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ORIGINAL ARTICLE

AfsR is an important regulatory factor for growth and butenyl-spinosyn biosynthesis of *Saccharopolyspora pogona*

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Abstract

Purpose To generate a AfsR-like (AfsR-L) overexpression strain *Saccharopolyspora pogona*-AfsR-L and investigate its effects on the morphology and metabolism of *S. pogona*.

Methods Firstly, we generated the overexpression vector pOJ260-P_{ermE}-*afsR-L* via overlap extension PCR. Then, the recombination strain *S. pogona*-AfsR-L was constructed via conjugal transfer. To monitor the growth and morphology, mycelia and sporulation were observed. The distinctive proteins and butenyl-spinosyn biosynthesis were investigated by SDS-PAGE, HPLC, and mass spectrometry. And the transcriptional level of *afsR-L* and other relative functional genes in *S. pogona*-AfsR-L was analyzed by qRT-PCR. Western blot verified the increased amount of AfsR-L protein in the overexpression strain.

Result Growth curve and mycelia observation showed that *afsR-L* overexpression make the stationary phase of *S. pogona*-AfsR-L longer than that of wild *S. pogona* by approximate 3 days. Moreover, *S. pogona*-AfsR-L exhibited a more obvious white phenotype on the solid medium, which means *afsR-L* overexpression affects the sporulation ability of *S. pogona*. HPLC analysis revealed that the peak area of the butenyl-spinosyn yield of *S. pogona*-AfsR-L was 293.6, while that of *S. pogona* was 250.9. SDS-PAGE analysis showed that the two strains had different whole protein expression profiles, and the distinctive proteins were further identified by LC-MS/MS identification, which showed the possible control mechanism of *afsR-L* gene in *S. pogona*.

Conclusion We concluded that AfsR could directly or indirectly positively regulate the biosynthesis of butenyl-spinosyn and affect the growth features of *S. pogona*. We envisioned that this result can be expanded to other *Streptomyces* for strain improvement.

Keywords *Saccharopolyspora pogona* · AfsR · Regulatory factor · Butenyl-spinosyn biosynthesis · Strain morphology

Introduction

Butenyl-spinosyn is a macrolide secondary metabolite that is produced by *S. pogona* (Hahn et al. 2006; Lewer et al. 2009). This metabolite depolarizes insect nerve cells by binding to nicotinic acetylcholine receptors, thereby inducing the hyperactivity of the central nervous system that causes the rapid death of insects (Millar and Denholm 2007). Butenyl-spinosyn has extensive insecticidal activity against a wide spectrum of insect pests, including *Lepidoptera*, *Diptera*, and *Coleoptera* (Kirst 2010; Millar and Denholm 2007). However, wild *S. pogona* has very low capability for the biosynthesis of butenyl-spinosyn (Lewer et al. 2009; Li et al. 2018). Therefore, enhancing its biosynthesis is crucial for its wide application in the biocontrol of insect pests. Genetic engineering technology is a common and effective means for strain improvement. Liu reported that AtEDT1 overexpression in *Salvia miltiorrhiza* significantly increases salvianolic acid yield (Liu et al. 2017). Sun found that knocking out the negative control gene sav_742 in *Streptomyces avermitilis* increased abamectin production, compared with that of wild strain (Sun et al. 2016). Zhu utilized genetic engineering techniques to overexpress the *slnR* gene in *Streptomyces albus* and found that salinomycin yield is 25% higher than that of wild

Li Li and Liang Gong contributed equally to this work.

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strain (Zhu et al. 2017). Zhang reported that overexpressing the *milR* gene of *Streptomyces bingchenggensis* increases milbemycin A3/A4 production by 38% (Zhang et al. 2016). These studies showed that the use of genetic engineering technology to knock out or overexpress the genes related to the secondary metabolism in *Streptomyces* could effectively promote the biosynthesis of target secondary metabolites.

The global regulatory gene, not only participating in various life activities of organisms, but also involving in secondary metabolites biosynthesis, is located outside the biosynthetic gene cluster of secondary metabolites and regulates the expression of corresponding pathway-specific genes (Tatarko and Romeo 2001). Compared with other regulatory genes, the global regulatory gene is a more diverse, universal, and complex regulatory element of secondary metabolism in *Streptomyces* (Horinouehi et al. 1990; Xu et al. 2017).

AfsR is a global regulatory factor present in *Streptomyces*, which belongs to the *Streptomyces* antibiotic regulatory protein (SARP) family encoded by the *afsR* gene (Tanaka et al., 2007). The function of *afsR* gene has been extensively studied in *Streptomyces coelicolor*, which could activate the transcription of the *afsS* gene, regulate the biosynthesis of actinorhodin and undecylprodigiosin. AfsR protein, due to two domains containing ATP binding and DNA-binding domains, has an important influence on pigment phenotype of *S. coelicolor*. Furthermore, the DNA binding and ATPase activities of AfsR are physically separable, and these two functions are regulated by phosphorylation on serine and threonine residues (Sueharu Horinouehi, et al. 1990; Tanaka et al., 2007; Ping-Chin Lee, et al. 2002). Fernando Santos-Beneit found that AfsR and PhoP regulate the biosynthesis of actinorhodin and undecylprodigiosin by controlling the *afsS*, *pstS*, and *phoRP* transcription, PhoP may downregulate *afsS* expression by competitively binding to its promoter, and AfsR also competes with PhoP for binding to the promoters of *pstS* and *phoRP* (Fernando Santos-Beneit, et al. 2009).

AfsR regulates the biosynthesis of multiple secondary metabolites and activates different antibiotic biosynthetic gene clusters via different mechanisms. The *afsR-P* gene from *Streptomyces peucetius* ATCC 27952 had 60% similarity to that from *S. coelicolor*. When placing this gene under the strong promoter of P_{ermE}, the yield of γ-actinorhodin and clavulanic acid in *Streptomyces clavuligerus* increased by 260% and 150%, respectively (Parajuli, et al. 2005). Wang reported that *afsR* gene overexpression in *Streptomyces lomondensis* increases lorcomycin yield by 2.5-fold (Wang, et al. 2015). Sushila found that the overexpression of *afsR*-sp in *Streptomyces venezuelae* stimulated the expression of the pathway-specific regulatory genes, *pikD* and ketosynthase, which caused the production of pikromycin was increased by 2.6-fold (Maharjan, et al. 2008).

AfsR-L (orf06333-4034) protein in *S. pogona* shares 57% sequence similarity to AfsR in *Streptomyces sp.* NRRL WC-3742 (GenBank accession no. WP_078911560.1) (Supplementary Fig. 1). To study the regulation of *afsR-L* gene in *S. pogona*, it was overexpressed under the strong promoter of P_{ermE}. The effects of overexpression on the strain growth and secondary metabolite biosynthesis were also investigated.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains, plasmids, and primers used in this study are presented in Supplementary Table 1. The spores of *S. pogona* NRRL 30141 were inoculated in activation broth (glucose, 10 g/L; tryptic soy broth, 45 g/L; yeast extract, 9 g/L; $MgSO_4{\cdot}7H_2O$, 2.2 g/L) and cultivated at 30 °C with 300 rpm for 48 h, then 3 mL bacteria suspension was transferred to 30 mL fermentation broth (KNO_3,1 g/L; $FeSO_4$, 10 mg/L; $K_2HPO_4{\cdot}3H_2O$, 500 mg/L; $MgSO_4{\cdot}7H_2O$, 500 mg/L; glucose, 20 g/L; yeast extract, 4 g/L; tryptone, 4 g/L; pH, 7.2) and incubated at 30 °C with 300 rpm. The medium for the *S. pogona*-AfsR-L was the same as the wild strain and contained antibiotic of apramycin at 50 μg/mL). All *E. coli* strains were grown in LB broth at 37 °C. When necessary, the growth medium was supplemented with antibiotic (apramycin, 50 μg/mL).

Determination of *afsR* gene homologous sequences

The whole genome sequencing of *S. pogona* NRRL 30141 was completed by the third-generation genome sequencing technique (GenBank accession no. CP031142). AfsR-L protein was found from the sequencing results, and then the sequence homology analysis was performed for this protein by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). It was found that its homology with AfsR in *Streptomyces sp.* NRRL WC-3742 was 57%, and named as AfsR-L.

Construction of *afsR-L* overexpression recombinant strains

P_{ermE} is a strong promoter commonly used to initiate gene expression in the *Streptomyces* (Luo, et al. 2015). The strong constitutive promoter P_{ermE} was amplified from plasmid pOJ260-*cm*-P_{ermE} (Li, et al. 2018) using primers P_{ermE}-F and P_{ermE}-R (Supplementary Table 1), and the *afsR-L* gene was amplified using primers *afsR-L*-F and *afsR-L*-R from genomic DNA of *S. pogona* NRRL 30141 as template. Then, the two PCR fragments were fused by overlap extension PCR using primers P_{ermE}-F and *afsR-L*-R. The fusion fragment was excised using *Xba*I and *Eco*RV, and cloned into the

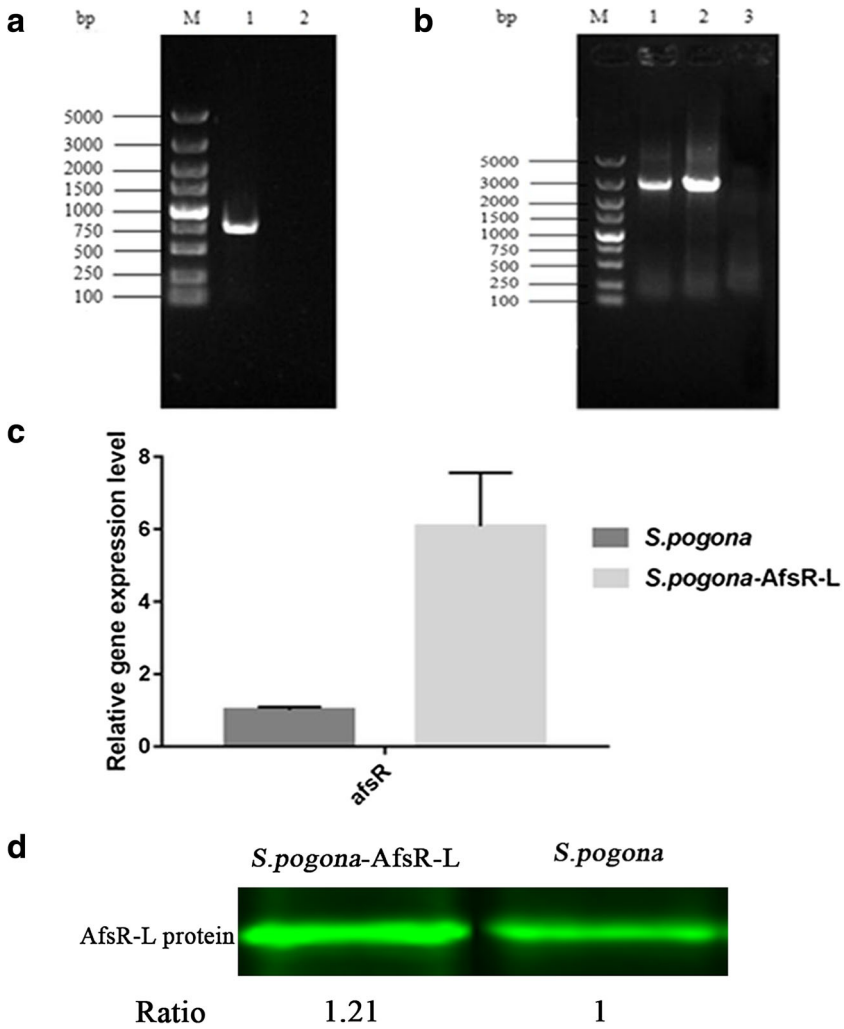

Fig. 1 Identification of recombinant strain *S. pogona*-AfsR-L. **a** PCR amplification of aac(3) IV gene (M, DNA marker; 1, PCR products of *S. pogona*-AfsR-L; 2, negative control *S. pogona* genome as template). **b** PCR detection of the fusion fragment P_{ermE}-*afsR-L* (M, DNA Marker; 1, positive control using pOJ260-P_{ermE}-*afsR-L* as template; 2, PCR products of *S. pogona*-AfsR-L; 3, negative control using *S. pogona* genome as template). **c** qRT-PCR results of *afsR-L* transcriptional level differences between the wild type and the engineering strain. **d** Western blot results of AfsR-L expression level differences between the wild type and the engineering strain

corresponding restriction sites of pOJ260 plasmid, producing the recombinant pOJ260-P_{ermE}-*afsR-L* plasmid (Supplementary Fig. 2). This recombinant plasmid was transferred into *S. pogona* by standard conjugation methods (Tang, et al. 2011) and integrated into the genome by single-crossover homologous recombination with *afsR-L* gene as homology region, yielding recombinant strain *S. pogona*-AfsR-L (Supplementary Fig. 3).

To confirm the recombinant strain, *S. pogona*-AfsR-L was constructed successfully, chromosomal DNA from the wild type and recombinant strains was isolated, the inserted gene was analyzed by PCR amplification, and its expression was further analyzed by qRT-PCR and Western blot.

Cultivation profile analysis of the wild type and recombinant strains

To monitor the growth profiles, the growth curve of wild type and recombinant strains was determined through measuring optical density of fermentation broth (OD_{600}) every 12 h (30 °C, 300 rpm). They were performed in triplicate. After cultivation for 2 days, 40 μL broth of wild type and recombinant strains was spread on BHI, R6, and TSB solid medium and incubated at 30 °C. The same amount of cultured strains was inoculated into 20 mL TSB medium culture for 2 days. Then, Hitachi SU8000 cold field emission scanning electron microscope (SEM) was used to observe the cell morphology. After fermentation for 10 days, the quantification of butenyl-spinosyn yield by UV-HPLC was performed as previously described with slight modification (Yang, et al. 2014). Butenyl-spinosyn was extracted by incubating the cell-free supernatant with acetone at a 1:1 volume ratio for 48 h. Cultures were centrifuged at 9000 rpm for 15 min, and the supernatants were filtered through 0.22-μm Millipore filters. After filtration, a 10 μL aliquot of each supernatant was loaded onto a C18 column (AQ12S05-1546WT) and eluted with the elution buffer at 1.0 mL/min. The elution buffer contained methanol, acetonitrile (ACN), and 2% aqueous ammonium acetate in a specific volumetric ratio (1:1). The detection wavelength was set at 250 nm during the analysis.

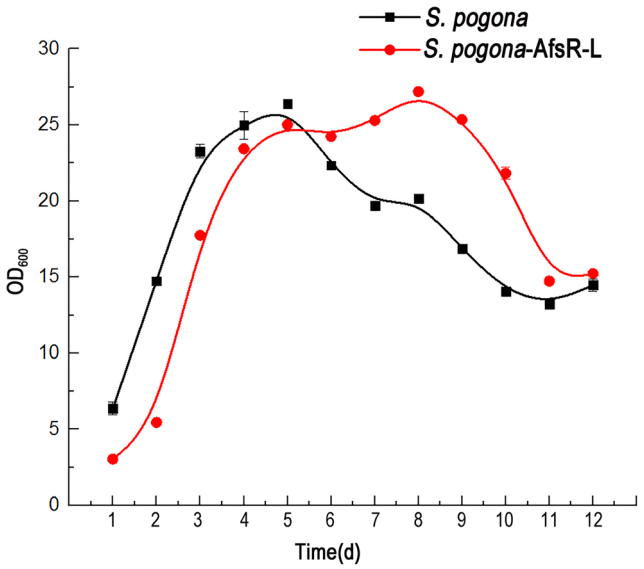

Fig. 2 Growth curve of *S. pogona*-AfsR-L and *S. pogona.* At the initial time, growth of *S. pogona* and *S. pogona*-AfsR-L was basically synchronized and the two strains entered stationary phase at 4 days. But as the time went by, the stationary phase of the wild-type strain *S. pogona* was short and the decline phase commenced at 5.5 days, whereas the engineered strain *S. pogona*-AfsR-L entered decline phase at 8 days. The highest biomass for *S. pogona*-AfsR-L was also higher than that for the wild-type strain *S. Pogona*

Protein extraction and SDS-PAGE analysis

To analyze the difference of the whole-cell protein between the wild type and recombinant strains, cells were harvested after 5 days and washed for four times with PBS (10 mM, pH 7.8, prechilled at 4 °C), resuspended in 200 μL of lysozyme (100 mg/mL) for 1 h, added with 600 μL lysis buffer (8 M urea; 2 M thiourea; 4% CHAPS; 75 mM NaCl; 50 mM Tris-HCl, pH: 8; 2 mM phenylmethylsul-fonyl fluoride; 4 μL of a protease inhibitor cocktail powder: P8465, Sigma, St. Louis, MO, USA) in each tube, and ultrasonically fragmented (JY92-II ultrasonic cell grinder, Ningbo Scientz biotechnology CO., LTD). After the quantitative analysis of the protein by Bradford assay, the components of protein samples were checked by SDS-PAGE.

Total RNA isolation and qRT-PCR analysis

To perform transcriptional analysis, total RNA from the wild type and recombinant strain was separately isolated following instructions of Total RNA Extractor Kit (Sangon Biotech) with slight modifications, and each strain was performed in triplicate. Briefly, bacteria, after incubated 5 days, were harvested from 1 mL suspension at 8000 rpm for 2 min, and immediately frozen in liquid nitrogen. Next, it was ground in liquid nitrogen and homogenized in 1 mL Trizol. The solution was mixed with 0.25 mL chloroform followed by an ice bath for 5 min. After centrifugation at 12000 rpm, 4 °C for 10 min, the aqueous phase was transferred to a new 1.5-mL tube and added equal volume of isopropanol with subsequent ice bath for 1 h. The mixture was centrifuged, and then the precipitation was washed twice with 75% ethanol and dissolved in RNase free water. RNA concentration and purity were established by measuring the ratio of OD_{260} nm to OD_{280} nm. DNase treatment and cDNA synthesis were performed by PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara) according to the manufacturer's instructions. The real-time qPCR amplification was performed on 7500 Real-Time PCR system instruments (Applied Biosystems, USA). The 16S rRNA gene was used as an internal control to quantify the relative expression of target genes.

Heterologous expression and Western blot analysis of AfsR protein

The *afsR* gene was amplified using primers *afsR-L* –F (WB) and *afsR-L* –R (WB) from genomic DNA of *S. pogona* (Supplementary Table 1). The amplified *afsR* gene was cloned into pET-28a using an inNovafast Assembly Cloning Kit (Innovagene), and then transferred to *E. coli* BL21 (DE3). The recombinant strain was inoculated into LB medium supplemented with 40 μg/mL kanamycin. After induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the cells were harvested by centrifugation and resuspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM $KHPO_4$). The cells were disrupted by sonication. Subsequently, the inclusion body was collected through centrifugation and the AfsR protein was purified by a Ni-NTA column. Anti-AfsR-L antibody was obtained through immunizing rabbit (Supplementary Table 2) as describe previously (Mikael, et al. 2017).

Twelve microgram of protein samples derived from *S. pogona* and *S. pogona*-AfsR-L were separated by SDS-PAGE gel and transferred to PVDF membrane. The membrane was incubated with primary antibody and then the secondary antibody (IRDye 680 Conjugated Goat Anti-Rabbit IgG, 1:10000). Odyssey infrared fluorescence detection system (Li-COR Biosciences, Lincoln, NE) was used to detect the results (Yang, et al. 2014; Stefanie Muller, et al. 2007).

Nano-LC-MS/MS analysis

Differential protein bands between the wild type and recombinant strains were cut out of the SDS-PAGE gel for in-gel tryptic digestion and proceed with liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Luo, et al. 2011, Yang, et al. 2014). 1D-LC-MS/MS analysis was performed as described previously (Huang, et al. 2012) by using LTQ XL mass spectrometer (Thermo Fisher, San Jose, CA, USA).

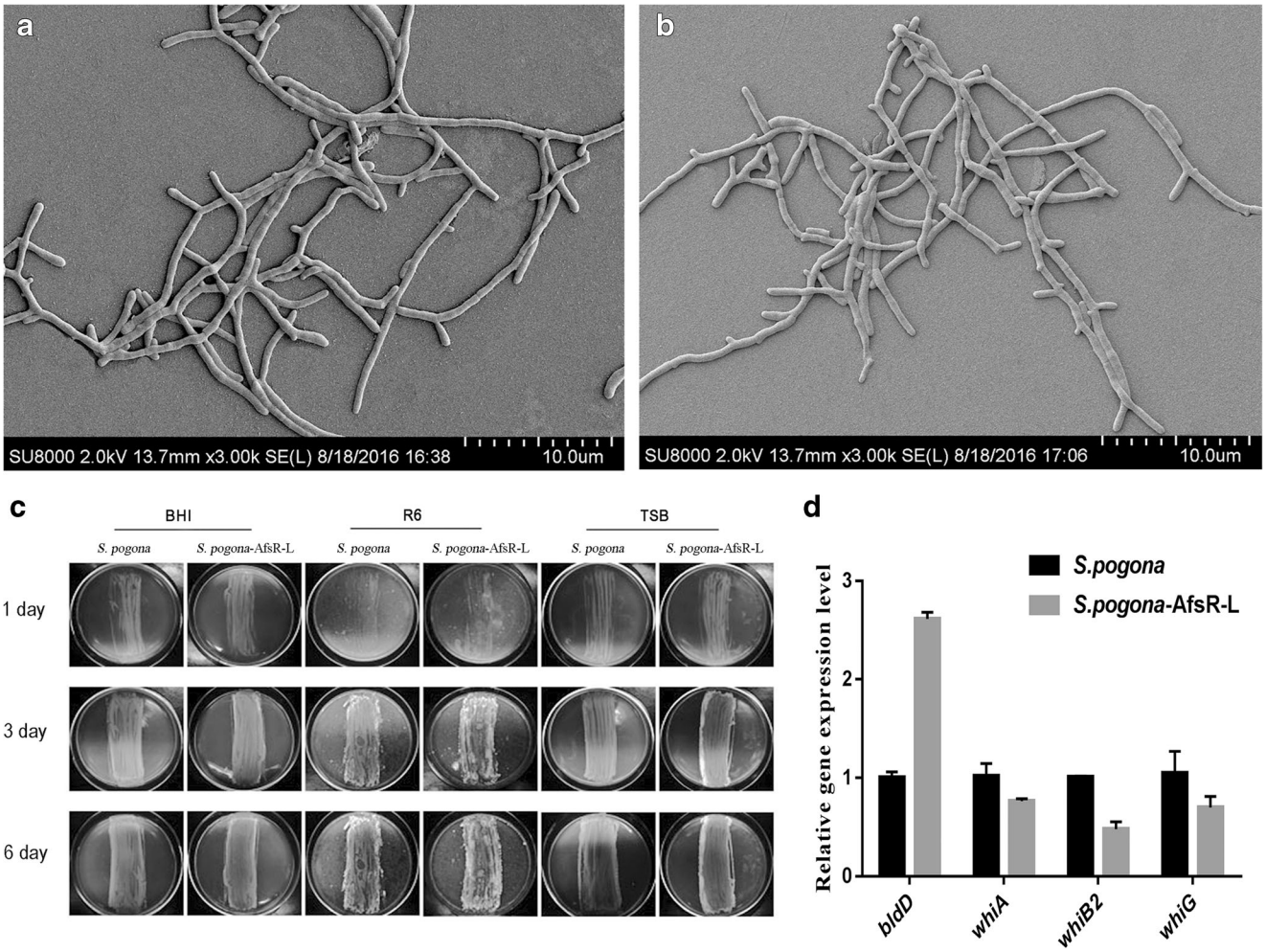

Fig. 3 The morphological comparison of wild type and the engineered strains. **a** Scanning electron microscopic observation of *S. pogona.* **b** Scanning electron microscopic observation of *S. pogona*-AfsR-L. The mycelia of *S. pogona*-AfsR-L grew faster than those of *S. pogona.* Its hyphae were more branched and developed than those of *S. pogona.* Bars, 10 μm. **c** The comparison of spore formation on different solid media. The two strains were grown on the three solid media plate and observed at days 1, 3, and 6 after inoculation. Spores were produced after 3 days on the three solid media. The sporulation ability of the two strains on BHI and TSB solid media was lower than on R6 solid medium. **d** Expression level of *whiA*, *whiB2*, *whiG*, and *bldD.* The mRNA samples were isolated after 3 days from the wild type and engineered strains. Expression of *bldD* exhibited a significant upregulation in the mutant, whereas *whiA*,*whiB2*, and *whiG* were downregulated, the 16S rRNA gene was used as an internal control to quantify the relative expression of target genes. Black bars represent *S. pogona*, and gray bars represent *S. pogona*-AfsR-L. Gene expression differences were shown by bars height. Error bars represent standard deviation of the mean

Results

Effects of *afsR-L* overexpression on the growth of *S. pogona*

S. pogona-AfsR-L strain was successfully obtained according to the proof of gene level, transcriptional level and protein expression level (Fig. 1). To find the difference between *S. pogona* and *S. pogona*-AfsR-L in fermentation broth, the growth characteristics were measured and controlled in real time (Fig. 2). The result showed that initial growths of *S. pogona* and *S. pogona*-AfsR-L were basically synchronized. The two strains entered stationary phase at 4 days (Fig. 2). However, the stationary phase of the wild-type strain *S. pogona* was short and the decline phase commenced at 5.5 days, whereas the engineered strain *S. pogona*-AfsR-L started decline phase at 8 days. In addition, the resultant biomass for *S. pogona*-AfsR-L was also higher than that for the wild-type strain *S. pogona.*

Effects of *afsR-L* overexpression on the morphology of *S. pogona*

The SEM observation of the two strains revealed that the mycelia of *S. pogona*-AfsR-L grew faster than those of *S. pogona*. Moreover, its hyphae were more branched and developed (Fig. 3a, b). To research the effect of *afsR-L* overexpression on sporulation in different solid culture

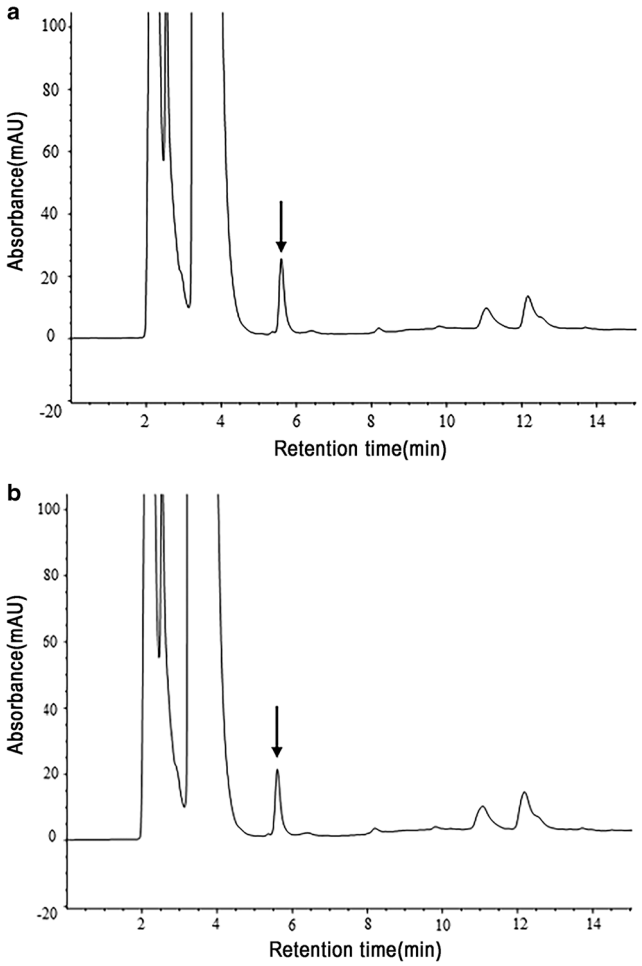

Fig. 4 The butenyl-spinosyn production analysis. **a** Butenyl-spinosyn production of *S. pogona*-AfsR-L. **b** Butenyl-spinosyn production of *S. pogona*. The peak area of the butenyl-spinosyn yield of *S. pogona*-AfsR-L and *S. pogona* was 293.6 and 250.9, respectively

medium, strains of *S. pogona*-AfsR-L and *S. pogona* were observed on BHI, R6, and TSB solid medium. *S. pogona*-AfsR-L obviously had a white phenotype on the three media compared with *S. pogona*, especially on the R6 solid medium (Fig. 3c). To identify the different phenotypes, several *whi-like* (*whi-L*) genes and *bldD* gene were selected through homology analysis to check the transcriptional level, which has a significant influence on strain phenotype and sporulation by *whi-L* family genes negative regulation and *bldD* gene positive regulation (Bush, et al. 2016; Elliot, et al. 2003; Kim, et al. 2006; Virginie Molle 2000; Yoshihiro Mouri 2017). Expression of *whiA*, *whiB2*, and *whiG* was lower in the mutant, especially *whiB2* that showed 52.2% downregulation. However, *bldD* gene expression level was increased by 160.5% in the mutant (Fig. 3d), which acts to repress expression of the *whi* genes (Bush, et al. 2016; Den Hengst, et al. 2010). These results provide an explanation for the special phenotype of the mutant.

Effects of *afsR-L* overexpression on butenyl-spinosyn biosynthesis

Butenyl-spinosyn was mainly found in the cell-free supernatant (Supplementary Fig. 4), and the extracted butenyl-spinosyn from the fermentation broth was subsequently examined by HPLC. The target peaks were harvested for MS identification; the results show that the (M+H)+ions at m/z = 634 contained 617[M+H]+(m/z) and a rhamnose ion fragment with a molecular mass of 189 (Supplementary Fig. 5). This finding proved that the substance was spinosyn αd, a butenyl-spinosyn component, which is consistent with previous data (Lewer, et al. 2009). The HPLC results showed that butenyl-spinosyn yield of *S. pogona*-AfsR-L was 293.6 and that of *S. pogona* was 250.9. Therefore, the total concentration of butenyl-spinosys produced by *S. pogona*-AfsR-L increased to 117% compared with that of *S. pogona* (Fig. 4), which implies *afsR-L* gene overexpression improves the butenyl-spinosyn yield of the engineered strain *S. pogona*-AfsR-L.

Analysis of whole-cell protein

After culture of 120 h, SDS-PAGE analysis showed that the two strains had significantly different whole protein profiles. Distinctive bands were identified by 1D-LC-MS/MS (Fig. 5a) and their expression level was further confirmed by qRT-PCR analysis (Fig. 5b). The results revealed that the protein band A was identified as polynucleotide phosphorylase and exhibited 1000.3% increase in *S. pogona*-AfsR-L. The protein bands B and C decreased by 32.2% and 84.7% in *S. pogona*-AfsR-L and were identified as the molecular chaperone GroEL (*groEL*) and senoyl-CoA hydratase (*ech*), respectively. The identified proteins were then categorized using UniProt (www.uniprot.org) and Gene Ontology (GO) for functional analysis. Polynucleotide phosphorylase (*pnp*) is involved in mRNA degradation by function analysis, molecular chaperone GroEL prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides that are generated under stress conditions, and senoyl-CoA hydratase is related with the pathway of fatty acid beta-oxidation, which is part of lipid metabolism (Table 1).

Discussion

The isolated secondary metabolites of *Streptomyces* have important application as antibiotics, enzymes, and immunosuppressive activity. The structure and function of secondary metabolites have been continuously selected and optimized over a long-term natural evolution, resulting in a unique chemical structure with superior activity. These secondary metabolites are an important biological resource

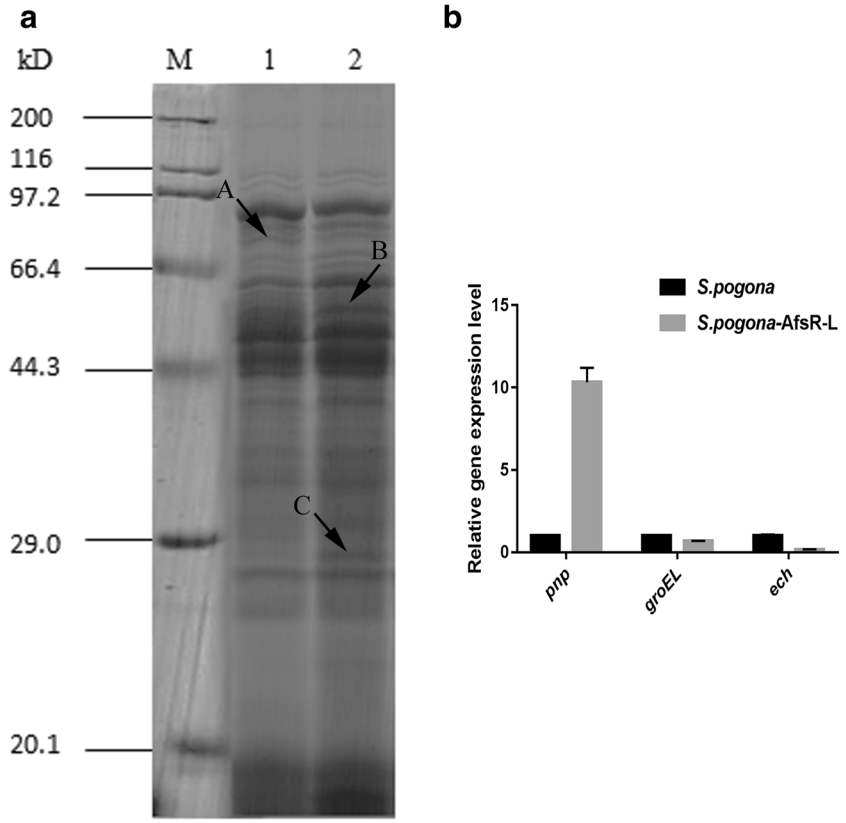

Fig. 5 Distinctive protein analysis. **a** SDS-PAGE gel analysis of total proteins. M, protein marker; 1, total proteins from *S. pogona*-AfsR-L at 5 days; 2, total proteins from *S. pogona* at 5 days. Three distinctive bands were observed between the two strains, which were identified via 1D-LC-MS/MS. **b** qRT-PCR results of *pnp*, *groEL*, and *ech* transcriptional level. The mRNA samples were isolated from *S. pogona*-AfsR-L and *S. pogona* at 120 h. The 16S rRNA gene was used as an internal control to quantify the relative expression of target genes. *pnp* expression level showed an increase, while *groEL* and *ech* expression levels were decreased in the mutant. Black bars represent *S. pogona*, and gray bars represent *S. pogona*-AfsR-L. Gene expression differences were shown by bars height. Error bars represent standard deviation of the mean

for the drug research and development. The development of gene sequencing and DNA recombination technologies has enabled the elucidation of the biosynthetic pathway and regulatory mechanism of various microbial natural products. Furthermore, these technologies provide an effective method for genetic modification of the *Streptomyces* genome. The yield of secondary metabolites can be increased based on the specificity of a regulatory pathway, overexpression of positive regulatory factors, knockout of negative regulatory factors, and doubling of secondary metabolic biosynthetic gene clusters (Li, et al. 2017; Olano, et al. 2008).

Butenyl-spinosyn is a spinosad analogue that is produced by *S. pogona*. However, the ability of the wild-type *S. pogona* to produce butenyl-spinosyn is low and insufficient for industrial production (Lewer, et al. 2009; Li, et al. 2018). Tang et al. directly cloned ~ 1.8 kb fragment containing *spnS*, *spnF*, and *spnQ* from spinosad biosynthetic gene clusters and then integrated these

Table 1 Proteins identified from SDS-PAGE gel analysis

Bands number	Protein description	Gene	MW (KDa)	Possible function
A	Polynucleotide phosphorylase	*pnp*	79.3	Involved in mRNA degradation. Catalyzes the phosphorolysis of single-stranded polyribo-nucleotides processively in the 3′- to 5′-direction.
B	Molecular chaperone GroEL	*groEL*	57.2	Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions.
C	Enoyl-CoA hydratase	*ech*	27.5	This protein is involved in the pathway fatty acid β-oxidation, which is part of lipid metabolism.

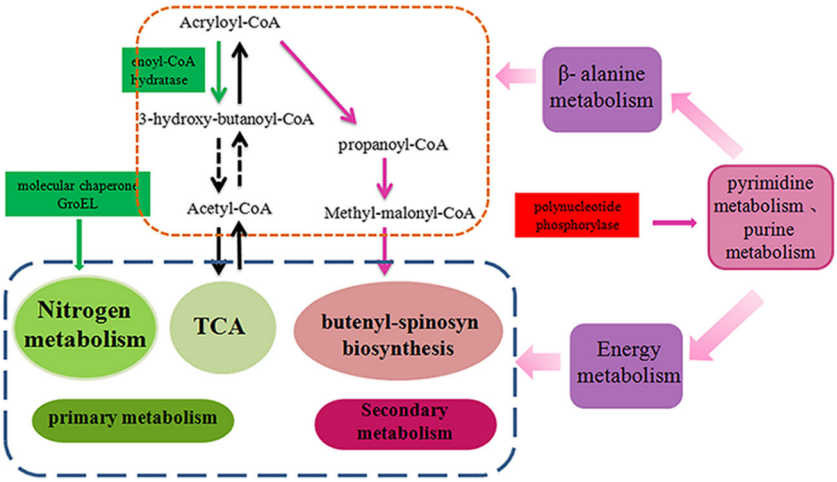

Fig. 6 Regulatory network schematic diagram of *afsR-L* gene in *S. pogona*. Green rectangle, downregulated protein; red rectangle, upregulated protein; green arrow, downregulatory pathway; mulberry arrow, upregulatory pathway; the downregulated metabolism described in green; the upregulated metabolism described in red or purple. The upregulated *pnp* gene could directly regulate pyrimidine metabolism and purine metabolism, which strengthened the energy metabolism and β-alanine metabolism, giving more energy to primary and secondary metabolism and also providing more precursor material to the CoA metabolism pathway. The downregulated *groEL* gene could reduce the nitrogen metabolism. The downregulated *ech* gene could generate more methyl-malonyl-CoA, as a key precursor for secondary metabolites synthesis, and reduced the TCA cycle at the same time

genes into the wild-type strain *S. spinosa* chromosome via single-crossover homologous recombination, and the spinosad yield of engineered strain increased by more than 3.0-fold (Tang, et al. 2011). By knocking out the *metE* gene in the chromosome of the wild-type strain, Yang et al. found that the *metE* gene was the key regulator of secondary metabolite production in *S. spinosa* (Yang, et al. 2015). In this study, the *S. pogona* genome was genetically modified. First, the *afsR-L* gene was identified as homologous to the global regulatory factor *afsR* in *Streptomyces*. The gene was placed under the control of the strong constitutive promoter P_{ermE}, to overexpress the *afsR-L* gene in *S. pogona*. The engineered strain *S. pogona*-AfsR-L was thus obtained.

The final results showed that the *afsR-L* gene overexpression affects the growth and development of *S. pogona*. Observation with cold field emission SEM showed that the growth and development of mycelia in *S. pogona*-AfsR-L were faster than that in the wild-type strain. The hyphae were highly branched and developed, thus increasing the uptake of dissolved oxygen level and promoting the growth of *S. pogona*. Hence, the stationary phase of *S. pogona*-AfsR-L was longer than that of *S. pogona* by approximately 3 days. Moreover, *S. pogona*-AfsR-L exhibited a white phenotype on the solid medium. Therefore, *afsR-L* overexpression affects the sporulation ability of *S. pogona*. HPLC analysis revealed that the butenyl-spinosyn yield increased to 117.0% compared with that of *S. pogona*. AfsR-L regulates the secondary metabolites biosynthesis in other *Streptomyces* strains by affecting the growth of the strains, which the secondary metabolites are usually carried out during stationary phase (Parajuli, et al. 2005; Wang, et al. 2015; Maharjan, et al. 2008). We speculated that AfsR-L as an important regulatory factor might promote the increase of butenyl-spinosyn yield by prolonging the stationary phase. This finding showed that AfsR-L affects the biosynthesis of secondary metabolites in *S.pogona*.

To explore the possible control mechanism of AfsR-L on butenyl-spinosyn production, the differentially expressed proteins of *S. pogona*-AfsR-L and *S. pogona* were analyzed and we found that PNPase was upregulated due to the overexpression of AfsR-L. It was reported that this protein regulates the amino acid metabolism, organic acid metabolism, cell biosynthesis, and transcription-related enzymes in *E. coli* (Nurmohamed et al. 2011). Our previous work also found that PNPase affected the growth and the biosynthesis of butenyl-spinosyns in *S. pogona*, and proved that the PNPase is a key regulatory factor in the metabolism of *S. pogona* (Li et al. 2018). The analysis of KEGG software reveals that the PNPase directly regulates the pyrimidine and purine metabolism, which produces UDP, CDP, ADP, and GDP that are then catalyzed into UTP, CTP, ATP, and GTP by relevant enzymes, respectively. These compounds participated as energy sources in the cell metabolism, and the UTP and CTP catabolism is β-alanine, which is further metabolized to generate pyruvate and promote acetyl-COA synthesis. Acetyl-COA is a substrate of butenyl-spinosyn biosynthesis, thus increasing butenyl-spinosyn production. Enoyl-CoA hydrolase catalyzes the formation of 3-hydroxy-butyryl-CoA via acryloyl-CoA and then further generates acetyl-CoA as a raw material for the basic carbon metabolism in the tricarboxylic acid cycle. GroEL affects the assembly and translation of the ribosomal protein factor in the nitrogen metabolism pathway. The biomass of *S. pogona*-AfsR-L is lower than that of the wild-type strain at the beginning of 5 days of cultivation (Fig. 2). This finding is due to the downregulation of *groEL* and *ech* in *S. pogona*-AfsR-L, which slow down the primary metabolism pathways of *S. pogona*-AfsR-L, such as TCA

and nitrogen metabolism. The downregulation of *ech* in *S. pogona*-AfsR-L decreases the conversion of acryloyl-CoA to acetyl-CoA, enhancing the synthesis of propanoyl-CoA and methyl-malonyl-CoA. In *Saccharopolyspora erythaea*, methyl-malonyl-CoA is a key precursor substance in the erythromycin biosynthetic pathway (Karnicar, et al. 2016). When cultured in a minimal medium, methyl-malonyl-CoA is transformed into succinyl-CoA to participate in the tricarboxylic acid cycle, which decreases the need for the biosynthesis of erythromycin as a precursor (Reeves, et al. 2006). The downregulation of acryloyl-CoA hydrolase in *S. pogona*-AfsR-L probably increases the conversion of acryloyl-CoA to methyl-malonyl-CoA, providing more precursors for butenyl-spinosyn biosynthesis and promoting the biosynthesis of these secondary metabolites (Fig. 6).

In summary, we first identified the presence of AfsR homolog named as AfsR-L in *S. pogona*. AfsR-L might function as an important regulatory factor which could render more initial raw materials into the secondary metabolism by decreasing the tricarboxylic acid cycle, nitrogen metabolism, and other primary metabolism, and thus promote the butenyl-spinosyn biosynthesis. At the same time, AfsR-L might indirectly regulate energy and amino acid metabolism, providing more energy and metabolic substances for the growth of *S. pogona*. Our study provided an important target for genetic engineering to improve butenyl-spinosyn biosynthesis in *S. pogona*.

Author contributions L. L. and S. Y. developed the bacterial recombinant and performed bacterial strain isolation. Y. S., L. G., and J. T. performed HPLC. J. R., X. D., and Z. L. performed LC-MS/MS and data analysis. L. G., H. H., Z. Y., and S. P. performed Total RNA isolation and qRT-PCR analysis. L. G. and H. H. performed Western blot. L. L., H. H., Y. S., and L. X. designed the study and wrote the draft of manuscript. L. G. and Y. S. modified and corrected this manuscript. All authors discussed the results and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals Ethical approval "All applicable international, national, and/or institutional guidelines for the care and use of animals were followed." All procedures performed in studies involving animals were in accordance with the ethical standards of the Animal Care Committee of Hunan Normal University at which the studies were conducted.

Informed consent Informed consent was obtained from all individual participants included in the study.

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