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Enhancing macrolide production in *Streptomyces* by coexpressing three heterologous genes

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ABSTRACT

Antibiotic production in *Streptomyces* can often be increased by introducing heterologous genes into strains that contain an antibiotic biosynthesis gene cluster. A number of genes are known to be useful for this purpose. We chose three such genes and cloned them singly or in combination under the control of the strong constitutive *ermE** promoter into a ϕC31-derived integrating vector that can be transferred efficiently by conjugation from *Escherichia coli* to *Streptomyces*. The three genes are *adpA*, a global regulator from *Streptomyces coelicolor*, *metK*, encoding S-adenosylmethionine synthetase from *S. coelicolor*, and, *VHbS*, hemoglobin from *Vitreoscilla*. The substitutions with GC in *VHbS* was intended to convert codons from lower usage to higher, yet causing no change to the encoded amino acid. Plasmids containing either one of these genes or genes in various combinations were introduced into *Streptomyces* sp. FR-008, which produces the macrolide antibiotic FR-008-III (also known as candicidin D). The largest increase in FR-008-III production was achieved by the plasmid containing all three genes. This plasmid also increased avermectin production in *Streptomyces avermitilis*, and is likely to be generally useful for improving antibiotic production in *Streptomyces*.

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1. Introduction

During the last two decades, significant advances have been made in strain improvement to meet the commercial requirements for the production of particular secondary metabolites. Recombinant DNA technology provides new strategies that can complement the traditional methods for improving fermentation titers. A range of strategies that can be employed to improve secondary metabolite production have been illustrated: overexpression of pathway-specific activators or global positive regulators; deletion of pathway-specific repressors or global negative regulators; and accumulation of targeted biosynthetic molecules [1,2]. In *Streptomyces*, most secondary metabolite biosynthetic pathways involve specific activator genes and/or global regulators, both of which are important targets to increase the production of secondary metabolites. Pleiotropic regulators, such as AfsR, PhoP, and AdpA, control pathway-specific regulators that switch on the transcription of antibiotic biosynthesis by influencing several secondary metabolic pathways [3,4]. Manipulation of global regulators affects many aspects of secondary metabolite production, including precursor supply and biosynthetic gene expression.

The transcriptional activator AdpA is an important regulator of morphological development and secondary metabolism in several streptomycetes. AdpA belongs to a subgroup of the AraC/XylS family [5]. AdpA is active in the A-factor regulatory cascade, and switches on a number of genes required for both morphological development and secondary-metabolite formation [6,7]. AdpA orthologs acting as positive regulators for antibiotic biosynthesis have been found in several *Streptomyces*. In *Streptomyces griseus*, where AdpA was first discovered, *adpA* mutants failed to produce streptomycin, and overexpression of *adpA* enhanced streptomycin production [8]. In *Streptomyces coelicolor*, an *adpA* mutant overproduced undecylprodigiosin, but failed to produce actinorhodin [5].

S-adenosylmethionine (SAM) is synthesized from methionine and adenosinetriphosphate by S-adenosylmethionine synthetase [9]. Recent studies have shown that exogenous addition of SAM or enhanced expression of SAM synthetase gene (*metK*) enhanced production of antibiotics in *Streptomyces* species, including actinorhodin from *S. coelicolor* [10] and *S. lividans* [11], nosiheptide from *S. actuosus* [12], avermectin from *S. avermitilis* [13], doxorubicin from *S. peucetius* [13]. It has been proposed that SAM activated transcription factors or served as a methyl donor directly in the biosynthesis of *Streptomyces* antibiotics.

Vitreoscilla hemoglobin (VHb) is an oxygen binding protein isolated from the Gram-negative bacterium *Vitreoscilla* [14]. Expression of VHb in various organisms has improved growth and increased metabolic productivity especially under oxygen-limiting

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conditions [15,16]. The beneficial effect of VHb for antibiotic production was demonstrated in a number of *Streptomyces* strains: it enhanced chlortetracycline yield in *Streptomyces aureofaciens* [17] and monensin yield in *Streptomyces cinnamonensis* under aeration-poor conditions [18].

Here we describe the construction of a series of plasmids containing three heterologous genes, *metK* (SAM synthetase from *S. coelicolor*), *VHbS* (high G + C version of hemoglobin gene from *Vitreoscilla*), and *adpA* (pleiotropic activator from *S. coelicolor*) under control of the strong constitutive *ermE** promoter. Overexpressing these heterologous genes in single, double, or triple leads to an increased production of the antibiotic FR-008-III in a mutant of *Streptomyces* sp. FR-008, and avermectin in *S. avermitilis* NRRL8165. These results illustrate an effective approach for improving the production of antibiotics by rational engineering of combined favorable factors.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

Streptomyces sp. FR-008 produces a mixture of antifungal polyene macrolides called FR-008 (also called candicidin) that differs in the oxidation state at four positions [19]. For our studies we used strain ZYJ-6 which is a mutant strain, accumulates only a single component, FR-008-III [20]. Strain HJ-5 is a derivative of *Streptomyces* sp. FR-008 from which the whole 137.2-kb FR-008 gene cluster was deleted (J. He, unpublished). *S. avermitilis* NRRL8165 (Northern Regional Research Laboratory, IL, USA) is a wild type producer of avermectin [21,22]. The non-methylating (*dam dcm hsd*) *Escherichia coli* ET12567/pUZ8002 [23] was used to introduce plasmids into ZYJ-6 and *S. avermitilis* NRRL8165 by conjugation.

pIB139 [24] is a shuttle vector that replicates in *E. coli* and integrates site-specifically into *Streptomyces* chromosomes. It contains the strong constitutive *ermE** promoter for the expression of cloned genes in *Streptomyces*, and an apramycin resistance gene for selection in *E. coli* and *Streptomyces*. pJTU968, a pRSET-B derivative containing the *ermE** promoter (H. Xu, unpublished), pBluescript II SK (+) (Stratagene), and pGH (Generay Biotechnology) were used for subcloning.

SFM (2% agar, 2% mannitol, 2% soybean powder, pH 7.2) was used for sporulation and solid fermentation. TSBY (3% TSB, 1% yeast extract, 10.3% sucrose, pH 7.2) and YEME (0.3% yeast extract, 0.5% Bacto-peptone, 0.3% malt extract, 1% glucose, 10.3% sucrose, pH 7.2) were used for mycelial growth and antibiotic production, respectively. *E. coli* was grown at 37 °C, and all *Streptomyces* strains were cultured at 30 °C.

2.2. Construction of metK, VHbS and adpA expressing plasmids singly and combinatorially

Cloning of the transcriptional activator *adpA* and the S-adenosylmethionine synthetase *metK* from *S. coelicolor* is as follows: the *adpA* gene and *metK* gene were amplified by PCR using two pairs of primers 5′-GGCTTAGCCATATGAGCCAC-3′and 5′-CGTTCATGCGGGCCACTTTA-3′ for *adpA*, 5′-CAGGGAGCCATATGTCCCGT-3′ and 5′-TCGCAAAGGCCACTGACAACA-3′ for *metK*, containing NdeI sites (indicated by underline) to produce 1.5-kb and 1.3-kb fragments. The fragments were inserted into pBluescript II SK (+), and then subcloned into the ϕC31-derived integrative vector pIB139 to be under the control of *ermE** promoter, via NdeI and EcoRI sites to generate pFAdpA and pFMetK, respectively (Fig. 1). The *adpA* and the *metK* sequences were confirmed by DNA sequencing.

Cloning of *VHbS* from *Vitreoscilla* is as follows: the promoter-less 456-bp hemoglobin gene *VHbS* gene was obtained from Majorbio Biotech Co., Ltd. The coding sequence was modified according to the codon usage frequency in *Streptomyces*, increasing the G + C content from 45.4% to 67.1%. The 456-bp synthetic *VHbS* DNA fragment end filled with NdeI and EcoRI sites was cloned into the SmaI site of pGH and subsequently a 441-bp NdeI–EcoRI DNA fragment was inserted into pIB139 with *ermE** promoter to generate pFVHb (Fig. 1).

Construction of plasmids pFMV expressing *metK* and *VHbS*, and pFMA expressing *metK* and *adpA* is as follows: the *VHbS* DNA fragment from pFVHb and *adpA* DNA fragment from pFAdpA via NdeI and EcoRI sites were cloned into pJTU968, and then inserted into EcoRI site of pFMetK via MunI and EcoRI digestion to generate pFMV and pFMA, respectively (Fig. 1).

Construction of plasmid pFMVA expressing *metK*, *VHbS* and *adpA* is as follows: the *adpA* fragment from pFAdpA via NdeI and EcoRI sites was cloned into pJTU968 and then *adpA* fragment with *PermE** via MunI and EcoRI digestion was cloned into the EcoRI site of pFMV to generate pFMVA (Fig. 1).

2.3. Conjugation

Intergeneric conjugation from *E. coli* to *Streptomyces* was performed as described previously [23]. Plasmids pFMetK, pFVHb, pFAdpA, pFMV, pFMA and pFMVA were introduced into strain ZYJ-6 or *S. avermitilis* NRRL8165, and exconjugants were confirmed by PCR using primers metKTF, 5′-GAACAGACCCACGGGCTCGG-3′ and metKTR, 5′-TGTCCCGTCGCCTGTTCACC-3′ for *metK*, VHbTF, 5′-GTGGACCAGCAGACCATCAA-3′ and VHbTR, 5′-ACTCGACCGCCTGGGCGTAC-3′ for *VHbS*, adpATF, 5′-CGCAGGGACTGGAGGCGATC-3′ and adpATR, 5′-CACCCGCTGGGTGATCAGCC-3′ for *adpA*.

2.4. Antibiotic FR-008-III production

Spores were prepared by growing the strains for 3 days on SFM at 30 °C. Fresh spores were inoculated into 25 ml of TSBY and incubated with shaking (220 rpm) for 20 h. This culture was then used as an inoculum for the antibiotic fermentation medium. Fermentation experiments were carried out in 250 ml flasks containing 50 ml of YEME medium (containing 10.3% sucrose). Flasks were inoculated with 1 ml inoculum culture and incubated with shaking for 84 h at 220 rpm. For the measurement of FR-800-III production, the fermentation liquid was extracted with n-butanol, and the supernatant was analyzed by detecting absorption at 380 nm using a PerkinElmer spectrophotometer. Extracts from strain HJ-5, which lacks the FR-008 gene cluster, were used to determine the non-specific background absorption. Each experiment was repeated at least three times.

2.5. Avermectin production

The inoculum was prepared as above, but avermectin production was measured using solid SFM agar instead of liquid medium. Equivalent amount of cell culture was transferred to 25 ml of SFM solid medium and incubated at 30 °C for 7 days. The agar cultures were extracted with 2 volumes of methanol, filtered, and concentrated by evaporation. The solid residue was dissolved in 1/10 volume of methanol and analyzed by high performance liquid chromatography (Agilent TC-C18, 4.6 mm × 250 mm, 5 μm; eluted at 0.5 ml/min using methanol:H_2O 90%:10%, v/v). UV detection of avermectin was at 246 nm. The whole process was performed at 25 °C.

3. Results and discussion

3.1. Expression of the transcriptional activator AdpA in Streptomyces sp. FR-008

Orthologs of the global transcriptional activator AdpA exist in many antibiotic-producing *Streptomyces* strains. An alignment of the AdpA sequences from *Streptomyces* sp. FR-008, *S. coelicolor*, *S. lividans*, *S. griseus*, and *S. avermitilis* showed that these orthologs had about 87% identity in sequence. This suggested that *adpA* from *S. coelicolor* might also function and increase antibiotic FR-008-III production in *Streptomyces* sp. FR-008 derived strain ZYJ-6. *S. coelicolor adpA* was cloned into the integrating plasmid pIB139 with *PermE** to give pFAdpA (Fig. 1). This plasmid was introduced by conjugation into strain ZYJ-6, a mutant which accumulates only a single component FR-008-III versus four components in the wild type FR-008, to facilitate detecting. Fig. 2a shows that in liquid YEME medium strain ZYJ-6::pFAdpA produced 1.9-fold more FR-008-III than ZYJ-6 without pFAdpA. Two repeat experiments using independent ZYJ-6::pFAdpA isolates confirmed that the increase of FR-008-III production was reproducible. Moreover, the increase of FR-008 production was also observed with solid cultures using HPLC to detect the antibiotic. This confirmed our hypothesis that AdpA from *S. coelicolor* was functional in *Streptomyces* sp. FR-008 and increased antibiotic FR-008-III production.

3.2. Expression of SAM synthetase in Streptomyces sp. FR-008

Previous studies have shown that exogenous addition of SAM or enhanced expression of *metK* (SAM synthetase) caused overproduction of several antibiotics in *Streptomyces* [25,26]. SAM is required for the production of the methylmalonate precursor used for many polyketides, and also for N- and O-methylations frequently found in antibiotics [27,28]. SAM may also serve as an effector for gene expression [29]. We wanted to test whether introducing an additional SAM methylase gene would increase the biosynthesis of the antifungal polyene macrolide FR-008 which features four propionate units that are derived from methylmalonate

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