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Multiplexed site-specific genome engineering for overproducing bioactive secondary metabolites in actinomycetes

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ABSTRACT

Actinomycetes produce a large variety of pharmaceutically active compounds, yet production titers often require to be improved for discovery, development and large-scale manufacturing. Here, we describe a new technique, multiplexed site-specific genome engineering (MSGE) via the 'one integrase-multiple *attB* sites' concept, for the stable integration of secondary metabolite biosynthetic gene clusters (BGCs). Using MSGE, we achieved five-copy chromosomal integration of the pristinamycin II (PII) BGC in *Streptomyces pristinaespiralis*, resulting in the highest reported PII titers in flask and batch fermentations (2.2 and 2 g/L, respectively). Furthermore, MSGE was successfully extended to develop a panel of powerful *Streptomyces coelicolor* heterologous hosts, in which up to four copies of the BGCs for chloramphenicol or anti-tumour compound YM-216391 were efficiently integrated in a single step, leading to significantly elevated productivity (2–23 times). Our multiplexed approach holds great potential for robust genome engineering of industrial actinomycetes and novel drug discovery by genome mining.

1. Introduction

Secondary metabolites produced by actinomycetes have historically made crucial contributions to health care and agriculture, and continue to be major sources for the discovery of novel drug leads (Barka et al., 2015; Harvey et al., 2015; Jensen et al., 2015). Recent extensive microbial (meta)genome sequencing efforts have revealed that ~90% of secondary metabolite biosynthetic gene clusters (BGCs) in actinomycetes are cryptic or silent in the laboratory setting and are thus regarded as a treasure chest of information awaiting linkage to chemical compounds (Baltz, 2016a; Cimermancic et al., 2014; Doroghazi et al., 2014). However, bioprospecting of novel bioactive small molecules or commercial production of important clinically used drugs often required improved biosynthetic performance. Typically, repeated cycles of random mutagenesis and screening have served as an efficient strategy for yield improvement, but this approach is laborious and time-consuming. Over the past decade, metabolic engineering has become a powerful strategy to optimize secondary metabolism in actinomycetes (Baltz, 2016b; O'Connor, 2015). The current metabolic engineering approaches that are generally used to facilitate product development and commercialization include: (1) enhancing precursor supply; (2) overexpressing or disrupting pleiotropic/pathway-specific regulatory genes; (3) modifying transcription/translation apparatus; and (4) duplication or higher-order amplification of rate-limiting enzyme encoding genes or complete BGCs (Baltz, 2016b; Zhang et al., 2016).

As a biotechnological tool, the amplification of bacterial genomic DNA plays an important role in strain improvement in a variety of pharmaceutical and industrial applications, including antibiotic biosynthesis, bioconversion and degradation of toxic compounds. Over the past decade, high-order amplification of complete BGCs has been developed as an efficient strategy to facilitate antibiotic biosynthesis. This approach was validated to a limited extent by findings from some overproducing strains derived from traditional mutagenesis screening, such as *Penicillium chrysogenum, Streptomyces lincolnensis* and *Streptomyces kanamyceticus*, which contain tandem amplifications of the corresponding antibiotic BGCs (Fierro et al., 1995; Peschke et al., 1995; Yanai et al., 2006). Murakami et al. discovered that the relaxase

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ZouA and two *oriT*-like recombination sites RsA/RsB mediated tandem amplification of the kanamycin BGC potentially via a rolling circle mechanism in *S. kanamyceticus* (Murakami et al., 2011). Using the ZouA-mediated DNA recombination system, 4–12 tandem copies of the actinorhodin (ACT) BGC and 3–5 copies of the validamycin A (VAL-A) BGC were successfully introduced into the genomes of *Streptomyces coelicolor* M145 and *Streptomyces hygroscopicus* 5008 under antibiotic selection, resulting in 20- and 0.34-fold increases in ACT and VAL-A production, respectively (Murakami et al., 2011; Zhou et al., 2014). However, the tandemly amplified BGCs would be gradually lost in the absence of antibiotic selection (Zhou et al., 2014), suggesting that this method is not suitable for constructing antibiotic hyperproducing strains for large-scale fermentation.

As an attractive alternative, bacteriophage (such as ΦC31, ΦBT1 and TG1) attachment/integration (Att/Int) systems have been widely exploited for the stable chromosomal integration of target BGCs, thereby enhancing the production of many antibiotics in actinomycetes (Baltz, 2012). In most cases, only one extra copy of target BGCs is introduced by a single Att/Int system, and thus the range of yield improvement is often limited. Recently, two compatible ΦC31 and TG1 Att/Int systems were used in a step-by-step manner to duplicate and triplicate the goadsporin BGC, resulting in 1.5- and 2.3-fold increases in goadsporin production of *Streptomyces* sp. TP-A0584, respectively (Haginaka et al., 2014). However, the entire procedure for BGC amplification using different Att/Int systems involves repeated rounds of integrating-plasmid construction and conjugative transfer, and the number of integrated BGCs is also limited by the number of resistance markers available for selection.

Here, we developed a simple and general method for the single-step, multi-copy chromosomal integration of target BGCs via the 'one integrase-multiple *attB* sites' concept. We designate this method MSGE (Multiplexed Site-specific Genome Engineering). As a proof-of-concept, multiple artificial *attB* sites (including three artificial ΦC31 sites and two artificial ΦBT1 *attB* sites) were inserted step by step into the genome of a pristinamycin II (PII) high-producing *Streptomyces pristinaespiralis* strain beforehand. Then, the ΦC31 Att/Int system was used to achieve three-copy integration of the pristinamycin II (PII) BGC. Subsequently, a new *in vitro* DNA editing method was established by combining the CRISPR/Cas9 system with Gibson assembly to rapidly introduce the compatible ΦBT1 Att/Int system for second-round amplification of the PII BGC. The final engineered *S. pristinaespiralis* strain carrying five extra copies of the PII BGC produced the highest PII titers thus far reported and showed a very high genetic stability over multiple generations in the absence of antibiotic selection. In addition, a series of reliable heterologous hosts derived from *S. coelicolor* M1146 and M1152 (Gomez-Escribano and Bibb, 2011) were developed by introducing different numbers of artificial ΦC31 *attB* sites. Using the engineered *S. coelicolor* hosts, the production of chloramphenicol and the anti-tumour compound YM-216391 was markedly enhanced by single-step, four-copy chromosomal integration of the corresponding cloned BGCs. We expect that MSGE not only can be an effective tool to facilitate the biosynthesis of important secondary metabolites in laboratory and industrial actinomycete strains, but also provides a new design mode to construct robust expression hosts for the discovery of novel natural products.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table S1. *S. pristinaespiralis* Δ*papR5+R4R6* (named SBJ1000) (Li et al., 2015) and its derivatives were grown at 30 °C on RP medium (g/L, soluble starch 20, soybean flour 10, valine 0.5, KH_2PO_4 0.5, $MgSO_4$•$7H_2O$ 1, NaCl 2, $CaCO_3$ 3 and agar 20) for spore preparation, conjugal transfer and phenotype observation. *S. coelicolor* M1146, M1152 and their derivatives were grown at 30 °C on MS medium (g/L, soybean flour 20, mannitol 20, and agar 20) for spore preparation, conjugal transfer and phenotype observation. *S. pristinaespiralis* and *S. coelicolor* were grown on M-Isp4 medium (g/L, soybean flour 5, mannitol 5, starch 5, tryptone 2, yeast extract 1, NaCl 1, $(NH_4)_2SO_4$ 2, K_2HPO_4 1, $CaCO_3$ 2, agar 20, and trace element solution 1 mL, pH 7.2) for conjugal transfer when the CRISPR/Cas9 genome editing tool was used to introduce artificial *attB* site into their chromosomes. *S. pristinaespiralis* HCCB10218 and *S.coelicolor* M145 were cultivated in liquid RP (g/L, tryptone 5, yeast extract 5, valine 0.5, NaCl 2, KH_2PO_4 0.5 and $MgSO_4$•$7H_2O$ 1, pH 6.4) and YEME medium (g/L, yeast extract 3, peptone, 5, malt extract 3, glucose 10, sucrose 340 and $MgSO_4$•$7H_2O$ 1.24), respectively, on an orbital shaker (200 rpm) at 30 °C for total DNA isolation.

Escherichia coli DH5α and EPI300 were used for DNA cloning. *E. coli* S17-1 and ET12567/pUZ8002 were used for conjugal transfer from *E. coli* to *S. pristinaespiralis* and *S. coelicolor*, respectively. *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) medium. Antibiotics (50 μg/mL of kanamycin and apramycin) were added when necessary.

2.2. DNA and RNA manipulation

An E.Z.N.A.® BAC/PAC DNA Kit (Omega Bio-Tek) was used to isolate bacterial artificial chromosome (BAC) vectors BAC-F1F15 and BAC-F1F15(BT-*aphII*) and cosmids pAH91 and pST85. Transcription templates for generating sgRNA (single guide RNA) were amplified using plasmid pCB003 (Jiang et al., 2015b) as the template with the primers listed in Table S2. sgRNA was *in vitro* transcribed using a MEGAscript Kit (Ambion) and then purified with a MEGAclear Kit (Ambion). The linearized DNA fragments from Cas9-digested BAC-F1F15 or pAH91 were obtained by ethanol precipitation. PFGE (Pulsed Field Gel Electrophoresis) was performed with 1% agarose gel in 0.5×TBE buffer using CHEF-DR® III System (Bio-Rad) set to auto-algorithm programme with 5–250 kb parameters (6 V cm^{-1}, 20 h, 120°) and with circulation at 14 °C. After PFGE, the gel was stained with ethidium bromide (EB) and DNA bands were visualized using a UVIpro Platinum gel documentation system. *S. pristinaespiralis* and *S. coelicolor* genomic DNA isolation and intergeneric conjugal transfer were performed as described previously (Kieser et al., 2000).

2.3. Refactoring BGCs by combining the in vitro CRISPR/Cas9 system with Gibson assembly

Plasmid BAC-F1F15 (containing the PII BGC) was obtained using the modified Gibson assembly method as described previously (Li et al., 2015). To obtain the recombinant plasmid BAC-F1F15(BT-*aphII*) by replacing the ΦC31 *int* & *acc(3)IV* cassette with the ΦBT1 *int* & *aphII* cassette, a new *in vitro* DNA editing method was developed by combining the CRISPR/Cas9 system with Gibson assembly. Briefly, BAC-F1F15 (3.2 μg) was digested with 1.6 μg of the RNA-guided endonuclease Cas9, 1 μg ΦC31-sgRNA-up and 1 μg ΦC31-sgRNA-down for 2 h at 37 °C, and the linearized plasmid was recovered by ethanol precipitation. Using plasmid pRT802 as the template, the ΦBT1 *int* & *aphII* cassette (4.1 kb) was obtained by PCR amplification using primers ΦBT1-cassette-fw/rev. Then, 5 μL of equimolar DNA (containing 0.8 μg Cas9-digested plasmid and the ΦBT1 *int* & *aphII* cassette) was added to the Gibson assembly mixture. The reaction mixture was incubated at 50 °C for 1 h and 1 μL of the assembled product was electrotransformed into *E. coli* EPI300. The cells were recovered for 2 h in LB liquid medium and then incubated on LB agar containing 50 μg/mL each of chloramphenicol and kanamycin at 37 °C for 18–24 h.

Next, two plasmids, pAH91Δ*cmlR* and pAH91*kasO*p*-*cmlR*, containing an in-frame deletion of *cmlR* and the strong promoter *kasO*p* in place of the *cmlR* promoter, respectively, were also constructed using the same DNA editing method. Briefly, the upstream and