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# Mining and fine-tuning sugar uptake system for titer improvement of milbemycins in *Streptomyces bingchenggensis*

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ABSTRACT

Dramatic decrease of sugar uptake is a general phenomenon in *Streptomyces* at stationary phase, when antibiotics are extensively produced. Milbemycins produced by *Streptomyces bingchenggensis* are a group of valuable macrolide biopesticides, while the low yield and titer impede their broad applications in agricultural field. Considering that inadequate sugar uptake generally hinders titer improvement of desired products, we mined the underlying sugar uptake systems and fine-tuned their expression in this work. First, we screened the candidates at both genomic and transcriptomic level in *S. bingchenggensis*. Then, two ATP-binding cassette transporters named TP2 and TP5 were characterized to improve milbemycin titer and yield significantly. Next, the appropriate native temporal promoters were selected and used to tune the expression of TP2 and TP5, resulting in a maximal milbemycin A3/A4 titer increase by 36.9% to 3321 mg/L. Finally, TP2 and TP5 were broadly fine-tuned in another two macrolide biopesticide producers *Streptomyces avermitilis* and *Streptomyces cyaneogriseus*, leading to a maximal titer improvement of 34.1% and 52.6% for avermectin $B_{1a}$ and nemadectin, respectively. This work provides useful transporter tools and corresponding engineering strategy for *Streptomyces*.

## 1. Introduction

Biosynthesis of secondary metabolites in *Streptomyces* occurs in stationary phase, when cell growth is almost stopped [1,2]. Despite the soluble sugars are widely used as main carbon source for both cell growth and secondary metabolite biosynthesis, they are primarily consumed during growth period rather than the stationary phase [3,4]. When entering stationary phase, a phenomenon of dramatic decrease of sugar uptake rate is usually observed in *Streptomyces* fermentations [5,6]. For a producing cell, carbon source uptake rates represent the input of materials available for downstream bioconversion [7]. Thus, sugar uptake can strongly influence the yield and titer of desired products during fermentations [7,8]. In addition, the residue sugar in *Streptomyces* fermentation broth also increases the difficulty in the downstream processes such as separation and wastewater treatment [9]. Therefore, overcoming the physiologically limited input of carbon source during stationary phase is an urgent task to improve production of secondary metabolites in *Streptomyces*.

As secondary metabolites of *Streptomyces*, milbemycins are currently-used insecticidal and acaricidal antibiotics in agriculture, owing to their advantages such as high activities, eco-friendly and safety for human and animal [10,11]. It is worth to note that derivatives from milbemycin A3/A4, such as milbemectin, possess higher insecticidal activity and lower toxicity than the well-known biopesticides avermectins [12,13]. Milbemycins were first discovered in *Streptomyces hygroscopicus* [14], and were later found in another producing strain *Streptomyces bingchenggensis* in 2007 by our group [15,16]. Using the established engineering strategies for production enhancement, such as random mutations [15,17], transcription regulator engineering [13,18] and biosynthetic pathway optimization [19], we have stepwise improved the titer of milbemycins during the past ten years. On the bases of these endeavors, *S. bingchenggensis* has been employed as industrial

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milbemycin producer in Zhejiang Hisun Pharmaceutical Co., Ltd [20]. However, the present titer and yield of milbemycins in *S. bingchenggensis* are much lower compared to the well-exploited biopesticide avermectin $B_{1a}$. By long-term and collective efforts from scientists and engineers from both academic and manufacturers, the titer of avermectin $B_{1a}$ in the producing *Streptomyces avermitilis* has elevated from 0.009 g/L up to more than 9 g/L, and the price has decreased to less than 500 RMB/kg [4,21–24]. Despite milbemycins show more potential than avermectins in plant protection, the current production cost still hinders the widespread applications in the agricultural field. Therefore, improvement of the milbemycin producing capacity of *S. bingchenggensis* is quite a necessary issue.

*S. bingchenggensis* uses sucrose from liquid medium as the main carbon source for milbemycin production. While at the end of fermentation, we observed that a considerable glucose and fructose generated from sucrose hydrolyzation still presented in the medium, indicating sugar uptake capability is inadequate at the milbemycin producing stage. To date, sugar uptake systems have been well investigated in the model microorganism, such as *Escherichia coli* and yeast [25–27]. However, in the well-known antibiotic producer *Streptomyces*, only a few literatures are available: namely, sugar uptake systems *in silico* analyzed in *Streptomyces coelicolor* [28,29] and the maltose transporter system *malEFG*-α engineered to increase starch utilization rate in *S. avermitilis* [30]. Hence, sugar uptake systems are remain poorly understood and manipulated in *Streptomyces* species. To fully exploit the underlying yet very crucial transport tools for glucose and fructose transportation at stationary phase, we decided to mine and engineer the uptake systems in *S. bingchenggensis* to promote sugar utilization as well as the titer and yield of milbemycins.

In the present work, we mined and engineered two previously unidentified sugar uptake systems for titer and yield improvement of macrolide biopesticide milbemycin A3/A4 in *S. bingchenggensis*. First, we analyzed the genome of *S. bingchenggensis* and screened putative sugar uptake systems. Next, based on transcriptome and experimental analyses, we identified two ATP-binding cassette (ABC) sugar uptake systems effective on titer improvement. Then, we optimized the yield and titer of milbemycin A3/A4 by fine-tuning the expression levels of the two uptake systems using the selected native promoters with appropriate temporal activities. Finally, the two identified sugar uptake systems were employed to enhance the titers of another two macrolide biopesticides (i.e., avermectin $B_{1a}$ and nemadectin) in the corresponding *Streptomyces* species, indicating the potential of broad application of these sugar uptake systems in *Streptomyces* species.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

All strains used in this study are summarized in Table S1. *Escherichia coli* JM109 was used to propagate all plasmids. *E. coli* ET12567/pUZ8002 was used to obtain un-methylated DNA from *E. coli* to *Streptomyces* for intergeneric conjugations. All *E. coli* strains were grown in Luria–Bertani (LB) medium at 37 °C. Unless stated, *Streptomyces* strains were cultivated at 28 °C statically or shaken at 250 rpm. For spore preparation, *S. bingchenggensis* BC-101-4 (the parent strain, low-yielding producer of milbemycins), BC04 (the high-yielding producer of milbemycins) and their mutants were grown on SKYM agar plate [13], *S. avermitilis* strains were maintained on MS agar plate [31], and *Streptomyces cyaneogriseus* strains were grown on ISP3 agar plate [32]. For intergeneric conjugations, all *Streptomyces* strains were grown on MS agar plates at 28 °C [31]. For milbemycin production, the spore suspensions of *S. bingchenggensis* were inoculated into 25 mL SSPY medium in 250 mL flasks and cultivated for 46 h. Then, 1.5 mL of seed culture was transferred into 250 mL flasks containing 25 mL fermentation medium and fermented for 9 days [13]. Seed medium, fermentation medium for *S. avermitilis* and *S. cyaneogriseus* were the same as previously described by Gao et al. and Li et al. [32,33], respectively. For avermectin fermentation, seeds were cultivated for 40 h in a rotary shaker. Then, 1.5 mL of seed culture was transferred into 25 mL fermentation medium and cultivated for 10 days. For nemadectin production, spore suspensions were inoculated into 25 mL seed medium and cultivated for 46 h. Then, 3 mL of seed culture was transferred into 50 mL fermentation medium and cultivated for 9 days.

### 2.2. Gene cloning and plasmid construction

All plasmids used in this work are listed in Table S1. All primers and oligonucleotides are listed in Table S2. The coding sequences of gene *sbi_03422 − sbi_03424*, *sbi_04112 − sbi_04114*, *sbi_06547 − sbi_06549*, *sbi_06565 − sbi_06567*, *sbi_06891 − sbi_06893*, *sbi_07017 − sbi_07019*, *sbi_07399 − sbi_07401*, and *sbi_04453* were amplified from the genomic DNA of *S. bingchenggensis* by PCR using the primer pairs TP1-F/TP1-R, TP2-F/TP2-R, TP3-F/TP3-R, TP4-F/TP4-R, TP5-F/TP5-R, TP6-F/TP6-R, TP7-F/TP7-R and TP8-F/TP8-R respectively (Table S2). The PCR products and the 500-bp promoter region upstream of gene *sbi_04864* (Table S3) amplified from the genome of *S. bingchenggensis* by primer pair P4864–F/P4864-R were cloned to the *Xba*I and *EcoR*I sites of pSET152 by Gibson DNA assembly to obtain P4864-TP*c* (Table S1). The selected native promoters (500-bp sequences upstream of the corresponding genes) were amplified from the genome of *S. bingchenggensis* using the corresponding primer pairs P1–F/P1-R, P2–F/P2-R, P3–F/P3-R, P4–F/P4-R, P5–F/P5-R, P6–F/P6-R, P7–F/P7-R, P8–F/P8-R, P9–F/P9-R, and P10–F/P10-R (Table S2), respectively. The *hrdB* promoter was cloned from plasmid pSET152::$P_{hrdB}$*milR* [13] using primer pair PhrdB-F/PhrdB-R (Table S2). The PCR products of these promoters were digested with *Not*I and *Spe*I, and ligated with *Not*I/*Spe*I double digested P4864-TP2 and P4864-TP5 to generate P*n*-TP2 or P*n*-TP5. The plasmid skeleton with *sfgfp* was cloned from plasmid pIJ-Potr [34] using primer pair PIJ-F/PIJ-R. The PCR product was digested with *Not*I and *Spe*I, and ligated with *Not*I/*Spe*I double digested P4864-TP2 to obtain P4864-GFP. Strain BC-101-4pG and BC04pG were obtained by integrated P4864-GFP into the genome of BC-101-4 and BC04, respectively.

### 2.3. Transporter analysis of S. bingchenggensis

Transporters of *S. bingchenggensis* were analyzed by local BLASTP (v. 2.6.0+) using transporter sequences deposited in Transporter Classification Database (TCDB) (by Otc. 26, 2018) [35]. The threshold for homologous genes was set as 30%, 70% and 1e-5 for identity, coverage, and e-value, respectively. The substrate of each transporter was predicted based on homology to functionally characterized transporters in the TCDB. All predicted transporters in *S. bingchenggensis* were classified according to the TC system and reported data [35,36].

### 2.4. Microarray data analysis

Cells of *S. bingchenggensis* BC-101-4 and BC04 were sampled at 0.75, 2, 3, 4, 6, and 8 day for experiments. Details for microarray experiments were described as previous report [37]. For each time-series transcriptome microarray dataset, the expression signals of each gene from different time points were normalized to that obtained from 0.75 day (the first sampling time point), resulting in a series of fold changes of gene expression. Transcript levels of genes whose fold changes are higher than two ($|\log_2{}^{\text{fold change}}| > 1$) are considered to be upregulated. Correlation analysis between gene transcript level and milbemycin production was conducted using the normalized data obtained from 2 to 4 day. The gene with a correlation coefficient more than 0.5 and an absolute value of linear slope more than 0.01 was thought to be correlated to milbemycin production enhancement.

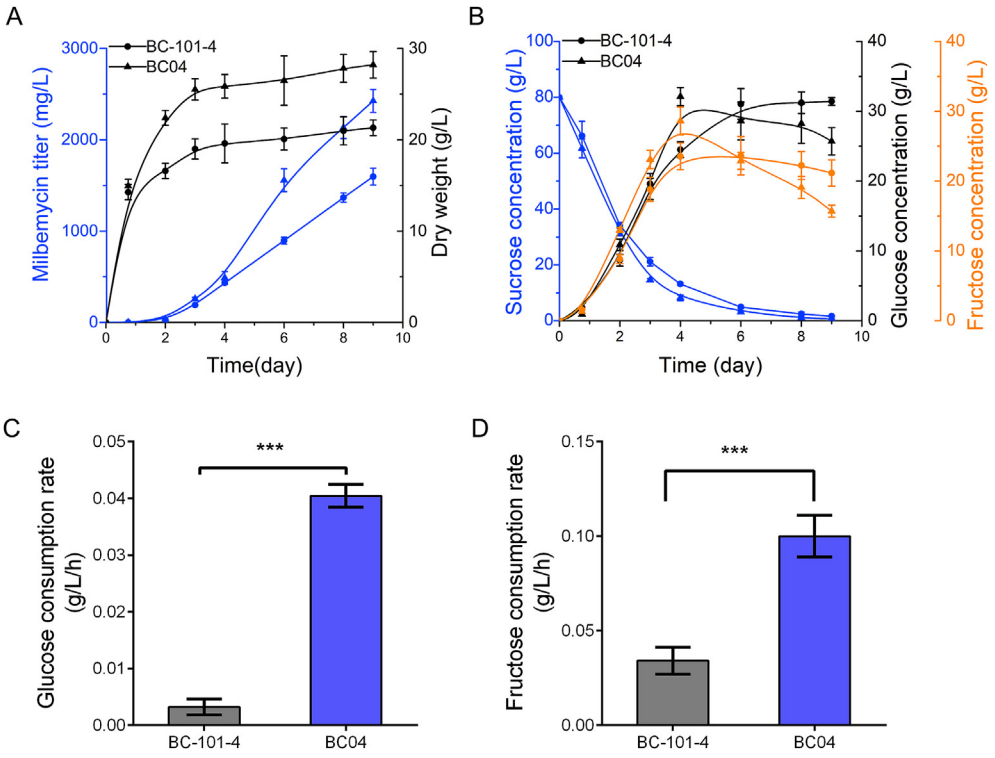


**Fig. 1. Fermentation profile of milbemycin production in *S. bingchenggensis* BC-101–4 and BC04.** (A) Time-course curves of cell growth and milbemycin production. (B) Time-course curves of sugar consumption. (C) and (D) Comparison of glucose and fructose consumption rate during milbemycin extensive production period (144–216 h). For (A–D), the data shown were the average and s.d. of three independent experiments. For (C–D), differences were analyzed by Student's *t*-test, and $p < 0.05$ was considered statistically significant. The levels of significance are $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$. All data were obtained from three independent experiments.

### 2.5. Promoter selection

Hierarchical clustering of time-course transcriptome data of *S. bingchenggensis* was used to select promoters with satisfied time-course profiles, and FPKM values of the corresponding genes were considered to choose promoters with appropriate strength. Promoter region was the 500-bp sequence upstream of the corresponding gene. Promoter profile was evaluated by determining the time-course transcriptional level of *sfgfp* it controlled using quantitative real time RT-PCR (qRT-PCR). For that, mycelia were harvested from fermentation broth of *S. bingchenggensis* at various time points (0.75, 2, 3, 4, 6, and 8 day). RNA extraction, quality examination, and quantification were performed as described previously [13]. The qRT-PCR experiments were implemented on a QuantStudio 5 (Applied Biosystems) using PowerUp SYBR Green Master Mix (Applied Biosystems). The running conditions were: 95 °C for 10 min, followed by 40 three-step amplification cycles consisting of denaturation at 95 °C for 15 s, annealing at 60 °C for 60 s and extension at 72 °C for 30 s.

### 2.6. Analysis of milbemycins, avermectins, and nemadectin

The fermentation broth of *S. bingchenggensis* and *S. cyaneogriseus* were extracted with three volumes of ethanol, while the fermentation broth of *S. avermitilis* was extracted with three volumes of methyl alcohol. The concentration of milbemycin A3/A4, avermectin $B_{1a}$ and nemadectin were quantitated by Agilent 1260 high-performance liquid chromatography (HPLC) with an SB-C18 column (Zorbax, 4.6 mm × 250 mm, 5 mm) at a flow rate of 1.0 mL/min. For milbemycin A3/A4, the following gradient of B was applied from 0 to 100% in 15 min (Solvent A: acetonitrile:water:methanol (7:1:2, v/v/v); Solvent B: methanol) and samples were detected at 242 nm. To determine the concentrations of avermectins and nemadectin, samples were detected at 246 nm and 240 nm with 90% and 85% methanol, respectively.

### 2.7. Analysis of sugars concentrations

Residual sugars in fermentation broth were extracted with two volumes of methanol, and concentration was quantified by HPLC (Shimadzu LC-20AT) equipped with a refractive index detector and a carbohydrate column (Zorbax, 4.6 mm × 250 mm, 5 mm). The 80% acetonitrile was used as mobile phase with a flow rate of 1.0 mL/min. Concentration of total sugar in *S. avermitilis* fermentation broth was assayed by the 3, 5-dinitrosalicylic acid and phenol sulfuric acid methods following the instruction of a total sugar content kit (Cominbio, China).

### 2.8. Determination of cell dry weight

Cells harvested from 2 mL fermentation cultures were washed three times by deionized water and then dried at 55 °C to a constant weight to determine the dry cell weight.

### 2.9. Statistical analysis

All experiments were executed independently at least three times, and data were presented as mean and standard derivations (s.d.). Significance was analyzed by Student's *t*-test or analysis of variance (ANOVA). $p < 0.05$ is considered as statistically significant standard criterion.

### 2.10. Data availability

Time-course transcriptome related to this article can be found in GEO database (GSE147644).

## 3. Results

### 3.1. Fermentation profile of S. bingchenggensis

Using sucrose as the main carbon source, milbemycin A3/A4 were extensively biosynthesized at stationary phase in both *S. bingchenggensis*

wild type BC-101-4 and high-yielding BC04 (Fig. 1A). When analyzing the fermentation profiles of the two strains, we observed that the concentration of residual sucrose in fermentation broth of both strains were lower than 1.5% at the end of fermentation, whereas the concentrations of residual glucose and fructose that generated from sucrose hydrolyzation were higher than 15 g/L (Fig. 1B), demonstrating the uptake and use of glucose and fructose were not efficient in both strain at the production stage. Notably, we found that the consumption rates of both glucose and fructose in BC04 were 12.5- and 2.9-fold of that in BC-101-4 during milbemycin A3/A4 extensively produced period (144–216 h) (Fig. 1C and D). These results indicate that the high-yielding strain BC04 possessed a better sugar uptake capability, and increasing sugar uptake efficiency during milbemycin production period might facilitate titer improvement in *S. bingchenggensis*. We thus tried to mine the unexploited sugar uptake systems to achieve this goal.

### 3.2. *In silico* analysis of sugar transporter candidates in *S. bingchenggensis*

To identify the sugar uptake systems contributing to the uptake discrepancy between *S. bingchenggensis* BC-101-4 and BC04, we designed a workflow to interrogate the candidates at genomic and transcriptomic level (Fig. 2A). First, putative sugar transporters of *S. bingchenggensis* were analyzed *in silico* [35]. A total of 1063 putative transport proteins were identified in *S. bingchenggensis* (Fig. 2B and Dataset S1). Among them, 206 proteins (19%) were predicted as carbon source uptake proteins, including 155 putative sugar uptake proteins (accounted for 75% of the predicted as carbon source uptake proteins) (Fig. 2B and Dataset S1), which belong to 86 predicted sugar uptake systems (Dataset S2).

Further, we selected sugar transporter candidates from the 155 proteins based on transcriptional level analyses of their encoding genes (GSE147644). First, we screened genes whose transcriptional levels were correlated with milbemycin production during early production stage (2–4 day) in both BC-101-4 and BC04 ($r^2 > 0.5$, $|slope| > 0.01$), and got 33 genes meeting such a criterion (Table S4). Then, we compared the transcriptional levels of these genes at early production stage in the two strains, and obtained 14 genes whose transcriptional levels were upregulated in the high-yielding strain BC04 (Fig. S1), indicating these genes may contribute to the better uptake of glucose and fructose in BC04. Finally, based on protein function analysis, we selected eight sugar uptake systems for further experimental tests, including seven

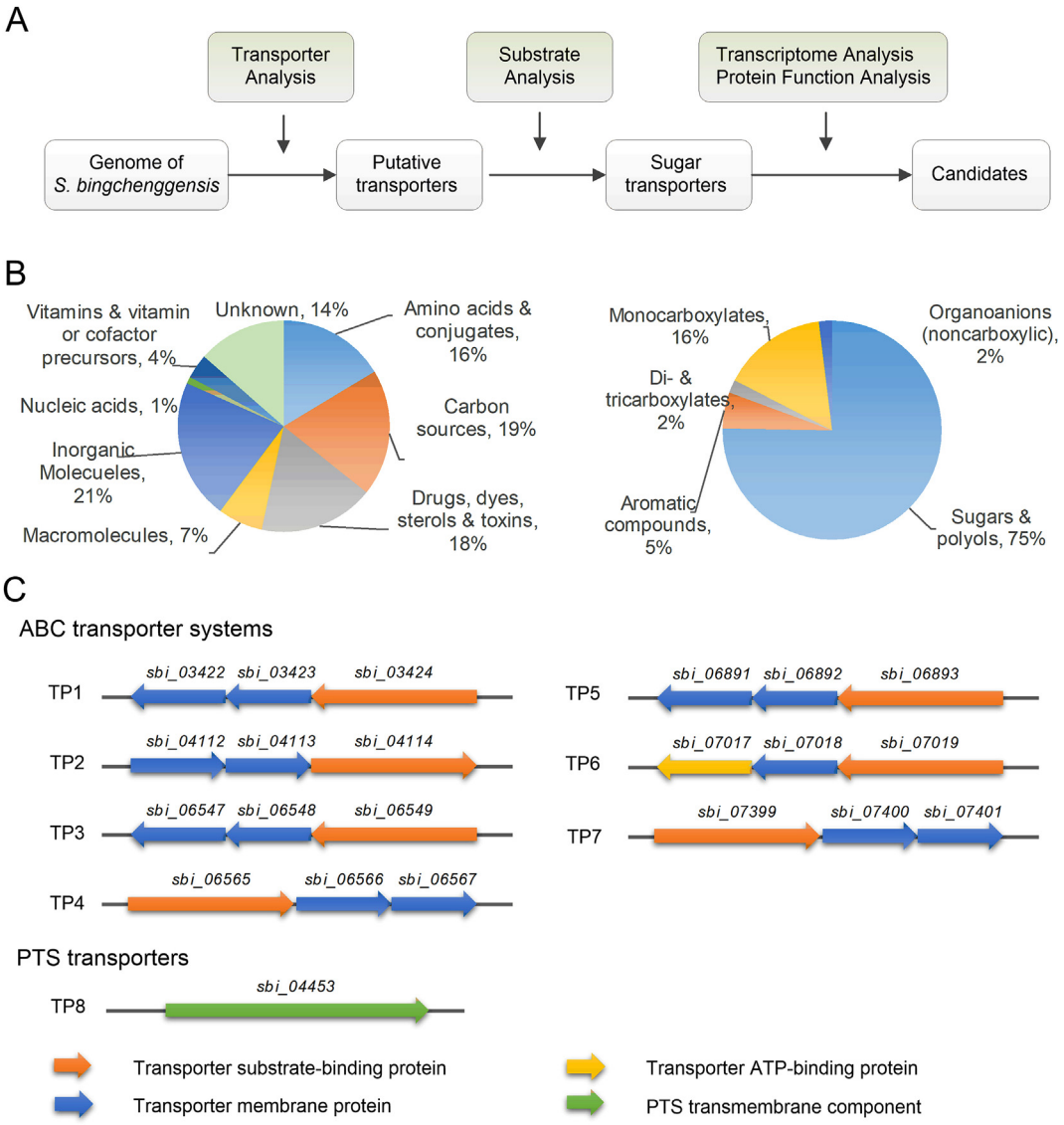


**Fig. 2. *In silico* identification of sugar uptake systems.** (A) Rational selection workflow of sugar uptake systems. (B) Putative substrate types of the *in-silico* selected uptake systems of *S. bingchenggensis*. (C) Operons of the selected putative sugar uptake systems.

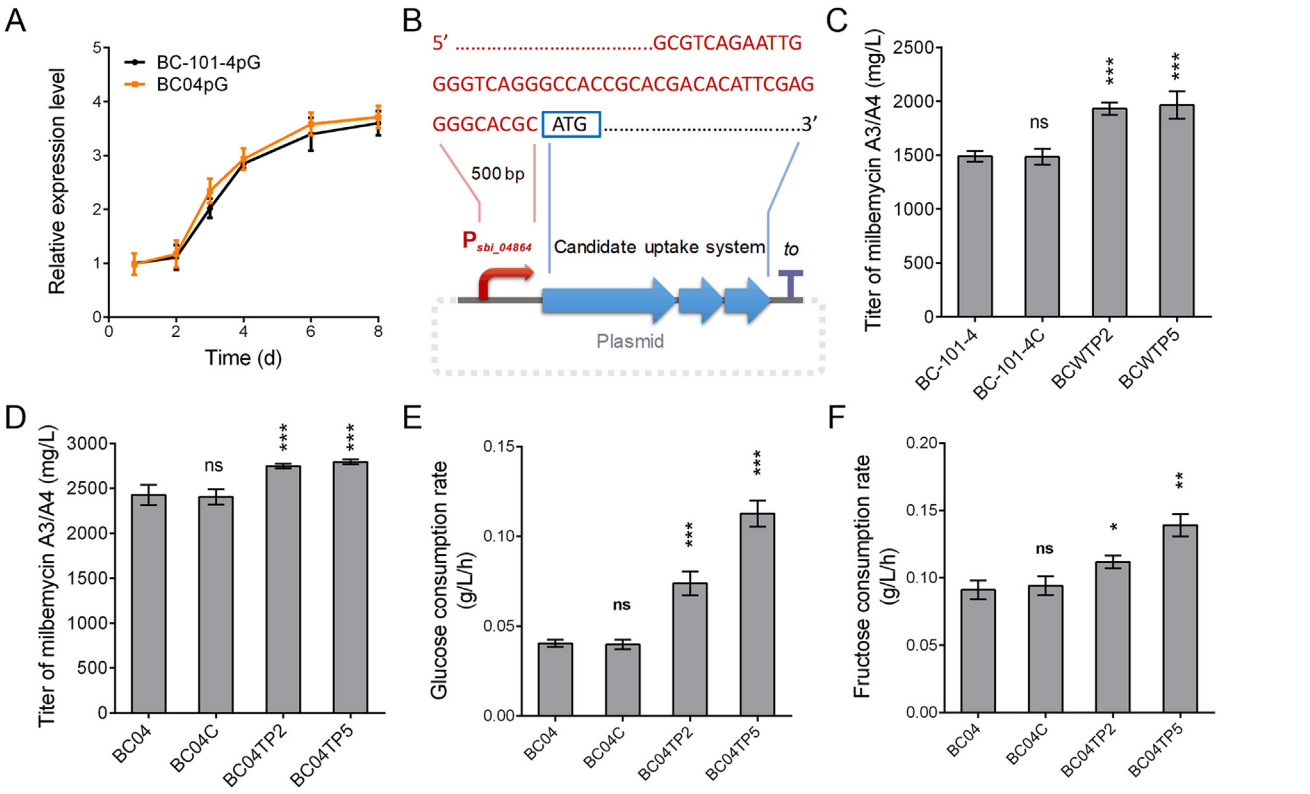


**Fig. 3. Evaluation of the candidates of sugar uptake system.** (A) Evaluation of promoter performance of $P_{sbi\_04864}$ in different *S. bingchenggensis* strains. (B) Schematic of the plasmid used for evaluation of the candidate sugar transporter system. (C) and (D) Effect of sugar uptake system TP2 and TP5 on milbemycin A3/A4 titer improvement, respectively. (E) and (F) Influence of TP2 and TP5 on glucose and fructose consumption rates (144–216 h), respectively. For (C–F), data of BC-101-4 (C) and BC04 (D–F) were set as control; BC-101-4C and BC04C were the strains integrated with the plasmid without tested transporter; overexpression of TP2 and TP5 were controlled by a native promoter $P_{sbi\_04864}$. Differences were analyzed by ANOVA, and $p < 0.05$ was considered statistically significant. The levels of significance are $***p < 0.001$, $**p < 0.01$, $*p < 0.05$, 'ns' means no significant difference. All data were obtained from three independent experiments.

ABC transporter systems (TP1, SBI_03422 – SBI_03424; TP2, SBI_04112 – SBI_04114; TP3, SBI_06547 – SBI_06549; TP4, SBI_06565 – SBI_06567; TP5, SBI_06891 – SBI_06893; TP6, SBI_07017 – SBI_07019; TP7, SBI_07399 – SBI_07401), and one phosphotransferase system (PTS; TP8, SBI_04453) (Fig. 2C).

### 3.3. Evaluation of the candidate sugar uptake systems

The effect of the eight sugar uptake systems on milbemycin production was experimentally evaluated in both parent and high-yielding strains. To avoid the fast sugar consumption during cell growth stage, we first chose a native temporal promoter $P_{sbi\_04864}$ with baseline strength at growth stage while a moderate elevated activity at stationary phase via time-course transcriptome analysis (Fig. S2 and Table S3; GSE147644). We further confirmed the consistent performance of this promoter at translational level in *S. bingchenggensis* BC-101-4 and BC04 using the reporter green fluorescent protein (sfGFP) (Fig. 3A). We thus used this promoter rather than constitutive strong promoters to control the expression levels of eight candidate sugar uptake systems (Fig. 3B). The results showed that, in the parent stain BC-101-4, all the tested candidates could facilitate milbemycin production (Fig. S3A); whereas in the high-yielding strain BC04, four of them exhibited titer improvement effect (Fig. S3B). Notably, two ABC transporter systems TP2 and TP5 showed the best results in both strains: TP2 improved the titer of milbemycin A3/A4 by 29.7% and 13.3% in BC-101-4 and BC04, respectively, while TP5 enhanced the titer by 32.0% and 15.2% in BC-101-4 and BC04, respectively (Fig. 3C and D).

We further tested the effect of overexpressing TP2 and TP5 on the consumption of glucose and fructose in BC04 during the period when milbemycins are largely produced. As expected, we observed that the consumption rate of glucose was increased by 82.4% and 178.5% in BC04, respectively; and the consumption rate of fructose was improved by 12.3% and 52.8%, respectively (Fig. 3E and F). The residual concentration of sucrose showed no significant changes in all tested strains (lower than 0.5 g/L). These results suggest that TP2 and TP5 might be valuable for titer improvement of milbemycins in the producing strain *S. bingchenggensis*.

### 3.4. Fine-tuning the expression levels of sugar transporters

Fine-tuning the expression level of targets is an effective strategy for titer improvement of desired metabolites, especially for that of the secondary metabolites [38–40]. We therefore tried to select appropriate native temporal promoters to optimize the expression levels of the two sugar transporters. Since the sugar consumption rates of the two strains began to show differences after four days in *S. bingchenggensis* BC-101-4 and BC04 (Fig. 1B), we selected native temporal promoters whose strength was upregulated after three days to ensure the increase of sugar consumption for milbemycin production. There were 419 and 391 genes showing desired profile in BC-101-4 and BC04 (Fig. 4A, Fig. S4 and Dataset S3), respectively. The intersection contained 87 qualified genes and 80 putative promoters (Table S5). Here, we selected 10 promoters with different strengths to fine-tune the expression of sugar transporter TP2 and TP5, respectively (Fig. 4B and C and Table S3). Meanwhile, the constitutive *hrdB* promoter was also used for comparison.

Overexpression of target sugar transporter system TP2 and TP5 by using the identified time-course promoters with different strengths in BC04 improved the titer of milbemycin A3/A4 by 30.3% and 36.9% to 3161 and 3321 mg/L, respectively (Fig. 4D and E). Generally, temporal

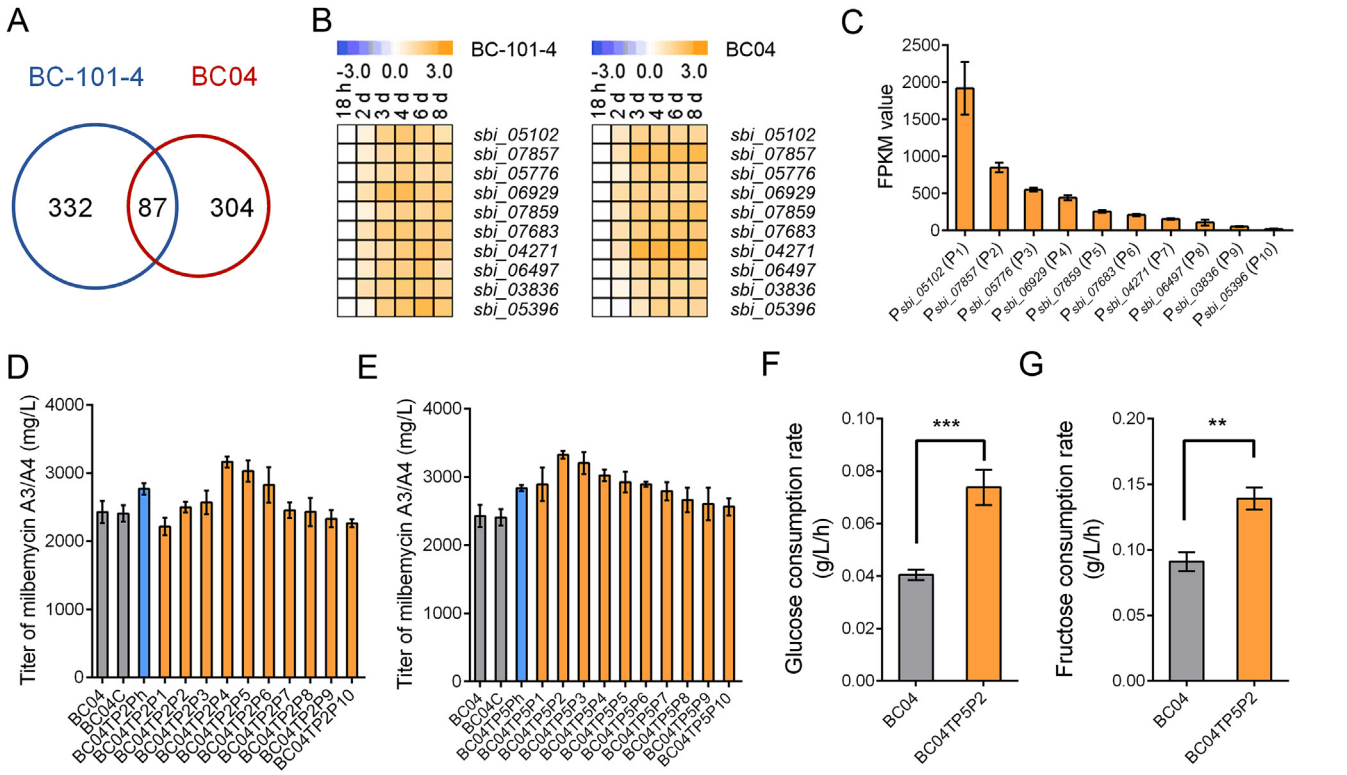


**Fig. 4. Fine-tuning the expression levels of sugar transporters for milbemycin titer improvement.** (A) Number of genes with upregulated transcription levels after three days. (B) Transcription profile of ten selected genes in BC-101-4 and BC04. (C) Relative strengths of the selected promoters. Promoter strength was indicated by FPKM of genes in the three day from transcriptome data of *S. bingchenggensis* (GSE147644). (D) Influence of sugar uptake system TP2 on titer of milbemycin A3/A4. (E) Influence of sugar uptake system TP5 on titer of milbemycin A3/A4. For (D) and (E), titer of milbemycins were obtained at 216 h; BC-101-4C and BC04C were the strains integrated with the plasmid without tested transporter; Ph, P2, and P4 indicated the *hrdB* promoter, native promoter $P_{sbi\_07857}$ and $P_{sbi\_06929}$, respectively. (F) Comparison of glucose consumption rate (144–216 h) between BC04 and BC04TP5P2. (G) Comparison of fructose consumption rate (144–216 h) between BC04 and BC04TP5P2. For (B–G), data were obtained from three independent experiments. Differences were analyzed by Student's *t*-test, and $p < 0.05$ was considered statistically significant. The levels of significance are $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

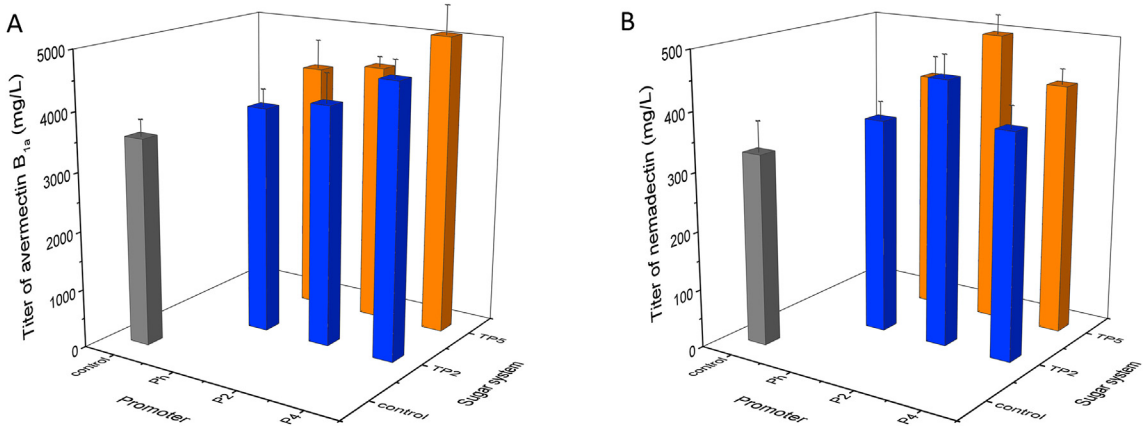


**Fig. 5. Broad application of the identified sugar uptake systems.** (A) Performance of overexpression of TP2 and TP5 with different promoters on titer improvement of avermectin $B_{1a}$. (B) Performance of overexpression of TP2 and TP5 with different promoters on titer improvement of nemadectin. Ph, P2, and P4 indicated the *hrdB* promoter, native promoter $P_{sbi\_07857}$ and $P_{sbi\_06929}$, respectively. *S. avermitilis* NEAU12 and *S. cyaneogriseus* NMWT1 were set as control. Data were obtained from three independent experiments.

promoters showed better effect on titer improvement of milbemycins than the constitutive promoter $P_{hrdB}$. In additional, promoters with moderate strengths rather than the strong ones were benefit to titer improvement (Fig. 4D and E). We further analyzed the residual sugars in fermentation broth of engineered *S. bingchenggensis* BC04TP5P2 with the highest milbemycin A3/A4 titer. It was shown that concentration of both glucose and fructose were reduced at the end of fermentation (Fig. S5). By calculating these data, we found that the glucose and fructose consumption rates of the strain BC04TP5P2 were 82.4% and 52.8% higher than those of the parental strain BC04 during the period when milbemycins were extensively produced (Fig. 4F and G). Meanwhile, yield of this strain was improved by 36.8% to 0.07 C-mol/C-mol. These results demonstrate that the increased sugar uptake efficiency during late fermentation stage was effective on milbemycins titer and yield improvement in *S. bingchenggensis*, which further highlight that fine-tuning the expression level of the target sugar transporter system is a

simple and feasible engineering approach.

### 3.5. Broad application of TP2 and TP5

Since overexpression of TP2 and TP5 could promote uptake of glucose and fructose as well as milbemycin production (Fig. 4), we thus speculated that these two sugar uptake systems might facilitate antibiotic fermentations whose culture medium contains glucose and/or fructose. To broadly test the effect of TP2 and TP5, here we focused on avermectin $B_{1a}$ producing strain *S. avermitilis* NEAU12 and nemadectin producing strain *S. cyaneogriseus* NMWT1. Based on previous results (Fig. 4), we here chose $P_{hrdB}$ (Ph), $P_{sbi\_07857}$ (P2) and $P_{sbi\_06929}$ (P4) to fine-tune the two sugar uptake systems in *S. avermitilis* NEAU12 and *S. cyaneogriseus* NMWT1, respectively. Results showed that expression of TP2 and TP5 also improved the titers of avermectin $B_{1a}$ and nemadectin (Fig. 5). Especially, overexpressing TP5 in NEAU12 using a temporal promoter $P_{sbi\_06929}$ gave the highest titer of avermectin $B_{1a}$ from 3533 to 5092 mg/L in NEAU12TP5P4 (Fig. 5A). Similarly, the highest titer of nemadectin was enhanced from 327 to 499 mg/L in ScyTP5P2, where TP5 was overexpressed under the control of a temporal promoter $P_{sbi\_07857}$ (Fig. 5B). Moreover, overexpression of TP5 improved the yields of avermectin $B_{1a}$ and nemadectin by 44.6% and 52.5% to 0.087 and 0.012 C-mol/C-mol in NEAU12TP5P4 and ScyTP5P2, respectively. These results demonstrated that TP2 and TP5 were not only advantageous to titer improvement of milbemycins, but also effective on other macrolide biopesticides, highlighting the two sugar transporter systems might be valuable to be applied in *Streptomyces* when using glucose related substrate as carbon source (i.e., glucose, maltose, sucrose, starch, etc.) during fermentations.

## 4. Discussions

Carbon sources, as we all know, are not only important nutrients for survival of the majority of heterotrophic bacteria [41], but also provide carbon atoms for microbial hosts to synthesize skeletons of desired products directly or indirectly [42]. Therefore, effective utilization of carbon sources (mainly sugars) is a prerequisite of high yield. Inadequate sugar utilization efficiency is a general knotty issue hindering further titer and yield improvement of desired products [25,42]. Especially in *Streptomyces* fermentations, sugar uptake capability is significantly reduced during secondary metabolite biosynthetic period [4]. Considering the significant contribution of sugar uptake transporter to the flux towards products, it is quite important to investigate sugar uptake transporters first [43]. At present, sugar transporter engineering for titer improvement meets two challenges in *Streptomyces* as well as in other species. One is the lack of identified sugar transporter; another one is the balance between transporter expression level and cell growth [8]. Hence, it is important to address these problems for strain engineering.

Different sugar transporters have been characterized in *E. coli* and yeast, whereas they have almost been a Cinderella subject in *Streptomyces*. Despite each *Streptomyces* strain is a rich transporter repository [36], few of them have been mined and applied. Our work provided two previously unidentified sugar uptake systems from *S. bingchenggensis* genome, which were demonstrated to be efficient for titer and yield improvement of milbemycin A3/A4, avermectin $B_{1a}$ and nemadectin. More importantly, we provided a simple workflow to discover useful transporters by establishing the relationship between time-course transcription levels of all putative sugar transporters and titers of desired products. In comparison to previous work depending on structural analogues of specific compounds [44] or omics data [45,46], our approach is less information required and more feasible to obtain unidentified candidates.

When obtaining transporter candidates, it is vital to fine-tune the expression levels of these targets to minimize the growth inhibition caused by overexpression. Besides, such a manipulation is quite necessary to avoid excessive sugar consumption by cell growth, which is advantageous to optimize carbon flux toward desired secondary metabolites in *Streptomyces* [4,47]. The optimal flux can be achieved via dynamic fine-tuning using small molecular responsive biosensors [40,48], inducible promoters [34] and native temporal promoters [38]. While considering the requirements of large-scale fermentations, appropriate native temporal promoters might be more preferable for dynamic previous control of gene expression level [38]. Here, we showed that native temporal promoters showed better effect than the constitutive promoters for titers improvement of three different macrolide biopesticides, which is consistent with previous investigations [49].

In summary, the present work provided two previously unidentified sugar uptake systems, which are useful transporter tools for metabolic engineering and synthetic biology in *Streptomyces*. Moreover, we demonstrated that fine-tuning the expression level of the identified sugar uptake system is a simple and effective approach for titer and yield improvement of three macrolide biopesticides (milbemycins, avermectins, and nemadectin) in the corresponding *Streptomyces* producers. Considering the importance of sugar uptake at stationary phase for biosynthesis of secondary metabolites, especially for the production of antibiotics in *Streptomyces*, the broad application potential of this work might be highlighted.

## CRediT authorship contribution statement

**Pinjiao Jin:** Methodology, Investigation, Writing - original draft, Project administration. **Shanshan Li:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition. **Yanyan Zhang:** Investigation, Visualization. **Liyang Chu:** Investigation, Visualization. **Hairong He:** Investigation, Visualization. **Zhuoxu Dong:** Investigation, Visualization. **Wensheng Xiang:** Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Wensheng Xiang, Shanshan Li, Pinjiao Jin, Yanyan Zhang have filed a patent 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.2020.07.001.

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