* and *actVA-ORF5* loci, utilizing the same protospacer sequences and 2 kb editing templates previously evaluated. Following conjugation and isolation of genomic DNA from multiple exconjugants, both loci of interest were sequenced. In four out of four strains evaluated, the desired deletion was observed at both loci, demonstrating the potential for multiplex genome editing. The ability to cut two loci in the genome simultaneously opens up the possibility for excision of larger chromosomal segments by introducing a DSB at both ends and bridging the gap with a plasmid-borne editing template (Figure 2a). To test this

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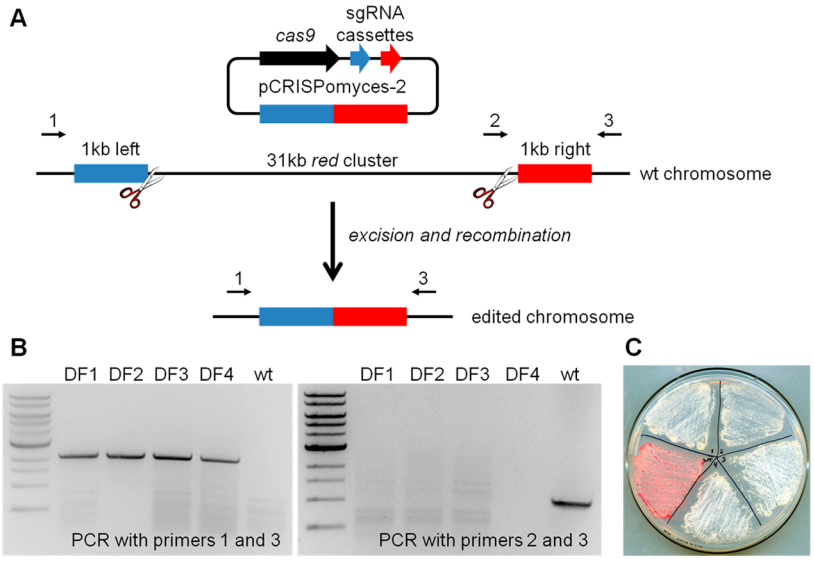

Figure 2. (a) Strategy for deletion of the 31kb *red* cluster. Two sgRNA transcripts guide Cas9 to introduce DSBs at both ends of the cluster, while a codelivered editing template bridges the gap via homologous recombination. (b) PCR evaluation of *red* cluster deletion from four exconjugants (DF1−DF4) with wild type control (wt). (c) Phenotypic evaluation of strains DF1−DF4 with wild type control.

method, a second dual-targeting construct was designed to delete the entire 31 kb *red* cluster. Protospacer sequences were selected within the genes at the start and end of the cluster (*redD* and *redF*, respectively), and a 2 kb editing template was constructed from the 1 kb sequences immediately flanking the cluster. In total, four exconjugants were picked and evaluated by PCR from genomic DNA with primers that anneal outside of the 1 kb homology arms. In all four cases, the 2.1 kb band indicative of cluster deletion was observed, while no band was observed when wild type genomic DNA was used as template (Figure 2b). Similarly, PCR amplification with a primer annealing within the deleted region only produced a 1.1 kb band from the wild type genomic DNA, but not from the strains in which the *red* cluster had been deleted. Phenotypic screening confirmed the loss of red pigmentation in all four edited strains (Figure 2c).

Outside of *S. lividans*, it would be desirable to utilize the pCRISPomyces plasmids in other *Streptomyces* strains to realize targeted editing of genes of interest, such as natural product gene clusters, in the native producers. To evaluate the possibility for broader applicability, two additional *Streptomyces* species were selected: *S. viridochromogenes* DSM 40736 and *Streptomyces albus* J1074. In *S. viridochromogenes*, two genes within the phosphinothricin tripeptide (PTT) gene cluster,[26] *phpD* and *phpM*, were chosen as individual targets. For both genes, the corresponding single-targeting pCRISPomyces-2 plasmid was constructed with an appropriate spacer and 2 kb editing template to introduce a short frame-shift deletion early in the coding sequence. Following an analogous conjugation and genotyping protocol as described for *S. lividans*, seven out of seven exconjugants isolated with the *phpD*-targeting plasmid were confirmed to possess the intended deletion. For *phpM*, a total of six exconjugants were screened, and four were found to exhibit the desired mutant genotype (Table 1; Supporting Information Figure S3). The other two exconjugants possessed the unedited wild type sequence.

In *S. albus*, the PKS-NRPS hybrid gene *sshg_05713* from a cryptic polycylic tetramic acid macrolactam gene cluster[27] was chosen to evaluate single locus targeting, again to introduce a short frame-shift deletion using a 2 kb editing template. Six out of six exconjugants evaluated by PCR amplification from genomic DNA and sequencing were confirmed to harbor the intended 67 bp deletion (Table 1), confirming Cas9 functionality in this strain. As a second trial, a dual-targeting plasmid was constructed to delete a full 13 kb lanthipeptide cluster encoded by genes *sshg_00040* to *sshg_00050*. After conjugation with the dual-targeting plasmid containing a 2 kb editing template, three *S. albus* exconjugants were isolated and evaluated by PCR. All three were found to yield the correct PCR product indicative of full-cluster deletion (amplified with primers 1 and 3 in Figure 2a), and the identity of the PCR product was confirmed by sequencing. However, one of the three also yielded the PCR product with a primer annealing within the deleted region (primers 2 and 3 in Figure 2a), indicating a mix of edited and wild type cells in this population. Nevertheless, the observed efficient genome editing of *S. viridochromogenes* and *S. albus* without strain-specific modification of the pCRISPomyces backbone suggests broader applicability of this system in various *Streptomyces* species.

In summary, we have demonstrated that the type II CRISPR/Cas system of *S. pyogenes* can be reconstituted successfully in multiple *Streptomyces* species to realize targeted, multiplex genome editing. To implement this system, we have developed pCRISPomyces plasmids amenable to insertion of custom spacers and editing templates via modern DNA assembly techniques. These tools reduce the time and labor needed to perform precise chromosomal manipulations compared to previous techniques. As a result, application of the pCRISPomyces system should facilitate a wide variety of future studies in *Streptomyces* species, such as analysis of biochemical pathways, activation of silent natural product gene clusters, pathway/metabolic engineering, and host design.

METHODS

Strains, Media, and Reagents. *E. coli* strain NEB5-alpha (New England Biolabs, Ipswich, MA) was used for plasmid cloning and maintenance. Yeast *in vivo* plasmid assembly was performed in *S. cerevisiae* HZ848.[28] *S. lividans* 66 was obtained

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from the Agricultural Resource Service Culture Collection (Peoria, IL), *S. albus* J1074 was a gift from Prof. Wenjun Zhang (University of California, Berkeley), and *S. viridochromogenes* DSM40736 and the *E. coli* conjugation strain WM6026[26] were gifts from Prof. William Metcalf (University of Illinois at Urbana−Champaign). *E. coli* strains were grown in LB medium supplemented with apramycin (50 μg/mL) and, for strain WM6026, diaminopimelic acid (19 μg/mL). *Streptomyces* strains were grown on modified MYG medium (10 g/L malt extract broth, 4 g/L yeast extract, 4 g/L glucose) with 50 μg/mL apramycin as needed for plasmid selection. Medium R2 (without sucrose) was used for conjugation.[8] All media components and supplements were purchased from Sigma-Aldrich (St. Louis, MO) with the exception of yeast extract (BD Biosciences, San Jose, CA) and LB broth (Fisher Scientific, Pittsburgh, PA). PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA), and PCR reactions were performed in FailSafe PCR PreMix G (Epicentre Biotechnologies, Madison, WI) with Q5 DNA polymerase (New England Biolabs, Ipswich, MA). All PCR products were purified using the DNA Clean & Concentrator or Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Plasmids were recovered using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Restriction enzymes and T4 ligase were purchased from New England Biolabs (Ipswich, MA).

Plasmid Construction. Plasmid pCRISPomyces-1 was constructed via yeast homologous recombination[28] from the following fragments: promoter rpsLp(XC),[18] synthesized as a gBlock (Integrated DNA Technologies, Coralville, IA) to remove *Bbs*I recognition sites; codon-optimized *Spcas9*, along with the wild-type fd terminator, synthesized by GenScript (Piscataway, NJ); promoter rpsLp(CF), PCR amplified from a previous construct;[18] tracrRNA, oop terminator, promoter gapdhp(EL),[18] a *lacZ* expression cassette flanked by *Bbs*I recognition sites and direct repeat sequences, and a T7 terminator, synthesized as a gBlock (IDT); yeast helper fragment containing *URA3* and CEN6/ARS4 flanked by *Xba*I recognition sites, PCR amplified from pRS416 (Stratagene, La Jolla, CA); and an *E. coli*/*Streptomyces* helper fragment containing origin colE1, selection marker *aac*(3)IV, pSG5 *rep* origin, and origin of transfer *oriT*, PCR amplified in two pieces from plasmid pJVD52.1[26] to remove a *Bbs*I recognition site in pSG5 *rep*. The resulting intermediate plasmid was then digested with *Xba*I to liberate the yeast helper fragment, and the backbone was religated to yield pCRISPomyces-1. Plasmid pCRISPomyces-2 was constructed via isothermal assembly of the *Eco*RI/*Xba*I-digested pCRISPomyces-1 backbone with two synthetic gBlocks (IDT) comprising a guide RNA expression cassette (with a *Bbs*I-flanked *lacZ* cassette in place of the spacer sequence). All targeting constructs were assembled by a combination of Golden Gate assembly[29] (for insertion of spacers) and traditional digestion/ligation or isothermal assembly[30] (for insertion of editing templates). Single spacer inserts were generated by annealing two 24 nt oligonucleotides (offset by 4 nt to generate sticky ends), while double spacer inserts were synthesized as gBlocks (IDT). The 1 kb left and right arms of each editing template were amplified from purified genomic DNA, spliced by overlap-extension PCR,[31] and ligated into the *Xba*I site of the desired plasmid. Correct plasmid assembly was confirmed by diagnostic digestion and sequencing (GeneWiz, South Plainfield, NJ). Plasmid maps were generated with Vector NTI (Invitrogen, Carlsbad, CA).

Transformation. *E. coli* NEB5-α was transformed by heat shock following the manufacturer's suggested protocol. Yeast transformation was performed as described elsewhere.[28] *E. coli* WM6026 was transformed by electroporation. Conjugation of plasmids into *Streptomyces* spores was performed using the modified protocol described elsewhere.[32]

Screening of *Streptomyces* Strains. Following conjugation, individual exconjugants were randomly picked and restreaked on MYG agar plates supplemented with 50 μg/mL apramycin and grown at 30 °C for 2−3 days. Single colonies were then picked to liquid MYG medium for genomic DNA isolation using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The locus of interest was PCR amplified and sequenced using primers annealing ∼300 bp upstream and downstream of the deletion site (GeneWiz, South Plainfield, NJ). Clearance of the plasmid was accomplished with high-temperature cultivation (37−39 °C) for 2−3 days, followed by replica plating on selective and nonselective plates to confirm restoration of apramycin sensitivity. For phenotypic screening of undecylprodigiosin-producing strains, saturated liquid cultures were streaked on nonselective R2 plates without sucrose and grown at 30 °C for 2−3 days.

■ ASSOCIATED CONTENT

Supporting Information

Additional methods, figures, and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

R.E.C. and H.Z. designed the experiments and wrote the manuscript. R.E.C. and Y.W. performed the experiments.

Notes

The authors declare no competing financial interest.

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