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Improvement of Rimocidin Biosynthesis by Increasing Supply of Precursor Malonyl-CoA via Over-expression of Acetyl-CoA Carboxylase in *Streptomyces rimosus* M527

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Abstract

Precursor engineering is an effective strategy for the overproduction of secondary metabolites. The polyene macrolide rimocidin, which is produced by *Streptomyces rimosus* M527, exhibits a potent activity against a broad range of phytopathogenic fungi. It has been predicted that malonyl-CoA is used as extender units for rimocidin biosynthesis. Based on a systematic analysis of three sets of time-series transcriptome microarray data of *S. rimosus* M527 fermented in different conditions, the differentially expressed acc_{sr} gene that encodes acetyl-CoA carboxylase (ACC) was found. To understand how the formation of rimocidin is being influenced by the expression of the acc_{sr} gene and by the concentration of malonyl-CoA, the acc_{sr} gene was cloned and over-expressed in the wild-type strain *S. rimosus* M527 in this study. The recombinant strain *S. rimosus* M527-ACC harboring the over-expressed acc_{sr} gene exhibited better performances based on the enzymatic activity of ACC, intracellular malonyl-CoA concentrations, and rimocidin production compared to *S. rimosus* M527 throughout the fermentation process. The enzymatic activity of ACC and intracellular concentration of malonyl-CoA of *S. rimosus* M527-ACC were 1.0- and 1.5-fold higher than those of *S. rimosus* M527, respectively. Finally, the yield of rimocidin produced by *S. rimosus* M527-ACC reached 320.7 mg/L, which was 34.0% higher than that of *S. rimosus* M527. These results confirmed that malonyl-CoA is an important precursor for rimocidin biosynthesis and suggested that an adequate supply of malonyl-CoA caused by acc_{sr} gene over-expression led to the improvement in rimocidin production.

Introduction

Streptomyces are soil-dwelling, gram-positive bacteria that produce several kinds of secondary metabolites [1–3]. Among them, polyketides are an outstanding group of secondary metabolites because of their structural diversity and the range of their applications, such as antibiotics (e.g., tetracycline, erythromycin), antifungals (e.g., amphotericin B), antivirals (e.g., balticolid), anticancer agents (e.g., bleomycin, doxorubicin), immunosuppressants (e.g., rapamycin, FK506), and antiparasitics (e.g., avermectins, milbemycins) [4]. However, their yields are too low to meet the requirements of industrial fermentation. Therefore, the genetic modification of producing strains is normally required to improve the production of these compounds [5–7].

Glycolysis, tricarboxylic acid cycle, and pentose phosphate pathway are the primary metabolic pathways involved in the production of precursors that are utilized for the biosynthesis of secondary metabolites [8, 9]. The precursors of polyketides come directly or indirectly from primary metabolism, so polyketide production is often limited by the availability of these essential precursors [10]. Therefore, precursor engineering has been applied to redesign and/or optimize the metabolic pathways to increase the intracellular pool of precursors and to redirect the flux toward the targeted polyketide biosynthesis [11–13]. Malonyl-CoA is usually involved in numerous essential primary metabolic pathways, as well as a starter and extender units for polyketide biosynthesis [14]. Malonyl-CoA is mainly synthesized

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through the carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC) [15]. It was reported that the introduction and heterologous expression/over-expression of genes encoding ACC are practicable approaches for the improvement of polyketides [16]. For example, increasing the copy number of the *acc* gene encoding ACC remarkably improved the FK520 titers in *Streptomyces hygroscopicus* [17]. Similarly, Ryu et al. [18] over-expressed the *acc* gene, which led to an increase in precursor supply for actinorhodin production in *S. coelicolor*.

Rimocidin is a 28-membered tetraene macrolide comprising a large lactone ring with a sugar moiety [19], and it is produced by type I modular polyketide synthases (PKS) in *S. rimosus* M527 [20]. Rimocidin is a promising fungicide utilized in controlling the occurrence of plant fungal diseases in agricultural field [21]. Constant efforts were always made to enhance the yield of rimocidin in *S. rimosus* M527 to meet the commercial requirements. Various strategies, such as medium optimization [22], addition of elicitors [23], genetic engineering [24], and ribosomal engineering [25], have been adopted to achieve this goal.

A rimocidin synthetic pathway was predicted [20]. Butyryl-CoA was used as a starter unit by the loading module; macrolactone rimocidin was formed through elongation steps in which acetate was mainly incorporated by the decarboxylative condensation of malonyl-CoA [20]. However, it is still unclear whether malonyl-CoA is a real and important precursor involved in rimocidin biosynthesis in *S. rimosus* M527. In a previous study, based on the analysis of comparative transcriptome data for rimocidin production under normal and optimized conditions, a differential expression gene that encodes ACC was found in *S. rimosus* M527.

Therefore, the aim of this study was to investigate the relationships between the differential expression gene, malonyl-CoA, and rimocidin in *S. rimosus* M527.

Materials and Methods

Materials

Q5 High-Fidelity Master Mix with GC-buffer was purchased from NEB. PCR reagents, restriction endonucleases, Miniprep kits, and gel extraction kits were purchased from TaKaRa Biotechnology Co., Ltd. Malonyl-CoA ELISA kit and ACC assay kit were purchased from Shanghai Jianglai Biotechnology Co., Ltd. Oligonucleotide primer synthesis and DNA sequencing of PCR products were performed by Beijing TSINGKE Biotechnology Co., Ltd., China.

Bacterial Strains and Plasmids

The strains and plasmids used in this study are listed in Table S1. *Streptomyces rimosus* M527, a rimocidin producer, was used as a host for gene expression and rimocidin production; it has been deposited at the CCTCC (M2013270), Wuhan, China. *Escherichia coli* JM109 was used as a general host for gene cloning and plasmid construction. The methylation-deficient strain *E. coli* ET12567/pUZ8002 was used as a donor for intergeneric conjugation. Plasmid pIB139 harboring a *perm*E* promoter is a shuttle vector that replicates in *E. coli* and integrates into *Streptomyces* chromosomes site specifically.

Media and Culture Conditions

E. coli strains were cultured in liquid or solid Luria–Bertani (LB) medium, containing appropriate antibiotics at 37 °C. Apramycin (50 μg/mL), chloramphenicol (25 μg/mL), ampicillin (100 μg/mL), kanamycin (50 μg/mL), and nalidixic acid (50 μg/mL) were added as needed.

S. rimosus M527 and its derivatives were incubated according to the method described by Zhao et al. [25]. *S. rimosus* M527 was incubated at 28 °C and grown in a solid mannitol soya flour (MS) medium (20 g/L soya flour, 20 g/L mannitol, 20 g/L agar, and tap water) for sporulation. Moreover, 2CMC solid media containing 10 g/L starch, 2 g/L tryptone, 1 g/L NaCl, 2 g/L $(NH_4)_2SO_4$, 2 g/L $MgSO_4{\cdot}7H_2O$, 2 g/L $CaCO_3$, 2 g/L casamino acids, 1 g/L $K_2HPO_4{\cdot}3H_2O$, 1 g/L FeSO4·$7H_2O$, 1 g/L $MgCl_2{\cdot}6H_2O$, 1 g/L $ZnSO_4{\cdot}7H_2O$, and 20 g/L agar (adjusted to pH 7.2 by NaOH) were used for conjugation [22].

S. rimosus M527 spores (1×10^6/mL) were inoculated into a 250-mL Erlenmeyer flask containing 50 mL of seed medium and was shaken at 28 °C and 180 r/min. The CP liquid medium used as the seed medium had the same composition as that described in an earlier study [26].

DNA Manipulations

The general procedures for DNA manipulation were conducted according to the method described by Sambrook and Russel [27]. The intergeneric conjugation of *Streptomyces* and *E. coli* was performed as described by Kieser et al. [28] with minor modifications. *S. rimosus* M527 spores were collected from solid medium, filtrated by sterile absorbent cotton in order to remove mycelia and then incubated at 50 °C for 10 min to induce germination. The donor strain, *E. coli* ET12567/pUZ8002 harboring constructed plasmid, was grown in LB with the appropriate antibiotics until an OD_{600} of 0.4–0.6. The cells were washed twice with LB and

resuspended in a final volume of 500 µL of LB. *E. coli* (10^8/mL) as donor cells and the *S. rimosus* M527 spores (10^8/mL) as recipients were mixed and spread on 2CMC agar plate containing 10 mmol/L $MgCl_2$. The plates were incubated at 28 °C for 10–20 h and overlaid with 500 µL fresh LB medium containing 100 µg/mL nalidixic acid and 300 µg/mL apramycin. The plates were further incubated at 28 °C for 3–4 days, and the exconjugants were counted [22].

Clone of acc_{sr} Gene and Construction of Recombinant Strains

Using the genomic DNA of *S. rimosus* M527 as a template, a 1524 bp acc_{sr} gene open reading frame (ORF) was amplified by PCR using primers P1 and P2 (Table S1). High-fidelity PCR was performed using Q5 DNA polymerase (NEB) to obtain DNA fragments to be used for plasmid construction. PCR amplification was started at 95 °C for 5 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72 °C for 45 s. After 30 cycles, a 5-min extension at 72 °C was done. Then, the PCR product was digested with *Nde*I and *Not*I and inserted into the corresponding site of plasmid pIB139, yielding a plasmid pIB139-acc_{sr}. Sequencing results confirmed that the gene did not contain any mutation.

Subsequently, the introduction of the constructed pIB139-acc_{sr} into *S. rimosus* M527 was performed by intergeneric conjugation [22] to the recombinant strain *S. rimosus* M527-ACC. Recombinant strains were confirmed using apramycin resistance and PCR.

Determination of Intracellular Malonyl-CoA and Enzyme Assay

Fermentation culture broth (1 mL) of *S. rimosus* M527 and *S. rimosus* M527-ACC was collected at 12 h, 24 h, 36 h, 48 h, 72 h, and 96 h. Each sample was centrifuged at 11,000×*g* for 5 min to obtain mycelium; then, each was washed twice with PBS buffer. For extraction and quantification of intracellular malonyl-CoA, the instructions described in the ELISA kits were followed [29]. The standard sample was diluted to different concentrations with assay buffer to prepare a standard curve. The samples for testing were diluted 1:5 by adding 10 µL of sample into 40 µL of Sample Diluent. The solutions were incubated at 37 °C for 60 min and the TMB chromogenic substrate was added. We measured the light absorption value at 450 nm and then measured intracellular concentration of malonyl-CoA in each sample according to the standard curve.

ACC activity was assayed using the ACC assay kit as described by the kit instructions. ACC activity was measured by determining the increased inorganic phosphorus content using the ammonium molybdate phosphate method; one unit of ACC activity is defined as the amount of inorganic phosphorus per hour per mg of tissue protein produced by 1 mol of inorganic phosphorus [30].

Analysis of Gene Transcriptional Level by qRT-PCR

We extracted RNA and analyzed the transcriptional level of acc_{sr} and some structural genes (*rimA*, *rimE*, *rimJ*, *rimK*) located in the rimocidin biosynthetic gene cluster between the recombinant strain *S. rimosus* M527-ACC and WT strain, as described by Zhao et al. [25]. The thermal profiles consisted of initial denaturation at 94 °C for 3 min, followed by previously indicated cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and a final step at 72 °C for 10 min. The 16S rRNA gene in *S. rimosus* M527 was used as a positive internal control for the quantitative RT-PCR (qRT-PCR) assay. The qRT-PCR experiments were carried out in triplicate using RNA samples from three independent experiments. The primers used to analyze transcriptional levels of acc_{sr} and some *rim* genes in WT strain M527 and recombinant strains *S. rimosus* M527-ACC are listed in Table S1.

Production and HPLC Analysis of Rimocidin

Rimocidin production and its analysis were performed according to the method described by Zhao et al. [25]. Rimocidin presence was analyzed and confirmed using high-performance liquid chromatography (HPLC) with a column of Supersil ODS2 (4.6×150 mm, 5 µm) maintained at 30 °C. The percentage volume of methanol was varied as follows: linearly increased from 5 to 83% (0–20 min), held at 83% (20–30 min), linearly increased to 100% (30–35 min), and then linearly decreased to 5% (35–40 min). The UV detection of rimocidin was conducted at 304 nm and the solvent flow rate was 1.0 mL/min.

Construction of the Phylogenetic Tree

Phylogenetic analysis of *acc* gene using NCBI BLAST. The nucleotide sequences of acc_{sr} from different prokaryotic species were collected from the NCBI database, and phylogenetic analysis of acc_{sr} was performed with MEGA 7.0, using the neighbor-joining method in the Jukes–Cantor model [31].

Statistical Analysis

All experiments were conducted at least three times, and the results are expressed as means ± standard deviations. Statistical analysis was performed using Student's *t* test.

Data Availability

The nucleotide sequence of acc_{sr} gene was submitted to the GenBank database under the accession number MT176432.

Results

Cloning of acc_{sr} from *S. rimosus* M527

It is predicted that malonyl-CoA is an important extender unit for rimocidin biosynthesis in *Streptomyces diastaticus* var. 108 [20, 32]. The *acc* gene encodes ACC which can catalyze the carboxylation of acetyl-coenzyme A (acetyl-CoA) to produce malonyl-CoA. In this context, two obvious questions are as follows: Which gene is responsible for malonyl-CoA formation in *S. rimosus* M527? Whether increase of malonyl-CoA is beneficial to overproduction of rimocidin in *S. rimosus* M527?

According to the analysis of comparative transcriptome data of *S. rimosus* M527 (GenBank accession No. GCA_004196335.1) in different conditions, a differential expression gene from *S. rimosus* M527 was cloned. The 1524 bp gene encodes a protein with 508 amino acids. As shown in Fig. 1, among these, it was identical to the *acc* from *S. rimosus* strain WT5260 and *S. rimosus* strain ATCC 10970. This gene was named acc_{sr}, and its nucleotide sequence was submitted to the GenBank database under the accession number MT176432.

Construction of *S. rimosus* M527-ACC

To verify the correlation among the differential expression gene acc_{sr}, malonyl-CoA synthesis, and rimocidin production, a DNA fragment containing the acc_{sr} gene was cloned as described in the Materials and methods section. The gene acc_{sr} was placed under the control of promoter $ermE^{*}$ in plasmid pIB139 to create pIB139-acc_{sr} (Fig. S1). Then, the plasmid pIB139-acc_{sr} was introduced into *S. rimosus* M527 by intergeneric conjugative transfer between *E. coli*/*Streptomyces* to generate the recombinant strain *S. rimosus* M527-ACC, which was resistant to 300 μg/mL apramycin (Fig. S2). The integration of plasmid pIB139-acc_{sr} into the chromosome of *S. rimosus* M527 was verified by PCR (Fig. S3).

Over-expression of acc_{sr} Gene Improved the Rimocidin Production in *S. rimosus* M527

To further investigate the effect of acc_{sr} gene on rimocidin production, a batch fermentation experiment in a 250-mL Erlenmeyer flask was performed using *S. rimosus* M527-ACC and the wild-type (WT) strain *S. rimosus* M527, as described in the Materials and methods section. As shown in Fig. 2a, a significant increase in the rimocidin production of *S. rimosus* M527-ACC was observed compared to that of *S. rimosus* M527 throughout the fermentation process. After 96 h, the amount of rimocidin produced by *S. rimosus* M527-ACC reached the highest level of 320.7 mg/L, which was 34.0% higher than the rimocidin produced by *S. rimosus* M527 (239.4 mg/L). There was no significant difference between *S. rimosus* M527-ACC and *S. rimosus* M527 in terms of dry cell weight (DCW) (Fig. 2b). In addition, the expression of empty plasmid pIB139 in *S. rimosus* M527 had no effect on rimocidin production (data not shown).

The transcriptional level of the acc_{sr} gene was quantified by RT-qPCR analysis using the total RNA isolated from the recombinant strain *S. rimosus* M527-ACC and WT strain *S. rimosus* M527 at three time points of fermentation (36 h, 48 h, and 72 h). RT-qPCR results indicated that

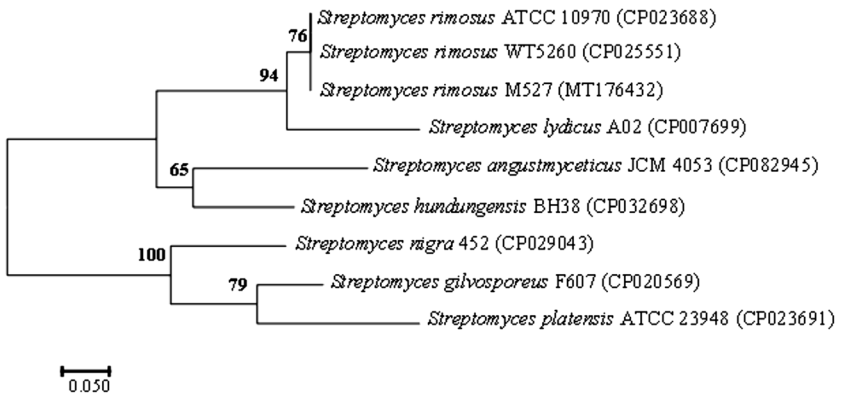

Fig. 1 Phylogenetic analysis of *acc* gene using NCBI BLAST. The nucleotide sequences of acc_{sr} from different prokaryotic species were collected from the NCBI database, and phylogenetic analysis of acc_{sr} was performed with MEGA 7.0, using the neighbor-joining method in the Jukes–Cantor model [31]. Bootstrap values (> 50%) based on 1000 replicates were shown at the branch nodes. Bar, 0.05 substitutions per nucleotide positions

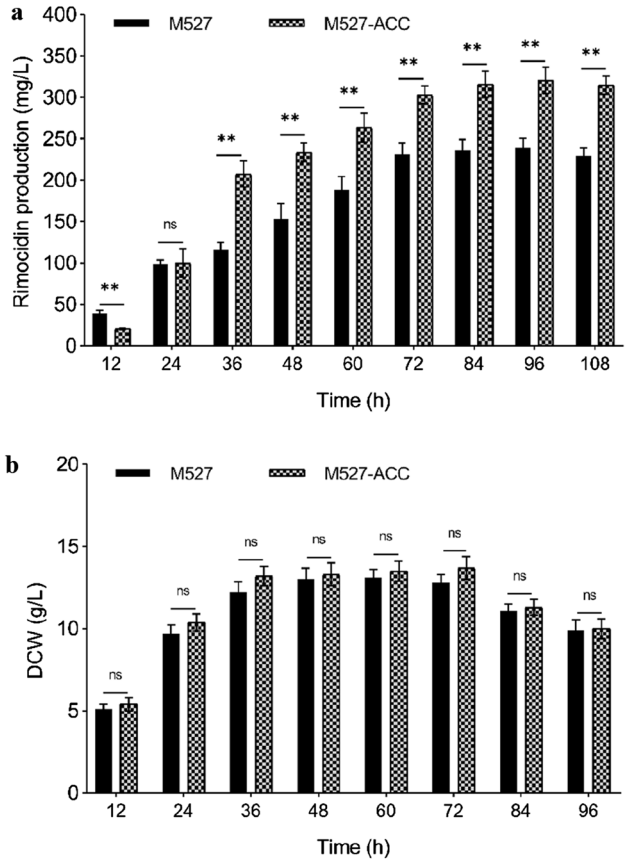

Fig. 2 Analysis and comparison of rimocidin concentration (**a**) and cell growth (**b**) of control strain M527 and recombinant strain M527-ACC. The error bars were calculated from three different batches of fermentation. **Indicates highly statistically significant results (P value < 0.01). ns indicates no statistically significant results (P value > 0.05)

the transcriptional level of the acc_{sr} gene was obviously increased in *S. rimosus* M527-ACC compared to that in *S. rimosus* M527 due to the introduction of an extra copy of acc_{sr} gene (Fig. 3a).

The effect of over-expression of gene acc_{sr} on transcriptional level of *rim* structural genes involved in rimocidin biosynthesis was also determined. As compared to those of the WT strain *S. rimosus* M527, recombinant strain *S. rimosus* M527-ACC exhibited higher transcriptional level of *rimA* and had no significant difference in transcriptional levels of *rimE*, *rimJ*, and *rimK* (Fig. 3b).

Enhancement of the Enzymatic Activity of ACC and Intracellular Malonyl-CoA in *S. rimosus* M527

We speculated that the increased transcription of acc_{sr} gene could improve the specific activity of ACC and intracellular concentration of malonyl-CoA in *S. rimosus* M527. To confirm this hypothesis, the specific activity of ACC and intracellular concentration of malonyl-CoA in *S. rimosus* M527-ACC and *S. rimosus* M527 was determined. The results confirmed that acc_{sr} over-expression promoted the enzymatic activity of ACC (Fig. 4a). The enzymatic activity of ACC in *S. rimosus* M527-ACC harboring the over-expression of acc_{sr} reached the highest level of 2.20 U/mg/DCW, which was onefold higher than that in WT strain *S. rimosus* M527 (1.08 U/mg/DCW).

In addition, this also indicated that higher intracellular concentration of malonyl-CoA was caused by the higher enzymatic activity of ACC (Fig. 4b). In *S. rimosus* M527-ACC, the intracellular concentration of malonyl-CoA was 547 ng/g/DCW, which was 1.5 times higher than that in *S. rimosus* M527 (364 ng/g/DCW).

Discussion

Rimocidin is a member of polyene macrolides that are commercially important because of their antifungal properties. Rimocidin, like all polyenes, is produced through the action of type I modular PKS. Based on its chemical structure, a model for the biosynthetic pathway of rimocidin was proposed. During its elongation process, acetate was incorporated as an elongation unit in all modules through the decarboxylative condensation of malonyl-CoA, except in modules 7 and 13. Thus, malonyl-CoA is an essential precursor for rimocidin biosynthesis. Increasing the supply of acyl-CoA, which is used as a starter unit or extender unit, has been an effective strategy for the enhancement of polyene macrolides [33, 34]. However, precursor engineering in *S. rimosus* M527 has not been performed so far. Malonyl-CoA is mainly synthesized by ACC carboxylation. In addition, the transcriptome microarray data of *S. rimosus* M527 fermented in different conditions were obtained; here, the differentially expressed gene called acc_{sr} that encodes ACC exhibited a higher transcriptional level in an optimized condition than in a normal condition. This provides a good opportunity to verify the relationships between acc_{sr} gene over-expression, intracellular malonyl-CoA concentrations, and rimocidin biosynthesis.

In this study, the acc_{sr} gene from *S. rimosus* M527 was cloned and over-expressed in its own host. The results showed that the over-expression of the acc_{sr} gene could enhance the specific enzymatic activity of ACC, increase the intracellular concentration of malonyl-CoA, and further promote the production of rimocidin. The results also confirmed that increasing the supply of malonyl-CoA is beneficial for the enhancement of rimocidin production. It is worth noting that we also attempted to knock out the acc_{sr} gene and confirm the correlation between the acc_{sr} gene, malonyl-CoA concentration, and rimocidin synthesis. However, these attempts remained unsuccessful after

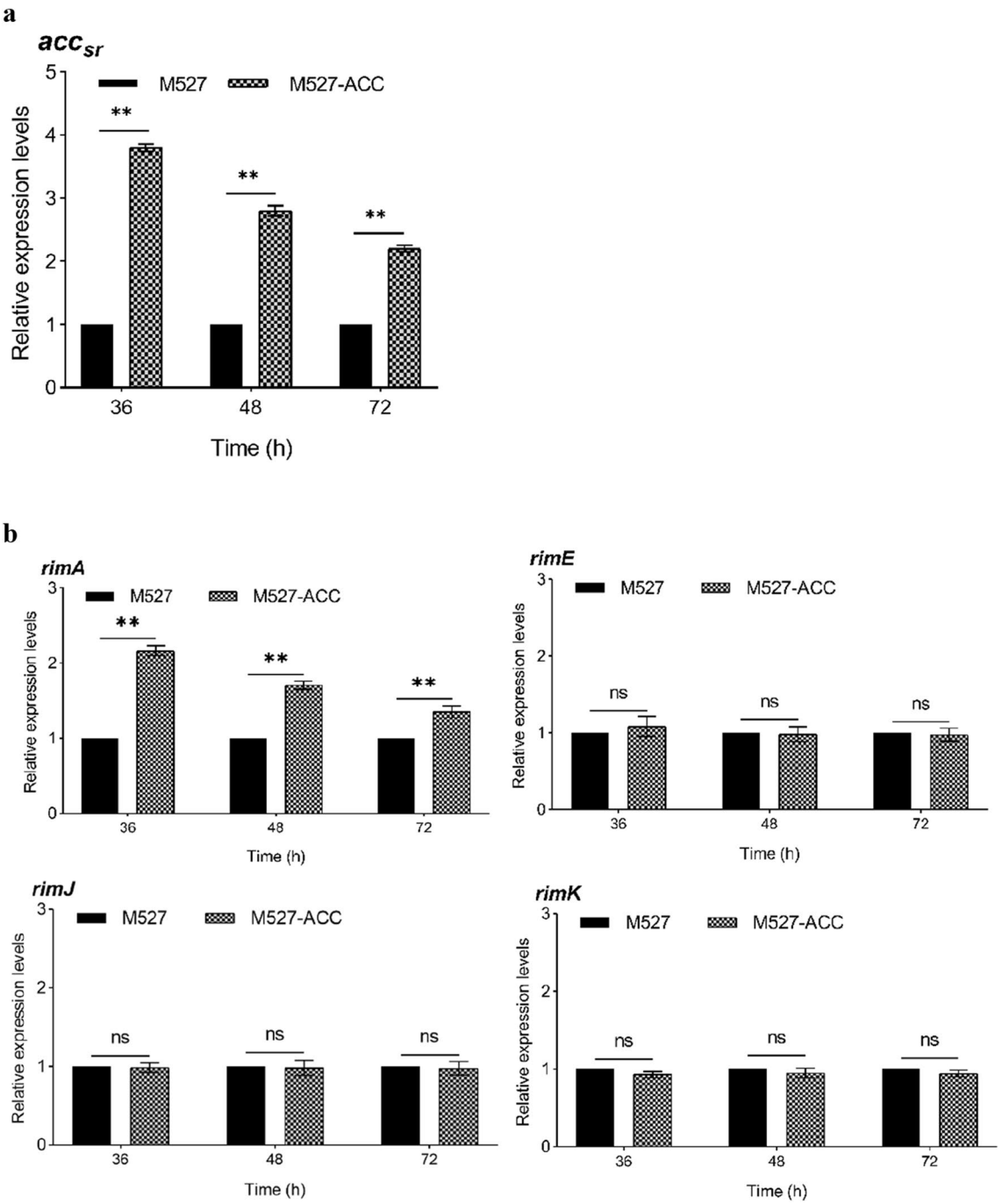

Fig. 3 Comparison of the transcription levels of acc_{sr} gene (**a**) and *rim* genes (*rimA*, *rimE*, *rimJ*, *rimK*) (**b**) in different strains obtained by quantitative reverse transcription-PCR. M527: *S. rimosus* M527; M527-ACC: *S. rimosus* M527-ACC. The cells were harvested from the fermentation 36 h, 48 h, and 72 h. Error bars were calculated by measuring the standard deviations of the data from three replicates of each sample. **Indicates highly statistically significant results (P value < 0.01). ns indicates no statistically significant results (P value > 0.05)

many trials. Because of poor growth, only the fermentation performance and cell characteristics of few acc_{sr} deletion mutants can be analyzed. A possible explanation for this phenomenon is the lack of adequate malonyl-CoA, which is involved in essential cell growth and primary metabolism, leading to difficulty in isolating mutant strains that harbor acc_{sr} gene deletion.

It is important to note that another tetraene, CE-108, which is a structural analog of rimocidin, was also found in the fermentation broth of *S. rimosus* M527 [35]. The two

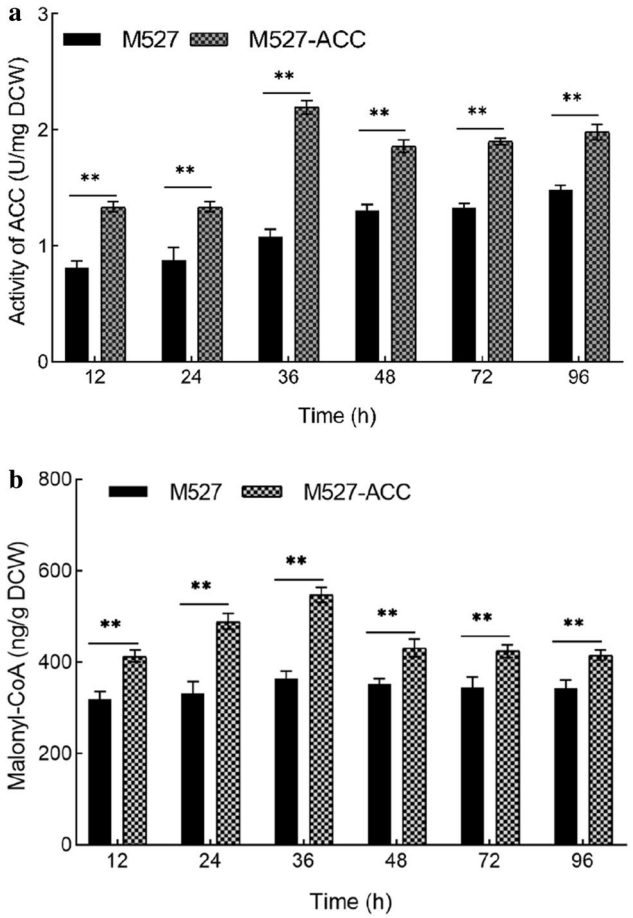

Fig. 4 Comparison of the acetyl-CoA carboxylase activity (**a**) and intracellular malonyl-CoA (**b**) in *S. rimosus* M527 and *S. rimosus* M527-ACC. Error bars were calculated by measuring the standard deviations of the data from three replicates of each sample. **Indicates highly statistically significant results (P value < 0.01)

tetraenes differed in aglycone moiety, with a methyl side chain in CE-108 instead of the propyl group in rimocidin. In the proposed model for rimocidin and CE-108 biosynthesis, the PKS loading module seems to play a crucial role in recognizing acetyl-CoA or butyryl-CoA as starter units, giving rise to methyl or propyl side chains and determining the biosynthesis of CE-108 and rimocidin [20]. There is a common elongation module that is responsible for polyketide chain formation in CE-108 and rimocidin biosynthesis [35], malonyl-CoA is also an important precursor for CE-108 formation during the elongation step. As a result, the over-expression of the acc_{sr} gene also resulted in the improvement of CE-108 (Fig. S4). On the contrary, CE-108 exhibited a weaker antifungal activity than rimocidin [32, 36]. Therefore, an individual-targeted increase of rimocidin instead of CE-108 is worth considering.

The significance of this study is that it provides a new method to promote rimocidin production by precursor engineering via acc_{sr} gene over-expression. To further specifically increase the rimocidin production, the specificity of the loading module to recognize butyryl-CoA as a starter unit can be improved by modifying the site-specific acyltransferase domain in the loading module. For further improvement of rimocidin production in *S. rimosus* M527, the supply of butyryl-CoA as a starter unit and malonyl-CoA as an extender can also be increased at the same time by metabolic engineering technology. Further investigations will be continued in our studies.

Conclusion

In this study, the gene called the acc_{sr} encoding ACC, which exhibits differential expression from comparative transcriptome data of *S. rimosus* M527 in different conditions, was cloned and over-expressed in original strain *S. rimosus* M527. The recombinant strain *S. rimosus* M527-ACC harboring the over-expressed acc_{sr} gene exhibited higher transcriptional level of acc_{sr} gene, enzymatic activity of ACC, malonyl-CoA concentration, and rimocidin production, compared to *S. rimosus* M527. These results not only verified the accuracy of transcriptome data in which a higher expression level of acc_{sr} gene was found in *S. rimosus* M527 under optimization condition but also illustrated the relationships between the acc_{sr} gene, malonyl-CoA, and rimocidin biosynthesis.

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Author Contributions ZJL, JYZ, and YS conducted experiments. ZM and YYZ designed the experiments and wrote this article. AB and XPY checked the final version.

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Declarations

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

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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