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Up-regulated spinosad pathway coupling with the increased concentration of acetyl-CoA and malonyl-CoA contributed to the increase of spinosad in the presence of exogenous fatty acid

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ABSTRACT

Polyketides are important compounds with a staggering range of biological and medicinal activities. Previous studies have demonstrated that the addition of fatty acids can increase polyketides production. However, a detailed metabolic explanation of this phenomenon has not been established. The aim of this study was to explain the positive effect of exogenous fatty acids on polyketides production. Spinosyns are polyketide-derived macrolides. In our study, spinosyns were used, as an example, to study the positive effect of exogenous fatty acids on their production. In the presence of exogenous fatty acids, gene expression assays indicated that the transcription of *de novo* fatty acid biosynthesis was significantly decreased and the transcriptions of β-oxidation and spinosad biosynthesis were up-regulated. The decreased *de novo* fatty acid synthesis transcription and the increased β-oxidation transcription resulted in the increase of acetyl-CoA and malonyl-CoA. It is the up-regulated spinosad pathway coupling with the enhanced concentration of acetyl-CoA and malonyl-CoA that contributed to the increase of spinosad. Taken together, a metabolic link among *de novo* fatty acid synthesis, β-oxidation, and spinosad biosynthesis at the presence of exogenous fatty acids was established. The results presented here enable researchers to better understand why added fatty acids can increase polyketides production.

1. Introduction

Polyketides are a remarkable group of structurally diverse natural compounds with a staggering range of biological and medicinal important activities including antibiotic, antifungal, anticholesterol, antiparasitic, anticancer, and immunosuppressive properties. Since their discovery, polyketides have been studied as attractive targets because of their extraordinary pharmacological, and biological properties [1]. Polyketides are synthesized from repetitive condensation reactions by multifunctional enzymes called polyketide synthases (PKSs). PKSs can be divided into three groups, type I PKSs, type II PKSs and type III PKSs. Although the structures of polyketides are extraordinary diversity, they can be synthesized from a relatively small subset of common building blocks such as acetyl-CoA, propionyl-CoA, malonyl-CoA and methylmalonyl-CoA [2].

Many studies have shown that the addition of fatty acids can increase polyketides production [3–5]. These studies indicate that it is the increased concentration of acetyl-CoA, butyryl-CoA or propionyl-CoA that contributed to the enhancement of polyketides. The β-oxidation of the exogenous fatty acids contributed to the increased concentration of these common building blocks [6]. However, they have overlooked the influence of the exogenous fatty acids on *de novo* fatty acid synthesis. *De novo* fatty acid synthesis need to be considered because of two reasons: the intermediates of exogenous fatty acids can be directly used in the synthesis of cell lipids [7,8]; *de novo* fatty acid synthesis and PKS systems share the same precursors [9,10]. So, a metabolic explanation from the perspective of β-oxidation, *de novo* fatty acid synthesis and PKS systems is needed to better understand why the addition of fatty acids can influence polyketides production.

Previous studies chose to add long chain fatty acids (C_{16}–C_{18}) at the beginning of the fermentation process [11,12]. However, polyketides are secondary metabolites who are mainly synthesized in the middle-to-late stages of fermentation [13]. If fatty acids are

Abbreviations: DCW, cell dry weight; PKSs, polyketide synthases; qPCR, reverse transcription quantitative PCR.

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added at the beginning of the fermentation process the majority of the fatty acids are involved in the primary metabolism [6]. There are just minor added fatty acids are left when polyketides begin to synthesis. How the residual and minor added fatty acids significantly influence polyketides biosynthesis? So, the examination of the addition time of fatty acids is important to better understand the positive effect of added fatty acids on polyketides production.

Spinosyns are polyketide-derived macrolides which are produced by *Saccharopolyspora spinosa* [14]. One molecule spinosyns is composed of a 21-carbon tetracyclic lactone, a tri-*O*-methylated rhamnose, and a forosamine. Spinosad, a mixture of spinosyns A and D, is the two major components in *S. spinosa* fermentation [15]. Spinosad is an environmentally friendly pesticide and has a broad-spectrum insecticidal activity [16–18]. In 1999, spinosad-based insect control pesticide was awarded the Presidential Green Chemistry Challenge Award because of its low risk to non-target species, low environmental impact, and low mammalian toxicity [19]. In the past few years, details of the spinosad biosynthesis pathway and the genome information of *S. spinosa* have been elucidated by various studies [20,21]. These results make the study about the positive effect of exogenous fatty acids on spinosad production from the perspective of gene expression assays and intermediates determination possible.

The goal of this study was to explain why exogenous fatty acids can increase the yield of spinosad. Specifically, we studied the addition time of fatty acids (a mixture of linoleic and oleic) and the addition dose of fatty acids. Linoleic and oleic were chosen based on previous studies. After that, the changes in the expressions of genes involved in *de novo* fatty acid synthesis, β-oxidation and spinosad biosynthesis were analyzed after fatty acids were added. Then the concentration of acetyl-CoA and malonyl-CoA between the control group and the experimental group was determined. Finally, a potential metabolic explanation was established based on these results. The group without fatty acids addition was set as the control group and the experimental group was added the suitable dose of fatty acids at the suitable time.

2. Materials and methods

2.1. Bacterial strains and culture conditions

S. spinosa strain LU104 was stored as a glycerol freezer stock at −80 °C [22]. The spores of *S. spinosa* LU104 were maintained at 30 °C on ABB13 agar plates containing (per liter) 5 g soytone, 5 g soluble starch, 3 g $CaCO_3$, 2.1 g MOPS, 20 g agar. 25 μl sprore (10^9/ml) was inoculated into 30 ml seed medium containing (per liter) 30 g tryptic soy broth, 3 g yeast extract, 3 g beef extract, 2 g $MgSO_4{\cdot}7H_2O$, 10 g glucose, 2.5 g corn steep liquor, pH 7.2. The seed medium was incubated at 30 °C for 72 h with constant shaking at 220 rpm, and then 3 ml was inoculated into 30 ml growth culture (WHC) containing (per liter) 40 g glucose, 10 g beef extract, 1 g $MgSO_4$, 2 g NaCl, 15 g soytone, 30 g soluble starch, 2.4 g $CaCO_3$, 0.34 g yeast powder, 6.34 g peptone, with or without a mixture of linoleic and oleic, as described. The ratio of linoleic and oleic in the mixture was 1:2. The final pH of WHC was adjusted to 7.2 with 1 M NaOH.

2.2. Extraction of intracellular acetyl-CoA and malonyl-CoA

Intracellular acetyl-CoA and malonyl-CoA were extracted according to the method described with some modifications [23]. An aliquot of 1 ml cell culture was collected, chilled on ice immediately, and centrifuged at 10,000 rpm, 4 °C for 10 min. Then cell pellets were immediately ground to powder in a porcelain mortar, which was pre-cooled to −80 °C, under liquid nitrogen protection. The frozen powder was stored in liquid nitrogen until used for analysis. Prior to use, 0.2 g frozen powder was weighed into 1 ml ice-cold 10% trichloroacetic acid (TCA) and mixed for 30 s. Precipitants were removed by centrifugation at 10,000 rpm, 4 °C for 10 min and then supernatants, which were filtered with a 0.45 μm syringe filter, were analyzed by HPLC. Standard acetyl-CoA and malonyl-CoA were purchased from Sigma–Aldrich.

2.3. RNA extraction and cDNA synthesis

For transcriptional studies, 5 ml samples taken at 120, 144, 168, 192, 216 and 240 h were mixed with 2 ml RNA protect reagent (Tiange, China) to preserve RNA integrity. Then they were stored in liquid nitrogen. RNA was extracted using the RNeasy minikit (Tiangen, China). The integrity of RNA was confirmed by gel electrophoresis and the ratio of A_{260}–A_{280}. The synthesis of cDNA for reverse transcription-quantitative PCR analysis (RT-qPCR) was carried out using Maxima H Minus First Strand cDNA Synthesis Kit (Fermentas, USA) in a total volume of 20 μl containing 4 μl of 5 × RT buffer, 0.25 μl random hexamer primer, 1 μl 10 mM dNTP Mix, 2 μl total RNA and 1 μl reverse transcriptase. Negative reactions were performed using the same mixture without the reverse transcriptase. The reactions were performed as follows: incubate for 10 min at 25 °C followed by 15 min at 50 °C and then reactions were terminated by heating at 85 °C for 5 min. The reverse transcription reaction products were stored at −80 °C until used.

2.4. qPCR

The quantification of transcriptional gene expression was determined by qPCR on the LightCycler 480. The primers used in qPCR were designed using Primer3 and are listed in Table 1 [24]. Triplicate qPCRs were performed on LightCycler 480 using Super PreMix Plus (SYBR Green) kit (Tiangen, China). The reaction volume was 20 μl containing 10 μl 2× SuperReal PreMix Plus, 0.6 μl each primer, 1 μl cDNA and 7.8 μl RNase-free ddH_2O. Quantitative PCR cycle parameters were as follows: initial denaturation at 95 °C for 15 min, followed by 40 cycles of 10 s denaturation at 95 °C and 20 s annealing and extension at 60 °C. Fluorescence measurements were taken between each cycle. Negative controls were analyzed to check the residual contaminating genomic DNA. C_T values of these seven target genes were normalized to two reference genes, 16S rRNA and rbL13. 16S rRNA and rbL13 were suitable reference genes in this study, which has been determined in another study. The data

Table 1
List of primers used in this study.

Primers	Sequence 5′ → 3′
fabI-F	ATCATCGAACCGGTGGTCTTG
fabI-R	CTGGGCTGGGACGTCAAC
fadG-F	ATCTTCGACCACACCGAAACC
fadG-R	GTGGAATTCGGCCATCAGCAG
fadA-F	TCGGCCTCTTCGAGATCAAC
fadA-R	CGATGCACATGGTCGTGATG
fadB1-F	CGACTTCGACAATGCCTC
fadB1-R	ACGCCTGAATCATGTTTG
fadE-F	GGCTCCAGGTCGACTTTTCC
fadE-R	AACTGCTGGTTACGGTCGAG
SpnA-F	ATCGGTCCTTCCGGGATTTTC
SpnA-R	GCTGGACGAACCGGTACATCTC
SpnG-F	GGGCACTTCTCACCGACCTAC
SpnG-R	ACGCAGGTACGCAGGAACAG
16S rRNA-F	CCTACGAGCTCTTTACGCCC
16S rRNA-R	AGAAGCACCGGCTAACTACG
rbL13-F	GGCGTAGACCTTGAGCTTC
rbL13-R	GCTCGAAAAGGCGATCAAG