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Enhanced salinomycin production by adjusting the supply of polyketide extender units in *Streptomyces albus*

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ABSTRACT

The anticoccidial salinomycin is a polyketide produced by *Streptomyces albus* and requires malonyl-CoAs, methylmalonyl-CoAs, and ethylmalonyl-CoAs for the backbone assembly. Genome sequencing of *S. albus* DSM 41398 revealed a high percentage of genes involved in lipid metabolism, supporting the high salinomycin yield in oil-rich media. Seven PKS/PKS-NRPS gene clusters in the genome were found to be actively transcribed and had been individually deleted, which resulted in significantly improved salinomycin production. However, a combined deletion of PKS-NRPS-2 and PKS-6 showed no further improvement. Whereas the concentrations of malonyl-CoA and methylmalonyl-CoA were increased, the concentration of ethylmalonyl-CoA remained low in the mutants. An endogenous crotonyl-CoA reductase gene (*ccr*) was overexpressed in the ΔPKS-NRPS-2/ΔPKS-6 mutant, resulting in improved production. Combination of cluster deletions and over-expression of *ccr* gene led to an overall titer improvement of salinomycin from 0.60 to 6.60 g/L. This engineering strategy can be implemented for various natural polyketides production.

1. Introduction

Salinomycin (Fig. 1A), a member of polyether antibiotics, has been widely used as veterinary medicine in animal husbandry as food additive and growth promoter for 30 years (Gumila et al., 1997). In addition, salinomycin has recently been identified as a potential agent to inhibit leukemia stem cells (Fuchs et al., 2010) and epithelial cancer stem cells (Kuo et al., 2012). It is produced by the *Streptomyces albus* DSM 41398 (hereafter abbreviated as *S. albus* DSM 41398 or strain DSM 41398) (Yurkovich et al., 2012) and its derived strains.

Some efforts have been devoted to generate high-yielding salinomycin strains and optimize fermentation media. Jia et al. improved the production of salinomycin by 70% using UV mutagenesis together with a high-throughput screening method (Jia et al., 2013). On the other hand, bean oil was proved to be essential for salinomycin overproduction, and it also took quite a large proportion in production cost. Zhang et al. screened the medium components for salinomycin production by Plackett–Burman design, and reduced the amount of bean oil from 12% to 10%, which resulted in an increased titer of salinomycin by 20% (Zhang et al., 2010). Even though a tremendous increase in production has been achieved in the past 30 years, there is yet lack of study on the enhancement of salinomycin production by metabolic engineering, and the detailed mechanism for salinomycin overproduction in oil-rich medium remains unknown.

Recently, the salinomycin biosynthesis gene cluster was identified, and the mechanism for salinomycin biosynthesis was proposed (Jiang et al., 2015, 2012; Yurkovich et al., 2012). As a typical type-I PKSs, there are nine large adjacent genes (*slnA1* to *slnA9*) collinearly arranged in the gene cluster, which are responsible for 14 condensation steps using 1 acetyl-CoA, 5 malonyl-CoAs (M-CoAs), 6 methylmalonyl-CoAs (MM-CoAs), and 3 ethylmalonyl-CoAs (EM-CoAs) for the biosynthesis of salinomycin polyketide skeleton (Fig. 1A). After several post modification steps, the fully modified polyether chain was released from PKS. Moreover, *orf11* and *orf12* in the salinomycin gene cluster are annotated as 3-hydroxybutyryl-CoA dehydrogenase and 3-oxoacyl-(acyl carrier protein) synthase III genes, respectively. They are considered to be involved in the dedicated supply of EM-CoA, whose disruptions decreased the titer of salinomycin by 90% and 74%, respectively (Jiang et al., 2012).

Polyketides are a large class of structurally diverse natural products and assembled by rounds of decarboxylative Claisen condensations between an acyl thioester and a thioesterified

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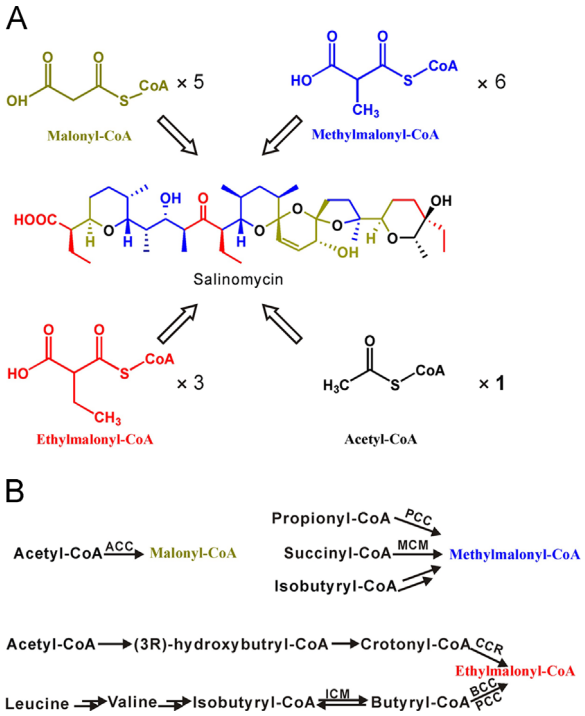

Fig. 1. Structures and biosynthetic pathways of extender units involved in salinomycin biosynthesis. (A) Chemical structures of salinomycin and its extender units. (B) Biosynthetic pathways of the extender units. ACC, acetyl-CoA carboxylase; PCC, propionyl-CoA carboxylase; BCC, butyryl-CoA carboxylase; MCM operon, methylmalonyl-CoA mutase; CCR, crotonyl-CoA reductase.

malonate derivative, catalyzed by polyketide synthases (PKSs). Three types of bacterial PKSs are known to date, and type-I and type-II PKSs are the most common ones. Type-I PKS assemblies are formed from giant multifunctional enzymes consisting of one or more modules, and each module contains a set of domains for β-ketoacyl synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) required for polyketide elongation, as well as optional β-keto processing domains, such as ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) (Park et al., 2010). Type-II PKSs have discrete enzymes functionally equivalent to the domains of type-I PKSs, but they contains KS_α and KS_β enzymes. The KS_α is equivalent to the KS domain seen in type-I PKSs, while the KS_β controls polyketide length (Otten et al., 1990).

There has been considerable interest in enhancing the yield of polyketides by increasing the supply of extender units (Reeves et al., 2006; Zabala et al., 2013), and it has been proposed that the shortage of particular extender units may lead to chain termination (Ding et al., 2015) and reduced production. Usually two strategies have been alternatively used for increasing the supply of extender units and the titer of polyketides. One is the improved expression of the biosynthetic genes for specific extender unit, e.g. the productions of actinorhodin, FK506, and epothilone B were respectively improved by 6-fold, 2-fold and 2.5-fold via this strategy (Mo et al., 2009; Ryu et al., 2006; Stassi et al., 1998; Zabala et al., 2013). The other strategy is the deletion of polyketide gene clusters putatively competing for the same extender units. Genes for the biosynthesis of avermectin's starter unit had been deleted, which resulted in an increased production of oligomycin from 0.1 to 2.3 g/L in *Streptomyces avermitilis* (Schwientek et al., 2012; Wei et al., 2006).

Among the three extender units for salinomycin biosynthesis, malonyl-CoA is mainly formed from the carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC). Methylmalonyl-CoA can be generated via three biosynthetic pathways, i.e. the carboxylation of propionyl-CoA, the isomerization of succinyl-CoA, or the catabolism of valine (Fig. 1B) (Chan et al., 2009). There are at least two ways leading to ethylmalonyl-CoA observed in *Streptomyces* spp. One route is initiated by the condensation of two acetyl-CoAs, and terminated with the reduction of crotonyl-CoA to ethylmalonyl-CoA (Liu and Reynolds, 2001). The other route involves the isomerization of the valine catabolite isobutyryl-CoA and the carboxylation of butyryl-CoA (Fig. 1B). Furthermore, previous works also supported that the class of crotonyl-CoA carboxylase-reductase (CCR) could catalyze the formation of some uncommon extender units (straight-/branched chain extender units), such as propylmalonyl-CoA (Mo et al., 2010), 2-(2-methyl-butyl)-malonyl-CoA (Umezawa et al., 2001), and isobutylmalonyl-CoA (Xu et al., 2011), which are respectively involved in the biosynthesis of FK506, polyoxypeptin A, and germicidin F/G.

Herein, the genome of salinomycin producer *S. albus* DSM 41398 was fully sequenced, which shed lights on the extremely high productivity of salinomycin. By monitoring the concentrations of intracellular acyl-CoA esters, tandem deletion of competing polyketide gene clusters and over-expression of rate-limiting gene(s) involved in extender unit biosynthesis were interactively performed and resulted in the overproduction of salinomycin not only in a low-yielding, but also in a high-yielding strain.

2. Materials and methods

2.1. Bacterial strains, culture conditions, and general techniques

The bacterial strains and plasmids used in this study are listed in Table. S1.

S. albus DSM 41398, BK3-25 (from Zhejiang Shenghua Biok Biology Co., Ltd.), and their derivatives were grown on ISP 4 agar plates (BD) for sporulation and conjugation. For the isolation of the total DNA, *S. albus* was cultivated in trypticase soy broth (TSB) (Kieser et al., 2000) supplemented with 10.3% sucrose and 0.5% yeast extract at 30 °C.

2.2. Genome sequencing and assembly

S. albus DSM 41398 genome was sequenced by 454 GS-FLX sequencer (Margulies et al., 2005), which generated 637,209 reads and provided 26-fold coverage. Plasmid library of 6-8 kb insert (pSmart) and fosmid library of 35–45 kb insert (pCC2FOS) were constructed with genomic DNA of DSM 41398, and end-sequenced to provide contig linkage information. Gaps were closed by PCR amplification using specifically designed PCR primers. Final sequence assembly of 637,812 reads was performed using phred/phrap/consed package, including reads from 454 GS-FLX, specific PCR products, and primer walking. Data of Solexa and Sanger re-sequencing were used to revise the homopolymer error in 454 raw data and the low-quality bases (phrap score < 40) in assembled sequence. Finally, an estimated error rate of < 1 per 10,000 bases (phrap score ≥ 40) was endued to the consensus sequence.

2.3. Genome annotation and analysis

Putative protein-coding sequences (CDSs) were predicted by Glimmer 3.02 software (Delcher et al., 1999). CDS annotation was based on the BLASTP program with NR, COG, and KEGG databases. The tRNA genes were directly predicted by tRNAscan-SE (Lowe and Eddy, 1997).