MilR3, a unique SARP family pleiotropic regulator in *Streptomyces bingchenggensis*

Yu-Si Yan¹ · Yun-Qi Yang¹ · Li-Sha Zhou¹ · Ling Zhang¹ · Hai-Yang Xia¹

Received: 10 January 2022 / Revised: 30 August 2022 / Accepted: 2 September 2022 / Published online: 19 September 2022
© The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2022

Abstract

*Streptomyces bingchenggensis* is the main industrial producer of milbemycins, which are a group of 16-membered macrocyclic lactones with excellent insecticidal activities. In the past several decades, scientists have made great efforts to solve its low productivity. However, a lack of understanding of the regulatory network of milbemycin biosynthesis limited the development of high-producing strains using a regulatory rewiring strategy. SARPs (*Streptomyces* Antibiotic Regulatory Proteins) 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*, *sbi_06845* and *sbi_06846*) gene cluster, which was proved to be responsible for producing a yellow pigment. The quantitative real-time PCR analysis proved that MilR3 positively affected the transcription of specific genes within milbemycin BGC and those from the type II PKS gene cluster. Unlike previous “small” SARP family regulators that played pathway-specific roles, MilR3 was probably a unique SARP family regulator and played a pleotropic role. MilR3 was an upper level regulator in the MilR3-MilR regulatory cascade. This study first illustrated the co-regulatory role of this unique SARP regulator. This greatly enriches our understanding of SARP and lay a solid foundation for milbemycin yield enhancement in the near future.

Keywords Milbemycins · Cpf1-CRISPRi system · Transcriptional regulation · SARP family regulator · *Streptomyces bingchenggensis*

Introduction

Milbemycins, a group of 16-membered macrolide antibiotics with a unique action mode on the nervous system of insects and parasites, have been widely used as acaricides, insecticides and anthelmintics for an excellent high and broad spectrum of activities, harmless to human and animal, as well as the environmentally friendly character (Danaher et al. 2012; Jacobs and Scholtz 2015). Milbemycins are structurally similar to the well-known anthelmintic agent avermectins (Chen et al. 2016). Currently, several products derived from milbemycin A3 and A4, including milbemectin, milbemycin oxime, lepimectin and latidectin, have been marketed and widely applied in agriculture, animal husbandry and the medical industry (Bienhoff et al. 2013; Kim et al. 2017; Nicastro et al. 2011; Pluschkell et al. 1999) (Fig. 1a). Significantly, milbemectin is an effective insecticide against insects resistant to avermectin (Romero-Rodríguez et al. 2015).

Milbemycins can be produced by strains from several *Streptomyces* species, including *Streptomyces hygroscopicus*, *Streptomyces griseochromogenes*, *Streptomyces cya-neoegriseus*, *Streptomyces nanchangensis* and *Streptomyces bingchenggensis* (Baker et al. 1996; Carter et al. 1988; Ono et al. 1983; Wang et al. 2010). Presently, *S. bingchenggensis* is the main industrial milbemycin producer in China (Wang et al. 2020). 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 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 proteins with unknown functions (Orf1 and Orf2) (Fig. 1b). Unlike avermectin biosynthetic gene cluster, the mil cluster is separated by a 62 kb DNA fragment between milR and milA1 which is not required for milbemycin biosynthesis. Its gene organization is similar to that of meilingmycin biosynthetic gene cluster, but different from that of avermectin biosynthetic gene cluster (He et al. 2010a, b; Nonaka et al. 1999a, b). The revealed biosynthetic pathways of avermectin and meilingmycin help in the understanding of milbemycin. As an extender unit, seven malonyl-CoA and five methylmalonyl-CoA are condensed to generate start units acetyl-CoA or propionyl-CoA, which are successively catalyzed by four giant polyketide synthases to form the milbemycin polyketide backbone. Consequently, the polyketide backbones are formed spontaneously as spiroketal and furan rings, which are catalyzed by the tailoring enzymes MilE and MilC. (Sun et al. 2013; Wang et al. 2020). Two tailoring enzymes, C5-keto reductase and C5-O-methyltransferase encoded by milF and milD, were responsible for the reduction of C5-keto groups and methylation of C5-hydroxyl of milbemycinA3/A4, respectively (Nonaka et al. 2010; Wang et al. 2014; Zhang et al. 2013).

Many strategies have been used in recent years to increase milbemycin production, including random mutation optimization of producing strains, metabolic engineering, combinational biosynthesis, and fermentation process enhancement. (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 still much lower than that of avermectins in S. avermitilis (Jin et al. 2020). So far, only a few regulators have been identified as being involved in milbemycin biosynthesis. MilR, as the pathway-specific regulator, positively regulates milbemycin biosynthesis by directly binding to the promoters of the milA4-milE operon and milF, respectively. Overexpression of milR with its native promoter increased milbemycin A3/A4 production by 34%. (Zhang et al. 2016). SbbR/SbbA, as the upper layer regulator, affects milbemycin biosynthesis through γ-butyrolactone signal pathway (He et al. 2018). The pleiotropic regulator NsdA represses the production of milbemycin in S. bingchenggensis (Wang et al. 2006). Recently, MilR2, a TetR family regulator, has been proved as an activator for 5-oxomilbemycin A3/A4 biosynthesis in S. hygroscopicus SIPI-KF (Wei et al. 2018). The limited

| Milbemycins | R1 | R2 |
|-------------|----|----|
| A3          | H  | CH₃|
| A4          | H  | C₂H₅|
| D           | H  | C₃H₇|
| B2          | CH₃| CH₃|
| B3          | CH₃| C₂H₅|
| G           | CH₃| C₃H₇|

Fig. 1 Chemical structures of the milbemycins 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 tailoring steps. Red for the regulator gene and gray for genes with unknown function.
knowledge on the regulatory network of milbemycin biosynthesis has bottlenecked the generation of high-producing strains by regulatory rewiring approaches.

*Streptomyces* antibiotic regulatory proteins (SARPs) have been exclusively found in actinobacteria and most of them within *Streptomyces*. SARP encoding genes are usually 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). SARPs can be divided into “small” and “large” groups according to the sizes and domains organization. “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 peucetius* (Sheldon et al. 2002), Aur1PR2 and Aur1PR3 from *Streptomyces aureofaciens* (Novakova et al. 2005), FdmR1 from in *Streptomyces griseus* (Chen et al. 2008), TyIS from *Streptomyces fradiae* (Bate et al. 2002), SrrY and SrrZ from *Streptomyces rochei* (Suzuki et al. 2010) are all known to be 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 “large” SARP-type activators including SanG for nikkomycin in *Streptomyces anschromogenes* (He et al. 2010a, b), PloR for poloxin 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 AsfR, a member of “large” SARPs, has been proved as a pleiotropic regulator. AsfR affects the expression of *afsS* gene, which encodes a small protein (63 amino acids residues) controlling the synthesis of “large” SARPs, has been proved as a pleiotropic regulator in *S. bingchengensis* in this study.

### Materials and methods

#### Bacterial strains, plasmid, and culture conditions

All strains and plasmids used in this study are listed in Table S1. *S. bingchengensis* TMB is derived from *S. bingchengensis* CGMCC 1734 by single colony isolation with stable milbemycin production. *S. bingchengensis* TMB and its derivatives were grown on manni tol soya flour (MS) medium (mannitol 20 g/L, soybean power 20 g/L, agar 20 g/L, pH 7.2) at 28 °C for sporulation. For *S. bingchengensis* fermentation and milbemycin A3/A4 production, strains were cultured in seed medium (sucrose 10 g/L, skim milk power 1 g/L, tryptone 3.5 g/L, yeast extract 5 g/L, K2HPO4·3H2O 0.5 g/L, pH 7.2) at 28 °C and 250 rpm on a rotary shaker for 44 h. Then, 4 ml of seed culture was transferred into 250 ml Erlenmeyer flasks containing 25 ml fermentation medium, including sucrose 80 g/L, soy bean power 20 g/L, skim milk power 1 g/L, CaCO3 3 g/L, K2HPO4 1 g/L, and FeSO4·7H2O 0.1 g/L, pH 7.2. The fermentation was cultured on the shaker at 28 °C and 250 rpm for 9 days. ISP4 medium was used for intergeneric conjugation between *Escherichia coli* S17-1 and *S. bingchengensis*. *E. coli* strains were grown on LB agar plates or in LB liquid medium at 37 °C. *E. coli* DH10B was used as a general cloning host. *E. coli* BL21(DE3) was used for protein overexpression. If necessary, the medium was supplemented with the proper amount antibiotics to the final concentration, including 50 μg/mL apramycin, 50 μg/mL kanamycin, nalidixic acid 25 μg/mL or 100 μg/mL ampicillin. Water containing apramycin and nalidixic acid at final concentrations of 25 μg/mL each was overlaid on the conjugation plates.

#### Construction of recombinant strains

pSETddCpf1 derived vectors for transcriptional 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′- GAC TAGTN22ATCTACAACAGTAGAAA-3′ (N22 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. bingchengensis* TMB by intergeneric conjugation.

The pKCCpf1 derived vectors for target genes deletion were constructed by amplification of crRNA expression cassette, upstream fragment and downstream fragment of the target gene and assembling with NdeI and SpeI-linearized

---

### Notes

- **ESR**: (Optional) Please provide any additional notes or references that are relevant to the content of the text.
- **Fig/Tab**: (Optional) If the text includes figures or tables, please specify any details or annotations that are important for understanding the data presented.
- **Data**: (Optional) If the text refers to any specific data or datasets, please include any necessary details for accessing or verifying the data.
pKCCPF1 according the procedure described by Li et al. (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 intergeneric conjugation. pKCCPF1 derived plasmids were cured from positive conjugants by culture under 37 °C. Mutants were verified by colony PCR and the amplified fragments were sequenced. For overexpression of genes in S. bingchenggensis TMB, target genes were amplified with proper primer pair by intergeneric conjugation. Positive conjugants were verified by amplification of apramycin resistance gene.

HPLC analysis of milbemycin A3/A4 production

1 mL fermentation culture broth was mixed with 4 mL methanol and extracted for 2 h at room temperature. The amount of milbemycin A3/A4 in the extract was analyzed by high-pressure liquid chromatography (HPLC: 1260 series; Agilent) using 4.6×150 mm Hesperil C18 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 °C. Metabolites were monitored at a wavelength of 240 nm. The production was calibrated with standard milbemycin A3/A4 provided by Zhejiang Hisun Pharmaceutical Inc. (Taizhou, China).

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

RNA was isolated from fermentation cultures using an EASY spin Plus bacterial RNA kit (Aidlab Biotechnologies, Beijing, China) according to the manufacturer’s protocol, and subsequently treated with RNA-free DNase I (Takara, Dalian, China) to remove the residual genomic DNA. The quantity and quality of RNA samples were analyzed with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

Reverse transcription of total RNA was performed using the PrimeScript™ RT reagent kit according to its instruction. Quantitative PCR was performed with TB green Premix Ex Taq™ II (Takara, Dalian, China) on a CFX96 Touch Real-Time System (Bio-Rad, USA). The gene sbi_03299 (encoding the principal house-keeping sigma factor) was used as the reference gene. All reactions were conducted in triplicate wells for each RNA sample and repeated with three independent samples of each strain. The relative transcriptional 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^−ΔΔCT method (Livak and Schmittgen 2001). Error bars indicate the standard deviations from three independent biological replicates. The primers used in qRT-PCR analysis are listed Table S2 in the Supplementary Materials.

Construction of gusA-encoding β-glucuronidase (GUS) reporter systems

An 1818-bp DNA fragment containing the complete gusA coding region was amplified using gusA-F and gusA-R primer pair, and digested with XbaI and BamHI. The result DNA fragment was cloned into the XbaI and BamHI linearized pSET152, thus generating the plasmid pSET152-gusA. The six predicted promoters of milA2, milR, milA4, sbi_06843, sbi_06844 and hrdB were amplified from the genomic DNA template of S. bingchenggensis TMB by PCR with primer pair milA2p-F/R, milA4p-F/R, milRp-F/R, sbi_06843p-F/R, sbi_06844p-F/R and hrdBp-F/R, respectively. P_mila2–P_mila4–P_milR, P_sbi_06843, P_sbi_06844 and P_gusA were introduced to pSET152-gusA between XbaI and NdeI sites to generate pSET152:P_mila2-gusA, pSET152:P_mila4−gusA, pSET152:P_milR−gusA, pSET152:P_sbi_06843−gusA, pSET152:P_sbi_06844−gusA, pSET152:P_hrdB−gusA. All plasmids were verified by DNA sequencing. These pSET152-derived plasmids were incorporated into S. bingchenggensis TMB and DmilR3 by intergeneric conjugation. The S. bingchenggensis TMB strain containing plasmid pSET152::P_hrdB-gusA was used as a negative control for β-glucuronidase (GUS) assays. Exconjugants were verified by PCR amplification of the apramycin resistant gene. For GUS assays, spores of S. bingchenggensis derived strains were spotted on 25 mL plates of MS agar containing 40 μL of 100 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronide and photographed after 2, 4 and 6 days at 28 °C (Sherwood and Bibb, 2013).

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 (Li et al. 2018). All 19 SARP candidates of S. bingchenggensis were investigated for their influence on milbemycin production by this ddCpf1-based CRISPRi system for SARP family regulators typically involved in secondary metabolite biosynthesis. The milbemycin A3/A4 production of all mutants were listed in Figure S1. 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 significantly decreased by 87.3% as compared to the TMB/pSETddCpf1.
These putative SARPs can be divided into three groups based on their phylogenetic relationship with numerous well-studied SARP family regulators. (Figure S2). 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, the encoding gene located in the 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 has 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 ΔmilR3) were constructed based on the parental strain *S. bingchenggensis* TMB with CRISPR-Cpf1 system. The ΔmilR3 mutant was further confirmed by PCR and DNA sequencing (Figure S3). 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 ΔmilR3 mutant and TMB, resulting in the complemented strain CmilR3 and the overexpression strain OmilR3. As controls, the plasmid pSET152 was introduced into ΔmilR3 and TMB generating DmilR3 and TMB/ pSET152, respectively. Milbemycin A3/A4 production of strains TMB/pSET152, DmilR3, CmilR3 and OmilR3 were presented in Fig. 2a. Milbemycin A3/A4 production was abolished in DmilR3 and restored in CmilR3. These results demonstrated that MilR3 played an indispensable activator for milbemycin production in *S. bingchenggensis* TMB. Notably, milbemycin A3/A4 production in OmilR3 was slightly lower than that of TMB/pSET152 (Fig. 2a).

Interestingly, a yellow pigment produced by *S. bingchenggensis* TMB was abolished in DmilR3 strain and restored in CmilR3 strain (Fig. 2b). The yellow pigment production patterns of these four strains on solid agar plates were comparable to those seen during liquid fermentation (Fig. 2c). For *milR3* was adjacent to a putative type II polyketide gene cluster, we proposed that this gene cluster was responsible for the production of the yellow pigment.

Noticeably, the SARP family protein MilR3 acted as a pleiotropic regulator to modulate milbemycins and a yellow pigment biosynthesis in *S. bingchenggensis* TMB.

**The type II PKS cluster is responsible for the biosynthesis of a yellow pigment***

Four genes (*sbi_06843, sbi_06844, sbi_06845* and *sbi_06846*) neighboring milR3 were predicted to encode putative type II PKS (Figure S6). 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 S5). This mutant, named Dsbi_06844, was incapable to produce the yellow pigment on MS plate (Fig. 3a), which indicated that the putative type II PKS encoding genes are responsible for the yellow pigment biosynthesis. The absorption of methanol extracts from Dsbi_06844, DmilR3 and TMB/pSET152 fermentation samples were analyzed through UV–Vis-spectroscopy. TMB/pSET152 sample showed an absorption peak from wavelength 400 nm to 500 nm (Fig. 3b). So, the optical density at 440 nm was taken to quantify this yellow pigment. The relative productions of yellow pigment from DmilR3, Dsbi_06844, OmilR3 and TMB/pSET152 strains were illustrated in Fig. 3c. OmilR3 strains can produce about 20% yellow pigment than TMB/pSET152.

A type-II polyketide biosynthetic gene cluster (from *sbi_06834 to sbi_06875*) can be predicted from *S. bingchenggensis* genome data by antiSMASH, which contains the minimal PKS unit (*sbi_06843, sbi_06844, sbi_06845* and *sbi_06846* encoding a cycloase, a beta-ketoacyl synthase, a chain-length factor and an acyl carrier protein, respectively) (Figure S6) (Blin et al. 2021). This type-II PKS cluster is proved to be involved in the biosynthesis of this yellow pigment. Based on a large-scale analysis of type II polyketide synthases, Chen et al. proposed that the chain-length factor protein could be a marker to predict both chemical class and molecular uniqueness of the type II polyketide biosynthetic product and estimated its structural diversity in bacteria (Chen et al. 2022). SBI_06844 and SBI_06845 showed 81.38 and 78.01% identity to TjhA1(KS) and TjhA2 (CLF) (responsible for glycosylated tetracyclines in *Streptomyces aureus*) (Ji et al. 2019). And there are 6 glycosyltransferase encoding genes in this type-II polyketide cluster in *S. bingchenggensis* (Figure S6). We proposed that this yellow pigment was glycosylated tetracyclic polyketide.

**Milbemycin biosynthesis is uncoupled with the yellow pigment producing***

For *milR3* is located adjacently to the biosynthetic gene cluster of yellow pigment and far from that of milbemycin, it was necessary to know whether the yellow pigment would involve in milbemycin production. The milbemycin production and transcription of *mil* cluster were examined in Dsbi_06844 and TMB strains. The result showed that Dsbi_06844 strain produced a similar amount of milbemycin.
Milbemycin production was affected by MilR3 in *S. bingchengensis* TMB. a Milbemycin A3/A4 production in TMB/pSET152, DmilR3, CmilR3 and OmilR3 strains. b Picture of methanol extracts of fermentation broth of TMB/pSET152, DmilR3, CmilR3 and OmilR3 strains. c The growth phenotypes of TMB/pSET152, DmilR3, CmilR3 and OmilR3 strains grown on MS plates for 2, 4, 6 or 8 days

Fig. 2 Milbemycin production was affected by MilR3 in *S. bingchengensis* TMB. a Milbemycin A3/A4 production in TMB/pSET152, DmilR3, CmilR3 and OmilR3 strains. b Picture of methanol extracts of fermentation broth of TMB/pSET152, DmilR3, CmilR3 and OmilR3 strains. c The growth phenotypes of TMB/pSET152, DmilR3, CmilR3 and OmilR3 strains grown on MS plates for 2, 4, 6 or 8 days

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 were examined by qRT-PCR analysis of time-course RNA samples from fermentation culture of TMB/pSET152, DmilR3, CmilR3 and OmilR3 strains (2, 4, 6 and 8 day). The results showed that transcription of milA2, milA4, milD and milR were almost undetectable in DmilR3, but restored in CmilR3 compared with those of TMB/pSET152 (Fig. 5a–d). Transcription of sbi_06844 in DmilR3 was expressed extremely lower than that in TMB/
pSET152, and restored in CmiIR3 (Fig. 5e). And the time-course transcription patterns of tested genes were all similar. These results indicated that MilR3 functioned as an indispensable activator for the transcription of both milbemycin and yellow pigment biosynthetic gene clusters.

However, the transcription of milR2, a previous reported TetR family activator involved in 5-oxomilbemycin A3/A4

Fig. 3 The type II PKS cluster was responsible for the yellow pigment biosynthesis in *S. bingchenggensis* TMB. a The growth phenotypes of TMB and Dsbi_06844 grown on MS plates at 7 days. b The UV–Vis spectrum absorption of methanol extractions of strains Dsbi_06844, DmiIR3 and TMB/pSET152 fermentation culture. c Relative production of yellow pigment from strains DmiIR3, Dsbi_06844, OmilR3 and TMB/pSET152 by optical density at 440 nm
biosynthesis, was increased in DmilR3, and its transcription level can be restored in the complementary strain CmilR3 (Fig. 5f). This indicates that milR3 had a negative effect on the transcription of milR2.

**MilR3 affects the expression of genes involved in milbemycin and the yellow pigment biosynthesis by binding to their promoter regions**

Soluble expression of MilR3 in *E. coli* has not been achieved after efforts with different vectors, fusion tags, chaperones and hosts. Therefore, in vivo β-glucuronidase (GUS) assays were employed to examine the targets of MilR3 (Sherwood and Bibb, 2013). GUS activities were clearly observed from TMB and Dsbi_06844 strain. All RNA samples were isolated from fermentation culture broth of TMB and Dsbi_06844 at 2, 4, 6 and 8 days, respectively. The relative transcriptional levels of each gene are calibrated with its transcription in TMB at 2 days.
Fig. 5  MilR3 regulated transcription of genes involved in milbemycin and yellow pigment biosynthesis. a milA2, b milA4, c milD, d milR, e sbi_06844, f milR2. The relative transcription levels of these genes were compared among TMB/pSET152, DmilR3, CmilR3 and OmilR3 strains and represented separately. RNA samples were isolated from fermentation culture broth of TMB/pSET152, DmilR3, CmilR3 and OmilR3 at 2, 4, 6 and 8 days.
The milbemycin biosynthesis is regulated by MilR3-MilR regulatory 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 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 OmilR (TMB strain carried an integrative plasmid pSET152 containing milR driven by a strong constitutive kasOp* promoter) and wild type strain in fermentation culture. The transcription of milR was up-regulated in OmilR at each time point. The transcription of previously reported target genes of MilR also showed the same tendency in the OmilR strain. The milbemycin A3/A4 production in OmilR was increased by 29% compared with that of TMB/pSET152, which was consistent with the results of qRT-PCR (Figure S9a).

Notably, the expression level of milR3 in OmilR was similar to that in TMB/pSET152 (Figure S9b). It was indicated that transcription of milR3 was not affected by milR. Probably the milbemycin production is regulated through MilR3 to MilR cascade.

Discussion

In this study, MilR3, a SARP family protein, was screened out for its critical involvement in milbemycin production using the ddCpf1-based CRISPR interference (CRISPRi) system in S. bingchenggensis. The milR3 was found near a putative type II PKS encoding gene cluster, which was around 7 Mb away from the milbemycin biosynthetic gene cluster and was responsible for the biosynthesis of yellow pigment. Further experiments showed that MilR3 was a unique pleiotropic regulator directly controlling the biosynthesis of milbemycin and yellow pigment in S. bingchenggensis. Based on the findings, we hypothesized that milbemycin production was most likely controlled by a MilR3 to MilR regulatory cascade.

Overexpression of transcriptional activators is an efficient approach to enhancing the production of target antibiotics (Du et al. 2013). However, over-production of milbemycin has not been reported whenever overexpression of milR3 is driven by its native promoter or constitutive promoters like kasOp* and ermEp* (Fig. 3a, data not shown), which suggested that MilR3 might not be the key rate-limiting factor in the tested strain. The OmilR3 strain generated a much higher amount of yellow pigment on MS plates in comparison to the TMB strain (Fig. 4c). Both milbemycin and yellow pigment were produced by PKS pathways and shared precursors like malonyl-CoA, so the competition between the clusters probably affected 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 transcriptome analysis could contribute to the investigation of alternative MilR3 targets and the prediction of the metabolic flux affected by MilR3. Elucidation of the action mechanism of MilR3 will help to provide new insights into the regulation network of milbemycin biosynthesis and lay a solid foundation for milbemycin production enhancement. Milbemycin synthesis may most likely be promoted by overexpression of MilR3 in the Dsbi_06844 strain.

SARP regulators, which are widespread in Streptomyces with varying numbers, 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 blasted on antiSMASH server with the sequenced genomes, several clusters showed conserved genes organization pattern (Figure S4) (Blin et al. 2021). Each SARP-regulators had an adjacent putative Type-II PKS cluster. We hypothesized that these “Small” SARP-type proteins probably played roles as pleiotropic activators like MilR3. Since, this situation is widely distributed in Streptomyces, further elucidating the role of milR3 will help to enrich the knowledge of “Small” SARP-type regulators.

Recently, with the 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. The present study provides an approach for rapidly and efficiently investigating genes involved in milbemycins production in a high throughput 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. The purpose of this study was to identify the novel role of “Small” SARP-type regulators in S. bingchenggensis. Taken together, this work lays a solid basis for future study of the regulatory mechanism of “Small” SARP-type pleiotropic regulators. Understanding their mechanism will promote their application to enhance
the production and new products mining by rewiring the regulatory pathway.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00203-022-03240-x.

**Acknowledgements** We thank the members of Institute of Biopharmaceuticals, Taizhou University for many fruitful discussions. Many thanks to Dr. Muhammad Hafeez (Zhejiang Academy of Agricultural Sciences, Hangzhou, China) for critical editing and suggestion.

**Author contributions** YY and HX: conceived and designed this study. YY, LZ, YY and LZ: performed all experimental work. HX: reviewed the results and provided critical feedback. The manuscript was drafted by YY. HX and YY: revised the manuscript. All authors read and approved the final manuscript.

**Funding** This study was funded the Natural Science Foundation of Zhejiang Province to HX (LY19C010002), the Scientific Research Foundation of Taizhou to HX (No. 2001xg07) and Taizhou University established scientific research and cultivation project (2018PY044).

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

**Declarations**

**Conflict of interest** All authors declare that they have no conflict of interest.

**Ethical approval** 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.

**References**

Antón N, Mendes MV, Martin JF, Aparicio FJ (2004) Identification of PimR as a positive regulator of pimaricin biosynthesis in *Streptomyces natatensis*. J Bacteriol 186(9):2567–2575. https://doi.org/10.1128/JB.186.9.2567-2575.2004

Arias P, Fernández-Moreno MA, Malpartida F (1999) Characterization of the pathway-specific positive transcriptional regulator for actinorhodin biosynthesis in *Streptomyces coelicolor* A3(2) as a DNA-binding protein. J Bacteriol 181:6958–6968. https://doi.org/10.1128/jb.181.22.6958-6968.1999

Baker GH, Blanchflower SE, Dorgen RJ, Everett JR, Manger BR, Reading CR, Readshaw SA, Shelley P (1996) Further novel milbemycin antibiotics from *Streptomyces* sp. E225: fermentation, isolation and structure elucidation. J Antimicrob Chemother 37(3):272–280. https://doi.org/10.1093/antimicrob/37.3.272

Bate N, Stretigopoulos G, Gundliffe E (2002) Differential roles of two SARP-encoding regulatory genes during tylosin biosynthesis. Mol Microbiol 42(2):449–458. https://doi.org/10.1046/j.1365-2958.2002.02756.x

Bibb MJ (2005) Regulation of secondary metabolism in streptomycetes. Curr Opin Microbiol 8(2):208–215. https://doi.org/10.1016/j.mib.2005.02.016

Bienhoff SE, Kok DJ, Roycroft LM, Roberts ES (2013) Efficacy of a single oral administration of milbemycin oxime against natural infections of *Ancylostoma braziliense* in dogs. Vet Parasitol 195(1–2):102–105. https://doi.org/10.1016/j.vetpar.2013.01.004

Blin K, Shaw S, Kloosterman AM, Charlop-Powers Z, van Wezel GP, Medema MH, Weber T (2021) antiSMASH 6.0: improving cluster detection and comparison capabilities. Nucleic Acids Res 49:W29–W35. https://doi.org/10.1093/nar/gkab335

Carter GT, Nietsche JA, Hertz MR, William DR, Siegl MM, Morton GO, James JC, Borders DB (1988) LL-F28249 antibiotic complex: a new family of antiparasitic macrolactam antibiotics. J Antibiot 41(4):519–529. https://doi.org/10.7164/antibiotics.41.519

Chen Y, Wendt-Pienkowski E, Shen B (2008) Identification and utility of FdmR1 as a *Streptomyces* antibiotic regulatory protein activator for fredericamycin production in *Streptomyces griseus* ATCC 49344 and heterologous hosts. J Bacteriol 190(16):5587–5596. https://doi.org/10.1128/JB.00592-08

Chen JS, Liu M, Liu XT, Miao J, Fu CZ, Gao HY, Müller R, Zhang Q, Zhang LX (2016) Interrogation of *Streptomyces avermitilis* for efficient production of avermectins. Synth Syst Biotechnol 1(1):7–16. https://doi.org/10.1016/j.synbio.2016.03.002

Chen S, Zhang C, Zhang L (2022) Investigation of the molecular landscape of bacterial aromatic polyketides by global analysis of type II polyketide synthases. Angew Chem Int Ed Engl 61:e202202286. https://doi.org/10.1002/anie.202202286

Danaher M, Radeke W, Kolar L, Keegan J, Cerkvenik-Flajs V (2012) Recent developments in the analysis of avermectin and milbemycin residues in food safety and the environment. Curr Pharm Biotechnol 13(6):936–955. https://doi.org/10.2174/1389201128003990068

Du DY, Zhu Y, Wei JH, Tian YQ, Niu GQ, Tan HR (2013) Improvement of goguerotin and nikkomycin production by engineering their biosynthetic gene cluster. Appl Microbiol Biotechnol 97(14):6383–6396. https://doi.org/10.1007/s00253-013-4836-7

He XH, Li R, Pan YY, Liu G, Tan HR (2010a) SanG, a transcriptional activator, controls nikkomycin biosynthesis through binding to the sanN-sanO intergenic region in *Streptomyces anschromogenes*. Microbiology 156(3):828–837. https://doi.org/10.1099/mic.0.033605-0

He YL, Sun YH, Liu TG, Zhou XF, Bai LQ, Deng ZX (2010b) Cloning of separate meilingmycin biosynthesis gene clusters by use of a cloning transferase-ketoeductase didomain PCR amplification. Appl Environ Microbiol 76(10):3283–3292. https://doi.org/10.1128/AEM.02262-09

He HR, Ye L, Li C, Wang HY, Guo XW, Wang XJ, Zhang YY, Xiang WS (2018) SbbR/SbbA, an important ArpA/AfsA-like system, regulates milbemycin production in *Streptomyces bingchengensis*. Front Microbiol 5(9):1064. https://doi.org/10.3389/fmicb.2018.01064

Horinouchi S (2003) AfsR as an integrator of signals that are sensed by multiple serine/threonine kinases in *Streptomyces coelicolor* A3(2). J Ind Microbiol Biotechnol 30(8):462–467. https://doi.org/10.1007/s10295-003-0063-z

Jacobs CT, Scholtz CH (2015) A review on the effect of macrolide lactones on dung-dwelling insects: toxicity of macrolide lactones to dung beetles. Uderstepoort J Vet Res 82(1):858. https://doi.org/10.4102/ojvr.v82i1.858

Ji ZY, Nie QY, Yin Y, Zhang M, Pan HX, Hou XF, Tang GL (2019) Activation and characterization of cryptic gene cluster: two series of aromatic polyketides biosynthesized by divergent pathways. Angew Chem Int Ed Engl 58(50):18046–18054. https://doi.org/10.1002/anie.201910882

Jin PJ, Li SS, Zhang YY, Chu LY, He HR, Dong ZX, Xiang WS (2020) Mining and fine-tuning sugar uptake system for titer improvement of milbemycins in *Streptomyces bingchengensis*. Synth Syst Biotechnol 5(3):214–221. https://doi.org/10.1016/j.synbio.2020.07.001

H. XU, Y. YU, B. N. LIN, Y. Y. CHEN, L. H. ZHANG, H. B. WANG, T. R. LIU, J. F. WANG, Z. F. YANG, Y. Q. TAO, J. F. ZHENG, B. N. YANG, L. W. ZHAO, Y. Y. CHEN, Y. Y. ZHANG, C. W. YANG, H. XU (2018) Activation and characterization of cryptic gene cluster: two series of aromatic polyketides biosynthesized by divergent pathways. Angew Chem Int Ed Engl 58(50):18046–18054. https://doi.org/10.1002/anie.201910882
Kim MS, Cho WJ, Song MC, Park SW, Kim K, Kim E, Lee N, Nam SJ, Oh KH, Yoon YJ (2017) Engineered biosynthesis of milbemycins in the avermectin high-producing strain Streptomyces avermitilis. Microb Cell Fact 16(1):9. https://doi.org/10.1186/s12934-017-0626-8

Kurniawan NY, Kitani S, Maeda A, Nihira T (2014) Differential contributions of two SARP family regulatory genes to indigoidine biosynthesis in Streptomyces lavendulae FRI-5. Appl Microbiol Biotechnol 98(23):9713–9721. https://doi.org/10.1007/s00253-014-5988-9

Lee CP, Umezaya T, Horinouchi S (2002) afsS is a target of AfsR, a transcriptional factor with ATPase activity that globally controls secondary metabolism in Streptomyces coelicolor A3(2). Mol Microbiol 43(6):1413–1430. https://doi.org/10.1046/j.1365-2958.2002.02840.x

Li R, Xie ZJ, Tian YQ, Yang HH, Chen WQ, You DL, Liu G, DENG ZX, Tan HR (2009) polIR, a pathway-specific transcriptional regulatory gene, positively controls polyoxin biosynthesis in Streptomyces cacaoi asos. Microbiology 155(6):1819–1831. https://doi.org/10.1099/mic.0.028639-0

Li L, Wei KK, Zheng GS, Liu XC, Jiang WH, Lu YY (2018) CRISPR-Cpf1 assisted multiplex genome editing and transcriptional repression in Streptomyces. Appl Environ Microbiol. https://doi.org/10.1128/AEM.00827-18

Liu G, Chater KF, Chandra G, Niu GQ, Tan HR (2013) Molecular regulation of antibiotic biosynthesis in Streptomyces. Microbiol Mol Biol Rev 77(1):112–143. https://doi.org/10.1128/MMBR.00054-12

Liu YQ, Wang HY, Li SS, Zhang YY, Cheng X, Xiang WS, Wang XJ (2013) Engineering of primary metabolic pathways for titer improvement of milbemycins in Streptomyces bingchengensis. Appl Microbiol Biotechnol 105(5):1875–1887. https://doi.org/10.1007/s00253-012-11164-7

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Method 25(4):402–408. https://doi.org/10.1006/meth.2001.1262

Ma DX, Wang C, Chen H, Wen JP (2018) Manipulating the expression of SARP family regulator BulZ and its target gene product to increase taconilin production. Appl Microbiol Biotechnol 102(11):4887–4900. https://doi.org/10.1007/s00253-018-8979-4

Narva KE, Feltlson JS (1990) Nucleotide sequence and transcriptional analysis of the redD locus of Streptomyces coelicolor A3(2). J Bacteriol 172(1):326–333. https://doi.org/10.1128/jb.172.1.326-333.1990

Nicastro RL, Sato ME, Silva MZ (2011) Fitness costs associated with milbemecin resistance in the two-spotted spider mite Tetranychus urticae. Int J Pest Manag 57(3):223–228. https://doi.org/10.1080/09670874.2011.574745

Nonaka K, Kumasaka C, Okamoto Y, Maruyama F, Yoshikawa H (1999a) Bioconversion of milbemycin-related compounds: biosynthetic pathway of milbemycins. J Antibiot 52(2):109–116. https://doi.org/10.7164/antibiotics.52.109

Nonaka K, Tsukiyama T, Sato K, Kumasaka C, Maruyama F, Yoshikawa H (1999b) Bioconversion of milbemycin-related compounds: isolation and utilization of non-producer, strain RNBC-5-51. J Antibiot 52(7):620–627. https://doi.org/10.7164/antibiotics.52.620

Nonaka K, Tsukiyama T, Okamoto Y, Sato K, Kumasaka C, Yamamoto T, Maruyama F, Yoshikawa H (2010) New milbemycins from Streptomyces hygroscopicus subsp. aureolacrimosus: fermentation, isolation and structure elucidation. J Antibiot 31(47):694–704. https://doi.org/10.7164/antibiotics.53.694

Ono M, Mishima H, Takiguchi Y, Terao M (1983) Milbemycins, a new family of macrolide antibiotics. Fermentation, isolation and physico-chemical properties of milbemycins D, E, F, G, and H. J Antibiot 36(5):509–515. https://doi.org/10.7164/antibiotics.36.509

Pluschke U, Horowitz AR, Ishaya I (1999) Effect of milbemec- tin on the sweetpotato whitefly, Bemisia Tabac Pygoparasitica 27(3):183–191. https://doi.org/10.1007/BF02981457

Romero-Rodriguez A, Robledo-Casados I, Sánchez S (2015) An overview on transcriptional regulators in Streptomyces. Biochim Biophys Acta 1894(8):1017–1039. https://doi.org/10.1016/j.bbabcm.2015.06.007

Sheldon PJ, Busarow SB, Hutchinson CR (2002) Mapping the DNA-binding domain and target sequences of the Streptomyces peucetius daunorubicin biosynthesis regulatory protein, DnrI. Mol Microbiol 44:449–460. https://doi.org/10.1046/j.1365-2958.2002.02886.x

Sherwood EJ, Bibb MJ (2013) The antibiotic planosporic acid coordinates differentiation in Streptomyces rochei. Appl Microbiol Biotechnol 98(23):9703–9712. https://doi.org/10.1007/s00253-018-9280-2

Sun P, Zhao Q, Yu F, Zhang H, Wu Z, Wang Y, Zhang Y, Zhang Q, Liu W (2013) Sporulation and formation and modification in avermectin biosynthesis involves a dual activity of AveC. J Am Chem Soc 135(4):1540–1548. https://doi.org/10.1021/ja311339u

Suzuki T, Mochizuki S, Yamamoto S, Arakawa K, Kinashi H (2010) Regulation of lankamycin biosynthesis in Streptomyces rochei by two SARP genes, srrY and srrZ. Biosci Biotechnol Biochem 74(4):819–827. https://doi.org/10.1271/bbb.90927

Tanaka A, Takano Y, Ohnishi Y, Horinouchi S (2007) AfsR recruits RNA polymerase to the afsS promoter: a model for transcriptional activation by SARPs. J Mol Biol 369(2):322–333. https://doi.org/10.1016/j.jmb.2007.02.096

Wang XJ, Guo SL, Guo WQ, Xiang WS (2006) Role of nsaD in negative regulation of antibiotic production and morphological differentiation in Streptomyces bingchengensis. J Antibiot 62:309–313. https://doi.org/10.1038/ja.2009.33

Wang XJ, Wang XC, Xiang WS (2009) Improvement of milbemycin-producing Streptomyces bingchengensis by rational screening of ultraviolet- and chemically induced mutants. World J Microbiol Biotechnol 25(6):1051–1056. https://doi.org/10.1007/s11274-009-9986-5

Wang XJ, Yan YJ, Zhang B, An J, Wang JJ, Tian J, Jiang L, Chen YH, Huang SX, Yin M, Zhang J, Gao AL, Liu CX, Zhu ZX, Xiang WS (2010) Genome sequence of the milbemycin-producing bacterium Streptomyces bingchengensis. J Bacteriol 192(17):4526–4527. https://doi.org/10.1128/JB.00586-10

Wang XJ, Zhang B, Yan YJ, An J, Zhang J, Liu CX, Xiang WS (2013) Characterization and analysis of an industrial strain of Streptomyces bingchengensis by genome sequencing and gene microarray. Genome 56(11):677–689. https://doi.org/10.1139/gen-2013-0098

Wang HY, Zhang J, Zhang YJ, Zhang B, Liu CX, He HR, Wang XJ, Xiang WS (2014) Combined application of plasma mutagenesis and gene engineering leads to 5-oxomilbemycins A3/A4 as main components from Streptomyces bingchengensis. Appl Microbiol Biotechnol 98(23):9703–9712. https://doi.org/10.1007/s00253-014-5970-6

Wang HY, Cheng X, Liu YQ, Li SS, Zhang YY, Wang XJ, Xiang WS (2020) Improved milbemycin production by engineering two Cytochromes P450 in Streptomyces bingchengensis. Appl Microbiol Biotechnol 104(7):2935–2946. https://doi.org/10.1007/s00253-020-10410-8

Wei KK, Wu YJ, Li L, Jiang WH, Hu JF, Lu YH, Chen SX (2018) M1R2, a novel TetR family regulator involved in 5-oxomilbe- mycin A3/A4 biosynthesis in Streptomyces hygroscopicus. Appl Microbiol Biotechnol 102:8841–8853. https://doi.org/10.1007/s00253-018-9280-2

Wietzorek A, Bibb M (1997) A novel family of proteins that regulates antibiotic production in Streptomyces appears to contain an
OmpR-like DNA-binding fold. Mol Microbiol 25(6):1181–1184. https://doi.org/10.1046/j.1365-2958.1997.5421903.x

Zhang J, An J, Wang JJ, Yan YJ, He HR, Wang XJ, Xiang WS (2013) Genetic engineering of Streptomyces bingchenggensis to produce milbemycins A3/A4 as main components and eliminate the biosynthesis of nanchangmycin. Appl Microbiol Biotechnol 97(23):10091–10101. https://doi.org/10.1007/s00253-013-5255-5

Zhang YY, He HR, Liu H, Wang HY, Wang XJ, Xiang WS (2016) Characterization of a pathway-specific activator of milbemycin biosynthesis and improved milbemycin production by its over-expression in Streptomyces bingchenggensis. Microb Cell Fact 15(1):152. https://doi.org/10.1186/s12934-016-0552-1

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.