MilR3, a Unique SARP Family Pleiotropic Regulator in *Streptomyces Bingchenggensis*

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Research Article

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Abstract

Streptomyces bingchenggensis is the main industrial producer of milbemycins, which are a group of 16-membered macrocylic lactones with excellent acaricidal, insecticidal and anthelmintic activities. In past decades, scientists tried a lot to solve its low productivity. But less understanding the regulatory network of milbemycin biosynthesis limited the generation of high producing strains with regulatory rewiring approach. SARPs（***S****treptomyces* **A**ntibiotic **R**egulatory **P**roteins）family regulators are widely distributed and play key roles in regulating antibiotics production in actinobacteria. In this paper, MilR3 (encoded by *sbi_06842*) has been screened out for significantly affecting milbemycin production from all the 19 putative SARP family regulators in *S. bingchenggensis* with the DNase-deactivated Cpf1-based integrative CRISPRi system. Interestingly, *milR3* is about 7 Mb away from milbemycin biosynthetic gene cluster and adjacent to a putative type II PKS （the core minimal PKS encoding genes are *sbi_06843, sbi_06844 and sbi_06845*）gene cluster, which was proved to be responsible for producing a yellow pigment. The quantitative real-time PCR (qRT-PCR) analysis proved that MilR3 positively affected the transcription of *milR*, *milA1*, *milA2*, *milA4*, *milF*, *milR3* and *sbi_06844* in both clusters. Unlike previous “small” SARP family regulators played pathway specific roles, MilR3 was probably a unique SARP family regulator and played a pleotropic role. MilR3 was an upper-level regulator in MilR3-MilR cascade. This study firstly illustrated the co-regulatory role of this unique SARP regulator. This greatly enrich our understanding of SARPs and lay a solid foundation for milbemycin yield enhancement in the near future.

Key Points

- A “small” SARP protein was firstly verified as a pleiotropic regulator.
- An indispensable activator was identified for producing milbemycin.
- Cpf1-CRISPRi system can be a high-throughput tool for mining new regulators.

Introduction

Milbemycins, a group of 16-membered macrolide antibiotics with unique action mode on nervous system of insects and parasites, have been widely used as acaricides, insecticides and anthelmintics for excellent high and broad spectrum of activities, harmless to human and animal, environmentally friendly character, as well as (Danaher et al. 2012; Jacobs and Scholtz 2015). Milbemycins are structurally and chemically similar to the well-known anthelmintic agent avermectins (Chen et al. 2016). Currently, several products derived from the most active components milbemycin A3 and A4, including milbemectin, milbemycin oxime, lepimectin and latidectin, have been marketed and applied in agriculture, animal husbandry and medical industry (Bienhoff et al. 2013; Kim et al. 2016; Nicastro et al. 2011; Pluschkell et al. 1999) (Fig. 1a). Significantly, due to higher insecticidal activity than avermectins, milbemectin is a more effective insecticide against insects resistant to avermectin (Romero-Rodríguez et al. 2015).

Aside from *Streptomyces hygroscopicus*, *Streptomyces griseochromogenes*, *Streptomyces cyaneogriseus* and *Streptomyces nanchangensis*, *Streptomyces bingchenggensis* has been developed and used as the

main industrial strain of milbemycins (Baker et al. 1996; Carter et al. 1988; Ono et al. 1983; Wang et al. 2010). Presently, the milbemycin biosynthetic gene cluster (*mil* cluster) has been identified in the genome of *S. bingchenggensis* (Kim et al. 2017; Wang et al. 2013). The *mil* cluster includes eleven genes encoding four milbemycin polyketide synthases (MilA1, MilA2, MilA3 and MilA4), four enzymes for polyketide modification (MilC, MilD, MilE and MilF), one LuxR family transcriptional factor (MilR) and two unknown function proteins (Orf1 and Orf2) (Fig. 1b). Unlike avermectin biosynthetic gene cluster, the *mil* cluster is separated by 62-kb DNA fragment between *milR* and *milA1* which is not required for milbemycin biosynthesis.

Based on some investigations of biosynthesis avermectin and meilingmycin, the biosynthetic pathway of milbemycin has been deduced (He et al. 2010; Nonaka et al. 1999a, b). Seven malonyl-CoA and five methylmalonyl-CoA moleculars are condensed to start units acetyl-CoA or propionyl-CoA, which are catalyzed by four giant polyketide synthases to form polyketide backbone in step-by-step procedure. Subsequently, the polyketide backbone is spontaneously formed spiroketal and furan ring, which are post-modified by tailoring enzymes to form the final milbemycin components. The two tailoring enzymes, C5-*O*-methyltransferase and C5-keto reductase encoded by *milD* and *milF*, were responsible for the methylation of C5-hydroxyl and reduction of C5-keto groups of milbemycinA3/A4 (Nonaka et al. 2010; Wang et al. 2014; Zhang et al. 2013). Recently, another tailoring enzyme MilE was identified to catalyze furan ring forming from precursors of milbemycin A3/A4 (Wang et al. 2020).

In past years, many engineering strategies have been made for milbemycin yield enhancement, such as optimization of fermentation, random mutations, metabolic engineering, as well as combinational biosynthesis (Kim et al. 2017; Liu et al. 2021; Wang et al. 2009; 2014; 2020; Wei et al. 2018). However, the current production of milbemycins in *S. bingchenggensis* is also much lower than that of avermectins in *S. avermitilis* (Jin et al. 2020). So far, only four regulators have been identified being involved to milbemycin biosynthesis in *Streptomyces*, including the clustered-specific activator MilR, two global regulators NsdA and SbbR, and a novel regulator MilR2 involved in 5-oxomilbemycin A3/A4 (He et al. 2018; Wang et al. 2006; Wei et al. 2018; Zhang et al. 2016). The limited knowledge about the regulation of milbemycin biosynthesis has caused some bottlenecks for milbemycin production enhancement by metabolic engineering approach.

Streptomyces antibiotic regulatory proteins (SARPs) have been exclusively found in actinobacteria and most of them within *Streptomycetes*. SARP encoding genes are mostly located within their target biosynthetic gene cluster (BGC) and act as pathway-specific activators (Bibb 2005). SARPs are characterized by a winged helix-turn-helix (HTH) DNA binding motif near the N terminus and bacterial activation domain (BTAD) at the C-terminal (Liu et al. 2013; Wietzorrek and Bibb 1997). The SARPs can be divided into “small” and “large” group according their lengths. “Small” SARP-type activators only contain the HTH DNA binding and BTAD domain. The ActII-ORF4 and RedD from *Streptomyces coelicolor* (Arias et al. 1999; Narva and Feitelson 1990), DnrI from *Streptomyces peucetitus* (Sheldon et al. 2002), Aur1PR2 and Aur1PR3 from *Streptomyces aureofaciens* (Novakova et al. 2005), FdmR1 from in *Streptomyces griseus* (Chen et al. 2008), TylS from *Streptomyces fradiae* (Bate et al. 2002), SrrY and SrrZ

from *Streptomyces rochei* (Suzuki et al. 2010) are all belonged to small group. And they have been proved as pathway specific regulators. The “large” SARPs have extra ATPase and TPR (Tetratricopeptide Repeat) domain at their C-terminal, which are responsible for sensing endogenous signals (such as ADP/ATP pool) and regulating the activity of SARPs (Liu et al. 2013). Most of “large” SARP-type activators including SanG for nikkomycin in *Streptomyces ansochromogenes* (He et al. 2010), PloR for polyoxin in *Streptomyces cacaoi* (Li et al. 2009), and PimR for pimaricin in *Streptomyces natalensis* (Antón et al. 2004) also act as pathway specific regulators. However, only AfsR, a member of “Large” SARPs, has been proved as a pleiotropic regulator. AfsR affects the expression of *afsS* gene, which encodes a small protein (63 amino acids) controlling the *act* and *red* cluster by binding the upstream of *actII-ORF4* and *redD* (Lee et al. 2002; Tanaka et al. 2007). Phosphorylation of AfsR at serine/threonine residues by the AfsK kinase significantly increases the binding affinity of AfsR to the *afsS* promoter. Also, AfsK/AfsR proteins serve to integrate the signal of S-adenosylmethionine (SAM) concentration level (Horinouchi 2003).

Li *et al* have developed CRISPR-Cpf1-assisted multiplex genome editing and transcriptional repression system in *Streptomyces* (2018). They proved that DNase-deactivated Cpf1(ddCpf1)-based integrative CRISPR interference system (CRISPRi) can work efficiently in the milbemycin producing strain. Here, this system was employed to screening crucial regulators of milbemycin biosynthesis with targeting each of 19 putative SARP family regulators in *S. bingchenggensis*. As a “Small” SARP-type activator, MilR3 has been figured out as a unique pleiotropic regulator of BGCs in *S. bingchenggensis*.

Materials And Methods

Bacterial strains, plasmid, and culture conditions

All strains and plasmids used in this study are listed in Table S1. *S. bingchenggensis* TMB is derived from *S. bingchenggensis* CGMCC 1734 by single colony isolation with stable milbemycin production.. *S. bingchenggensis* TMB and its derivatives were grown on mannitol soya flour (MS) medium (mannitol 20 g/l, soybean power 20 g/l, agar 20 g/l, pH 7.2) at 28 ℃ for sporulation. For *S. bingchenggensis* fermentation and milbemycin A3/A4 production, strains were cultured in seed medium (sucrose 10 g/l, skim milk power 1g/l, tryptone 3.5 g/l, yeast extract 5 g/l, K_2HPO_4 $3H_2O$ 0.5 g/l, pH 7.2) at 28 ℃ for 44 h on a rotary shaker at 250 rpm. Then, 4 ml of seed culture was transferred into 250 ml Erlenmeyer flasks containing 25 ml fermentation medium, including sucrose 80 g/l, soybean power 20 g/l, skim milk power 1 g/l, $CaCO_3$ 3 g/l, K_2HPO_4 1 g/l, and $FeSO_4$ $7H_2O$ 0.1 g/l, pH 7.2. The fermentation was carried out at 28 ℃ for 9 days at 250 rpm. ISP4 medium was used for intergeneric conjugation between *Escherichia coli* S17-1 and *S. bingchenggensis*. *E. coil* strains were aerobically grown on LB agar plates or in LB liquid medium at 37 ℃. *E. coil* DH10B was used as a general cloning host. *E. coil* BL21(DE3) was used for protein overexpression. If necessary, the medium was supplemented with the proper amount antibiotics to final concentration, including 50 μg/ml apramycin, 50 μg/ml kanamycin, nalidixic acid 25 μg/ml or 100 μg/ml ampicillin. For the conjugation plates, each over flood with 1 ml water containing 1.25 mg apramycin and 500 μg nalidixic acid.

Construction of recombinant strains

pSETddCpf1 derived vectors for expression repression were constructed as described (Li et al. 2018). The crRNA expression cassettes for 19 putative SARP family regulators were amplified using the CRISPRi-*X*-F primer 5'- GACTAGTN$_{22}$ATCTACAACAGTAGAAA-3' (N_{22} represents the 22-nt gene-specific spacer sequence) and ddCpf1-crRNA-R primer pair. All the primers were listed in Table S2 in the supplementary material. The derived pSETddCpf1-*X* (*X* represented the target gene) was transferred into *S. bingchenggensis* TMB by intergeneric conjugation.

The pKCCpf1 vectors for deletion target genes were constructed by amplification of crRNA expression cassette, upstream fragment and downstream fragment of target gene and assembling with *Nde*I and *Spe*I-linearized pKCCPf1 by Gibson method according procedure described by Li et al (2018). All primer pairs were listed in Table S2 in the supplementary material. The pKCCpf1 derived vectors were introduced into *S. bingchenggensis* TMB by intergenic conjugation. pKCCpf1 derived plasmids were cured from positive conjugants by culture under 37 ºC. Mutants were verified by colony PCR with the proper primer pairs and sequencing the amplified fragment.

For overexpression of genes in *S. bingchenggensis* TMB, target genes were amplified with proper primer pair and cloned into restriction enzyme digested pSET152. The pSET152 derived plasmids was introduced into *S. bingchenggensis* by intergeneric conjugation. Positive conjugants were verified by amplification the apramycin resistance gene.

HPLC analysis of milbemycin A3/A4 production

One-milliliter culture broth was sampled from each fermentation flask at 9 days and extracted with 4 ml methanol for 2 h. The production of milbemycin A3/A4 in the extract was analyzed by high-pressure liquid chromatography (HPLC; 1260 series; Agilent) using 4.6 × 150 - mm Hypersil C_{18} column. The column was developed at a flow rate of 1 mL/min for 15 min with methanol-water (90:10 [vol/vol]) under 28 ℃. Metabolites were monitored at a wavelength of 240 nm. The production was calibrated with standard milbemycin A3/A4 from Zhejiang Hisun Pharmaceutical Inc. (Taizhou, China).

RNA extraction, preparation of cDNA, and quantitative real-time PCR analysis

The RNA was extracted from fermentation cultures at four time points (2 days, 4 days, 6 days and 8 days). RNA was isolated using EASY spin Plus bacterial RNA kit (Aidlab Biotechnologies, Beijing, China) according to the manufacturer's protocol, and subsequently treated with RNA-free DNase (Takara, Dalian, China) to remove the residual genomic DNA. The quantity and quality of RNA samples was analyzed with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

Reverse transcription of total RNA was performed using the PrimeScript^{TM} RT reagent kit according its instruction. Quantitative PCR was performed with TB green *Premix Ex Taq*^{TM} II (Takara, Dalian, China) on a CFX96 Touch Real-Time System (Bio-Rad, USA) by using. The gene *hrdB* (*sbi_03299*, encoding the

principal sigma factor) was used as the internal control. All reactions were conducted in triplicate wells for each RNA sample and repeated with three independent samples of each strain. The relative expression levels of tested genes were normalized to those of *hrdB*. The relative fold changes in the transcription of each gene were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Error bars indicate the standard deviations from three independent biological replicates. The primers used in transcriptional analysis are listed Table S2 in Supplementary Materials.

Results

Identification of SARP family regulators involved in milbemycin biosynthesis in *S. bingchenggensis*

Li *et al* developed a highly efficient transcriptional repression system by CRISPR-ddCpf1 in *S. bingchenggensis* (2018). For SARP family regulators usually involved in biosynthesis of secondary metabolites, all the 19 SARP candidates of *S. bingchenggensis* were tested for their effect on milbemycin production by this ddCpf1-based CRISPRi system. The milbemycin A3/A4 production of all mutants were listed in Fig. 2a. Notably, the titer of milbemycin A3/A4 in TMB/pSETddCpf1-*milR3* (targeting the *sbi_06842*,*sbi_06842* was designed as *milR3* for its effect on the production of milbemycin) was significantly decreased by 87.3% compared to that of TMB/pSETddCpf1.

Phylogenetic relation of these SARPs with several well-studied SARP family regulators showed that these putative SARPs could be divided into three groups (Fig. 2b). MilR3 was close to three well-studied SARP family regulatory proteins: SrrZ from *S. rochei*, FarR3 from *Streptomyces lavendulae* and BulZ from *Streptomyces tsukubaensis* (Kurniawan et al. 2014; Ma et al. 2018; Suzuki et al. 2010). SrrZ and FarR3 have been proved as specific-pathway positive regulators of their adjacent biosynthetic gene clusters. BulZ, encoding gene located in GBL gene cluster, influenced tacrolimus production through regulation of the biosynthesis of GBL by directly binding to the upstream region of *bulS2* (GBL synthetase). Interestingly, *milR3* is located 7.16 MB from the *mil* cluster (*sbi_00726 - sbi_00790*) and adjacent to a putative type II PKS genes (*sbi_06843 - sbi_06846* encoding minimal PKS synthetase). MilR3 probably was a unique SARP regulator in *S. bingchenggensis*.

MilR3 is a pleiotropic regulator in *S. bingchenggensis*

The *milR3* in-frame deletion mutants (designed as △*milR3*) were constructed on basis of the parental strain *S. bingchenggensis* TMB with CRISPR-Cpf1 system. The △*milR3* mutant was further confirmed by PCR and DNA sequencing (Figure S1). The complemented plasmid pSET152-*milR3*, in which *milR3* was cloned in the integrative plasmid pSET152 and its expression was driven by its native promoter, was constructed and then transferred into the △*milR3* mutant and TMB, resulting in the complemented strain C*milR3* and the overexpression strain O*milR3*. As controls, the plasmid pSET152 was introduced into △*milR3* and TMB generating D*milR3* and TMB/pSET152, respectively. Milbemycin A3/A4 production of strains TMB/pSET152, D*milR3*, C*milR3* and O*milR3* were presented in Fig. 3a. Milbemycin A3/A4 production was abolished in D*milR3* and can be restored in C*milR3*. These results clearly demonstrated

that MilR3 played as an indispensable activator for milbemycin production in *S. bingchenggensis* TMB. Milbemycin A3/A4 production in O*milR3* was slightly lower than that of TMB/pSET152(Fig. 3a).

Interestingly, a yellow pigment produced in *S. bingchenggensis* TMB was abolished in D*milR3* strain and restored in C*milR3* strain (Fig. 3b). The yellow pigment production patterns of these four strains were similar both under liquid fermentation and on solid agar plates (Fig. 3c). It seemed that more yellow pigment was produced in the *milR3* overexpression strain. For the *milR3* was adjacent to a putative type II polyketide gene cluster, we proposed that this gene cluster was responsible for production of the yellow pigment.

Obviously, the SARP family protein MilR3 acted as a pleiotropic regulator to modulate not only milbemycin biosynthesis but also the yellow pigment biosynthesis in *S. bingchenggensis* TMB.

The type II PKS was responsible for the biosynthesis of yellow pigments

Four genes (*sbi_06843*, *sbi_06844*, *sbi_06845* and *sbi_06846*) neighboring to *milR3* were predicted to encode putative type II PKS (Fig. 7). The 3297-bp internal fragment in the putative type II PKS encoding genes (from *sbi_06843* to *sbi_06845*) was deleted by CRISPR-Cpf1 system in *S. bingchenggensis* TMB (Figure S2). This mutant was named as D*sbi_06844*., D*sbi_06844* was incapable to produce the yellow pigment on MS plate (Fig. 4a). This situation was in consistent with D*milR3*, indicating that the putative type II PKS encoding genes are responsible for the yellow pigment biosynthesis. The milbemycin A3/A4 yield of D*sbi_06844* strainwas nearly same to that in TMB/pKCCpf1 (Fig. 4b). Meanwhile, the transcription of milbemycin biosynthesis and type II PKS cluster were analyzed through qRT-PCR. The transcriptional time course pattern of *milA2*, *milD*, *milE*, *milF*, *milR* and *milR3* in D*sbi_06844* were similar to that in TMB/pKCCpf1 (Fig. 4c). These results demonstrated that the yellow pigment was uncoupled with milbemycin biosynthesis. And the biosynthesis of yellow pigment was regulated by MilR3.

MilR3 regulates the transcription of targeted genes involved in milbemycin biosynthesis and the yellow pigment biosynthesis

The dynamic transcription of milbemycin and yellow pigment biosynthetic genes of was investigated by quantitative real-time PCR (RT-qPCR) with time course RNA samples of TMB/pSET152, D*milR3*, C*milR3* and O*milR3* strain fermentation culture (2 day, 4 day, 6 day and 8 day). The results showed that transcription of *milA1*, *milA2*, *milA3*, *milA4*, *milC*, *milD*, *milE*, *milF* and*milR* were almost undetectable in D*milR3*, but restored in C*milR3* compared with those of TMB (Fig. 5). Transcription of *sbi_06844* in D*milR3* was expressed extremely lower than that in TMB/pSET152, and restored in C*milR3*. And the time course transcription patterns of tested genes were all similar. These results indicated that MilR3 functions as an indispensable activator for both milbemycin and yellow pigment biosynthetic gene cluster.

However, the transcription of *milR2*,aprevious reported TetR family activator involved in 5-oxomilbemycin A3/A4 biosynthesis was increased in D*milR3.* And its transcription level can restore in the complementary strain C*milR3* (Fig. 5). This indicates that *milR3* had negative effect on the transcription of *milR2*.

The milbemycin biosynthesis was regulated by MilR3-MilR cascade

MilR has been reported as a LAL family (large ATP-binding regulator of the LuxR family) pathway-specific transcriptional activator of milbemycin biosynthesis through specifically binding the promoters of the *milA4-milE* operon and of *milF* with its C-terminal HTH domain (Zhang et al. 2016). The transcription of representative genes involved in milbemycin and yellow pigment biosynthesis was investigated by qRT-PCR analysis of MilR overexpression strain O*milR* (TMB strain carried an integrative plasmid pSET152 containing *milR* driven by a strong constitutive *kasOp** promoter) and wildtype strain in fermentation culture. The transcriptional level of *milR* was up-regulated in O*milR* at each time points. The transcription of previously reported target genes of MilR also showed same tendency in the O*milR* strain. The milbemycin A3/A4 production in O*milR* was increased by 29% compared with that of TMB/pSET152, which was consistent with the results of qRT-PCR (Fig. 6a).

Notably, the expression level of *milR3* in O*milR* was similar to that in TMB/pSET152 (Fig. 6b). It was indicated that transcription of *milR3* was not affected by *milR*. Probably the milbemycin production was regulated through MilR3 to MilR cascade.

Discussion

In this study, MilR3, a SARP family protein, was screened out for its crucial role involved in milbemycin biosynthesis by the ddCpf1-based CRISPR interference (CRISPRi) system in *S. bingchenggensis*. *milR3* was adjacent to a putative type II PKS encoding gene cluster, which was about 7 Mb from the biosynthetic gene cluster of milbemycin and responsible for the biosynthesis of yellow pigment. Further experiments showed that MilR3 was a unique pleiotropic regulator controlling the biosynthesis of milbemycin and yellow pigment in *S. bingchenggensis*. From all these results, we proposed that milbemycin production was probably controlled by MilR3 to MilR regulatory cascade.

The qRT-PCR showed that all genes in the *mil* cluster and *sbi_06844* involved in the yellow pigment biosynthesis were affected by *milR3*. Even using different expression vectors, fusion tag and host strains, we still failed to obtain soluble protein of MilR3 in *E.coil* overexpression. It is still unclear whether *milR3* regulates the transcription of these genes in a direct or indirect way. *In vivo* examination of *β*-glucuronidase (GUS) activities were carried out in mutants with *gusA* gene driven by *milA2*, *milR*, *milA4*, *sbi_06843*, *sbi_06844* and *hrdB* promoter, respectively. Results showed that the promoters of *milA2*, *milR*, *milA4*, *sbi_06843* and *sbi_06844* are probably the targets of MilR3 (Figure S3).

Overexpression of transcriptional activators is an efficient approach to enhance the production of the corresponding antibiotics (Du et al. 2013). But over-production of milbemycin has not been observed whenever overexpression of *milR3* driven by its native promoter or constitutive promoters like *kasOp** and *ermEp** (Fig. 3a, data not shown). In fact, the O*milR3* strain seemingly produced more the yellow pigment on MS plates than TMB strain (Fig. 3a). But yellow pigment can't be quantified for lack of structure information. For both compounds produced by PKS pathway, Milbemycin and yellow pigment shared precursors like malonyl-CoA, so the competition between the clusters probably affect their production.

Furthermore, MilR3 probably has other targets in the genome of *S. bingchenggensis* except for *mil* and the yellow pigment biosynthetic gene cluster. In future, comparative transcriptomic analysis could help to investigate other targets of MilR3 and predict the metabolic flux affected by MilR3. Elucidation of the action mechanism of MilR3 will help to understand the regulation network of milbemycin biosynthesis and lay solid foundation for milbemycin production enhancement. Probably milbemycin production can be promoted by overexpression of MilR3 in the D*sbi_06844* strain.

SARP regulators, which are widespread in *Streptomyces* with varying number, mostly act as pathway-specific activators for both “Small” SARPs and “Large” SARPs except AfsR. Three well-studied “Small” SARP-type regulators, SrrZ, FarR3 and BulZ, are closely homologous to MilR3 (Fig. 2b). SrrZ and FarR3 positively regulate lankamycin and indigoidine biosynthesis through the γ-butyrolactone dependent regulatory cascade in *S. rochei* and *S. lavendulae* FRI-5, respectively (Kurniawan et al. 2014; Suzuki et al. 2010). Interestingly, *srrZ* is adjacent to the *roc* cluster for an unknown type II polyketide compound in the genome of *S. rochei* (Suzuki et al. 2010). When the sequence of *sbi_06840* to *sbi_06846* was blast on antiSMASH server with the sequenced genomes, several clusters showed conserved genes organization pattern (Fig. 7) (Blin et al. 2021). Each SARP-regulators had an adjacent putative Type-II PKS clusters. We hypothesized that these “Small” SARP-type proteins probably played roles as pleiotropic activators like MilR3. For this situation are widely distributed in *Streptomyces*, further elucidating the role of *milR3* will help to enrich the knowledge of “Small” SARP-type regulators.

Recently, with rapid development of the promising CRISPR-based technologies, several CRISPR-based genetic manipulation tools have been developed in *Streptomyces* as well. The DNase-deactivated Cpf1(ddCpf1)-based integrative CRISPR interference system (CRISPRi) showed great potential to investigate a panel of genes like these putative SARP family regulators in *S. bingchenggensis*. Present study provides an approach for rapidly and efficiently investigating genes involved in milbemycins production in a high through-put way.

In summary, the “Small” SARP-type regulator MilR3 has been identified as a novel pleiotropic activator that co-regulates milbemycin biosynthesis and the yellow pigment biosynthesis in *S. bingchenggensis*. This study renovates to recognize the novel role of “Small” SARP-type regulators in *S. bingchenggensis.* This work lays a solid basis for future study of regulatory mechanism of “Small” SARP-type pleiotropic regulators. Understanding their mechanism will promote their application to enhance production and new products mining by rewiring regulatory pathway.

Declarations

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Author contributions

YYan and HX conceived and designed this study. YYang, LZ, YYan and LZhang performed all experimental work. HX reviewed the results and provided critical feedback. The manuscript was drafted by YYan. HX and YYan revised the manuscript. All authors read and approved the final manuscript.

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Data availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Conflict of interest

All authors declare that they have no conflict of interest.

Ethical statement

This manuscript is in compliance with ethical standards. This manuscript does not contain any studies with human participants or animals performed by any of the authors.

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Figures

Figure 1

Chemical structures of the milbemcyins and physical map of their biosynthetic gene cluster. **a** Chemical Structure of milbemycins; **b** Genetic organization of *mil* cluster in *S. bingchenggensis*. Arrows indicate separate ORFs. The PKS genes (*milA1*, *milA2*, *milA3*, *milA4*) encoding backbone synthases were showed in green. Yellow arrows for genes involved in post-modification steps. Red for the regulator gene and gray for the gene with unknown function.

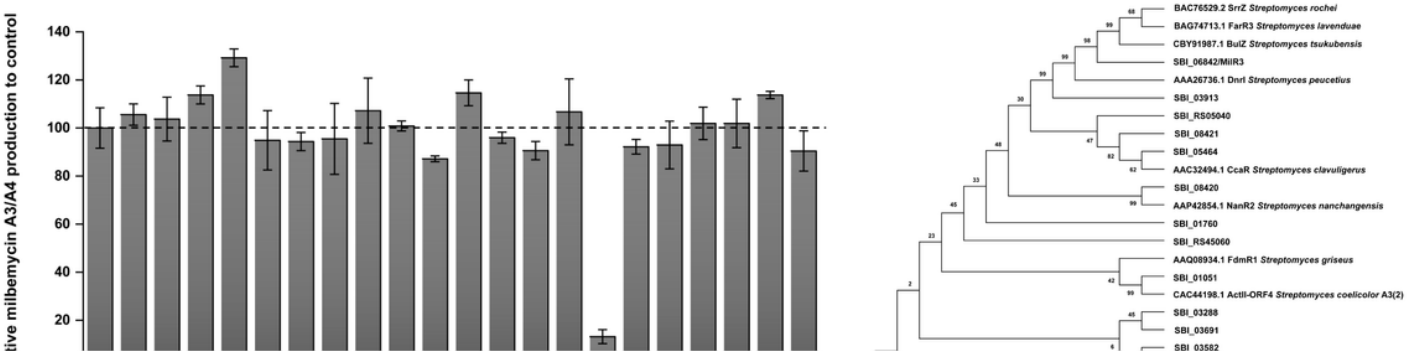

Figure 2

The 19 putative SARP family regulators in *S. bingchenggensis*. **a** Milbemycin A3/A4 production of CRISPRi mutants of each 19 putative SARP family regulators. **b** The phylogenetic tree of the 19 putative SARP family regulators with reported SARP family proteins. The phylogenetic tree was constructed with neighbor-joining methods. The confidence of the tree was tested with the bootstrap approach (500 replicates).

Figure 3

Milbemycin production was affected by MilR3 in *S. bingchenggensis* TMB. **a** Milbemycin A3/A4 production in TMB/pSET152, D*milR3*, C*milR3* and O*milR3* Strains **b** Picture of methanol extracts of fermentation broth of TMB/pSET152, D*milR3*, C*milR3* and O*milR3* strains. **c** The growth phenotypes of TMB/pSET152, D*milR3*, C*milR3* and O*milR3* grown on MS plates for 2, 4, 6 or 8 days.

Figure 4

The core minimal type II PKS genes was involved in yellow pigment biosynthesis in *S. bingchenggensis* TMB. **a** The growth phenotypes of TMB/pSET152 and D*sbi_06844* grown on MS plates at 7 days. **b** Milbemycin A3/A4 production in TMB/pSET152 and D*sbi_06844* strains. **d** qRT-PCR analysis of *milR*, *milF*, *milA2*, *milD*, *milE*, *milR3* and *sbi_06844* in TMB/pKCCpf1 and D*sbi_06844* strain. All RNA samples were isolated from culture broth of TMB/pKCCpf1 and D*sbi_06844* in fermentation medium at 2, 4, 6 and 8 days, respectively. The relative transcriptional levels of each gene are calibrated with its transcription in TMB/pKCCpf1 at 2 days.

Figure 5

Transcription of genes involved in milbemycin and yellow pigment biosynthesis were regulated by MilR3. The relative transcription levels of *milA1*, *milA2*, *milA3*, *milA4*, *milC*, *milD*, *milE*, *milF*, *milR*, *milR2*, *milR3* and *sbi_06844* were compared among TMB/pSET152, D*milR3*, C*milR3* and O*milR3* strains and

represented separately. RNA samples were isolated from 2, 4, 6 and 8 days culture broth of each strain in fermentation medium.

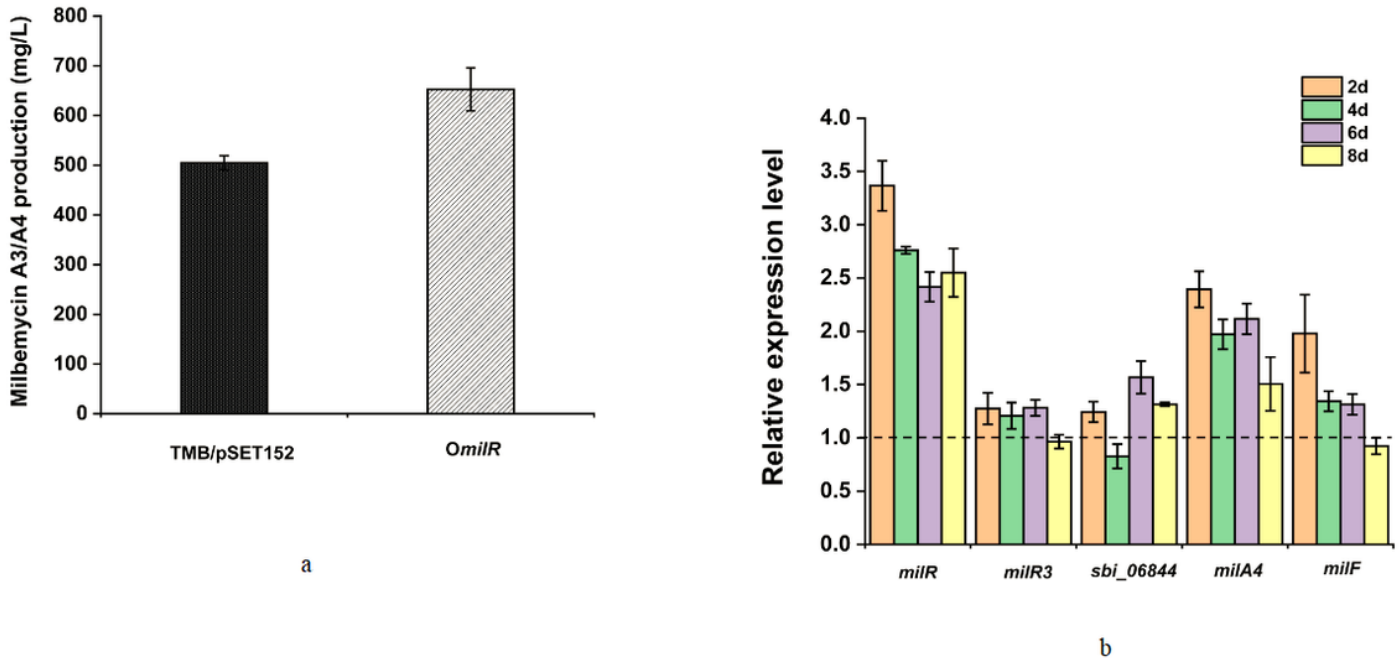

Figure 6

The regulatory cascade between MilR3 and MilR in *S. bingchenggensis* TMB. **a** Milbemycin A3/A4 production in *S. bingchenggensis* TMB/pSET152 and O*milR* strain. **b** Relative expression levels of *milR*, *milR3*, *sbi_06844*, *milA4* and *milF* in TMB/pSET152 and O*milR*. All RNA samples were isolated from culture broth of TMB/pSET152 and O*milR* strain in fermentation medium at 2, 4, 6 and 8 days, respectively.

Figure 7

The distribution of gene clusters conserved to *sbi_06840* to *sbi_06846* cluster. Cluster blast was analyzed on antiSMASH server.

Supplementary Files

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- Supplementarymaterials0111.pdf