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Original Research Article

Engineering the TetR-family transcriptional regulator XNR_0706 to enhance heterologous spinosad production in *Streptomyces albus* B4 chassis

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ABSTRACT

The TetR family of regulators are an important group of transcription regulators that regulate diverse cellular processes in prokaryotes. In this study, we found that XNR_0706, a TetR family regulator, controlled the expression of *XNR_0345*, *XNR_0454*, *XNR_0513* and *XNR_1438* putatively involved in fatty acid β-oxidation by interacting with the promoter regions in *Streptomyces albus* B4. The transcription level of these four genes was downregulated in *XNR_0706* deletion strain (ΔXNR_0706) and restored by *XNR_0706* complementation in Δ0706/pIB-*0706*, demonstrating that XNR_0706 was a positive transcriptional regulator of the genes. With toxic long-chain fatty acids addition in TSB media, deletion of *XNR_0706* caused significantly poor growth, whereas *XNR_0706* complementation increased the utilization of additional fatty acids, resulting in restored growth. Fatty acid β-oxidation is one source of acetyl- and malonyl-CoA precursors for polyketides biosynthesis in actinobacteria. Overexpression of *XNR_0706* in B4/spnNEW, a spinosad heterologous expression strain derived from *S. albus* B4, increased spinosad yield by 20.6 %. Additionally, supplement of 0.3 g/L fatty acids resulted in a further 42.4 % increase in spinosad yield. Our study reveals a regulatory mechanism in long-chain fatty acids metabolism in *S. albus* and these insights into the molecular regulation of β-oxidation by XNR_0706 are instrumental for increasing secondary metabolites in actinobacteria.

1. Introduction

Polyketides produced through actinomycete fermentation processes are important sources of therapeutics and agrochemicals, including antibacterial antibiotics, antifungals, cholesterol-lowering drugs, immunosuppressants, antitumor agents, anthelmintic agents, and insecticides [1–3]. Most of these compounds are isolated from *Streptomyces*, the largest actinomycete genera [3]. However, several valuable compounds were produced by non-*Streptomyces* actinomycetes, known as rare actinomycetes, such as spinosad from *Saccharopolyspora spinosa* [4]. Considering rare actinomycetes are not always genetically tractable and require strict control of fermentation conditions, combination of heterologous expression with further genetic engineering seems to be an attractive alternative [5]. The model strain *Streptomyces albus* J1074 is widely used for heterologous production of natural compounds. It exhibits properties of clear genetic background, short growth cycle, relaxed culture conditions, and simple genetic manipulation [6,7]. Many published studies have documented the heterologous expression of natural products, including spinosad [8–10], pamamycin [2,11], and thaxtomins [12] in *S. albus* J1074 chassis. *S. albus* B4 (B4) was derived from *S. albus* J1074 *via* deleting 15 gene clusters and introducing additional *phi*C31 *attB* sites into the genome. It was thought that the yield of heterologously expressed products would be higher with the use of this B4 chassis [13].

Spinosad is an environment-friendly insecticide that is highly valued commercially [14]. It is a mixture of spinosyns A and D and is composed of a tetracyclic polyketide aglycone as well as tri-O-methyl-L-rhamnose and D-forosamine moieties [15]. To date, the production of spinosad by *S. spinosa* still cannot meet the huge global demand [4]. In our early work, spinosad production was detected in the recombinant strain

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B4/spnNEW which was constructed by introducing the entire artificial biosynthesized gene cluster into B4 [16]. To generate one molecule of spinosad, 1 propionyl-CoA is required as a starter unit, along with 9 malonyl-CoA and 1 methylmalonyl-CoA as extender units to form aglycone [4]. Therefore, intracellular malonyl-CoA may play a significant role in spinosad biosynthesis. In bacteria, malonyl-CoA is generally produced through two different pathways [17]. The first involves the activation of malonate, which derives from the degradation of pyrimidines, with CoA by malonyl-CoA synthetase. The second and more predominant pathway involves the direct carboxylation of acetyl-CoA by acetyl-CoA carboxylase and cells generate acetyl-CoA from several catabolic pathways, including the glycolysis, the catabolism of amino acids, and the fatty acids β-oxidation. Among them, long chain fatty acids undergo β-oxidation to generate acetyl-CoA, a process catalyzed successively by fatty-acyl-CoA synthase, long-chain-acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and acetyl-CoA acetyltransferase [18]. Several studies have reported on engineering the β-oxidation pathway to increase the yield of secondary metabolites in actinomycetes, such as spinosad (*S. spinosa*) [19] and avermectin (*Streptomyces avermitilis*) [1]. Furthermore, many previous studies have demonstrated that the positive influence of additional fatty acids on polyketide yield is attributed to fatty acids β-oxidation. For instance, Jung et al. demonstrated that the utilization of exogenous fatty acids elevated propionyl-CoA concentrations, which allowed for enhanced rapamycin production by *Streptomyces hygroscopicus* [20]. Butyryl-CoA concentration increased, which led to enhanced monensin biosynthesis in *Streptomyces cinnamonensis* when fermented with oil-based media [21]. Nevertheless, the regulatory mechanism underlying fatty acids β-oxidation in actinobacteria remains poorly understood, even in some model *Streptomyces*.

In this study, we identified a TetR-family transcriptional regulator XNR_0706 in B4, which could bind directly to the promoter regions of *XNR_0345* (*fadD3*), *XNR_0454* (*fadC1*), *XNR_0513* (*fadD5*) and *XNR_1438* (*fadA1*) associated with fatty acids β-oxidation and positively regulate their expression. The novel spinosad-producing strain with *XNR_0706* overexpression (B4/spnNEW-0706) produced 20.6 % higher spinosad yield in comparison with the initial (B4/spnNEW) in SFM fermentation media, and exogenous fatty acids (a mixture of linoleic and oleic) addition further led to a 42.4 % increase in spinosad production.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

All strains and plasmids in this study are listed in Table S1. *Escherichia coli* strains were cultivated in LB liquid media or on LB agar plate at 37 °C. B4 and its derivatives were grown in Trypticase soy broth (TSB) liquid media, and mannitol soy flour (MS) solid media (20 g/L soybean powder, 20 g/L mannitol, 20 g/L agar and pH adjusted to 7.5) was used for spore production. The spores of B4 and its derivatives were inoculated into a 250-mL flask containing 50 mL of CSM media [22] for 48 h for seed-stock preparation. Next, a small aliquot of seed culture was then transferred into a 250-mL flask containing 50 mL of TSB or SFM media [22] supplemented with or without additional fatty acids. The ratio of linoleic and oleic in the fatty acids mixture was 1:2 as a previous report described [23]. The initial OD_{600} value was set to 0.05 in TSB or SFM. All fermentations were carried out in shake flasks at 220 rpm and 30 °C. The fermentation samples were harvested at the indicated time points for analysis.

2.2. RNA extraction and real-time RT-PCR

Cells of the B4 strain, *XNR_0706* deficient strain (ΔXNR_0706), and *XNR_0706* complementation strain (Δ0706/pIB-0706) grown in TSB liquid media were harvested at 48 h. Total RNA extraction was performed using the Bacterium Total RNA Kit (Simgen, Hangzhou, China). RNA concentration was determined by microplate reader (BioTek Reader). RNA was reverse transcribed into cDNA using the HyperScript III 1st Strand cDNA Synthesis Kit with gDNA Remover (EnzyArtisan, Shanghai, China). PCR was performed using the Universal SYBR qPCR Mix (EnzyArtisan, Shanghai, China). 50 ng of cDNA and primers (Table S2) with final concentration of 0.25 μM were contained in a final PCR reaction volume of 20 μL. Real-time RT-PCR experiments were carried out using a Stepone Real-Time System (ABI, USA) and the parameters were as follows: 95 °C for 30 s, then 40 cycles of 95 °C for 10 s, 58 °C for 30 s and 72 °C for 30 s), with a final extension step at 72 °C for 10 min *hrdB* (*XNR_1043*) served as reference gene.

2.3. Overexpression and purification of His-XNR_0706

For His-XNR_0706 overexpression in *E. coli*, the *XNR_0706* sequence was amplified from the genomic DNA of B4 by PCR using the primer pair pET 0706-F/R (Table S2) and cloned into pET28a (+) generating the pET-0706 plasmid. *E. coli* BL21 (DE3) cells harbouring pET-0706 were grown in LB media at 220 rpm and 37 °C until the OD_{600} value approached 0.6. The protein expression was then induced with IPTG supply of 0.6 mM final concentration at 18 °C for 24 h. His-XNR_0706 protein was purified with the Ni-NTA Superflow Column (Qiagen, Germany). The purified protein was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. S1) and then quantitative determined using the Enhanced BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China).

2.4. Electrophoretic mobility shift assay (EMSA)

EMSA was performed using the Chemiluminescent EMSA Kit (Beyotime Biotechnology, Shanghai, China) in accordance with the kit instruction manual. DNA fragments containing the promoter regions (350 bp, from −300 to +50) of the target genes involved in β-oxidation were PCR-amplified using the primers listed in Table S2. PCR products with biotin-labeled were obtained using a universal primer (5′biotin-AGCCAGTGGCGATAAG-3′). DNA probes containing various truncated promoter regions of *fadD3* were also PCR-amplified using this method. It is noteworthy that probes 4 and M-P_{fadD3} were synthesized using the oligonucleotides probe 4-F/R and M-P0345-F/R, respectively (Table S2). Equimolar amounts of the two complementary single-strand oligonucleotides were placed in boiling water and then allowed to cool naturally to room temperature in annealing buffer (10 mM Tris, 50 mM NaCl, 1 mM EDTA; pH 8) [24].

Purification of biotin-labeled probes employed the EasyPure PCR Purification Kit (TransGen Biotech, Beijing, China). The incubations were carried out in 10 μL reaction volume. Labeled DNA probes of XNR_0706 protein with a gradient concentration were incubated at room temperature for 20 min. Samples were separated on 8 % polyacrylamide gels in ice-cold 0.5 × Tris-Borate-EDTA (TBE) buffer at 100 V. The bands were detected using the BeyoECL Plus (Beyotime Biotechnology, Shanghai, China).

2.5. Construction of B4-derived strains

For *XNR_0706* deletion, DNA fragments (about 2.0 kb) upstream and downstream of the *XNR_0706* gene were amplified from B4 genomic DNA by PCR using the primer pairs pJTU0706up-F/R and pJTU0706dw-F/R, respectively (Table S2). The two fragments were inserted into the EcoRI and HindIII sites of pJTU1278, generating plasmid pJTU1278-0706 (Table S1). The deletion plasmids were then transferred into B4. Exconjugants were picked and underwent two rounds of nonselective growth in TSB media. After that, thiostrepton-sensitive mutants were selected. The double-crossover mutants were confirmed by PCR and DNA sequencing (Fig. S2). Primers are listed in Table S2.

For *XNR_0706* complementation in ΔXNR_0706 mutant, the *XNR*_0706 gene was amplified by PCR with the primer pair pIB0706-F/R

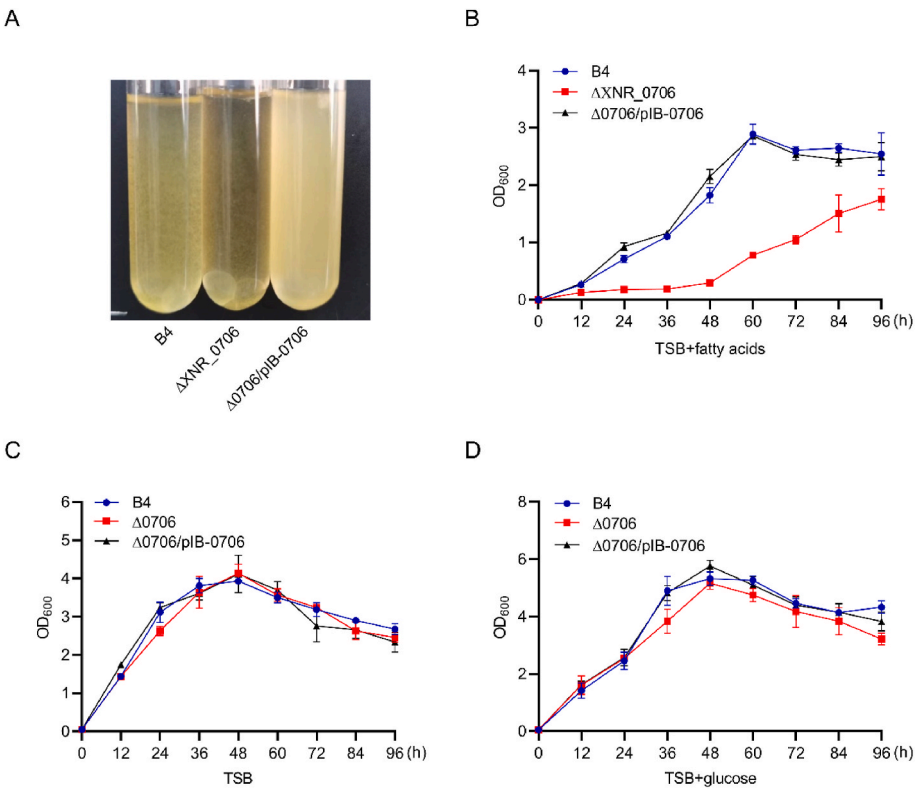

Fig. 1. Growth of strains B4, ΔXNR_0706 and Δ0706/pIB-0706 grown in TSB supplemented with additional carbon source or not. **A** Images were captured at 48 h (exponential growth phase). **B** Growth curves in TSB with 0.3 g/L fatty acids. **C** TSB. **D** TSB with 2.5 % glucose.

(Table S2). The fragment was then inserted into the NdeI and XbaI sites of pIB139, generating plasmid pIB-0706 (Table S1). pIB-0706 was transformed into the strain ΔXNR_0706 and the complementation strain Δ0706/pIB-0706 was thus obtained. By introducing plasmid pBAC-spnNEW [8] into B4, the original spinosad-producing strain B4/spnNEW was constructed [16]. pBC-0706 was transformed into B4/spnNEW and the B4/spnNEW-0706 strain was thus obtained. Plasmids were all transformed into *S. albus* by biparental conjugation according to the literature [11].

2.6. Determination of intracellular concentrations of acyl-CoAs

Intracellular acyl-CoAs were quantified by HPLC according to the method previously described, with modifications [25]. Cells of the strains B4, ΔXNR_0706 and Δ0706/pIB-0706 grown in TSB media were harvested at 48 h 2 mL of fermentation broth was collected and centrifuged for 10 min at 4 °C, cells were then washed twice with PBS buffer and resuspended in 800 μL lysis buffer (10 % trichloroacetic acid and 90 % 2 mM dithiothreitol) followed by freeze-thaw cycles until complete lysis. The supernatant was added into Sep-Pak column (1 mL, 50 mg tC18; Milford, MA, USA) and washed with 1 mL H_2O. Acyl-CoAs were eluted by 400 μL of 40 % acetonitrile. After which, lyophilization was performed. Samples were dissolved with 100 μL acetonitrile and directly analyzed by HPLC with an injection volume of 20 μL. HPLC detections were carried out on Agilent 1260 system, using DiKMA C18-A column (5 μm, 180 × 4.6 mm) with a column temperature of 30 °C. The detector wavelength was set to 254 nm. Mobile phase A was 75 mM KH_2PO_4 with the pH adjusted to 5.5 and mobile phase B was 80 % 75 mM KH_2PO_4 with the pH adjusted to 5.0, mixed with 20 % acetonitrile. Acyl-CoAs were separated using linear gradient as follow: 0–4 min, 10%–11 % B; 4–7 min, 11%–13 % B; 7–10 min, 13%–15 % B; 10–15 min, 15%–18 % B; 15–20 min, 18%–23 % B; 20–23 min, 23%–28 % B; 23–28 min, 28%–33 % B; 28–30 min, 33%–39 % B; 30–50 min, 39%–48 % B; 50–55 min, 48%–54 % B; 55–65 min, 54%–10 % B and 65–70 min, 10 % B, with a constant flow rate of 1 mL/min. To quantify the extracted acyl-CoAs, standard curves were constructed using acetyl- and malonyl-CoA standards (Sigma-Aldrich, USA).

2.7. Q-TOF analysis of spinosad production

For Q-TOF analysis, ESI positive ion mode (Agilent 6530C Q-TOF) was used, equipped with a ZORBAX Eclipse XDB-C18 column (5 μm, 250 × 4.6 mm). 1 mL of culture broth was mixed with 2 mL acetonitrile after adjusting the broth pH to 5. The mixture was sonicated for 10–15 min, followed by centrifugation 10,700×*g*, 10 min at room temperature. The supernatant was filtered with 0.22-μm syringe filter after eliminating the cell pellets. Then, the crude extract was injected into Q-TOF with a 5.0 μL volume. Mass spectra were acquired ranging from 100 to 1700 *m/z*. Mobile phase A (water with 0.1 % formic acid) and B (acetonitrile) were used with the following gradient: 0–10 min, 10%–54 % B; 10–16 min, 54%–62 % B; 16–20 min, 62%–90 % B and 20–25 min, 90%–10 % B, with a constant flow rate of 0.8 mL/min. To quantify the spinosad yield, the acquisition was carried out in targeted MS/MS mode, and a standard curve was constructed using the spinosad standard (Sigma-Aldrich, USA).

2.8. Computational analysis

The sequence alignments were generated using the GeneDoc software [26], and the MEGA11 was used to build phylogenetic trees [27]. Using MAST/MEME tools [28], putative XNR_0706 binding sites were searched within the promoter sequences of *fadC1*, *fadD5* and *fadA1*.

2.9. Statistical analysis

Three independent replicates were performed for each culture sample. Data with error bars represent the means and standard deviations. An unpaired two-tailed Student's t-test by GraphPad Prism 8 was

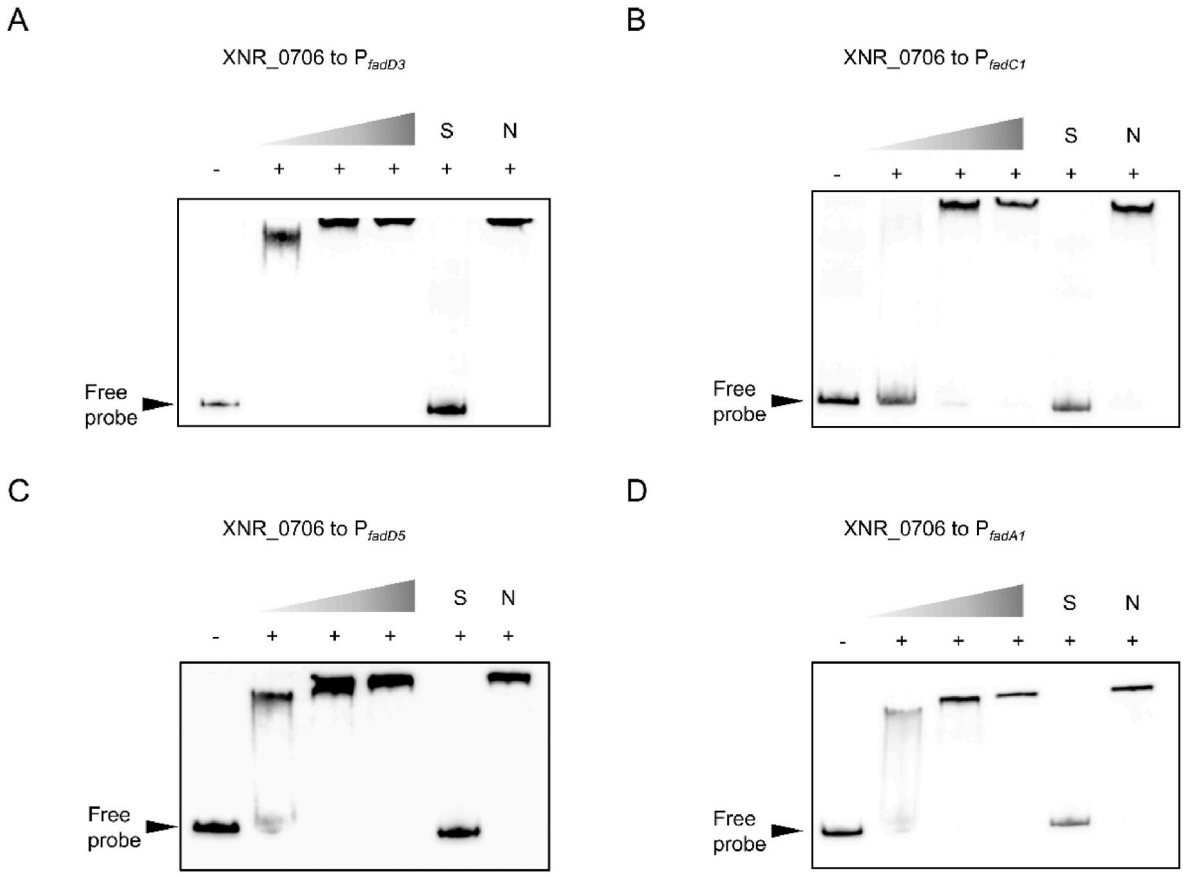

Fig. 2. Binding of XNR_0706 to the promoter regions of *fadD3* (**A**), *fadC1* (**B**), *fadD5* (**C**), and *fadA1* (**D**), as determined by EMSA. The biotin-labeled DNA probe (about 20 ng in each 10 μL reaction system) was incubated with gradient concentration of His-XNR_0706 (0, 0.5, 1, and 3 μM). EMSAs with a 200-fold excess of unlabeled specific probes as the competitor (S) or sonicated salmon sperm DNA, which serves as non-specific competitor (N), were performed as controls. The free probes without protein binding are shown by arrowheads.

performed to compare the statistical significance of the data (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

3. Results

3.1. Deletion of XNR_0706 represses the growth of B4 in TSB media containing 0.3 g/L of fatty acids

XNR_0706 encodes a 191-amino-acid protein with an N-terminal TetR-family helix-turn-helix DNA binding domain. To assess the *in vivo* biological significance of XNR_0706, we constructed the *XNR_0706* null mutant strain of B4 (ΔXNR_0706) and *XNR_0706*-complemented strain (Δ0706/pIB-0706). In certain instances, long-chain fatty acids have been observed to exhibit toxicity to microbial chassis, in addition to serving as a carbon source [29–31]. Actually, long-chain fatty acids (a mixture of linoleic and oleic) demonstrated strong toxicity to the B4 chassis, severely hindering its growth (Fig. S3). As shown in Fig. 1A and B, weaker growth of ΔXNR_0706 in comparison to B4 was observed in TSB media with additional 0.3 g/L of fatty acids, while complemention with *XNR_0706* could recover the growth of the *XNR_0706* null mutant. Additionally, when cultured in TSB media with 2.5 g/L glucose or not, the growth curves of strains B4, ΔXNR_0706 and Δ0706/pIB-0706 exhibited no discernible difference (Fig. 1C and D). Taken together, these observations indicate that XNR_0706 plays an important role in long-chain fatty acids metabolism in *S. albus*.

3.2. XNR_0706 binds to the promoter regions of four fatty acid β-oxidation genes

Given the significant influence of XNR_0706 on the effect of toxic exogenous fatty acids on the growth of *S. albus*, it is reasonable to presume that the expression of several genes involved in fatty acids metabolism is regulated by XNR_0706. Four genes, including *fadD3*, *fadC1*, *fadD5* and *fadA1*, were tentatively selected for testing. To examine whether XNR_0706 protein bound to promoter regions of *fadD3*, *fadC1*, *fadD5* and *fadA1* directly, EMSAs were performed. As shown in Fig. 2, obvious band shifts were observed, as the entire promoter regions of the four genes were incubated with purified recombinant His-tagged XNR_0706, respectively. The results indicate that XNR_0706 was able to directly bind to the promoter regions of these four genes.

To further define the binding sites of XNR_0706, we designed five Biotin-labeled DNA probes (probes 1 to 5) containing various truncated promoter regions of *fadD3* for EMSAs (Fig. 3A). Of the five probes tested, probe 1 (300 bp, from −300 to −1), probe 2 (241 bp, from −191 to +50), and probe 5 (191 bp, from −191 to −1) gave retarded signals, whereas specific DNA-protein interactions were not observed with probe 3 (109 bp, from −109 to −1) or probe 4 (50 bp, from +1 to +50), which did not include the *XNR_0344-XNR_0345* spacer region (Fig. 3B). Indeed, the fragment containing the *XNR_0344-XNR_0345* spacer region was sufficient for interaction with the purified His-tagged XNR_0706, therefore confirming the XNR_0706-binding site was located at the *XNR_0344-XNR_0345* spacer region. Within the *XNR_0344-XNR_0345* spacer, a palindromic sequence (TGCGGGGCGCN$_{35}$GCCCCGCGCT) was observed. Subsequent EMSA analysis confirmed that it was exactly the binding site of XNR_0706 (Fig. 3C and D), with further truncation of the

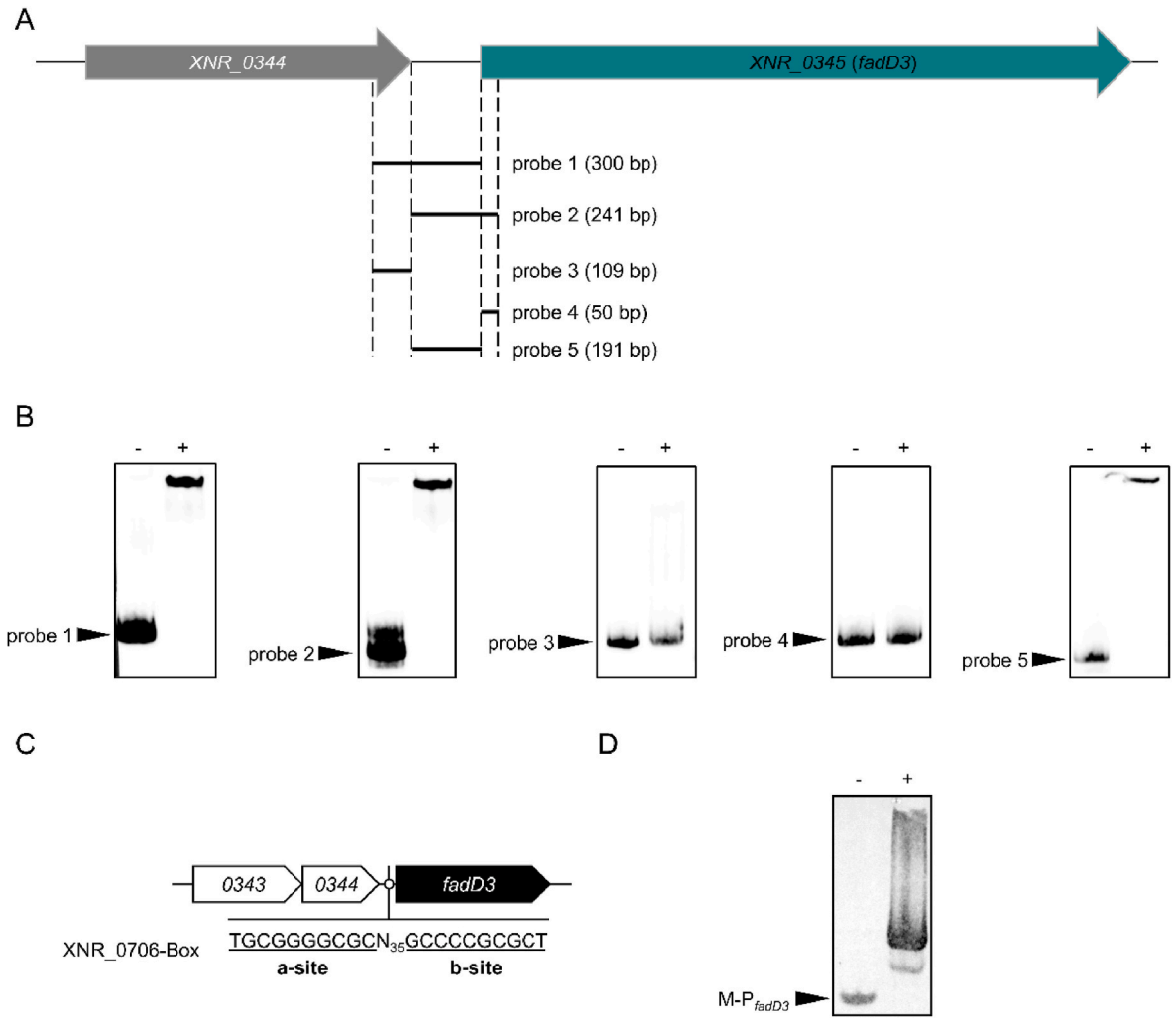

Fig. 3. EMSA for determination of the XNR_0706 binding site within the *XNR_0344—XNR_0345* spacer. **A** Schematic representation of the probes utilized for EMSAs. The lengths and positions of the probes are illustrated. **B** EMSAs with probes 1 to 5. The biotin-labeled DNA probe (about 20 ng in each 10 μL reaction system) was incubated with 3 μM His-XNR_0706. **C** Genetic organization of *fadD3* in B4 and the XNR_0706-binding site. **D** Verification of XNR_0706-binding site by EMSA. Synthetic probe M-P_{fadD3} contains the XNR_0706-binding site in the promoter region of *fadD3*.

palindromic sequence leading to the loss of its ability to bind XNR_0706 *in vitro* (data not shown). The putative XNR_0706-binding sites within the promoter sequences of *fadC1*, *fadD5* and *fadA1* were shown in Fig. S4.

3.3. *XNR_0706 positively regulates the transcription of the four fatty acid β-oxidation genes*

To investigate the effect of XNR_0706 on expression of *fadD3*, *fadC1*, *fadD5*, and *fadA1 in vivo*, the transcription levels of the four genes in B4, ΔXNR_0706, and *XNR_0706*-complemented (Δ0706/pIB-0706) strains grown in TSB media were quantified by real-time RT-PCR. As shown in Fig. 4A, deletion of *XNR_0706* resulted in a 29.6 %, 27.6 %, 59.8 % and 47.7 % decrease in expression levels of *fadD3*, *fadC1*, *fadD5*, and *fadA1*, respectively. By contrast, complementation of the mutant strain with the *XNR_0706* gene resulted in transcription levels much higher than those of the ΔXNR_0706 strain. Furthermore, we determined the intracellular concentrations of acetyl- and malonyl-CoA in strains B4, ΔXNR_0706 and Δ0706/pIB-0706 grown in TSB media. In contrast to B4, cytosolic acetyl- and malonyl-CoA levels in ΔXNR_0706 were found to be decreased, whereas those of Δ0706/pIB-0706 demonstrated comparable levels to B4 (Fig. 4B). Altogether, the four genes involved in β-oxidation are subjected to transcriptional activation by XNR_0706 in B4.

3.4. *Overexpression of XNR_0706 increased heterologous spinosad yield by precursor malonyl-CoA accumulation*

Based on the above results, we overexpressed the *XNR_0706* gene in the initial spinosad-producing *S. albus* strain (B4/spnNEW), thereby generating the B4/spnNEW-0706 strain. We determined the intracellular concentrations of malonyl-CoA in strains B4/spnNEW and B4/spnNEW-0706 grown in TSB media with 0.3 g/L of exogenous fatty acids. As shown in Fig. 5A, malonyl-CoA level in B4/spnNEW-0706 was higher than that in B4/spnNEW. The result indicates that XNR_0706 exerts a positive effect on cytosolic malonyl-CoA accumulation *via* acetyl-CoA in spinosad-producing *S. albus*. Moreover, Q-TOF tests revealed that the B4/spnNEW-0706 strain exhibited a higher spinosad yield (669.7 μg/L) than the B4/spnNEW strain (589.47 μg/L) in TSB media. Lastly, 0.3 g/L of fatty acids were added into the TSB media and the B4/spnNEW-0706 strain further increased spinosad yield with a titer of 993 μg/L (Fig. 5B).

We tested spinosad yield of strains B4/spnNEW and B4/spnNEW-0706 with SFM fermentation media. The yield of spinosad was upgraded from 1498.9 μg/L by B4/spnNEW to 1807.85 μg/L by B4/spnNEW-0706, with an increase of 20.6 %. Furthermore, when 0.3 g/L of fatty acids were added into SFM media, the spinosad yield of B4/spnNEW-0706 reached 2574.1 μg/L, with a further 42.4 % increase (Fig. 5C).

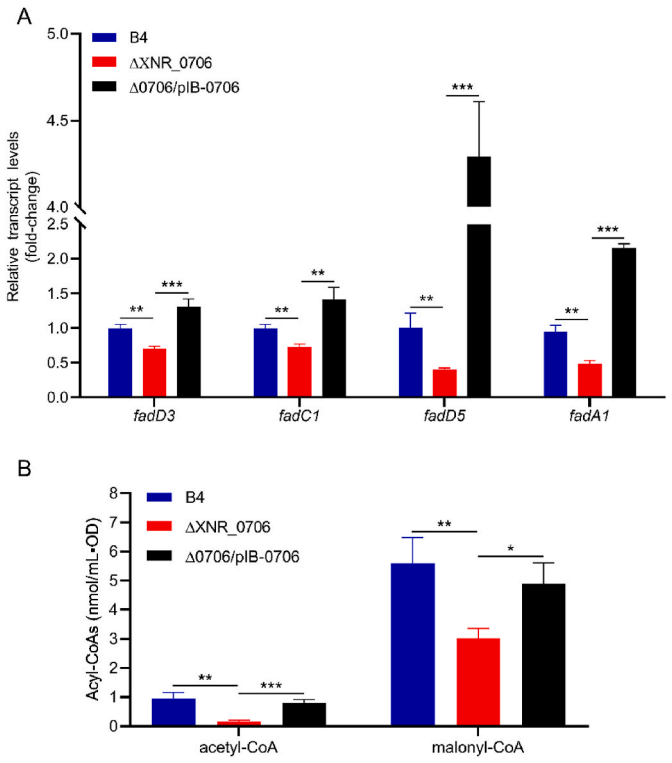

Fig. 4. XNR_0706 is an activator of the *fadD3*, *fadC1*, *fadD5* and *fadA1* genes. **A** Relative transcript levels of *fadD3*, *fadC1*, *fadD5* and *fadA1* quantified by real-time RT-PCR analysis in strains B4, ΔXNR_0706, and Δ0706/pIB-0706 grown in TSB media. **B** Intracellular acetyl- and malonyl-CoA concentrations of strains B4, ΔXNR_0706 and Δ0706/pIB-0706 grown in TSB media.

4. Discussion

Acetyl- and malonyl-CoA are important building blocks for biosynthesis of polyketides. In bacteria, long-chain fatty acids undergo β-oxidation to produce acetyl-CoA, which can be assimilated by acetyl-CoA carboxylase to form malonyl-CoA. Fatty acids are commonly used as an additional carbon source to increase polyketides production. For example, when exogenous octadecanoic acid was present, the simultaneous use of β-oxidation and *de novo* fatty acid synthesis pathways resulted in increased rhamnolipid yield in *Pseudomonas aeruginosa* [32]. For *S. spinosa* strain LU104, spinosad biosynthesis was facilitated by enhanced concentration of acetyl- and malonyl-CoA with exogenous fatty acids addition [23]. However, the toxicity of fatty acids to microorganisms has held back their utilization [29–31]. Accordingly, the potential for cell death in response to fatty acids should be considered when developing fatty acids as carbon source. The strengthening of the β-oxidation pathway would allow for a greater conversion of toxic fatty acids to downstream acyl-CoA esters, thereby further enhancing the synthesis of polyketides and reducing the toxic effects of fatty acids on microbial chassis. The regulators of bacterial fatty acid metabolism are of great physiological significance, and thus engineering these regulators is one of the most effective ways to achieve the goal [33–35]. TetR family regulators are widely distributed in the bacterial genome and involved in various biological processes [36,37]. They generally act to repress transcription of the target genes. However, there are also a limited number of instances where TetR family regulators act as positive regulators [38–40]. In this study, we found that the TetR family transcriptional regulator XNR_0706 positively controlled the expression of four genes involved in fatty acid β-oxidation through binding to palindromic sequences in upstream regions of these target genes. Exogenous fatty acids (a mixture of linoleic and oleic) were highly toxic to *S. albus* chassis, with this effect being further exacerbated by knockout of the *XNR_0706* gene and restored by *XNR_0706* complementation. These observations indicate that XNR_0706 is a good target for strengthening the β-oxidation pathway for heterologous polyketides production in *S. albus*. Biochemically, spinosad biosynthesis requires 1 propionyl-CoA

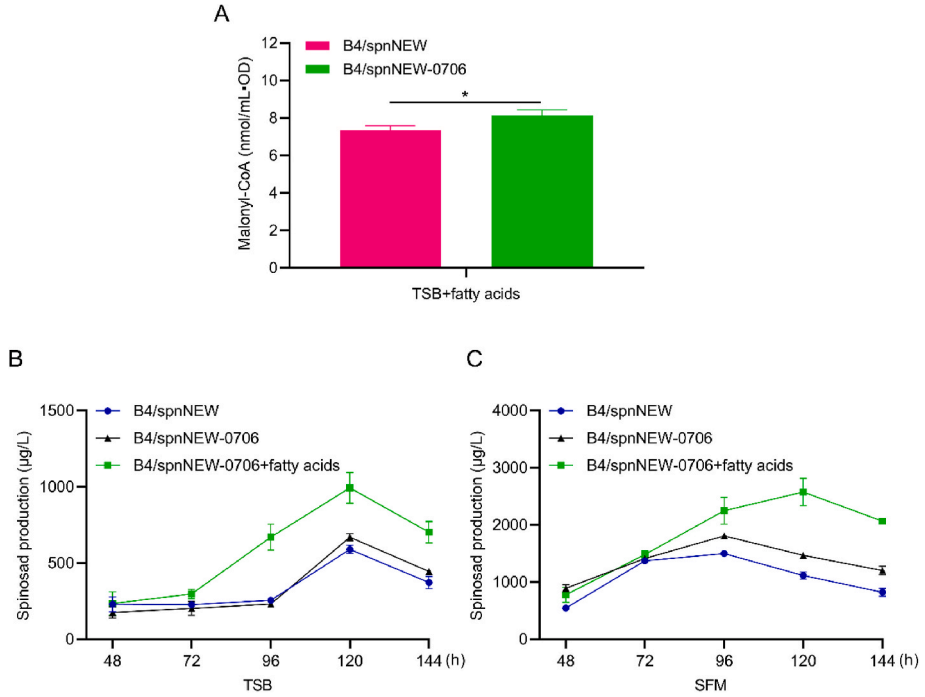

Fig. 5. Obvious enhancement of heterologous spinosad production by overexpression of *XNR_0706*. **A** Intracellular malonyl-CoA concentrations of strains B4/spnNEW and B4/spnNEW-0706 grown in TSB media with 0.3 g/L of exogenous fatty acids. **B** Heterologous spinosad yields of strains B4/spnNEW and B4/spnNEW-0706 grown in TSB media with 0.3 g/L of exogenous fatty acids or not. **C** Spinosad yields in SFM media with 0.3 g/L of exogenous fatty acids or not.

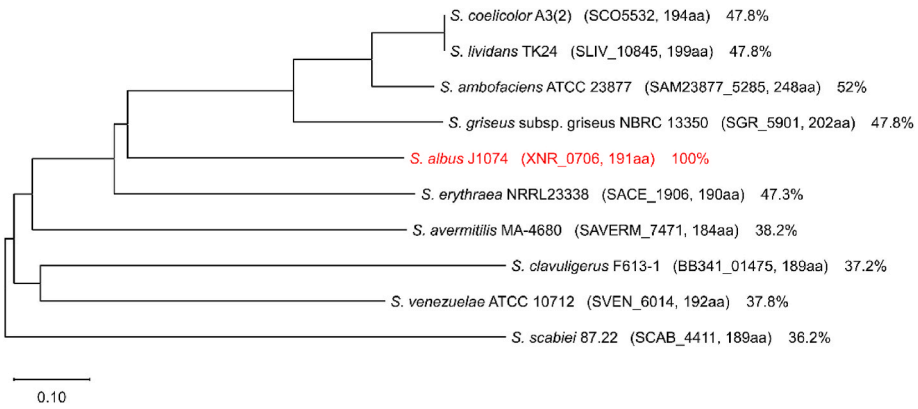

Fig. 6. Neighbor-joining distance tree of XNR_0706 with corresponding homologs from *Saccharopolyspora erythraea* and *Streptomyces* species.

as the starter unit as well as 9 malonyl-CoA and 1 methylmalonyl-CoA as extender units. Therefore, it is reasonable to assume that the intracellular malonyl-CoA pools play a significant role in spinosad biosynthesis. As was expected, the higher level of heterologous spinosad in the B4/spnNEW-0706 strain was observed compared with B4/spnNEW strain in this study. Admittedly, it also has to realize that, compared to *S. spinosa* NRRL 18395, the current yield of spinosad in B4/spnNEW-0706 was still relatively low.

In a previous study, SAVERM_7471, an ortholog of XNR_0706 in *S. avermitilis*, affected cellular metabolic flux, thereby indirectly regulating avermectin biosynthesis through the negative regulation of genes involved in CoA metabolism [1]. Four proteins related to β-oxidation, encoded by genes *fadD3*, *fadC1*, *fadD5* and *fadA1* in *S. albus*, exhibited high similarity to proteins encoded by genes involved in β-oxidation, whose transcription is regulated by SAVERM_7471 in *S. avermitilis* (Fig. S5). Indeed, exactly through searching for orthologs of proteins encoded by genes transcriptionally regulated by SAVERM_7471, we rationally identified potential target genes (*fadD3*, *fadC1*, *fadD5* and *fadA1*) that might be regulated by XNR_0706 in *S. albus*. However, the sequence of XNR_0706-binding site ($TGCGGGGCGCN_{35}GCCCCGCGCT$) displays no similarity to that of SAVERM_7471 ($GAGAACN_3CGTTCTC$) [1]. In typical antibiotic-producing actinobacteria, XNR_0706 shares high sequence identities with homologs of *Streptomyces coelicolor* (SCO5532, 47.8 %), *Streptomyces lividans* (SLIV_10845, 47.8 %), *Streptomyces ambofaciens* (SAM23877_5285, 52 %), *Streptomyces griseus* (SGR_5901, 47.8 %), *Saccharopolyspora erythraea* (SACE_1906, 47.3 %), *Streptomyces clavuligerus* (BB341_01475, 37.2 %), *Streptomyces venezuelae* (SVEN_6014, 37.8 %), and *Streptomyces scabiei* (SCAB_4411, 36.2 %), inferring that XNR_0706 homologs are widely distributed in antibiotic-producing actinobacteria (Fig. 6). Therefore, there is likely to be a beneficial application of our study on XNR_0706 regulation to the engineering of other industrial antibiotic-producers. Our work reveals a regulatory mechanism in long-chain fatty acids metabolism and suggests new possibilities for designing metabolic engineering to increase heterologous spinosad yield in *S. albus* chassis. Importantly, this study also reveals a potential for broader industrial applications, such as the yield improvement of target secondary metabolites by engineering of relevant regulatory systems in other actinobacteria.

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CRediT authorship contribution statement

Xingjun Cui: Data curation, Formal analysis, Investigation, Visualization. **Hao Tang:** Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. **Wenzong Wang:** Investigation. **Wenping Wei:** Writing – review & editing. **Jing Wu:** Investigation. **Bang-Ce Ye:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix. ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.08.008.

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