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Research Article

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Development of a *pyrF*-based counterselectable system for targeted gene deletion in *Streptomyces rimosus*

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Abstract: *Streptomyces* produces many valuable and important biomolecules with clinical and pharmaceutical applications. The development of simple and highly efficient gene editing tools for genetic modification of *Streptomyces* is highly desirable. In this study, we developed a screening system for targeted gene knockout using a uracil auxotrophic host (Δ*pyrF*) resistant to the highly toxic uracil analog of 5-fluoroorotic acid (5-FOA) converted by PyrF, and a non-replicative vector pKC1132-pyrF carrying the complemented *pyrF* gene coding for orotidine-5'-phosphate decarboxylase. The *pyrF* gene acts as a positive selection and counterselection marker for recombinants during genetic modifications. Single-crossover homologous integration mutants were selected on minimal medium without uracil by reintroducing *pyrF* along with pKC1132-pyrF into the genome of the mutant Δ*pyrF* at the targeted locus. Double-crossover recombinants were generated, from which the *pyrF* gene, plasmid backbone, and targeted gene were excised through homologous recombination exchange. These recombinants were rapidly screened by the counterselection agent, 5-FOA. We demonstrated the feasibility and advantage of using this *pyrF*-based screening system through deleting the *otcR* gene, which encodes the cluster-situated regulator that directly activates oxytetracycline biosynthesis in *Streptomyces rimosus* M4018. This system provides a new genetic tool for investigating the genetic characteristics of *Streptomyces* species.

Key words: Counterselectable system; *pyrF*; 5-Fluoroorotic acid (5-FOA); Gene deletion; *Streptomyces rimosus*

1 Introduction

Streptomyces is a genus of more than 900 species of filamentous actinobacteria, most of which can produce many kinds of antibiotics and other natural bioactive products (Barka et al., 2016; Wang et al., 2020; Xia et al., 2020). However, the increasing occurrence of multidrug resistant bacteria is creating an urgent need to increase the yield of approved natural drugs and to discover more novel classes of antibiotics with remarkable antimicrobial effects. Modern genome sequencing, alignments, and annotations of *Streptomyces* species have revealed that most natural products encoded in their genomes remain undiscovered, because of numerous cryptic secondary metabolite gene clusters encoding products that are either silent or not identified as natural products during growth in the laboratory (Niu et al., 2016; Li et al., 2019). Novel and efficient gene editing tools for the genetic manipulation of *Streptomyces* genes are highly desired to exploit these treasure troves (Zeng et al., 2015).

The pyrimidine biosynthesis pathway is highly conserved from bacteria to humans and provides an interesting target for molecular genetic manipulation due to the selection role of the bactericidal pyrimidine analog 5-fluoroorotic acid (5-FOA) (Yano et al., 2005; Capone et al., 2007). Orotate phosphoribosyl transferase (OPRTase, EC 2.4.2.10, encoded by *pyrE*) catalyzes the conversion of orotic acid to the normal

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product orotidine-5'-monophosphate (OMP), but 5-FOA used as substrate is converted to form 5-fluoroorotidine-5'-monophosphate (5-FOMP) (Fig. 1). 5-FOMP is converted into the product 5-fluorouridine-5'-monophosphate (5-FUMP) instead of uridine-5'-monophosphate (UMP) by the catalytic action of orotidine-5'-monophosphate decarboxylase (OMPDCase, EC 4.1.1.23, encoded by *pyrF*), which catalyzes a key step in de novo pyrimidine biosynthesis (Brockman et al., 1960; West, 2012). Although 5-FOA is not directly responsible for toxicity, the accumulation of cellular components containing 5-FUMP often leads to cell death due to their toxicity (O'Donovan and Neuhard, 1970; Boeke et al., 1984; Kurniyati and Li, 2016). Thus, mutants with deletion of the *pyrF* gene are uracil auxotrophs but resistant to 5-FOA, creating an opportunity whereby the ectopic expression of *pyrF* with a non-replicative plasmid can be selected or counterselected (Boeke et al., 1984; D'Enfert, 1996). The *pyrF*-based gene disturbance system has been successfully adapted in various organisms, such as yeasts (Staab and Sundstrom, 2003), fungi (Fujii et al., 2012), archaea (Liu et al., 2011), and other bacterial species. However, to the best of our knowledge, no study has reported the application of *pyrF*-based counterselectable gene knockout systems to *Streptomyces* species.

The aim of this study was to explore the potential for using *pyrF* as a counterselectable marker to establish an efficient screening system in *Streptomyces* species. The genetic manipulation of *Streptomyces* strains is considerably more difficult than that of other traditional model microorganisms, such as *Escherichia coli* and *Saccharomyces cerevisiae*, because few gene knock-in and knock-out methods are available (Zhao et al., 2018). Few genetic manipulation tools work well in industrial *Streptomyces* strains (Liu et al., 2018). Industrial strains that have undergone multiple rounds of exposure to ultraviolet (UV) radiation or chemical mutagens (e.g. nitrosoguanidine) are often resistant to several antibiotics; hence, no suitable antibiotic-selection marker is available for genetic manipulation. *Streptomyces rimosus* is characterized as the best industrial streptomycete producer of oxytetracycline (OTC) and other tetracycline antibiotics, with the highest OTC production yield of 35 g/L in large-scale fermentations. However, genetic modification of *S. rimosus* is considerably more difficult than that of other species due to its intrinsic high resistance to many commonly used antibiotics (Song et al., 2019). The minimal inhibitory concentrations in *S. rimosus* M4018 are 1000 μg/mL for spectinomycin, 400 μg/mL for kanamycin, and 500 μg/mL for apramycin (Yu et al., 2012; Yin et al., 2016, 2017; Wang et al., 2019). Therefore, most gene disruption tools are inefficient due to the lack of suitable selectable markers and a low homologous recombination (HR) frequency in industrial microbiology.

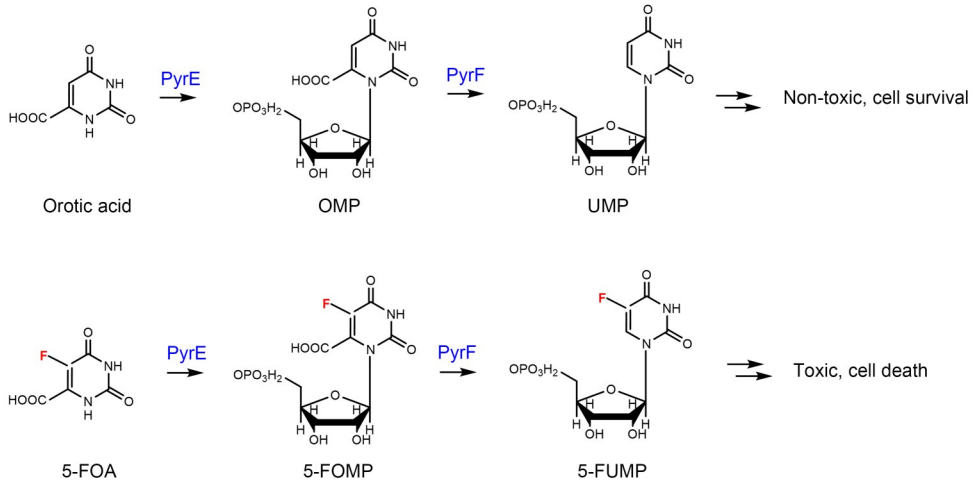

Fig. 1 Partial steps of the pyrimidine de novo biosynthesis pathway. PyrE, orotate phosphoribosyl transferase, converts orotic acid to OMP; PyrF, orotidine-5'-phosphate decarboxylase, catalyzes the synthesis of UMP. 5-FOA as an analog of pyrimidine can be converted to 5-FOMP by PyrE, and then to 5-FUMP by PyrF. 5-FUMP is toxic and often leads to cell death. 5-FOA: 5-fluoroorotic acid; 5-FOMP: 5-fluoroorotidine-5'-monophosphate; 5-FUMP: 5-fluorouridine-5'-monophosphate; OMP: orotidine-5'-monophosphate; UMP: uridine-5'-monophosphate.

Conventional gene editing in *Streptomyces* is based mainly on HR using a double-crossover integration, site-specific recombination system, or I-*Sce*I endonuclease system with an antibiotic selection marker. Usually, hundreds of clones must be screened, requiring a multistep process or leaving a recombinase target site in the chromosome to select the desired mutants, resulting in a time-consuming or arduous process (Zeng et al., 2015; Niu et al., 2016; Kong et al., 2019). The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system is a powerful gene-editing technology with high efficacy for site-specific gene targeting. The application of the CRISPR/Cas9 system to genome editing in *Streptomyces* is highly developed. Diverse approaches are widely used to edit or refactor biosynthetic gene clusters for natural drug discovery and characterization (Jia et al., 2017; Tao et al., 2018). However, potential off-target effects have raised major concerns regarding their application (Zhang et al., 2015). In this study, we developed a *pyrF*-based gene disruption screening system in *S. rimosus* M4018 and demonstrated its efficient application to targeted gene deletion. This new system should facilitate genetic manipulation of other *Streptomyces* species.

2 Materials and methods

2.1 Strains and culture conditions

The strains and plasmids used in this study are listed in Table 1. *E. coli* strain DH5α (Invitrogen, Waltham, Massachusetts, USA) was used for plasmid DNA manipulation; *E. coli* ET12567/pUZ8002 was used as a host for transferring DNA from *E. coli* to *Streptomyce*s by conjugation (Kieser et al., 2000). *E. coli* strains were grown in Luria-Bertani (LB) medium supplemented with chloramphenicol (25 μg/mL), kanamycin (25 μg/mL), or apramycin (50 μg/mL) when necessary. The *S. rimosus* transformants were screened and selected on minimal medium (MM) plates containing nalidixic acid (25 μg/mL) and apramycin (500 μg/mL) when necessary. Spores of *S. rimosus* were obtained on mannitol soya flour (MS) medium (Kieser et al., 2000) after incubation at 30 ℃ for 5 d. 5-FOA and uracil were purchased from the Sigma Chemical Company (St. Louis, USA) and solubilized in dimethyl sulfoxide (DMSO) before being added to the growth medium. DNA restriction endonucleases and DNA polymerases (Phusion, Q5, and Taq) were purchased from New England Biolabs, Inc. (NEB,

Table 1 Strains and plasmids used in this study

Name	Description	Source
Streptomyces		
S. rimosus M4018	Used for commercial production of oxytetracycline (OTC)	Tang et al., 2011
Δ*pyrF*	*pyrF* gene disruption mutant, derived from M4018	This study
Δ*pyrF::pyrF*	*pyrF* complemented strain, derived from Δ*pyrF*	This study
Δ*pyrF* Δ*otcR*	*otcR* gene disruption mutant, derived from Δ*pyrF*	This study
Δ*pyrF* Δ*otcR::otcR*	*otcR* complemented strain, derived from Δ*pyrF* Δ*otcR*	This study
Escherichia coli		
DH5α	General cloning host for plasmid manipulation	Invitrogen
ET12567 (pUZ8002)	Donor strain for conjugation between *E. coli* and *Streptomyces*	Kieser et al., 2000
Plasmids		
pKC1132	Conjugative vector, Am^R (*aac(3)IV*), *lacZ*, ori^{pUC18}, *oriT*	Yin et al., 2015
pKC1132-pyrFDM	For deletion of the *pyrF* gene, containing upstream and downstream homologous arms of *pyrF*	This study
pSET152	*E. coli-Streptomyces* shuttle vector, Am^R (*aac(3)IV*), *lacZ*, ori^{pUC18}, *attp* (*φC31*), *oriT*, and *int*	Bierman et al., 1992
pSET152-pyrF	Conjugative, integrative vector derived from pSET152, containing the *pyrF* gene driven by the promoter of SF14	This study
pKC1132-pyrF	Conjugative, derived from pKC1132, containing the *pyrF* gene driven by the promoter of SF14	This study
pOtcRDM	Conjugative vector, containing upstream and downstream homologous arms of *otcR*	Yin et al., 2015
pKC1132-otcRDM1	Conjugative, derived from pKC1132-SFpyrF, containing upstream and downstream homologous arms of *otcR*	This study
pSF14-otcR	Derived from pGusT-SF14, SF14-driven *otcR*	Yin et al., 2015

Ipswich, Massachusetts, USA). The other chemicals and reagents, unless noted, were obtained from local commercial sources.

2.2 Construction of plasmids

All the primers used in this work are listed in Table 2.

2.2.1 Construction of the vector for deletion of *pyrF*

To construct the *pyrF* deletion vector plasmid pKC1132-pyrFDM, the upstream homologous arm fragment (3080 bp) of *pyrF* was amplified with primers pyrF-UP-F/pyrF-UP-R, and the downstream homologous arm fragment (3237 bp) was amplified with primers pyrF-DN-F/pyrF-DN-R from the genomic DNA template of M4018. These two fragments were joined with pKC1132 (digested with *Bam*HI/*Hin*dIII) using the recombination method described by Gibson et al. (2009). To construct the complementation plasmid pSET152-pyrF, the *pyrF* fragment was amplified with primers SFpyrF1-F/SFpyrF1-R from the template of genomic DNA, and inserted into the *Nde*I/*Xba*I sites of pSF14-otcR following the Gibson assembly method.

2.2.2 Construction of the non-replicative and *pyrF*-complementary plasmid for deletion of *otcR*

A 1029-bp fragment containing both the SF14 promoter and *pyrF* was amplified from the template pSET152-pyrF with primers SFpyrF2-F/SFpyrF2-R. Subsequently, this fragment was inserted into the *Hin*dIII/*Xba*I sites of pKC1132 to generate plasmid pKC1132-pyrF. To construct the *otcR* disruption plasmid pKC1132-otcRDM1, the upstream and downstream fragments were amplified from pOtcRDM using primers otcR-UPDN-F/otcR-UPDN-R, and then inserted into the *Eco*RI/*Xba*I sites of pKC1132-pyrF.

2.3 Deletions of *pyrF* and *otcR*

The plasmid pKC1132-pyrFDM was introduced into the strain M4018 to obtain apramycin-resistant transconjugants that had integrated pKC1132-pyrFDM into the chromosome as a result of one crossover. Then, the transconjugants were subcultured in MM medium with 100 μmol/L 5-FOA and 200 μmol/L uracil, and grown for 4 d at 30 °C to select against the *pyrF* wild-type (WT) allele. The *pyrF* gene was eliminated along with the non-replicative plasmid skeleton

Table 2 Primers used in this study

Name	Sequence (5' to 3')	Use
pyrF-UP-F	**CGACGTTGTAAAACGACGGCCAGTGCCAAGCTT**CGTCCTACGACGAGGAGAGCG	Construction of pKC1132-pyrFDM
pyrF-UP-R	**TCGTCGGCACATCGGACTGCAG**GAAGGCGGACTGCGGCTTGAG	
pyrF-DN-F	**AAGCCGCAGTCCGCCTTCCTGCAG**TCCGATGTGCCGACGAGGTGC	
pyrF-DN-R	**TTACGAATTCGATATCGCGCGCGGCCGCGGATC**CCCGATCATGAGGTAGGAGG	
Test1-F	GAGCACAAGAAGGTGACCGC	Identification of the recombinant Δ*pyrF*
Test1-R	TGACGTCATGAAGGGAGGCG	
ExTest1-F	TGGCTGATCATTGGCGGAAG	Identification of the recombinant Δ*pyrF*
Extest1-R	TCGTCGACCAGCTCTTCCTG	
otcR-UPDN-F	**GCCGCCTCATCAAGGTCAAGGC**TCTAGAGTGGCCATTGCGGACTTCTACC	Construction of pKC1132-otcRDM1
otcR-UPDN-R	**GCCGTACCTCTACGACTACTTGAATTC**GTAATCATGTCATAGCTGTTTCC	
Test2-F	CAGAGGGACATATTGCCGCC	Identification of the recombinant Δ*pyrF* Δ*otcR*
Test2-R	TCATGACCTGGTTCCTGCGC	
SFpyrF1-F	**CACAGAACCACTCCACAGGAGGACCCATATG**ACGGCCCCGACCCCCTTCG	Construction of pKC1132-pyrF
SFpyrF1-R	**CGGCCCTGGTTCCTTCTTGTTGTCTAGA**CTAAAGTCCCCGTCGTTCGACCGC	
SFpyrF2-F	**GTTGTAAAACGACGGCCAGTGCCAAGCTT**CTAAAGTCCCCGTCGTTCGAC	
SFpyrF2-R	**GATATCGCGCGCGGCCGCGGATCCTCTAGA**GCCTTGACCTTGATGAGGCG	

The introduced overlapping sequences used for Gibson assembly are shown in bold.

from the chromosome as a result of a second crossover. The extracted DNA samples were then subjected to polymerase chain reaction (PCR) amplification. The correct isolation of Δ*pyrF* was confirmed using internal primers Test1-F/Test1-R (found on both the plasmid and the genome). The PCR program included: initial denaturation (5 min, 95 ℃) followed by 30 cycles of denaturation (30 s, 95 ℃), annealing (30 s, 56 ℃), elongation (1.5 min, 68 ℃), and then final extension (10 min, 68 ℃). The disruption of *pyrF* was also confirmed by external primers ExTest1-F/ExTest1-R (found only in the genome). The PCR elongations were performed at 68 ℃ using the extension time of 7.5 min (1 min per 1000 bp).

Deletion of the *otcR* gene was performed using the *pyrF*-based gene knockout systems. Briefly, the Δ*pyrF* strain conjugated with pKC1132-otcRDM1 (Table 1) was grown in uracil-free MM medium for about 3 to 4 d to select for uracil prototrophy. Then, to screen the transconjugants of Δ*pyrF* Δ*otcR* mutants, the above uracil auxotrophic strains were collected and cultured using solid MM medium supplemented with 100 μmol/L 5-FOA and 200 μmol/L uracil. All the 5-FOA resistant colonies were screened by PCR using the primers Test2-F/Test2-R (Table 2). PCR extensions were normally performed at 68 ℃ using the extension time of 1.5 min.

2.4 Bioinformatic analysis

The selected putative OMPDCase sequences from *Streptomyces* were aligned by Clustal Omega (Conway Institute UCD Dublin, Dublin, Ireland) (Sievers et al., 2011). The sequence similarities and secondary structure information from aligned sequences were rendered and displayed with the server ESPript 3.0 (https://espript.ibcp.fr/ESPript/ESPript/index.php) (Robert and Gouet, 2014).

2.5 Determination of 5-FOA susceptibility

All the strains were incubated on MS medium plates at 30 ℃ for 5 d, and then the spores were collected and diluted with sterilized water to a final concentration from 1×10^6 to 1×10^3 mL^{-1} using plate colony-counting methods. Afterwards, 5 μL of the spore suspension was spread on MM culture plates supplemented with increasing amounts of 5-FOA and uracil (0 and 0, 0 and 250, 250 and 250, 500 and 250 μmol/L, respectively). The growth and morphology of the colonies incubated on the plates were used to evaluate the tolerance of the strains to 5-FOA.

2.6 Fermentation and detection of OTC production

The spore suspensions (the total number of spores is 1.0×10^8) of *S. rimosus* were inoculated into 40 mL of R5 medium with 1 mmol/L uracil on a rotary shaker (250 r/min) for 24 h at 30 ℃, and then 2 mL of the seed cultures were transferred into 40 mL fresh R5 medium and incubated at 30 ℃ for 4 d. The OTC production was quantified by high-performance liquid chromatography (HPLC), as previously described (Yin et al., 2015). First, the fermentation samples were adjusted to pH 1.5−2.0 with 9 mol/L HCl, and 1-mL samples were centrifuged at 12 000 r/min for 10 min. Then, the supernatants were subjected to HPLC analysis on a Shimadzu Prominence HPLC system (Kyoto, Japan) with a dual λ UV detector and YMC polymer C18 column (4.6 mm×250 mm; Kyoto, Japan). Separation was performed with the following conditions: 60% H_2O, 10% methanol, 20% acetonitrile, and 10% phosphoric acid (2 mmol/L) at a constant flow rate of 1 mL/min. The OTC peak areas detected at 350 nm were used to calculate the concentration of OTC.

3 Results

3.1 Sensitivity of *Streptomyces* strains to 5-FOA

Gene knockout systems based on *pyrF* as the selection marker have been successfully applied to *Treponema denticola* (Kurniyati and Li, 2016), *Shewanella livingstonensis* (Ito et al., 2016), *Caldicellulosiruptor hydrothermalis* (Groom et al., 2014), *Haloarcula hispanica* and *Haloferax mediterranei* (Liu et al., 2011), *Candida albicans* (Staab and Sundstrom, 2003), and some other bacterial species. However, no study has reported the application of this system to *Streptomyces*. To develop a *pyrF*-based selection and counter-selection system, the WT strain must be sensitive to 5-FOA, and the strain with deletion of *pyrF* must confer resistance to 5-FOA. Thus, as an initial step, we tested the sensitivities of *Streptomyces coelicolor* M145, *Streptomyces venezuelae* ISP 5230, *S. rimosus* M4018, and *Streptomyces aureofaciens* ATCC 10762 to 5-FOA. The cells grew well in MM medium without supplementation of 5-FOA (Fig. 2). However, 5-FOA at a concentration of 5 μmol/L inhibited the

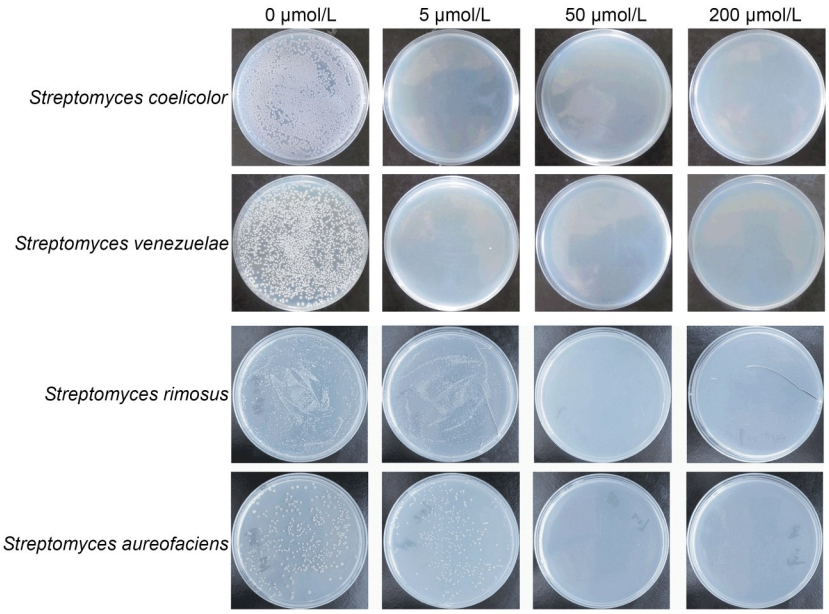

Fig. 2 Sensitivities of *Streptomyces coelicolor* M145, *Streptomyces venezuelae* ISP 5230, *Streptomyces rimosus* M4018, and *Streptomyces aureofaciens* ATCC 10762 to 5-FOA at concentrations of 0, 5, 50, and 200 μmol/L. 5-FOA: 5-fluoroorotic acid.

growth of *S. coelicolor* M145 and *S. venezuelae* ISP 5230, and the growth of all *Streptomyces* strains was completely inhibited when the concentration reached 50 μmol/L. These results indicated that *Streptomyces* strains were extremely susceptible to 5-FOA, and therefore highly likely to be good candidates for the development of an efficient gene disruption system using 5-FOA as the screening factor.

3.2 Identification of OMPDCase and creation of *S. rimosus* Δ*pyrF* host

The sensitivity to 5-FOA indicates the presence of genes related to the pyrimidine synthesis pathway in *Streptomyces*. The OMPDCase ortholog (*pyrF*) was annotated on the genome of *S. rimosus* provided by the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). All the information corresponding to the protein, source, and database accession number was given by a search with the basic local alignment search tool (BLAST). The deduced PyrF polypeptide (GenBank No. ELQ81494.1) from *S. rimosus* exhibits high similarity with those of a wide range of *Streptomyces* species, such as *S. avermitilis* (GenBank No. KUN53591.1), *S. coelicolo*r (GenBank No. TYP05411.1), *S. venezuelae* (GenBank No. WP_150188059.1), and *S. aureofaciens* (GenBank No. WP_030290267.1) (Fig. 3 and Table S1) (Chenna et al., 2003; Robert and Gouet, 2014). The crystal structure of putative OMPDCase from *S. avermitilis* (protein data bank (PDB) accession number: 3V75) comprises 12 α-helices (48.21% of amino acid, 135 residues) and 8 β-strands (17.86% of amino acid, 50 residues). Interestingly, the putative OMPDCase of *Streptomyces* exhibits low similarity with the PyrF (GenBank No. ACT29365.1) of *E. coli*, which contains a 265-amino acid polypeptide (Table 3). Multiple sequence alignment through Clustal Omega revealed that the OMPDCase amino acid sequence of *E. coli* exhibits about 20% sequence pair identity and less than 30% similarity with that of *Streptomyces* species (Sievers et al., 2011).

Alignment analysis of the whole genome sequence revealed that only one copy of the putative OMPDCase gene *pyrF* exists on the genome of *S. coelicolor* A3, *S. venezuelae* ISP 5230, *S. rimosus* M4018, and *S. aureofaciens* ATCC 10762. In the genome of *S. rimosus* M4018, *pyrF* is encoded in an operon between a conserved hypothetical protein and a predicted dihydroorotate dehydrogenase. Fig. 4a shows a schematic of the strategy used to delete the *pyrF* gene in *S. rimosus*. The pKC1132-pyrFDM vector was constructed (Fig. 4a) and conjugated into the WT strain *S. rimosus* M4018 as described in Section 2.2.1. The suicide plasmid, pKC1132-pyrFDM, which contained the apramycin resistance genes and the *pyrF* flanking sequences, was introduced into the WT strain *S. rimosus*

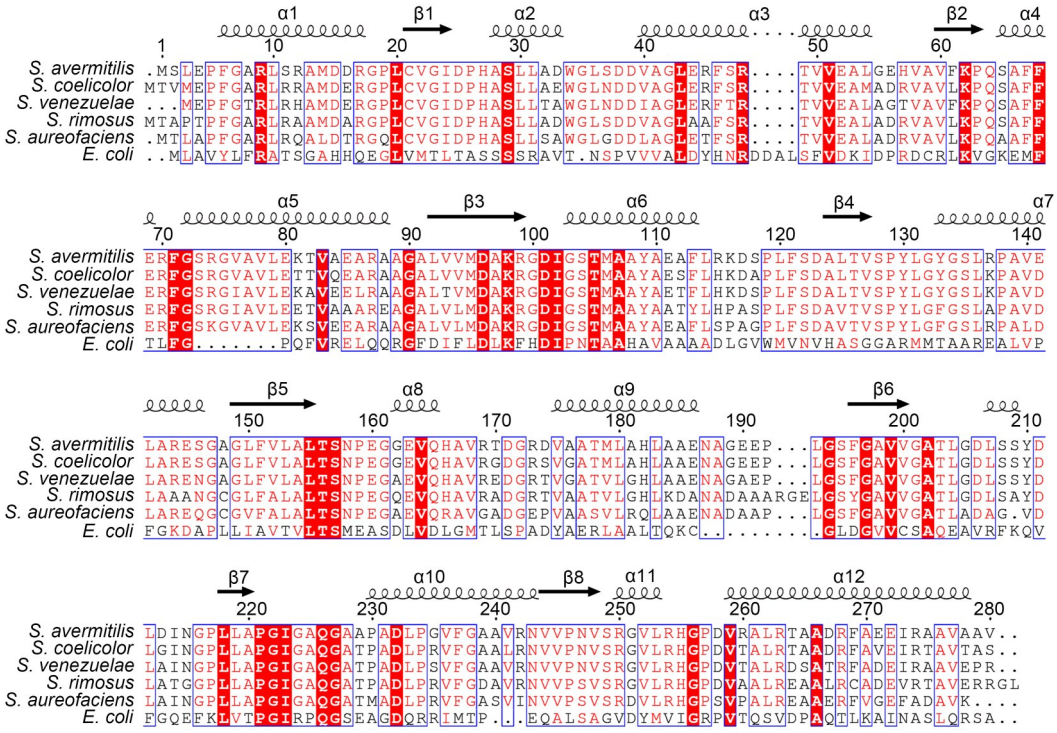

Fig. 3 Multiple amino acid sequence alignment among the deduced PyrF amino acid sequences. The numbers of amino acids and their positions within the respective proteins are indicated. The conserved amino acid residues are shaded in red, and the spiral coils and arrows represent the secondary structures of α-helices and β-sheets, respectively. The sequence homology of the deduced PyrF amino acid sequence among *Streptomyces* species exceeds 80%, but is low when compared with the putative orotidine-5'-phosphate decarboxylase ortholog of *Escherichia coli* (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Table 3 Similarity and identity values of orotidine-5'-phosphate decarboxylase amino acid sequences between *Escherichia coli* and *Streptomyces* species

Source	Amino acid residue	GenBank accession No.	Similarity (%)	Identity (%)
Escherichia coli	265	ACT29365.1		
Streptomyces avermitilis	280	KUN53591.1	29.8	20.8
Streptomyces coelicolor	281	TYP05411.1	28.7	19.9
Streptomyces venezuelae	278	WP_150188059.1	26.5	19.5
Streptomyces rimosus	286	ELQ81494.1	31.0	19.3
Streptomyces aureofaciens	277	WP_030290267.1	23.0	16.7

M4018. Plasmids harboring recombinants of a single-crossover were selected on apramycin plates. Through plating the apramycin-resistant transconjugants on MM medium containing 100 μmol/L 5-FOA and 200 μmol/L uracil, the *pyrF* gene was eliminated along with the non-replicative plasmid skeleton from the genome of M4018 by the second HR. The Δ*pyrF* strain was generated and selected by 5-FOA (Fig. 4a), because PyrF is responsible for uracil biosynthesis and its deletion confers resistance to 5-FOA (Fig. 4b). Among the 50 colonies resistant to 5-FOA, 49 strains were found to be uracil auxotrophs. The correct construction of the Δ*pyrF* mutant was confirmed by PCR using primers Test1-F/Test1-R, where the 600-bp difference between these DNA fragments of the WT strain and the Δ*pyrF* strain was consistent with the loss of *pyrF* (Figs. 4a and 4c). The disruption of *pyrF* was also confirmed by external primers ExTest1-F/ExTest1-R (found only in the genome). The length of predicted product detected by PCR was 6640 bp (Δ*pyrF*) instead of 7140 bp (M4018), indicating that the *pyrF* gene had been deleted from the chromosome (Fig. S1).

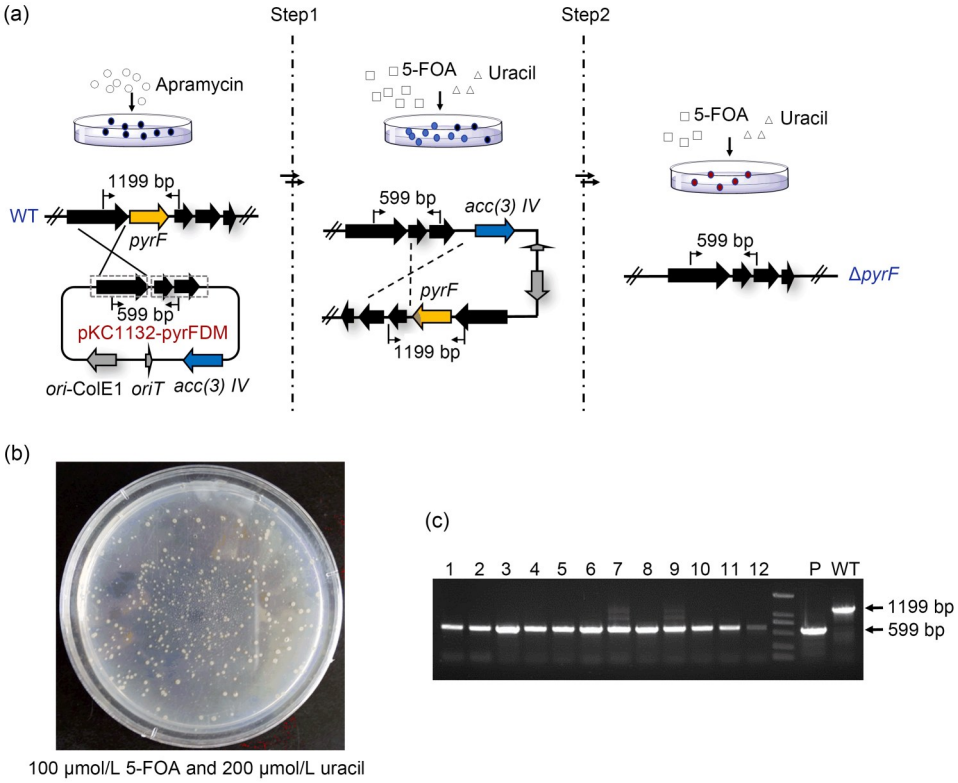

Fig. 4 Schematic diagram depicting the steps for deleting the *pyrF* gene from the *Streptomyces rimosus* genome and identification of the *pyrF*-deficient mutant. (a) To delete *pyrF*, the wide-type (WT) strain *S. rimosus* M4018 was conjugated with a non-replicative allelic exchange vector pKC1132-pyrFDM, followed by screening for apramycin resistant clones (Step 1). The Δ*pyrF* strain was selected through plating the apramycin-resistant transconjugants on MM medium using 5-FOA as the screening agent (Step 2). (b) Characteristics and phenotype of 5-FOA-resistant colonies screened on solid MM medium containing 5-FOA. (c) The double recombination event creating 5-FOA-resistant colonies was identified by PCR amplification. The expected amplicon length was 1199 bp for the WT and 599 bp for the Δ*pyrF* strain. The plasmid pKC1132-pyrFDM (P) and WT strain M4018 were positive and negative controls, respectively. 5-FOA: 5-fluoroorotic acid.

3.3 Characterization and complementation of the *S. rimosus* Δ*pyrF* strain

The WT, Δ*pyrF*, and *pyrF*-complementing (Δ*pyrF*::*pyrF*) strains were tested on solid uracil-free defined medium with or without supplementation of 5-FOA and uracil, to evaluate the characteristics of 5-FOA sensitivity/resistance and uracil auxotrophy/prototrophy. The Δ*pyrF* strain could not grow well in synthetic MM medium without uracil because of the incomplete pyrimidine synthesis pathway, whereas the growth of parental and *pyrF*-complementing strains was uninhibited (Fig. 5). The Δ*pyrF* strain proliferated when supplemented with 250 μmol/L uracil, and the morphology and sporulation of the clones were similar to those of the WT strain. Thus, deletion of the *pyrF* gene did not hamper growth when enough uracil was provided (Fig. 5). The pSET152-pyrF plasmid was transformed and integrated into the *pyrF* mutant at the φC31-mediated site-specific integration locus to reintroduce the *pyrF* gene into the genome of the Δ*pyrF* mutant. The pyrimidine biosynthetic pathway in the mutant Δ*pyrF* was complemented and restored as in the parental strain by constitutive expression of the OMPDCase gene. When the concentration of 5-FOA was increased, the *pyrF*-complementing strain and parental strain became sensitive to 5-FOA, and their growth was completely suppressed, whereas the mutants (Δ*pyrF*) lacking *pyrF* could still grow well in the presence of 5-FOA, indicating that deletion of *pyrF* conferred 5-FOA resistance in the mutants. All these results confirmed that the OMPDCase gene *pyrF* could be used as a counterselection marker in the Δ*pyrF* host. The catalytic activity of PyrF is required for 5-UMP toxicity, so the lack of toxicity under conditions of excess uracil likely reflects the activity of the

salvage pathways of pyrimidine, possibly due to the downregulation of de novo pyrimidine synthesis when exogenous uracil is available (Capone et al., 2007).

3.4 Constitution and principle of the *pyrF*-based screening system

A *pyrF*-based gene knockout system should contain a uracil auxotrophic host (Δ*pyrF*) and a non-replicative plasmid vector carrying the complementary gene *pyrF*, which could act as a positive selection and counterselection marker for generation of knockout mutants (Tripathi et al., 2010). This non-replicative plasmid vector pKC1132-pyrF for HR was constructed as described in the Section 2.2.1. The vector included an apramycin resistance cassette (*aac(3)IV*), an OMPDCase gene *pyrF* from *S. rimosus* M4018 driven by the SF14 promoter, a segment of plasmid pKC1132 containing the origin of replication (*ori*-colE1) from *E. coli*, an origin of plasmid DNA transfer element (*oriT*) for RK2-mediated mobilization, and multiple cloning sites for facilitating cloning of the desired gene (Fig. 6a).

First, a single-crossover transformant was constructed by reintroducing *pyrF* along with pKC1132-pyrF derivatives into the chromosome of the *S. rimosus* Δ*pyrF* host at the specific locus of the chosen target gene. The transformant can be obtained by plating spores on MM medium without uracil, because the obtained single-crossover mutation restores the uracil synthesis capability and susceptibility to 5-FOA. Next, given that only the targeted gene knockout mutants lacking PyrF activity can grow well in the medium containing 5-FOA, a clean marker-free mutant, from which the *pyrF* gene, antibiotic resistance marker, and the targeted gene have been excised through allelic exchange recombination, must be created to screen for 5-FOA-resistant colonies by using culture medium supplemented with 5-FOA.

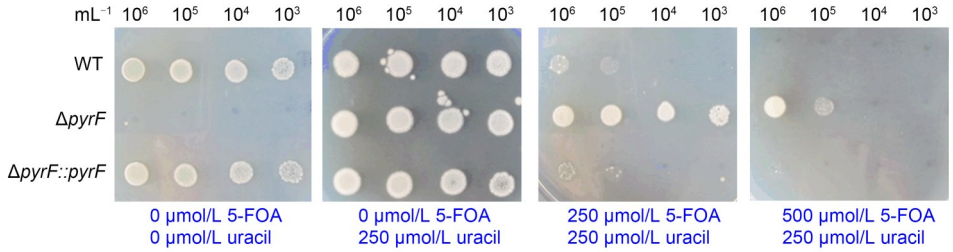

Fig. 5 Characteristics of 5-FOA sensitivity/resistance and uracil auxotrophy/prototrophy among the wide-type (WT), Δ*pyrF*, and *pyrF*-complementing strains. The spores of the WT, Δ*pyrF*, and Δ*pyrF::pyrF* strains were diluted from 1×10^6 to 1×10^3 mL^{-1} with plate colony-counting methods, and a 5-μL sample was taken and pointed on MM medium plates supplemented with increasing amounts of 5-FOA and uracil. All the plates were incubated at 30 °C for 5 d before photographs were taken. 5-FOA: 5-fluoroorotic acid.

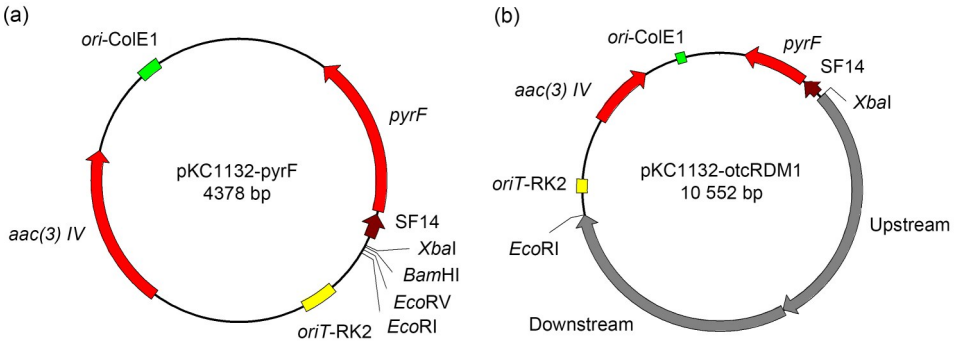

Fig. 6 Construction of the *pyrF*-based suicide vector pKC1132-pyrF and derived plasmid pKC1132-otcRDM1 to delete the *otcR* gene of *Streptomyces rimosus*. (a) Plasmid pKC1132-pyrF was constructed to reintroduce the *pyrF* gene into the chromosome. *aac(3)IV*, apramycin resistance-conferring gene; *ori*-ColE1, origin of replication in *E. coli*; *oriT*, an origin of plasmid DNA transfer element for RK2-mediated mobilization; SF14, constitutively stronger promoter; *pyrF*, the gene encoding orotidine-5'-phosphate decarboxylase from *S. rimosus* M4018. (b) pKC1132-otcRDM1 was constructed to delete the *otcR* gene using the *pyrF*-based gene knockout system in *S. rimosus*. Upstream represents the upstream homologous fragment of *otcR*; downstream represents the downstream homologous fragment of *otcR*.

3.5 Targeted gene deletion in *S. rimosus* with *pyrF*-based gene knockout systems

To demonstrate the feasibility of using the non-replicative plasmid pKC1132-pyrF in gene deletion in *S. rimosus* Δ*pyrF*, we used it to delete the gene *otcR*, identified in our previous research as a coding gene of a cluster-situated pathway specific activator for OTC biosynthesis (Yin et al., 2015). The plasmid pKC1132-otcRDM1 (Fig. 6b) containing two internal upstream and downstream fragments of *otcR* was introduced into *S. rimosus* Δ*pyrF*, and MM medium lacking uracil was used to select the Δ*pyrF* mutant clones restored to uracil prototrophy as a result of the transformation of plasmid pKC1132-otcRDM1 containing the *pyrF* gene by single crossover (Fig. 7a). Counterselection for a *pyrF* excision with a double crossover, targeting gene *otcR*-knockout recombinants (Δ*pyrF* Δ*otcR*) was performed by screening for 5-FOA-resistant colonies. More than 99% of the colonies screened by 5-FOA indicated the high accuracy of HR at the *otcR* locus. Strains in which *otcR* was knocked out were discernible by a PCR amplicon 862 bp shorter than that of the WT. The expected amplicon was 1462 bp for the *S. rimosus* Δ*pyrF* and 600 bp for the Δ*pyrF* Δ*otcR* strain (Fig. 7b). OtcR as the activator of the OTC biosynthesis pathway is essential for OTC production in *S. rimosus*. OTC production was completely abolished in the fermentation broth of the Δ*pyrF* Δ*otcR* strain analyzed by HPLC. Meanwhile, complementation of the Δ*pyrF* Δ*otcR* mutant with a single copy of *otcR* (Δ*pyrF* Δ*otcR*::*otcR*) restored the production of OTC to a level similar to that of the parental strain (Fig. 7c). With the counterselection marker applied in this screening system, the time taken to obtain the double-crossover recombinants from single-crossover mutants was reduced from more than two weeks to about only one week.

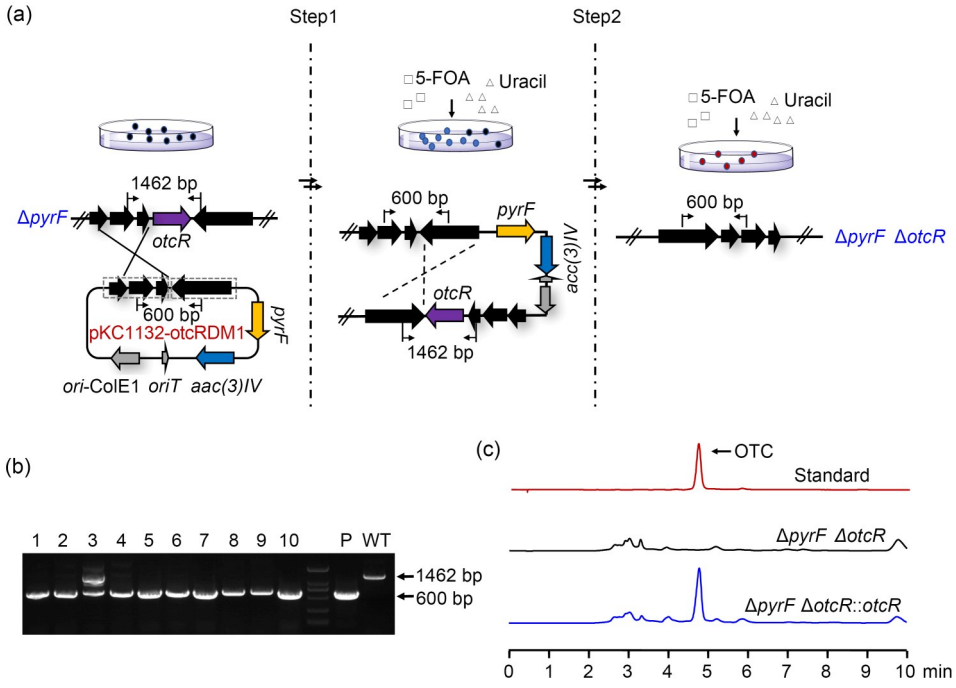

Fig. 7 Schematic diagram depicting the steps for *otcR* deletion using the *pyrF*-based counterselectable system, identification, and OTC production profiles of this *otcR*-deficient mutant. (a) To delete *otcR*, the Δ*pyrF* host was transformed with a non-replicative allelic exchange vector pKC1132-otcRDM1, to screen for single-crossovers on MM medium lacking uracil (Step 1). The uracil prototrophy mutants were plated to screen for 5-FOA-resistant colonies and obtain the double-crossover Δ*pyrF* Δ*otcR* strain on MM medium with 5-FOA and uracil (Step 2). (b) The double recombination event of 5-FOA-resistant colonies was identified by PCR amplification. The expected amplicon length was 1462 bp for the parental strain and 600 bp for the Δ*otcR* strain. The plasmid pKC1132-otcRDM1 (P) and wild-type (WT) strain M4018 were positive and negative controls, respectively. (c) OTC production profiles of Δ*pyrF* Δ*otcR* and Δ*pyrF* Δ*otcR*::*otcR* were analyzed by HPLC (UV 350 nm). 5-FOA: 5-fluoroorotic acid; OTC: oxytetracycline; HPLC: high-performance liquid chromatography; UV: ultraviolet.

A series of concentration gradients were used to obtain the appropriate concentrations of 5-FOA and uracil for counterselection. We determined that concentrations of 5-FOA from 50 to 100 μmol/L were sufficient for inhibiting the growth of the WT strain and *pyrF* plasmid-curing mutants. When the concentration of uracil in the medium gradually increased from 100 and 200 μmol/L to 300 μmol/L, the desired *otcR* elimination strain grew from a "clear" to "not clear" background (Figs. 4 and S2). False positive clones (*pyrF*-assisted plasmid was not lost) appeared and increased at high concentrations of uracil (>300 μmol/L) (Fig. S2). The surviving false positive clones could use the highly concentrated uracil via a salvage pathway and reduce the de novo synthesis pathway. The size of the predicted positive clones was large; however, the false-positive clones were very small, numerous ("not clear" background), and sensitive to 5-FOA after subculture.

4 Discussion

In this work, we developed a simple *pyrF*-based counterselectable system to allow efficient homologous gene disruption in *S. rimosus*. The de novo pyrimidine biosynthesis and salvage pathways are essential processes in organisms. If the required substrates in the surrounding environment are available and adequate, the salvage pathways are usually preferred over de novo synthesis. The *pyrF* gene encoding OMPDCase is involved in the de novo biosynthesis of pyrimidine-related metabolites, such as UMP, uridine-5'-diphosphate (UDP), and uridine-5'-triphosphate (UTP), and also can metabolize 5-FOA to toxic metabolites (Suzuki et al., 2012). Deficiency in PyrF makes a mutant strain uracil auxotrophic and 5-FOA resistant. Thus, the Δ*pyrF* mutant was expected to allow the use of *pyrF* as a counterselection marker on a non-replicating or suicide plasmid with 5-FOA as the selection agent (Capone et al., 2007). Most of the plasmids used for recombinant cloning technology in bacteria are derived by using antibiotic resistance genes as selection markers. However, given the sensitive issues of the appearance of antibiotic-resistant strains and legal requirements, these antibiotics must be avoided as much as possible in the industrial process. Thus, different antibiotic marker-free approaches, such as the complementation of auxotrophic strains, have been developed (Vandermeulen et al., 2011; Sevillano et al., 2017). With the application of the *pyrF*-based counterselectable screening system, two steps are followed to obtain the desired mutant (Fig. 7a). In the first step, a single recombination event is selected by uracil prototrophy. In the second step, a double recombination event is selected by resistance to 5-FOA (Galvão and de Lorenzo, 2005). With the assistance of this *pyrF*-screening marker, the non-replicative plasmid-integrated colonies can be rapidly selected by one-plate screening in a single step instead of the conventional multi-step continuous screening operation, and with a more certain outcome. This makes successive gene disruption highly accomplishable with improved stability and efficiency (Tripathi et al., 2010). Another advantage of this screening system is that the genome is unmarked after *pyrF* selection and counterselection, so additional genetic manipulations with the same plasmid derivatives are possible and convenient.

The regulation of pyrimidine de novo biosynthesis genes has been well studied in *E. coli* and *Bacillus subtilis*, and the attenuator PyrR can repress the expression of *pyr* genes at the transcription level (Tanaka et al., 2015). To increase the sensitivity of 5-FOA, Suzuki et al. (2012) constructed the double mutant strain Δ*pyrF* Δ*pyrR* in *Geobacillus kaustophilus*, which is prototrophic for uracil and 5-FOA sensitive. This double-knockout strain (Δ*pyrF* Δ*pyrR*) was constructed because the single-gene deletion mutant Δ*pyrF* is auxotrophic for uracil and resistant to 5-FOA. This complementary strain with *pyrF* is prototrophic for uracil, but still insensitive to 5-FOA in the presence of uracil (Suzuki et al., 2012). In our study, to reduce the inhibitory effect of PyrR and improve the screening efficiency, we deliberately used the non-replicative vector containing *pyrF* driven by the constitutively expressed and stronger promoter SF14 instead of its native promoter (Labes et al., 1997; Wang et al., 2013).

In our study, the frequencies of 5-FOA-resistant clones and false positives were analyzed in *S. rimosus* using the proposed screening system for the deletion of *otcR*. The *pyrF*-based counterselection system was very effective and enabled us to obtain the desired mutant by analyzing only a small number of clones. The screening rate of positive clones was higher than

that of the traditional method. The frequencies of false positives and revertants could be significantly reduced by adding appropriate proportions of uracil (200 μmol/L) and 5-FOA (100 μmol/L) to the MM medium. In summary, we successfully constructed a novel genetic manipulation system to perform targeted gene manipulation in *S. rimosus*. When counterselection was applied in this *pyrF*-based system, double-crossover recombinants were obtained from single-crossover recombinants much more quickly than by using conventional methods. Our results suggest that this *pyrF*-based counterselection system can also be applied to other *Streptomyces* species and will greatly improve our ability to explore the potential of *Streptomyces*.

5 Conclusions

Streptomyces produces a remarkably diverse array of secondary metabolites, including antibiotics, antitumor compounds, immunosuppressants, and antivirals. However, targeted gene disruption can be inefficient in industrial *Streptomyces* strains due to the lack of suitable selectable markers and a low frequency of HR. Here, we have demonstrated the feasibility and advantage of using the *pyrF* gene as a counterselectable marker to create marker-free mutants of *S. rimosus*. This *pyrF*-based counterselectable system was very effective and enabled us to obtain the target gene knockout mutants by analyzing a small number of clones, and the time to obtain the desired mutants was greatly shortened. The advantage of this system is that the marker does not remain in the genome, so additional deletion and/or insertion using the same vector system is possible. This *pyrF*-based genetic manipulation system will contribute greatly to fundamental and applied studies of *Streptomyces*.

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Author contributions

Yiying YANG, Qingqing SUN, and Yang LIU were responsible for the methodology, data curation, and formal analysis. Hanzhi YIN, Wenping YANG, and Yang WANG contributed to strain resources and supervision. Ying LIU and Yuxian LI contributed to the software and sequence alignment. Shen PANG, Wenxi LIU, Qian ZHANG, Fang YUAN, and Shiwen QIU contributed to the strain fermentation and composition analysis by HPLC. Jiong LI and Xuefeng WANG performed the fermentation experiments. Keqiang FAN, Weishan WANG, Zilong LI, and Shouliang YIN contributed to the investigation, project administration, supervision, writing, review, and editing. All authors have read and approved the final manuscript and, therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Yiying YANG, Qingqing SUN, Yang LIU, Hanzhi YIN, Wenping YANG, Yang WANG, Ying LIU, Yuxian LI, Shen PANG, Wenxi LIU, Qian ZHANG, Fang YUAN, Shiwen QIU, Jiong LI, Xuefeng WANG, Keqiang FAN, Weishan WANG, Zilong LI, and Shouliang YIN declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Barka EA, Vatsa P, Sanchez L, et al., 2016. Taxonomy, physiology, and natural products of actinobacteria. *Microbiol Mol Biol Rev*, 80(1):1-43. https://doi.org/10.1128/MMBR.00019-15

Bierman M, Logan R, O'Brien K, et al., 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene*, 116(1):43-49. https://doi.org/10.1016/0378-1119(92)90627-2

Boeke JD, la Croute F, Fink GR, 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol Gen Genet*, 197(2):345-346. https://doi.org/10.1007/bf00330984

Brockman RW, Davis JM, Stutts P, 1960. Metabolism of uracil and 5-fluorouracil by drug-sensitive and by drug-resistant bacteria. *Biochim Biophys Acta*, 40:22-32. https://doi.org/10.1016/0006-3002(60)91311-1

Capone RF, Ning Y, Pakulis N, et al., 2007. Characterization of *Treponema denticola pyrF* encoding orotidine-5'-monophosphate decarboxylase. *FEMS Microbiol Lett*, 268(2):261-267. https://doi.org/10.1111/j.1574-6968. 2006.00589.x

Chenna R, Sugawara H, Koike T, et al., 2003. Multiple sequence alignment with the clustal series of programs. *Nucleic Acids Res*, 31(13):3497-3500. https://doi.org/10.1093/nar/gkg500

D'Enfert C, 1996. Selection of multiple disruption events in *Aspergillus fumigatus* using the orotidine-5'-decarboxylase gene, *pyrG*, as a unique transformation marker. *Curr Genet*, 30(1):76-82. https://doi.org/10.1007/s002940050103

Fujii T, Iwata K, Murakami K, et al., 2012. Isolation of uracil auxotrophs of the fungus *Acremonium cellulolyticus* and the development of a transformation system with the *pyrF* gene. *Biosci Biotechnol Biochem*, 76(2):245-249. https://doi.org/10.1271/bbb.110498

Galvão TC, de Lorenzo V, 2005. Adaptation of the yeast *URA3* selection system to Gram-negative bacteria and generation of a Δ*betCDE Pseudomonas putida* strain. *Appl Environ Microbiol*, 71(2):883-892. https://doi.org/10.1128/AEM.71.2.883-892.2005

Gibson DG, Young L, Chuang RY, et al., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods*, 6(5):343-345. https://doi.org/10.1038/nmeth.1318

Groom J, Chung D, Young J, et al., 2014. Heterologous complementation of a *pyrF* deletion in *Caldicellulosiruptor hydrothermalis* generates a new host for the analysis of biomass deconstruction. *Biotechnol Biofuels*, 7:132. https://doi.org/10.1186/s13068-014-0132-8

Ito T, Gong CJ, Kawamoto J, et al., 2016. Development of a versatile method for targeted gene deletion and insertion by using the *pyrF* gene in the psychrotrophic bacterium, *Shewanella livingstonensis* Ac10. *J Biosci Bioeng*, 122(6):645-651. https://doi.org/10.1016/j.jbiosc.2016.06.004

Jia HY, Zhang LM, Wang TT, et al., 2017. Development of a CRISPR/Cas9-mediated gene-editing tool in *Streptomyces rimosus*. *Microbiology (Reading)*, 163(8):1148-1155. https://doi.org/10.1099/mic.0.000501

Kieser T, Bibb MJ, Buttner MJ, et al., 2000. Practical *Streptomyces* Genetics: A Laboratory Manual. The John Innes Foundation, Norwich, UK.

Kong DK, Wang X, Nie J, et al., 2019. Regulation of antibiotic production by signaling molecules in *Streptomyces*. *Front Microbiol*, 10:2927. https://doi.org/10.3389/fmicb. 2019.02927

Kurniyati K, Li CH, 2016. *pyrF* as a counterselectable marker for unmarked genetic manipulations in *Treponema denticola*. *Appl Environ Microbiol*, 82(4):1346-1352. https://doi.org/10.1128/AEM.03704-15

Labes G, Bibb M, Wohlleben W, 1997. Isolation and characterization of a strong promoter element from the *Streptomyces ghanaensis* phage I19 using the gentamicin resistance gene (*aacC1*) of Tn*1696* as reporter. *Microbiology*, 143(5):1503-1512. https://doi.org/10.1099/00221287-143-5-1503

Li ZY, Bu QT, Wang J, et al., 2019. Activation of anthrachamycin biosynthesis in *Streptomyces chattanoogensis* L10 by site-directed mutagenesis of *rpoB*. *J Zhejiang Univ-Sci B (Biomed & Biotechnol)*, 20(12):983-994. https://doi.org/10.1631/jzus.B1900344

Liu HL, Han J, Liu XQ, et al., 2011. Development of *pyrF*-based gene knockout systems for genome-wide manipulation of the archaea *Haloferax mediterranei* and *Haloarcula hispanica*. *J Genet Genomics*, 38(6):261-269. https://doi.org/10.1016/j.jgg.2011.05.003

Liu R, Deng ZX, Liu TG, 2018. *Streptomyces* species: ideal chassis for natural product discovery and overproduction. *Metab Eng*, 50:74-84. https://doi.org/10.1016/j.ymben.2018.05.015

Niu GQ, Chater KF, Tian YQ, et al., 2016. Specialised metabolites regulating antibiotic biosynthesis in *Streptomyces* spp. *FEMS Microbiol Rev*, 40(4):554-573. https://doi.org/10.1093/femsre/fuw012

O'Donovan GA, Neuhard J, 1970. Pyrimidine metabolism in microorganisms. *Bacteriol Rev*, 34(3):278-343. https://doi.org/10.1128/BR.34.3.278-343.1970

Robert X, Gouet P, 2014. Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Res*, 42(W1):W320-W324. https://doi.org/10.1093/nar/gku316

Sevillano L, Díaz M, Santamaría RI, 2017. Development of an antibiotic marker-free platform for heterologous protein production in *Streptomyces*. *Microb Cell Fact*, 16:164. https://doi.org/10.1186/s12934-017-0781-y

Sievers F, Wilm A, Dineen D, et al., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol*, 7:539. https://doi.org/10.1038/msb.2011.75

Song ZQ, Liao ZJ, Hu YF, et al., 2019. Development and optimization of an intergeneric conjugation system and analysis of promoter activity in *Streptomyces rimosus* M527. *J Zhejiang Univ-Sci B (Biomed & Biotechnol)*, 20(11):891-900. https://doi.org/10.1631/jzus.B1900270

Staab JF, Sundstrom P, 2003. *Ura3* as a selectable marker for disruption and virulence assessment of *Candida albicans* genes. *Trends Microbiol*, 11(2):69-73. https://doi.org/10.1016/s0966-842x(02)00029-x

Suzuki H, Murakami A, Yoshida KI, 2012. Counterselection system for *Geobacillus kaustophilus* HTA426 through disruption of *pyrF* and *pyrR*. *Appl Environ Microbiol*, 78(20):7376-7383. https://doi.org/10.1128/AEM.01669-12

Tanaka Y, Teramoto H, Inui M, 2015. Regulation of the expression of *de novo* pyrimidine biosynthesis genes in *Corynebacterium glutamicum*. *J Bacteriol*, 197(20):3307-3316. https://doi.org/10.1128/JB.00395-15

Tang ZY, Xiao CY, Zhuang YP, et al., 2011. Improved oxytetracycline production in *Streptomyces rimosu*s M4018 by metabolic engineering of the *G6PDH* gene in the pentose phosphate pathway. *Enzyme Microb Technol*, 49(1):17-24. https://doi.org/10.1016/j.enzmictec.2011.04.002

Tao WX, Yang AN, Deng ZX, et al., 2018. CRISPR/Cas9-based editing of *Streptomyces* for discovery, characterization, and production of natural products. *Front Microbiol*, 9:1660. https://doi.org/10.3389/fmicb.2018.01660

Tripathi SA, Olson DG, Argyros DA, et al., 2010. Development of *pyrF*-based genetic system for targeted gene deletion in *Clostridium thermocellum* and creation of a *pta* mutant. *Appl Environ Microbiol*, 76(19):6591-6599. https://doi.org/10.1128/AEM.01484-10

Vandermeulen G, Marie C, Scherman D, et al., 2011. New generation of plasmid backbones devoid of antibiotic resistance marker for gene therapy trials. *Mol Ther*, 19(11): 1942-1949. https://doi.org/10.1038/mt.2011.182

Wang WS, Li X, Wang J, et al., 2013. An engineered strong promoter for *Streptomycetes*. *Appl Environ Microbiol*, 79(14):4484-4492. https://doi.org/10.1128/AEM.00985-13

Wang WS, Li SS, Li ZL, et al., 2020. Harnessing the intracellular triacylglycerols for titer improvement of polyketides in *Streptomyces*. *Nat Biotechnol*, 38(1):76-83. https://doi.org/10.1038/s41587-019-0335-4

Wang XF, Yin SL, Bai J, et al., 2019. Heterologous production of chlortetracycline in an industrial grade *Streptomyces rimosus* host. *Appl Microbiol Biotechnol*, 103(16): 6645-6655. https://doi.org/10.1007/s00253-019-09970-1

West TP, 2012. Pyrimidine biosynthesis in *Pseudomonas veronii* and its regulation by pyrimidines. *Microbiol Res*, 167(5): 306-310. https://doi.org/10.1016/j.micres.2011.10.002

Xia HY, Li XF, Li ZQ, et al., 2020. The application of regulatory cascades in *Streptomyces*: yield enhancement and metabolite mining. *Front Microbiol*, 11:406. https://doi.org/10.3389/fmicb.2020.00406

Yano T, Sanders C, Catalano J, et al., 2005. *sacB*–5-fluoroorotic acid–*pyrE*-based bidirectional selection for integration of unmarked alleles into the chromosome of *Rhodobacter capsulatus*. *Appl Environ Microbiol*, 71(6):3014-3024. https://doi.org/10.1128/AEM.71.6.3014-3024.2005

Yin SL, Wang WS, Wang XF, et al., 2015. Identification of a cluster-situated activator of oxytetracycline biosynthesis and manipulation of its expression for improved oxytetracycline production in *Streptomyces rimosus*. *Microb Cell Fact*, 14:46. https://doi.org/10.1186/s12934-015-0231-7

Yin SL, Li ZL, Wang XF, et al., 2016. Heterologous expression of oxytetracycline biosynthetic gene cluster in *Streptomyces venezuelae* WVR2006 to improve production level and to alter fermentation process. *Appl Microbiol Biotechnol*, 100(24):10563-10572. https://doi.org/10.1007/s00253-016-7873-1

Yin SL, Wang XF, Shi MX, et al., 2017. Improvement of oxytetracycline production mediated via cooperation of resistance genes in *Streptomyces rimosus*. *Sci China Life Sci*, 60(9):992-999. https://doi.org/10.1007/s11427-017-9121-4

Yu L, Cao N, Wang L, et al., 2012. Oxytetracycline biosynthesis improvement in *Streptomyces rimosus* following duplication of minimal PKS genes. *Enzyme Microb Technol*, 50(6-7):318-324. https://doi.org/10.1016/j.enzmictec.2012.03.001

Zeng H, Wen SS, Xu W, et al., 2015. Highly efficient editing of the actinorhodin polyketide chain length factor gene in *Streptomyces coelicolor* M145 using CRISPR/Cas9-CodA(sm) combined system. *Appl Microbiol Biotechnol*, 99(24):10575-10585. https://doi.org/10.1007/s00253-015-6931-4

Zhang XH, Tee LY, Wang XG, et al., 2015. Off-target effects in CRISPR/Cas9-mediated genome engineering. *Mol Ther Nucleic Acids*, 4:E264. https://doi.org/10.1038/mtna.2015.37

Zhao YW, Li L, Zheng GS, et al., 2018. CRISPR/dCas9-mediated multiplex gene repression in *Streptomyces*. *Biotechnol J*, 13(9):1800121. https://doi.org/10.1002/biot.201800121

Supplementary information

Table S1; Figs. S1 and S2