Original Research Article

Transcriptome-guided identification of a four-component system, SbrH1-R, that modulates milbemycin biosynthesis by influencing gene cluster expression, precursor supply, and antibiotic efflux

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ABSTRACT

Streptomyces can produce numerous antibiotics and many other bioactive compounds. Recently, the molecular mechanisms of transcriptional regulators in control of antibiotic production by influencing the expression of biosynthetic gene clusters (BGCs) have been extensively studied. However, for regulators that affect both antibiotic production and cell growth, the way to influence antibiotic production may be diverse, but related studies are limited. Here, based on time-course transcriptome analysis, a four-component system, SbrH1-R, consisting of the two-component system SbrKR (SBI_03479/3478) and two hypothetical proteins SbrH1 (SBI_03481) and SbrH2 (SBI_03480) potentially related with the biosynthesis of milbemycins was identified in *Streptomyces bingchenggensis* BC-101-4. Deletion of *sbrH1-R* resulted in weakened cell growth but a 110% increase of milbemycin production compared with that in BC-101-4. Comparative transcriptome analyses of the *sbrH1-R* mutant and BC-101-4 revealed that SbrH1-R not only indirectly represses milbemycin BGC expression, but also inhibits milbemycin production by modulating expression levels of genes related to precursor supply and antibiotic efflux. Further genetic experiments identified several new targets, including five precursor supply-associated reactions/pathways (e.g., the reaction from pyruvate to acetyl-CoA, the reaction from acetyl-CoA to citrate, the fatty acid β-oxidation process, and the branched chain amino acid and phenylalanine acid degradation pathways) and a milbemycin exporter system (MilEX2) that can be engineered for milbemycin overproduction. These results shed new light on the understanding of regulation of milbemycin biosynthesis and provide useful targets for future metabolic engineering of the native host to improve milbemycin production.

1. Introduction

Streptomyces species produce a wealth of antibiotics and other bioactive natural products, many of which have been used in the medicine, veterinary and agriculture fields [1,2]. In streptomycetes, antibiotic production is tightly controlled by hierarchical regulators (cluster-situated regulators [CSRs] and pleiotropic or global regulators) and environmental and physiological cues [3]. Elucidating the

regulatory mechanism of antibiotic biosynthesis is critical for developing effective regulatory strategies to achieve antibiotic overproduction. In recent decades, the regulatory mechanisms of CSRs or pleiotropic regulators in the control of antibiotic production by influencing the expression levels of biosynthetic gene clusters (BGCs) have been extensively studied [1,4]. Hence, regulator-based engineering methods such as activator overexpression, repressor deletion, promoter replacement and cluster refactoring and amplification are also widely

used to promote overproduction of known antibiotics and activation of cryptic antibiotic BGCs [5–8]. As is well known, for commercially valuable antibiotics, generation of a high-titer strain is crucial for their large-scale industrial production and wide use. However, the existing regulator-based construction process of high-yield strains is usually focused on enhancing antibiotic biosynthetic pathways and rarely refers to optimization of other key factors (precursors or cofactors) influencing antibiotic production. Moreover, due to the regulator's pleiotropic function and the interweaved regulatory network [4], simple combined manipulation of multiple beneficial regulatory targets often fails to further increase the titer of antibiotics [9]. These shortages make it difficult for regulator-based strategies to play a substantial role in promoting the industrial production of antibiotics. With the increasing understanding of antibiotic biosynthesis, researchers gradually realize that the effects of regulators (particularly the pleiotropic regulators that influence both antibiotic production and cell growth) on production levels of antibiotics are a coordinative result of multiple physiological processes, not just the expression change of BGCs [10]. Therefore, it is supposed that identifying the physiological processes and related genes controlled by certain regulators will help to mine more targets beneficial for antibiotic overproduction and fully reveal the multiple regulatory mechanisms, and finally provide a solid theoretical foundation for construction of industrial strains with excellent productivities. Luckily, the rapid development of omics and genetic manipulation technologies has provided great convenience for us to reveal the multiple ways via which regulators control antibiotic production.

Milbemycins, a series of 16-membered macrolide antibiotics with potent insecticidal and anthelminthic activities, have been applied widely as insecticides and anthelmintics in the agriculture and veterinary fields [11]. Milbemectin, a mixture of milbemycin A3 and A4, is an important acaricide. Notably, other semi-synthetic derivatives of milbemycin A3/A4, such as lepimectin, latidectin and milbemycin oxime are also commercially valuable drugs. *Streptomyces bingchenggensis*, isolated by our research group, is an important industrial producer of milbemycins [12]. Initially, using traditional random mutagenesis strategies such as treatment with N-methyl-N′-nitro-N-nitrosoguanidine (NTG) [13], ultraviolet mutagenesis and atmospheric and room temperature plasma (ARTP) mutation [13,14], a significant increase of milbemycin titer was achieved and strain productivities reached the level for industrial production [13,14]. However, the titer of milbemycins is still much lower than that of another well-exploited polyketide pesticide, avermectin B1a (9 g/L), and it is necessary to fully explore the milbemycin-producing potential of *S. bingchenggensis* [15–17]. In recent years, with the developments of genome sequencing, omics, gene editing and synthetic biology technologies, many efforts have been made to understand the biosynthetic pathway and the transcriptional regulation of milbemycins [11,14,18]. The genome sequence of *S. bingchenggensis* was published and the BGC of milbemycins was identified (*sbi_00726–sbi_00790*) [19]. Several genes or systems including four tailoring enzyme genes (*milD*, *milE*, *milF* and *cyp41*) [14,20,21], a CSR gene (*milR*) [11], an *arpA/afsA*-like system (*sbbR/sbbA*) [18], and two sugar uptake systems (TP2 and TP5) have been shown to participate in or influence milbemycin production [17]. Notably, individual overexpression of *milE*, *milR*, TP2, and TP5 alone could increase the titer of milbemycins [11,17,21]; deletion of *sbbA* or *cyp41* could also improve milbemycin production [18,21]. In spite of these findings, our knowledge of the complicated transcriptional and metabolic regulation network is still very limited and fragmentary, hindering the progress of construction of superior milbemycin-producing strains using rational engineering strategies.

Two-component systems (TCSs), which typically exist in pairs consisting of a membrane-bound histidine kinase (HK) and a cognate response regulator (RR), are widespread in *Streptomyces*, and many of them have been reported to play important roles in antibiotic production and/or morphological development [1]. Careful analysis of the *Streptomyces* genome revealed that many genes encoding hypothetical

proteins (HPs) are found located alongside the TCS genes. For instance, in the vicinity of 17 of the 67 TCS loci from *Streptomyces coelicolor*, one to several HP genes are located that may co-transcribe with TCS genes (Table S1). Similarly, in *S. bingchenggensis* (the main object of this study), 25 paired TCSs were also found to be flanked by HP genes (Table S1). Genes clustered together might share related functions, so whether these HPs play a role in cellular processes and whether they have relationships with the TCS function in signal transduction pathways need further investigation.

In this work, based on the time-course transcriptome data, we identified a previously uncharacterized four-component system SbrH1-R in *S. bingchenggensis*. It contains a typical TCS, SbrKR (SBI_03479/3478) and two HPs SbrH1 (SBI_03481) and SbrH2 (SBI_03480). SbrH1-R is an activator of morphological development but a repressor of milbemycin biosynthesis. Transcriptome analysis of the *sbrH1-R* mutant (high titer of milbemycins) and the parental strain BC-101-4 revealed that SbrH1-R not only indirectly inhibits expression of the milbemycin BGC, but also represses milbemycin biosynthesis by affecting genes related to precursor supply and antibiotic efflux. Further gene overexpression and CRISPRi experiments determined six new targets that are beneficial for milbemycin overproduction, including five reactions/pathways associated with precursor supply (e.g., the reaction from pyruvate to acetyl-CoA, the reaction from acetyl-CoA to citrate, the fatty acid β-oxidation process, and the branched chain amino acid and phenylalanine acid degradation pathways) and one milbemycin exporter MilEX2. This work provides new insight into the regulatory network of milbemycin biosynthesis and provides useful targets for the construction of high-producing strains of milbemycins and other polyketide antibiotics.

2. Materials and methods

2.1. Strains, plasmids and culture conditions

All strains and plasmids used in the present work were summarized in Table S2. *S. bingchenggensis* BC-101-4 is a wild-type milbemycin producer, and BC04 is an industrial milbemycin producer. *Escherichia coli* was grown in/on Luria Bertani (LB) medium supplemented with antibiotics as required at 37 °C. For spore collection, BC-101-4, BC04 and their derivatives were grown at 28 °C on SKYM agar plates [11]. SKYM agar medium was also used for morphological differentiation analysis. The media and methods used for milbemycin production were the same as previous report [11].

2.2. Construction of recombinant strains

The related primers used in this work were listed in Table S3. For construction of *sbrH1-R* disruption mutant, two homologous fragments sbrH1R-L (2043-bp) and sbrH1R-R (2351-bp) flanking the *sbrH1-R* operon were amplified from the genomic DNA of BC-101-4 by PCR using sbrH1RD-LF/R and sbrH1RD-RF/R as primer pairs. The two PCR products were cloned into pKC1139 digested by EcoRI/XbaI by Gibson assembly to obtain pKC1139::ΔsbrH1-R. Then, the resultant plasmid was introduced into strain BC-101-4 to construct *sbrH1-R* null mutant (ΔsbrH1-R) via homologous recombination as previously described [14]. The yielding strain ΔsbrH1-R was confirmed by PCR with primer pairs ver1-F/R and ver2-F/R followed by DNA sequencing.

For construction of *sbrH1-R* complementation plasmids, one fragment containing the coding and upstream regions of *sbrH1-R* was amplified from the genomic DNA of BC-101-4 by PCR using primer pairs CsbrH1-R-F/R. Then the fragment was inserted into the EcoRI/XbaI sites of pSET152 by Gibson assembly to generate pSET152::sbrH1-R, The construction process of *sbrH1H2* complementation plasmid (pSET152::sbrH1H2) was similar to that of constructing *sbrH1-R* complementation plasmid.

For construction of *sbrKR* complementation plasmid, the coding region of *sbrKR* and the corresponding upstream *sbrH1* promoter region

were amplified from the genomic DNA of BC-101-4 using CsbrKR-F/R and P$_{sbrH1}$-F/R as primer pairs, respectively. The two PCR products were ligated to the EcoRI/XbaI sites of pSET152 to generate pSET152::sbrKR. Subsequently, the three complementation plasmids were introduced into ΔsbrH1-R by intergenic conjugation to obtain the complemented strains CsbrH1H2 (ΔsbrH1-R containing pSET152::sbrH1H2), CsbrKR (ΔsbrH1-R containing pSET152::sbrKR) and CsbrH1-R (ΔsbrH1-R containing pSET152::sbrH1-R).

For construction of the pyruvate metabolism module, the coding sequences of *sbi_05443-sbi_05445* and the 521-bp *sbi_03553* promoter region (P$_{sbi\_03553}$) were amplified using the genomic DNA of BC-101-4 with primer pairs sbi_05443-5445-F/R and P$_{sbi\_03553}$-Pyr1-F/R, respectively. The two resulting fragments were ligated to the EcoRI/XbaI digested pSET152 by Gibson assembly to obtain pSET152::Pyr1. Then the coding sequences of *sbi_05430–5432*, *sbi_07787* and P$_{sbi\_03553}$ were amplified from the genomic DNA of BC-101-4 using the primer pairs sbi_05430-5432-F/R, sbi_07787-F/R and P$_{sbi\_03553}$-Pyr2-F/R, respectively. The three PCR products were inserted into pSET152:Pyr1 digested by PacI by Gibson assembly to obtain pSET152::Pyr.

For construction of fatty acid degradation and BCAA catabolism module, the coding sequences of *sbi_06919-sbi_06922* and P$_{sbi\_03553}$ were amplified from the genomic DNA of BC-101-4 using the primer pairs sbi_06919–sbi_06922-F/R and P$_{sbi\_03553}$-FBCAA1-F/R, respectively. The two resulting fragments were ligated to the EcoRI/XbaI digested pSET152 to obtain pSET152::FBCAA1. Then the coding sequences of *sbi_08383, sbi_02642–2643, sbi_06924* and P$_{sbi\_03553}$ were amplified from the genomic DNA of BC-101-4 using the primer pairs sbi_08383-F/R, sbi_02642–2643-F/R, sbi_06924-F/R and P$_{sbi\_03553}$-FBCAA2-F/R, respectively. The four PCR products were inserted into pSET152:FBCAA1 digested by PacI to obtain pSET152::FBCAA2. Subsequently, the coding sequences of *sbi_03090, sbi_04601, sbi_08381, sbi_08661* and P$_{sbi\_03553}$ were amplified from the genomic DNA of BC-101-4 using the primer pairs sbi_03090-F/R, sbi_04601-F/R, sbi_08381-F/R, sbi_08661-F/R and P$_{sbi\_03553}$-FBCAA3-F/R, respectively. The five resulting fragments were inserted into PacI-digested pSET152::FBCAA2 to give pSET152::FBCAA3. Finally, the coding sequences of *sbi_07561, sbi_07154* and P$_{sbi\_03553}$ were amplified from the genomic DNA of BC-101-4 using the primer pairs sbi_07561-F/R, sbi_07154-F/R and P$_{sbi\_03553}$-FBCAA4-F/R, respectively. The resultant fragments were ligated to PacI-digested pSET152::FBCAA3 to obtain pSET152:FBCAA.

For construction of the phenylalanine degradation module, the coding sequences of *sbi_01968–sbi_01969, sbi_02832, sbi_08941* and P$_{sbi\_03553}$ were amplified from BC-101-4 genomic DNA using the primer pairs sbi_01968–1969-F/R, sbi_02832-F/R, sbi_08941-F/R and P$_{sbi\_03553}$-Phe1-F/R, respectively. The four resulting fragments were ligated together with EcoRI/XbaI digested pSET152 to obtain pSET152::Phe1. Then the coding sequences of *sbi_00715, sbi_05418–5422* and P$_{sbi\_03553}$ were amplified from the genomic DNA of BC-101-4 using the primer pairs sbi_00715-F/R, sbi_05418–5422-F/R and P$_{sbi\_03553}$-Phe2-F/R, respectively. The three PCR products were inserted into pSET152::Phe1 digested by PacI to obtain pSET152::Phe.

For construction of TCA cycle inhibition plasmid, ddCpf1-based integrative CRISPRi system was used to repress *sbi_07003* transcription [22]. P$_{ermE*}$ was amplified from pSETddCpf1 using primer pair Cpf1 (ermE)-F/R, and P$_{sbi\_03553}$ containing *sbi_07003* specific crRNA was amplified from BC-101-4 genomic DNA using primer pair P$_{sbi\_03553}$-sbi_07003-F/R. Then the two PCR products were ligated with NdeI/SpeI-digested pSETddCpf1 to generate pSETddCpf1::RgltA.

Finally, the four resultant modules (pSET152:Pyr, pSET152:FBCAA, pSET152:Phe and pSETddCpf1::RgltA) were individually integrated into the industrial milbemycin producer BC04 via intergenic conjugation to obtain strains BC04/Pyr, BC04/FBCAA, BC04/Phe and BC04/RgltA. Meanwhile, the empty plasmid pSET152 and pSETddCpf1 were also introduced into BC04 to generate control strains BC04/C1 and BC04/C2, respectively.

For construction of exporter overexpression plasmids, the *hrdB* promoter was amplified from plasmid pSET152::P$_{hrdB}$milR [11] by primer pair P$_{hrdB}$-F/R. Then the PCR product was ligated with EcoRI/XbaI-digested pSET152 by Gibson assembly to obtain pSET152::P$_{hrdB}$. Subsequently, genes *sbi_00861, sbi_01690−sbi_01691, sbi_05053−sbi_05054, sbi_07249, sbi_02810, sbi_06224* and *sbi_07695−sbi_07696* were amplified from the genome of strain BC-101-4 with primer pairs MilEX1-F/R, MilEX2-F/R, MilEX3-F/R, MilEX4-F/R, MilEX5-F/R, MilEX6-F/R and MilEX7-F/R, respectively. The resulting DNA fragments were individually inserted into PacI-digested pSET152::P$_{hrdB}$ by Gibson assembly, yielding pSET152::MilEX1, pSET152::MilEX2, pSET152::MilEX3, pSET152::MilEX4, pSET152::MilEX5, pSET152::MilEX6 and pSET152::MilEX7, respectively. Then the resulting plasmids were separately introduced into strain BC04 to obtain BC04/MilEX1 (overexpression of *sbi_00861*), BC04/MilEX2 (overexpression of *sbi_01690−sbi_01691*), BC04/MilEX3 (overexpression of *sbi_05053−sbi_05054*), BC04/MilEX4 (overexpression of *sbi_07249*), BC04/MilEX5 (overexpression of *sbi_02810*), BC04/MilEX6 (overexpression of *sbi_06224*) and BC04/MilEX7 (overexpression of *sbi_07695−sbi_07696*).

2.3. Microscopic analyses

To visualize the hyphal morphology differences among strains BC-101-4, ΔsbrH1-R, CsbrH1H2, CsbrKR and CsbrH1-R, equal amounts of spores/mycelia of each strain were inoculated on SKYM agar plates and cultured at 28 °C for 9 days. After cultivation, hyphae were observed with an Olympus BX63 microscope. Images were captured and processed using the cellSens Standard software.

2.4. Extraction and HP LC analysis of milbemycin A3/A4

The total and extracellular milbemycin A3/A4 were extracted and analyzed as described previously [11,23].

2.5. Determination of cell dry weight

Cells harvested from 2 mL fermentation cultures by vacuum filtration were then dried at 55 °C to a constant weight.

2.6. RNA extraction, RT-PCR and quantitative real-time RT-PCR analysis

Total RNAs were harvested from the fermentation broth of BC-101-4 and ΔsbrH1-R at the 3rd and 6th day. RNA extraction and purification, examination of RNA quality and quantity, synthesis of cDNA, RT-PCR and qRT-PCR were essentially performed as previous report [11]. The related primers used in the present work were listed in Table S3.

2.7. SbrR^{Gst} expression and purification

To prepare the GST-tagged protein SbrR^{Gst}, the coding region of *sbrR* was amplified from the genome of BC-101-4 using the primer pair proSbrR-F/R (Table S3). The PCR product was inserted into the EcoRI/XhoI sites of pGEX-4T-1 to obtain pGEX-4T-1:sbrR. The subsequent SbrR^{Gst} overexpression and purification were the same as described previously [8].

2.8. Electrophoretic Mobility Shift Assays (EMSAs)

EMSAs were carried out as reported previously [8]. The promoter probes were amplified from the genomic DNA of strain BC-101-4 by PCR with primer pairs listed in Table S3.

2.9. RNA-seq analysis

RNA-seq was performed by Novogene Bioinformatics Technology Co. Ltd. Three independent biological replicates were analyzed at day 3,

while at day 6, three independent RNA samples from the fermentation broth of each strain were pooled as one sample used for transcriptomic analysis. Gene expression levels were calculated using the expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced (FPKM). *P*-value threshold was determined using the false discovery rate (FDR) in multiple testing. The significantly differentially expressed genes were selected based on |log$_2$ (fold change) | > 1 and padj. < 0.05.

2.10. Samples preparation and analysis of the intracellular acyl-CoA esters (acetyl-CoA, malonyl-CoA, propionyl-CoA and methylmalonyl-CoA)

The fermentation cultures of BC-101-4 and ΔsbrH1-R were collected at day 2, 4 and 6. The methods for cell collection, quenching and grinding, together with extraction and analysis of acyl-CoA esters were the same as previous report [24,25].

2.11. Sequence analysis

The sequence alignment and domains of potential protein-coding sequences (CDSs) were documented by publicly available databases and their application tools, including Pfam (http://www.pfam.xfam. org/) and SMART (http://www.smart.embl-heidelberg.de/). Heat maps were created by the Mev software. KEGG enrichment analysis was carried out by KOBAS and OmicShare Tool (https://www.omicshare. com/tools/Home/Soft/pathwaygseasenior).

2.12. Statistical analysis

All experiments were performed independently at least three biological triplicates and data were presented as mean values ± standard deviation (s.d.). The Student's *t*-test (two-tail) was used to demonstrate the statistical significance, with *P < 0.05, **P < 0.01, ***P < 0.001 and "ns" means not significant.

2.13. Data availability

The raw datasets of time-course transcriptome and comparative transcriptome between BC-101-4 and ΔsbrH1-R used in this study have been deposited in the GEO (Gene Expression Omnibus) database (GSE147644 and GSE186852).
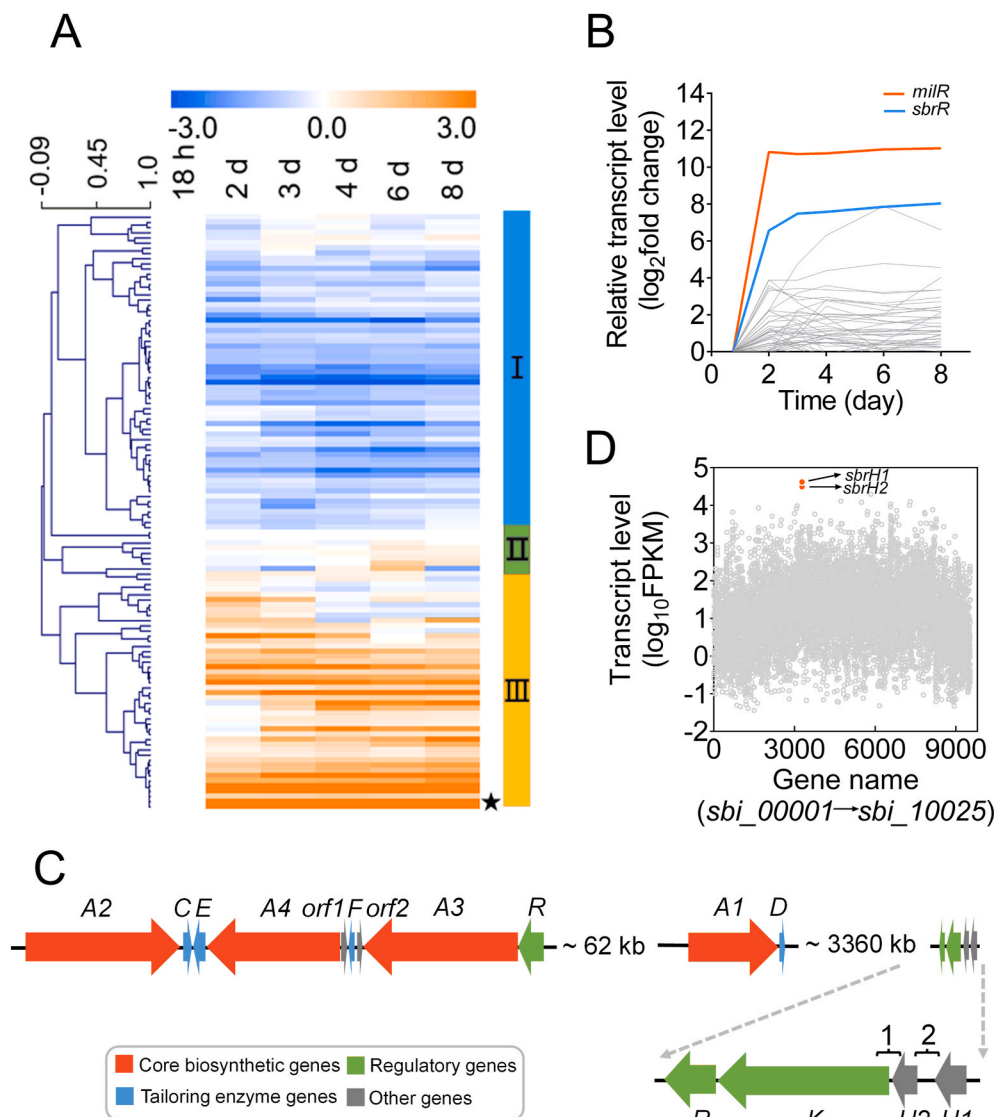
Fig. 1. Time-course transcriptome data-based identification of a four-component system SbrH1-R in *S. bingchenggensis*. (A) Heatmap analysis and clustering of RR genes and *milR* of *S. bingchenggensis*. The star represents *milR*. (B) The transcriptional profiles of the RR genes in Group III. (C) Schematic representation of the relative position of the *sbrH1-R* and the milbemycin BGC. Each arrow indicates a separate open reading frame (ORF) and orientation of transcription. "1" and "2" indicate the corresponding intergenic regions, which would be subjected to RT-PCR analysis. (D) Transcript abundances of genes measured by RNA-Seq in *S. bingchenggensis* BC-101-4 at day 3. All genes (FPKM>0) were shown by gray circles, the arrow pointed red dots represented genes *sbrH1* and *sbrH2*. Transcript abundance was shown in a log$_{10}$ FPKM scale.

3. Result

3.1. Identification of a novel four-component system SbrH1-R in S. bingchenggensis

To search for novel TCS regulators of milbemycin biosynthesis, all putative TCS-encoding genes in the genome of *S. bingchenggensis* were statistically and transcriptionally analyzed. Bioinformatics analysis showed that there were 91 pairs of classical TCS genes, 23 orphan RR genes and 23 unpaired HK genes in the genome of *S. bingchenggensis* (Table S4). Based on the time-course transcriptome data of RR genes in *S. bingchenggensis*, hierarchical clustering was used to select TCSs showing similar transcriptional profiles to *milR*, a cluster-situated activator gene for milbemycin biosynthesis in milbemycin BGC [11]. As shown in Fig. 1A, according to the expression patterns, 91 RRs were clustered into three groups: in group I, transcription was high at day 0.75, and then decreased onward; in group II, transcription was relatively constant throughout the fermentation process; and in group III, transcription was very low at day 0.75, but reached a high level at day 2 and remained high thereafter. Among them, group III RRs share a similar transcriptional pattern with *milR*, and of note, *sbi_03478* shows the most similar expression pattern to *milR* (Fig. 1B). *sbi_03478* is situated about 3360 kb upstream of the *milR* locus, and encodes a 23.6-kDa polypeptide featured with an N-terminal REC (CheY-homologous receiver) domain and a C-terminal LuxR-like DNA-binding domain. Situated upstream of *sbi_03478*, *sbi_03479* encodes the cognate sensor kinase of 713 amino acids and overlaps *sbi_03478* with 4 bp. *sbi_03478* and *sbi_03479* constitute a typical TCS. Two convergently transcribed genes, *sbi_03480* and *sbi_03481*, are located upstream of *sbi_03479* (Fig. 1C) and encode hypothetical proteins with unknown domains. The transcript profiles of these two genes are quite similar; their transcription increased dramatically and maintained a very high level expression during the fermentation process. Transcriptome data showed that the two genes presented the highest FPKM values among all the detected genes during the milbemycin production stage (day 3–8) (Fig. 1D and Fig. S1). Furthermore, RT-PCR analysis indicated that *sbi_03481* and *sbi_03480* are co-transcribed with *sbi_03479/03478* (Fig. S2). Thus, we speculated that this four-component system, referred to as SbrH1/H2/K/R or SbrH1-R, might play a regulatory role in the biosynthesis of milbemycins.

3.2. SbrH1-R activates morphological development but represses milbemycin production

To investigate the function of *sbrH1-R*, an *sbrH1-R* null mutant (ΔsbrH1-R) was constructed via homologous recombination in the parental strain BC-101-4 (Fig. S3). The resulting mutant ΔsbrH1-R was confirmed by PCR (Fig. S3) and DNA sequencing (data not shown). Compared with BC-101-4, ΔsbrH1-R exhibited obviously weakened aerial growth and reduced yellow pigment production on SKYM agar medium (Fig. 2A). Spiral spore chains were evident in BC-101-4, but only spare aerial and substrate mycelia were observed in ΔsbrH1-R (Fig. S4). HPLC analysis showed that the total titer of milbemycin A3/A4 (the main effective components) of ΔsbrH1-R was 110% higher than that of BC-101-4 (from 1515.3 to 3136.7 mg/L) (Fig. 2B). However, the final cell biomass of ΔsbrH1-R was significantly decreased and was only 45% that of BC-101-4 (Fig. 2C). To confirm whether these phenotypes were due to the absence of *sbrH1-R*, a native copy of the *sbrH1-R* operon was cloned into the integrative plasmid pSET152 to generate pSET152::sbrH1-R, which was further introduced into ΔsbrH1-R to obtain the complementation strain CsbrH1-R. In this strain, spore formation and yellow pigment production, as well as cell growth in fermentation
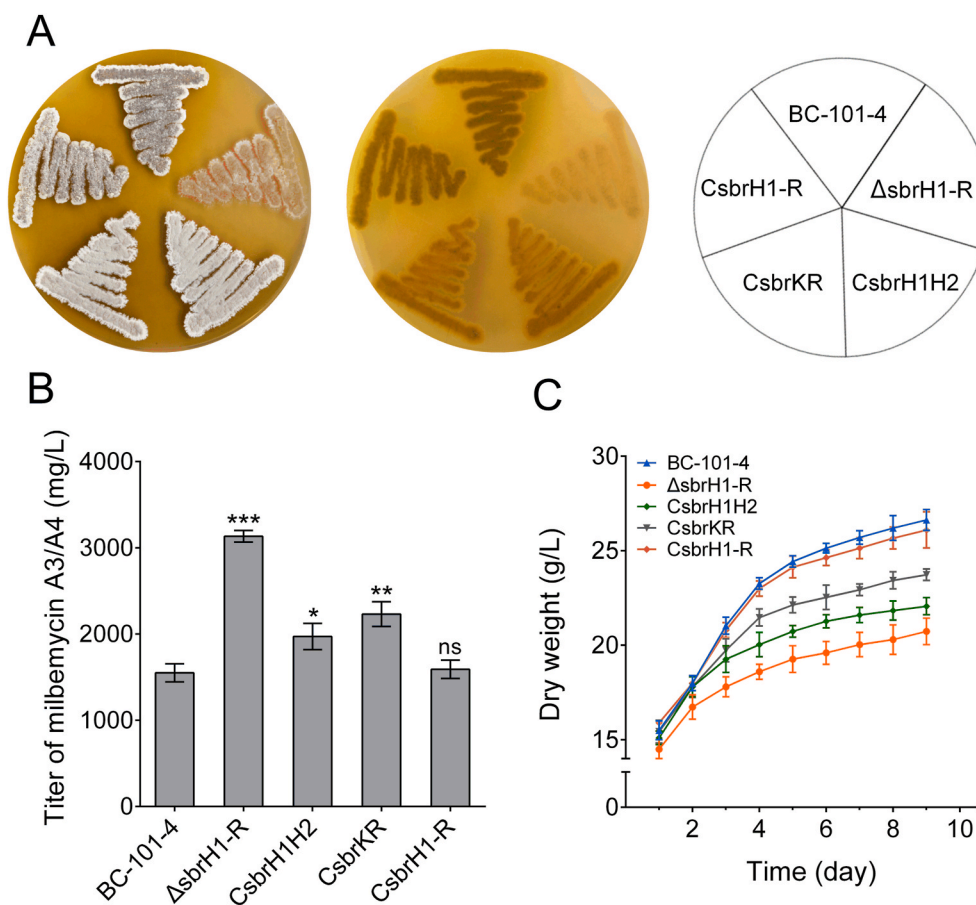
Fig. 2. Effects of SbrH1-R on morphological development, milbemycin production and cell growth. (A) Morphological differentiation among strains BC-101-4, ΔsbrH1-R, CsbrH1H2, CsbrKR and CsbrH1-R. (B) Comparative milbemycin A3/A4 production in BC-101-4, ΔsbrH1-R, CsbrH1H2, CsbrKR and CsbrH1-R cultured in fermentation medium. Error bars show standard deviations. *P*-values were determined by Student's t-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ns, not significant. (C) Growth curves of BC-101-4, ΔsbrH1-R1, CsbrH1H2, CsbrKR and CsbrH1-R cultured in fermentation medium. Biomass is expressed as dry cell weight.

medium were basically similar to that of the wild-type (Fig. 2A, C and Fig. S4); milbemycin production was also decreased to the BC-101-4 level (Fig. 2B), suggesting that *sbrH1-R* is essential for morphological development and negatively controls milbemycin production. To further determine the actual contributions of the two HP-encoding genes *sbrH1H2* and the TCS *sbrKR*, ΔsbrH1-R was complemented individually by pSET152::sbrH1H2 and pSET152::sbrKR, in which a single copy of *sbrH1H2* or *sbrKR* was driven by their native promoters, yielding complementation strains CsbrH1H2 (equivalent to the *sbrKR* mutant) and CsbrKR (equivalent to the *sbrH1H2* mutant). In the two complementation strains, production of aerial hyphae was obviously restored and small amounts of spores were produced (Fig. 2A and Fig. S4); meanwhile, the titer of milbemycins and cell biomass were also partially restored to the BC-101-4 levels (Fig. 2B and C), indicating that both *sbrKR* and *sbrH1H2* are necessary for *sbrH1-R* function and they may act synergistically in the control of morphological development and milbemycin production.

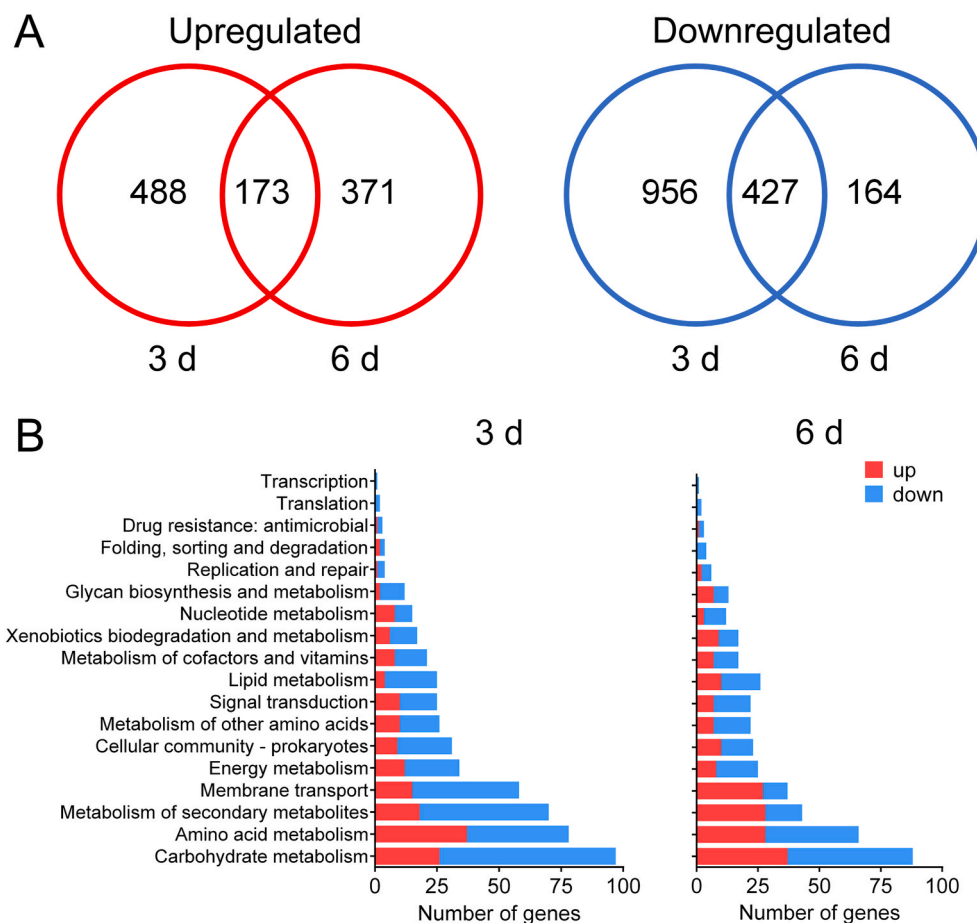### 3.3. Comparative transcriptome analysis to explore SbrH1-R targets that may influence milbemycin production

3.3.1. Preliminary transcriptome analysis of the sbrH1-R mutant

As demonstrated above, SbrH1-R is a pleiotropic regulatory system controlling morphological development, cell growth and milbemycin production, so it is speculated that SbrH1-R may control milbemycin biosynthesis via multiple pathways. To gain detailed insights into the regulatory ways by which SbrH1-R regulates milbemycin production and mine beneficial targets for milbemycin production, comparative transcriptome analysis was performed using total RNAs isolated from BC-101-4 and ΔsbrH1-R after cultivation for 3 and 6 days. Statistical analysis revealed that a total of 2044 genes showed significant differences in expression ($|log_2$ (fold change) $|>1$, $p < 0.05$) in ΔsbrH1-R at day 3, with 661 upregulated and 1383 downregulated, while 1135 genes were obviously differentially expressed at day 6, with 544 upregulated and 591 downregulated (Fig. 3A). KEGG analysis revealed that these significantly differentially expressed genes (SDEGs) are mostly enriched in pathways such as carbohydrate metabolism, amino acid metabolism, metabolism of secondary metabolites, membrane transport and lipid metabolism (Fig. 3B and Dataset S1). Notably, at both days, the number of downregulated genes was significantly higher than that of the upregulated genes in these enriched metabolic pathways such as carbohydrate metabolism, energy metabolism and lipid metabolism, which might explain the poor growth of ΔsbrH1-R during fermentation (Fig. 3B).

3.3.2. SbrH1-R indirectly represses transcription of milbemycin BGC

Consistent with the overproduction of milbemycin A3/A4 in ΔsbrH1-R, transcription of all milbemycin biosynthetic genes was upregulated, with six of them (*milE*, *milA4*, *orf1*, *milF*, *orf2* and *milD*) showing significant upregulation at day 3 and/or 6 ($|log_2$ (fold change) $|>1$, $p < 0.05$) (Dataset S2). Moreover, the transcription levels of *milR*, *milA1*, *milA2*, and *milF* as revealed by the RNA-seq data were basically consistent with those detected by qRT-PCR (Fig. S5), indicating the reliability of the RNA-seq results. However, no binding was detected between SbrR (the DNA-binding protein of this four-component system) and the promoter regions of the milbemycin BGC (Fig. S6), indicating that SbrH1-R may indirectly repress transcription of the milbemycin BGC.

Fig. 3. Transcriptome analysis of strains BC-101-4 and ΔsbrH1-R. (A) Comparative transcriptome analysis of the SDEGs between strains BC-101-4 and ΔsbrH1-R after cultivation for 3 and 6 days. (B) Functional categories of annotated SDEGs between BC-101-4 and ΔsbrH1-R according to the KEGG pathway.

3.3.3. Effect of sbrH1-R deletion on central carbon metabolism and its proximal precursor pathway-related genes

As is well known, building precursors for antibiotic biosynthesis are mainly derived from the primary metabolic pathways [26]. Moreover, milbemycin fermentation uses glucose as the only carbon source, so we then focused on the central carbon metabolism and its proximal metabolic pathway-related genes. We first found that a series of genes encoding putative pyruvate dehydrogenase (PDH) components, which are responsible for catalyzing pyruvate into acetyl-CoA, were upregulated at day 3 or 6 (Fig. 4 and Dataset S3). *sbi_07787* and *sbi_05444* (both are *pdh aceE* components) were upregulated at day 6. *sbi_05430* (*aceE*), *sbi_05431* (*aceF*), and *sbi_05432* (*aceE2*) were upregulated at day 3; notably, although these three showed a downregulation at day 6, their corresponding isozyme genes *sbi_05443–sbi_05445* were about 100% upregulated at the same time (*sbi_05443* by 98%; *sbi_05444* by 152%; *sbi_05445* by 97%), possibly counteracting the impact caused by the downregulation of *sbi_05430–sbi_05432*. Collectively, the total expression levels of PDH genes were obviously increased at both time points, implying the enhancement of the flux from pyruvate to acetyl-CoA. Acetyl-CoA is also the substrate for the synthesis of the extender unit malonyl-CoA, which can be generated by carboxylation of acetyl-CoA

performed by acetyl-CoA carboxylase (ACC). Interestingly, the ACC component gene *sbi_06921* also exhibited an obvious upregulation at day 3 (Fig. 4 and Dataset S3).

For the tricarboxylic acid (TCA) cycle, the citrate synthase (GltA) gene *sbi_07003* was significantly downregulated at day 3 in ΔsbrH1-R (Fig. 4 and Dataset S3); GltA is a rate-limiting enzyme responsible for the first step of the TCA cycle and consumes acetyl-CoA to generate citrate. Besides *sbi_07003*, the *S. bingchenggensis* genome also carries another GltA gene, *sbi_04868*, which showed a weak downregulation in ΔsbrH1-R (Dataset S3). Expression of *sbi_07003* was 15 and 8 times as high as that of *sbi_04868* at day 3 and 6, respectively, indicating that *sbi_07003* is the major functional GltA gene in *S. bingchenggensis*. Therefore, it can be concluded that the reactivity of this step, which is mainly performed by SBI_07003, is significantly downregulated, reducing the acetyl-CoA flux into TCA, which may be beneficial for milbemycin biosynthesis; this may also be one of the key reasons for the significant decrease of cell biomass during milbemycin production. Additionally, expression of two genes (*sbi_04139* and *sbi_04140*) encoding succinic dehydrogenase components, which can catalyze the oxidation of succinate to generate fumarate, was decreased at day 3 but increased at day 6 (Fig. 4 and Dataset S3). Notably, succinyl-CoA, the product of the fourth reaction
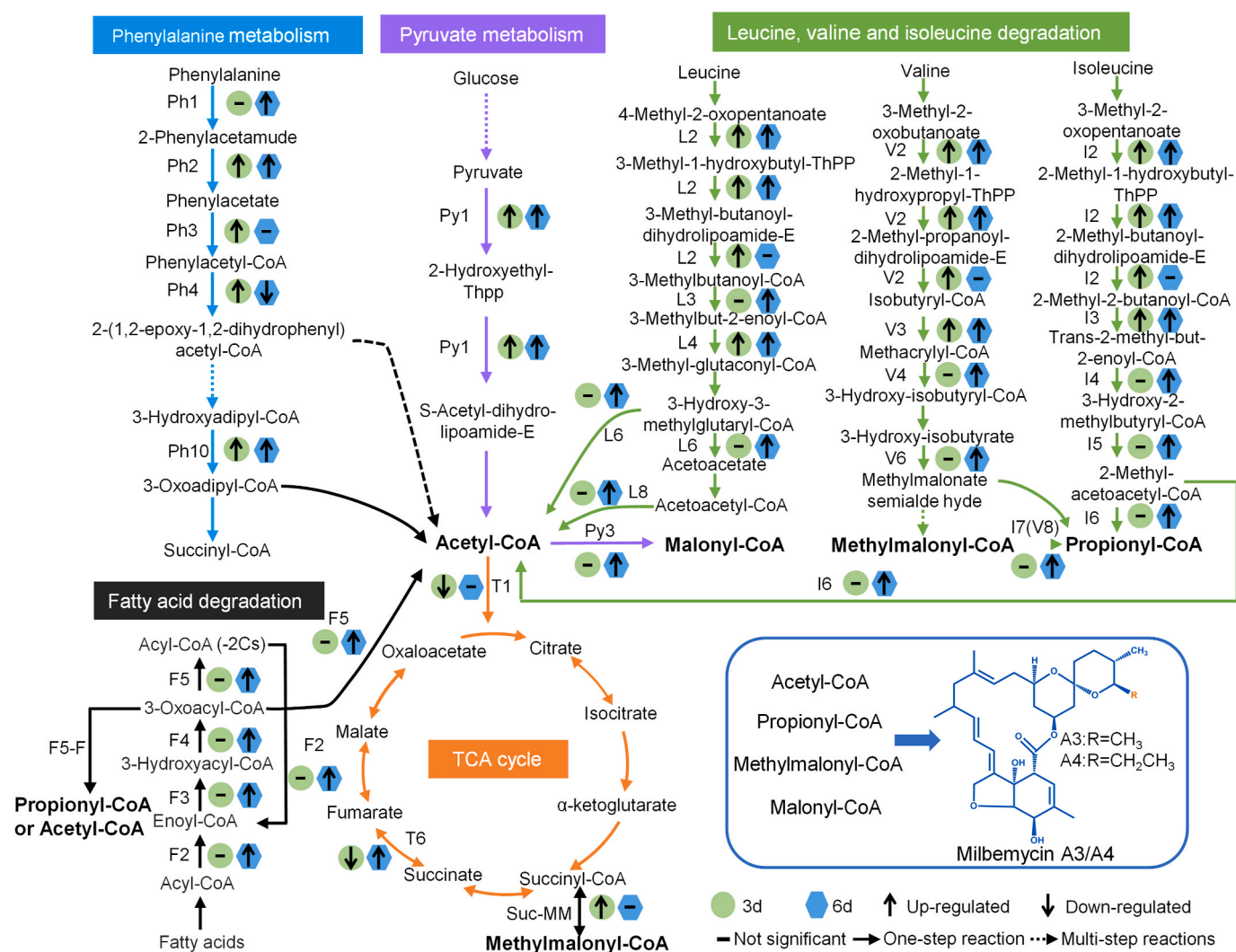

Fig. 4. Schematic view of alterations in the primary metabolism, in which SDEGs are involved, based on the transcriptome analysis of strains BC-101-4 and ΔsbrH1-R. Some reaction steps and primary metabolites are omitted for simplification. Precursors for milbemycin biosynthesis were in bold. Ph, Py, L, V, I, F and T represent the reaction steps involved in phenylalanine metabolism pathway, pyruvate metabolism pathway, leucine, valine and isoleucine catabolism pathway, fatty acid degradation pathway and TCA cycle, respectively. F5–F means the acyl-CoA (acetyl-CoA for even fatty acid or propionyl-CoA for odd fatty acid) generated after the final β-oxidation cycle.

step of the TCA cycle, can also be isomerized by methylmalonyl-CoA mutase (MCM) to generate methylmalonyl-CoA, another major extension unit of milbemycin biosynthesis. Indeed, we found that the MCM genes *sbi_01774*, *sbi_01775*, and *sbi_04768* were significantly upregulated at day 3 (Fig. 4 and Dataset S3), potentially leading to increased supply of methylmalonyl-CoA into milbemycins.

For other central carbon pathways including glycolysis and the pentose phosphate pathway (PPP), although *sbrH1-R* deletion resulted in significant changes in the expression of some genes, most of these trends are generally inconsistent and their effects on the corresponding pathways are not significant (Dataset S4). Glycolysis is essential for glucose degradation and utilization, while the PPP is the major pathway for NADPH generation. Therefore, it is possible that when the *sbrH1-R* mutant begins to produce milbemycins, the reduced carbon flux toward the TCA cycle caused the slow cell growth and directed more acetyl-CoA flux into the milbemycin biosynthesis pathway; meanwhile, the active glycolysis and PPP pathways ensured efficient supply of acetyl-CoA and NADPH, thus facilitating efficient production of milbemycins.

3.3.4. Effect of sbrH1-R deletion on genes related to fatty acid and amino acid metabolism

Except for the central carbon metabolism, other primary metabolic pathways such as fatty acid and amino acid metabolism are also closely related to the generation and consumption of polyketide precursors.

In ΔsbrH1-R, for the fatty acid degradation pathway, two genes (*sbi_07154* and *sbi_08381*) responsible for activating fatty acids by thioesterification and most of the genes involved in the β-oxidation cycle were significantly upregulated at day 6 (Fig. 4 and Dataset S3). Fatty acid degradation is known to provide large amounts of acetyl-CoA, propionyl-CoA (the product generated from the last step of odd fatty acid degradation) and NADH. Therefore, it is possible that the enhancement of the fatty acid degradation pathway could provide precursors and cofactors for milbemycin biosynthesis. Additionally, propionyl-CoA generated from odd fatty acid catabolism can also be catalyzed by propionyl-CoA carboxylase (PCC) to produce the extender unit methylmalonyl-CoA. We found that genes *sbi_04601* and *sbi_06921*, encoding PCC components were also significantly upregulated at the 6th day in ΔsbrH1-R (Fig. 4 and Dataset S3), indicating that the reaction from propionyl-CoA to methylmalonyl-CoA was also enhanced.

Fatty acid biosynthesis pathway can compete with milbemycin biosynthesis for the same precursors, such as acetyl-CoA (for even carbon fatty acids), propionyl-CoA (for odd chain fatty acids), and malonyl-CoA. A total of 26 genes in this pathway were significantly differentially expressed. However, of the 26 genes, 14 were upregulated and 12 were downregulated, making it difficult to evaluate the effect of *sbrH1-R* deletion on fatty acid biosynthesis (Dataset S4).

The amino acid metabolism pathways, particularly the branched chain amino acid (BCAA: leucine, valine, and isoleucine) degradation, phenylalanine degradation and arginine biosynthesis pathways were significantly affected by deletion of *sbrH1-R*. BCAA degradation could provide precursors such as acetyl-CoA, methylmalonyl-CoA, and propionyl-CoA for polyketide antibiotics. The transcriptome data showed that more than half of the genes participating in BCAA degradation pathways showed a significant upregulation pattern in ΔsbrH1-R (Fig. 4 and Dataset S3), indicating the strengthened catabolism and a speculated accumulation of biosynthesis precursors. According to the KEGG analysis of the *S. bingchenggensis* genome, phenylalanine degradation can produce acetyl-CoA and succinyl-CoA, and thus may generate the carboxylated malonyl-CoA and the isomerized methylmalonyl-CoA. This pathway requires a total of 11 reaction steps. We found that in ΔsbrH1-R, among the 11 steps, five showed significant upregulation; one showed a weak upregulation (here it means that the total expression level of the isoenzyme genes in this step was upregulated), although one gene (*sbi_06292*) involved in this reaction was significantly downregulated (Fig. 4 and Dataset S3). We also noticed that in the fourth reaction step, all isozyme genes were upregulated at day 3 but down-

regulated at day 6 (Dataset S3). We speculate that the effective accumulation of gene products in the early fermentation stage could guarantee the smooth process of degradation and might not affect the overall enhancement of the phenylalanine degradation pathway. Collectively, the phenylalanine degradation pathway was enhanced in ΔsbrH1-R, which might increase the supply of acetyl-CoA and succinyl-CoA and indirectly increase the levels of malonyl-CoA and methylmalonyl-CoA (Fig. 4 and Dataset S3).

In ΔsbrH1-R, most of the genes involved in the pathway from glutamate to arginine and fumarate biosynthesis were significantly upregulated at day 6 (Dataset S4). In fact, the aforementioned step from succinate to fumarate in the TCA cycle was also significantly upregulated at day 6. Both pathways suggested an enhancement of fumarate production, but the physiological effect of such a phenomenon needs further investigation.

3.3.5. Effects of sbrH1-R deletion on other PKS/PKS-NRPS genes

Generally, PKS/PKS-NRPS genes may also compete with milbemycin biosynthesis for acyl-CoA precursors. In ΔsbrH1-R, 20 PKS/PKS-NRPS genes (except for PKS genes of the milbemycin BGC) belonging to seven clusters exhibited significant changes in their expression levels (Dataset S5); among them, two were upregulated, 17 were downregulated and one showed an opposite expression trend (downregulated at day 3 and upregulated at day 6), indicating the major activator role of SbrH1-R in the expression of PKS/PKS-NRPS genes. Downregulation of PKS/PKS-NRPS genes may direct precursor flux toward milbemycin biosynthesis, so we carefully analyzed the possible effects of the 17 downregulated PKS/PKS-NRPS genes on milbemycin production. We first noted that one PKS-NRPS cluster gene *sbi_00151* showed high expression level in the parental strain, while no transcripts were detected after *sbrH1-R* deletion. So *sbi_00151* may compete for precursors with milbemycin biosynthesis; indeed, *sbi_00151* deletion promoted milbemycin production (our unpublished data). Among the 17 downregulated PKS/PKS-NRPS genes, 10 are nanchangmycin biosynthesis genes, while a previous report showed that deletion of the nanchangmycin biosynthesis genes does not influence milbemycin production [20]. Moreover, five PKS/PKS-NRPS genes involved in oxazolomycin production and one PKS-NRPS gene were completely unexpressed after *sbrH1-R* deletion; however, expression of them was extremely low (FPKM < 6), thus their effects on milbemycin production could be negligible. Collectively, these results indicate that SbrH1-R differentially regulates expression of some PKS/PKS-NRPS clusters and may repress milbemycin production via promoting expression of *sbi_00151*.

3.3.6. Construction of various precursor supply modules and their effects on milbemycin production

The abovementioned comparative transcriptome data analysis revealed the enrichment of genes related to several major primary metabolic pathways and one PKS biosynthesis pathway that could generate or consume precursors after *sbrH1-R* deletion. In short, genes related to reaction steps such as from pyruvate to acetyl-CoA, from acetyl-CoA to malonyl-CoA, from succinyl-CoA to methylmalonyl-CoA, and from propionyl-CoA to methylmalonyl-CoA and those involved in pathways such as fatty acid catabolism, BCAA catabolism and phenylalanine degradation pathways were significantly upregulated (Fig. 5A and Dataset S3). Enhancement of these pathways may facilitate the accumulation of various acyl-CoA precursors. Moreover, abolished expression of the PKS gene (*sbi_00151*) and the obvious downregulation of *gltA* (*sbi_07003*), encoding a rate-limiting enzyme for the first step of the TCA cycle, may reduce the consumption of acyl-CoA precursors and direct the flux toward antibiotic production. We speculate that these significant changes in the corresponding metabolic pathways (steps) may optimize the precursor flux, allowing cells to produce milbemycins efficiently. To verify our speculation, the concentrations of the four main precursor molecules for milbemycin biosynthesis in strains BC-101-4
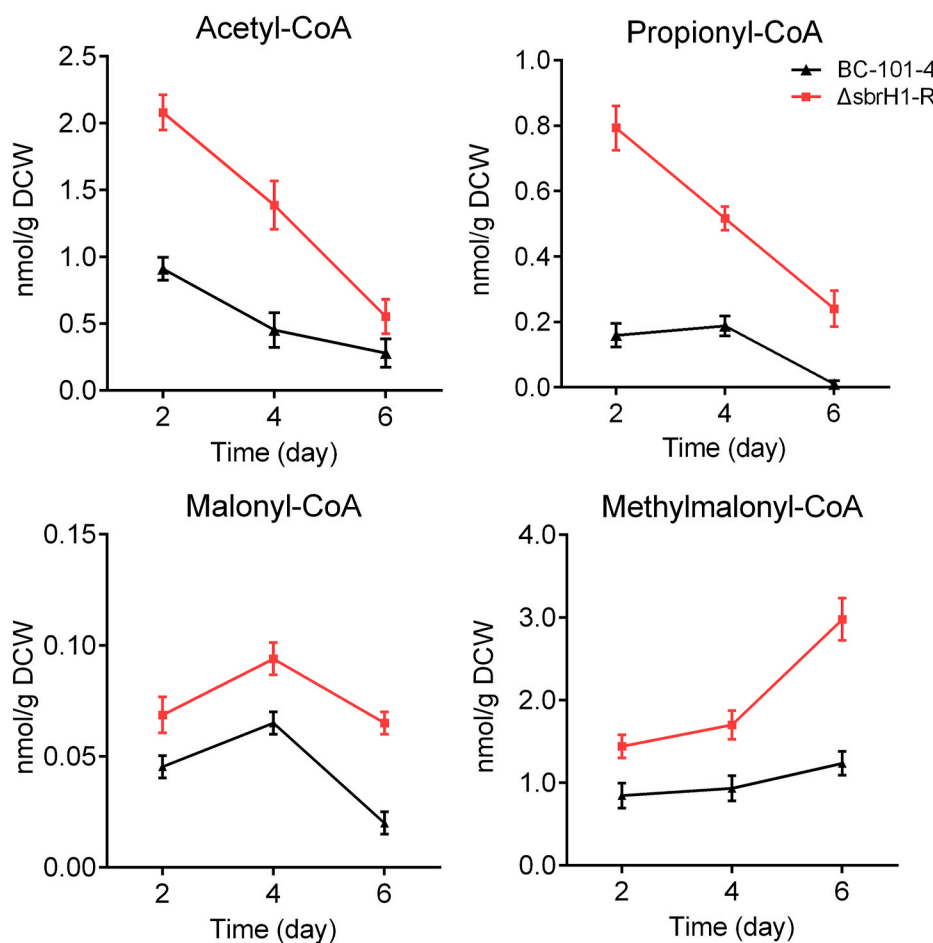
Fig. 5. Comparison of concentrations of milbemycin biosynthesis precursors between strains BC-101-4 and ΔsbrH1-R. All data were obtained from three independent experiments.

and ΔsbrH1-R were determined. As expected, the levels of four precursors were all significantly higher in ΔsbrH1-R than in BC-101-4 (Fig. 5), further confirming that the primary pathways of ΔsbrH1-R were indeed tuned to efficiently supply precursors for milbemycin biosynthesis. Therefore, these significantly altered metabolic pathways may be candidate targets to improve milbemycin overproduction.

In fact, individual enhancement of the reaction steps such as from acetyl-CoA to malonyl-CoA [27], from succinyl-CoA to methylmalonyl-CoA, and from propionyl-CoA to methylmalonyl-CoA and deletion of the PKS gene (*sbi_00151*) have been proved useful in improving milbemycin production (our unpublished data), while the impact of the other pathways on milbemycin production needs further confirmation. To determine whether these precursor-associated metabolic pathways could influence milbemycin production, we intended to construct the pathway modules. In each module, the expression levels of multiple genes from the same pathway were either enhanced or suppressed. Prior to construction of these modules, we optimized the construction process at three aspects: (1) some reaction steps may have multiple isozyme genes, but we only selected those with both significant changes and a relatively high expression (FPKM > 20) for module construction; (2) the reaction from acetyl-CoA to citrate could compete with polyketide production for acetyl-CoA, so we intended to use CRISPRi to build a module that could supress the reaction; (3) according to KEGG metabolic pathway analysis, most of the genes involved in the fatty acid β-oxidation process might also participate in BCAA catabolism, so the β-oxidation process and BCAA catabolism can be included in one module. The promoter of *sbi_03553* (P$_{sbi\_03553}$) with a moderate expression strength and a similar transcription profile to that of milbemycin BGC

was used to drive gene expression (Fig. S7). A total of four modules were designed and the detailed construction diagram was shown in Fig. 6A; these four modules were then individually introduced into the high-producing strain BC04 to obtain strains BC04/Pyr, BC04/FBCAA, BC04/Phe, and BC04/RgltA. Meanwhile, plasmids pSET152 and pSETddCpf1 were also introduced into BC04 to construct the control strains BC04/C1 and BC04/C2. These strains together with the parental strain BC04 were fermented and tested for milbemycin production. The results showed that the titer of milbemycin A3/A4 was improved by 14.7%, 13%, 26% and 21.3%, respectively, indicating that the enhancement or suppression of these pathways could contribute to milbemycin production (Fig. 6B). These results showed that SbrH1-R could modulate milbemycin production via influencing multiple primary metabolic pathways.

3.3.7. Effects of sbrH1-R deletion on milbemycin exporter genes

In the process of investigating the effect of SbrH1-R on milbemycin production, we also found that deletion of *sbrH1-R* resulted in a significant increase of extracellular milbemycin titer in the fermentation medium, reaching 443.3 mg/L, compared with 13.3 mg/L in BC-101-4 (Fig. 7A). This suggested that there may be some significantly upregulated exporter systems capable of excreting milbemycins after deletion of *sbrH1-R*. Based on substrate analysis, a total of 12 putative multiple/specific drug exporter genes (log$_{10}$ FPKM > 1) belonging to eight exporter systems (e.g., SBI_00839/840, SBI_00861, SBI_02810, SBI_01690/1691, SBI_05053/5054, SBI_06224, SBI_07249 and SBI_07695/7696) showed significant upregulation after *sbrH1-R* deletion (Fig. 7B and Dataset S6). Among the eight exporter systems, the
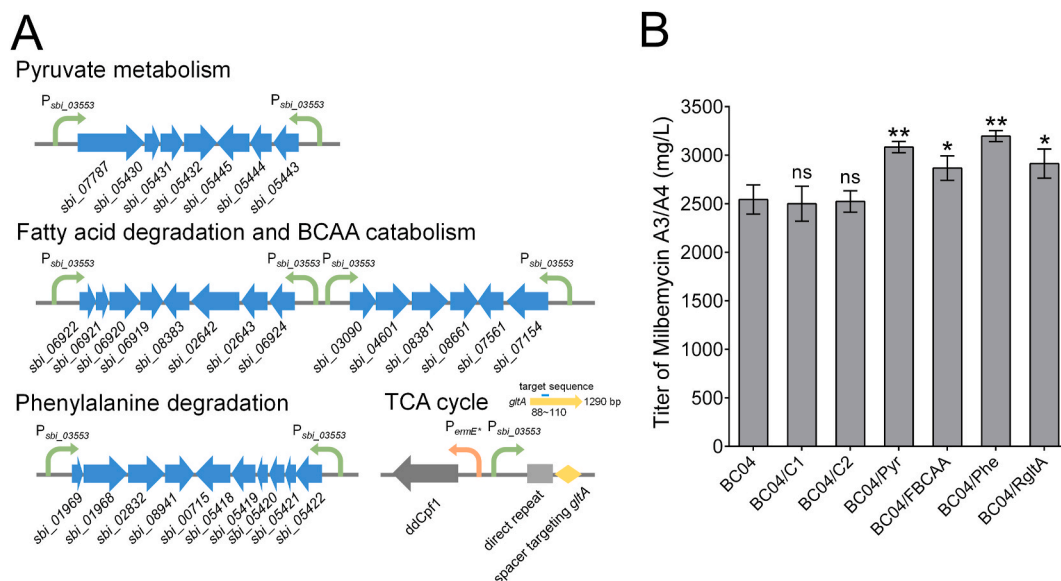
Fig. 6. Effects of overexpression of primary metabolic pathway modules on milbemycin production. (A) Schematic illustration of construction of primary metabolic pathway modules under the control of *sbi_03553* promoter. (B) Performance of pathway engineering on titer improvement of milbemycins. BC04/C1, BC04 with pSET152; BC04/C2, BC04 with pSETddCpf1; BC04/Pyr, overexpression of "pyruvate metabolism module" in BC04; BC04/FBCAA, overexpression of "fatty acid degradation and BCAA catabolism module" in BC04; BC04/Phe, overexpression of "phenylalanine degradation module" in BC04; BC04/RgltA, repression of *gltA* in BC04. All data were obtained from three independent experiments. *P*-values were determined by Student's t-test. *$*P < 0.05$, **$**P < 0.01$ and ns, not significant.

ATP-binding cassette (ABC) exporter system SBI_00839/840 (MiltAB2) has proved useful in promoting the total and extracellular milbemycin production [23], while the functions of the others were unknown. We then individually overexpressed the remaining seven exporter systems using the strong constitutive *hrdB* promoter (P_{hrdB}) and evaluated their contribution to milbemycin efflux. The results showed that only overexpression of SBI_01690/1691 (named MilEX2), an ABC exporter system pair, showed an obvious contribution to milbemycin production, leading to 23.9% and 250% increases in total and extracellular milbemycin A3/A4 production, respectively. These results indicated that SbrH1-R also regulates milbemycin production by influencing the expression of milbemycin exporter system genes.

4. Discussion

The regulation of antibiotic biosynthesis in *Streptomyces* is rigorous and is mainly performed by different families of regulatory proteins (CSRs and pleiotropic regulators) and various environmental and physiological factors [1]. The molecular mechanisms of regulators in control of the transcription levels of antibiotic BGCs have been extensively studied, thus providing ways for antibiotic overproduction or activation [1,4,6,28]. It is generally acknowledged that regulators, especially the pleiotropic ones that affect both antibiotic production and cell growth, may regulate antibiotic production through not only the control of expression of BGCs but also many other ways, such as control of precursor and/or cofactor supply, nutrient absorption, and antibiotic resistance [10]. However, studies on these aspects are relatively scarce, hampering the practical use of regulator-based strategies in the construction of industrial higher-titer strains. In this work, we screened a four-component system SbrH1-R, including the TCS SbrKR and the HPs SbrH1 and SbrH2, by analyzing the time-course transcriptome data collected during milbemycin production. This system showed a similar expression profile as the milbemycin BGC, and the expression levels of *sbrH1* and *sbrH2* were the highest among all the annotated genes from the genome. We found that this four-component system is essential for morphological development and can negatively regulate milbemycin biosynthesis by controlling expression of BGCs, precursor supply-associated pathway genes, and milbemycin exporter genes

(Fig. 8).

Transcriptomics can be applied to comprehensively identify SDEGs and the corresponding cellular physiological processes controlled by a specific transcriptional regulator, providing clues for mining candidate targets affecting desired product production from the complex regulatory networks. Further genetic manipulation of selected SDEGs will help to determine targets influencing production levels. Therefore, transcriptome analysis and genetic manipulation can be used together to systematically mine factors affecting antibiotic production, which will improve our understanding of the complex regulatory network underlying antibiotic production. Interestingly, comparative transcriptome analyses combined with genetic manipulation have been successfully applied to identify target genes that are beneficial for the biosynthesis of desired product. In *E. coli*, three free fatty acids (FFA)-overproducing strains (*ihf*A^{L-}, *fad*R^{H-} and *fad*R^{+}) and the control strain were applied for comparative RNA-seq analysis to explore potential gene targets associated with FFA production [29]. Fifteen differentially expressed genes in these three high FFA-producing strains were selected and tested for FFA production. The results showed that 10 of the 15 genes enhanced FFA production by more than 20% compared with that in the parental strain [29]. RNA-seq analysis of *Nannochloropsis gaditana* cultured in nitrogen-limited (lipid overproduction) and nitrogen-replete (a relatively low level of lipid accumulation) media was also performed. Twenty transcriptional regulators showed significant downregulation in nitrogen starvation conditions, among which, a $Zn(II)_2Cys_6$-encoding gene was confirmed to be a repressor of lipid accumulation, and modulating the expression level of this regulator doubled lipid production without decreasing cell growth [30]. In the gram-positive filamentous bacteria *Streptomyces avermitilis*, an important industrial avermectin producer, comparative transcriptome analysis of the high avermectin producer 76-02-e and the wild-type strain ATCC31267 combined with traditional genetic experiments identified three negative regulators (SAV151, SAV577 and SAV576) and one positive regulator (SAV4189) of avermectin biosynthesis, providing new targets that can be engineered to enhance avermectin production [31,32]. Here, RNA-seq analysis of the *sbrH1-R* mutant and the parental strain BC-101-4 combined with experimental confirmation identified five previously unidentified precursor supply-associated
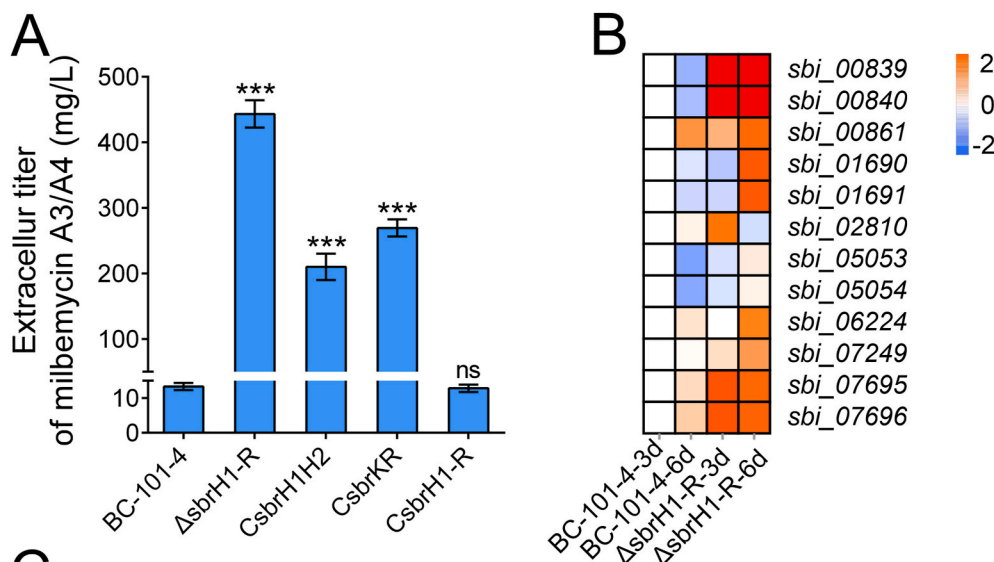
Fig. 7. Mining and confirmation of MilEX2 beneficial for milbemycin production. (A) Comparison of extracellular milbemycin production among BC-101-4, ΔsbrH1-R, CsbrH1H2, CsbrKR and CsbrH1-R. (B) Heatmap analysis of 12 putative multiple/specific drug exporter genes showing significant upregulation in ΔsbrH1-R. (C) Effects of candidate exporter overexpression on total and extracellular titer of milbemycin A3/A4. BC04/MilEX1, overexpression of *sbi_00861* in BC04; BC04/MilEX2, overexpression of *sbi_01960/1961* in BC04; BC04/MilEX3, overexpression of *sbi_05053/5054* in BC04; BC04/MilEX4, overexpression of *sbi_07249* in BC04; BC04/MilEX5, overexpression of *sbi_02810* in BC04; BC04/MilEX6, overexpression of *sbi_06224* in BC04; BC04/MilEX7, overexpression of *sbi_07695/7696* in BC04. Data shown were the averages and standard deviations. All data were obtained from three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ns, not significant.
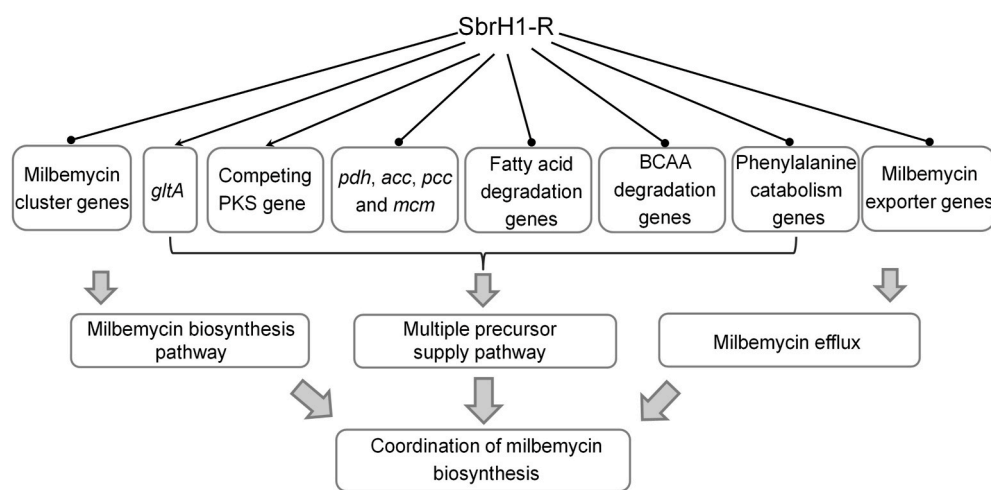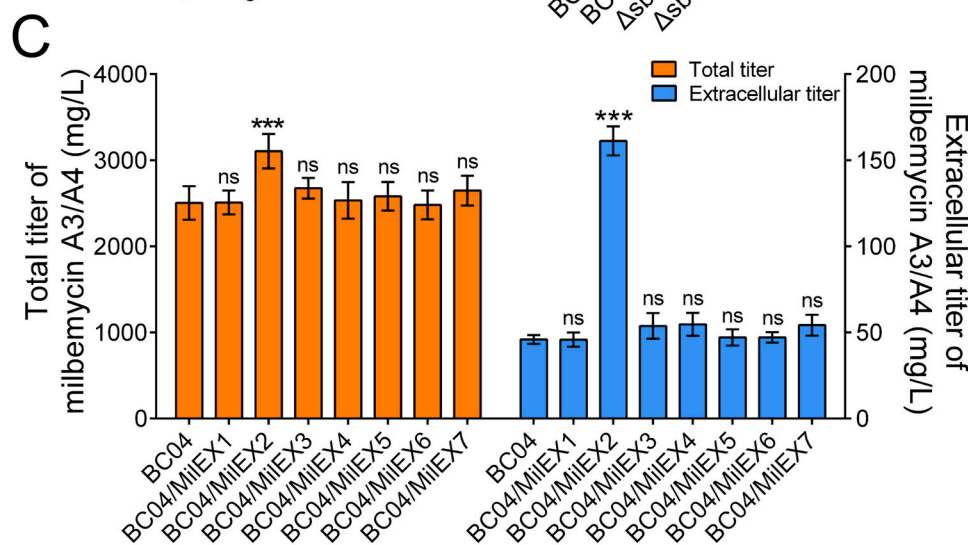
Fig. 8. SbrH1-R modulates milbemycin biosynthesis via multiple pathways. Black line with a dot at the end represents the repressive effect, while black line with an arrow at the end represents the positive effect.

reactions/pathways, including the reaction from pyruvate to acetyl-CoA, the reaction from acetyl-CoA to citrate, the fatty acid β-oxidation pathways, the BCAA degradation process, and the phenylalanine acid degradation pathway, which can influence milbemycin production. We also identified a previously unreported milbemycin exporter, MilEX2, whose overexpression contributes to

milbemycin overproduction. In sum, these results reveal multiple ways via which SbrH1-R influences antibiotic production, deepen our understanding of the complex regulatory networks underlying milbemycin production, and finally provide effective targets and corresponding engineering approaches to boost the production of milbemycins and other polyketide antibiotics.

In *Streptomyces*, many TCSs play important roles in antibiotic biosynthesis and even cell growth, such as the highly conserved and well-studied TCSs PhoRP, DraR-K, and MtrAB, among others [33–35]. In most reports, researchers focused on deciphering the downstream regulatory cascades of TCSs; the DNA-binding protein RR of the TCS binds to the promoter region of CSR genes and controls antibiotic production by directly influencing expression of BGCs. For example, DraR of DraR-K positively controls actinorhodin production by binding to the promoter of the CSR gene *actII-ORF4* in *S. coelicolor* [36]. However, *Streptomyces* genomes still contain large numbers of functionally unknown TCS-coding genes [33,37]. Moreover, *Streptomyces* genomes also contain numerous HP genes with unknown functions [38,39]. For example, there are 3694 (47% of the total annotated genes) HP genes in *S. coelicolor* and 3604 (36% of the total genes) in *S. bingchenggensis* [39, 40]. Identifying functions of these HPs would strengthen our knowledge about *Streptomyces* genetics. After careful analysis, we found that some HP genes are found located alongside the TCSs (Table S1). Genes in one operon or clustered together may have similar or related functions, so what are the biological functions of these TCS-adjacent HPs and whether their roles are associated with the function of TCS require further investigation. In the present work, we found that both the HPs SbrH1H2 and the TCS SbrKR are indispensable for *sbrH1-R*'s function, and they may act synergistically in the control of morphological development and milbemycin production. The individual contributions of SbrH1 and SbrH2 in this synergistic regulatory way and the possible interactions among the four components are unclear and remain to be determined. Collectively, these results expand our understanding of the TCS regulatory networks and provide methodological guidance for dissecting the functions of HP genes that are widely distributed in *Streptomyces*.

It is clear that SbrH1-R drastically affects morphological development, milbemycin production and cell growth in *S. bingchenggensis*. Repression of milbemycin production by SbrH1-R should be the coordinative effects of changes in multiple physiological processes, including at least the milbemycin biosynthetic pathway, various precursor supply-associated pathways, and antibiotic exporters (Fig. 8). However, most of the targets identified here that are beneficial for milbemycin production are restricted to primary metabolism and are already presumed to be relevant to the generation and consumption of precursors before experimental confirmation. Moreover, only a relatively small portion of SDEGs in ΔsbrH1-R are predicted to be involved in primary metabolism. The functions of most SDEGs are unknown, and their correlation with milbemycin production could not be inferred by bioinformatics analysis, but their potential roles should not be ignored. In the future, omics analysis of strains with different performance together with a rapid and high-throughput screening method (such as the use of a genome-scale sgRNA library) is needed to determine the functions of these unknown SDEGs and to evaluate the corresponding effects on milbemycin production. This is of major importance to fully decipher the SbrH1-R regulatory network and to explore the strain's potential for milbemycin overproduction.

CRediT authorship contribution statement

Lan Ye: Methodology, Investigation, Writing – original draft, Project administration. **Yanyan Zhang:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **Shanshan Li:** Investigation, Visualization, Funding acquisition. **Hairong He:** Investigation, Visualization. **Guomin Ai:** Performed the GC–MS analyses. **Xiangjing Wang:** Investigation, Visualization, Funding acquisition. **Wensheng**

Xiang: Conceptualization, Resources, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Wensheng Xiang, Yanyan Zhang, Lan Ye, Shanshan Li have filed two patents related to the data in this manuscript. All other authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2022.02.003.

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