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APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

A previously unidentified sugar transporter for engineering of high-yield *Streptomyces*

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Abstract

Sugar transporters have significant contributions to regulate metabolic flux towards products and they are general potential targets for engineering of high-yield microbial cell factories. *Streptomyces*, well-known producers of natural product pharmaceuticals, contain an abundance of sugar transporters, while few of them are well characterized and applied. Here, we report a previously unidentified ATP-binding cassette (ABC) sugar transporter TP6568 found within a *Streptomyces avermitilis* transposon library, along with its key regulator GM006564. Subsequent in silico molecular docking and genetic experiments demonstrated that TP6568 possessed a broad substrate specificity. It could not only promote uptake of diverse monosaccharides and disaccharides, but also enhance the utilization of industrial carbon sources such as starch, sucrose, and dextrin. Constitutive overexpression of TP6568 resulted in decrease of residual total sugar by 36.16%, 39.04%, 38.40%, and 30.21% in engineered *S. avermitilis* S0, *Streptomyces caniferus* NEAU6, *Streptomyces bingchenggensis* BC-101-4, and *Streptomyces roseosporus* NRRL 11379 than their individual parent strain, respectively. Production of avermectin B_{1a}, guvermectin, and milbemycin A3/A4 increased by 75.61%, 56.89%, and 41.13%, respectively. We then overexpressed TP6568 in combination with the regulator GM006564 in a high-yield strain *S. avermitilis* S45, and further fine-tuning of their overexpression levels boosted production of avermectin B_{1a} by 50.97% to 7.02 g/L in the engineering strain. Our work demonstrates that TP6568 as a promising sugar transporter may have broad applications in construction of high-yield *Streptomyces* microbial cell factories for desirable natural product pharmaceuticals.

Key points

- *TP6568 from Streptomyces avermitilis was identified as a sugar transporter*
- *TP6568 enhanced utilization of diverse industrially used sugars in Streptomyces*
- *TP6568 is a useful transporter to construct high-yield Streptomyces cell factories*

Keywords Sugar transporter · Transporter engineering · *Streptomyces* · Strain engineering · Titer improvement

Introduction

Uptake of carbon into cells across biological membranes is a fundamental process for vital biological functions in all domains of life (Chen et al. 2015; Dills et al. 1980). Bacteria and fungi have evolved a large number of carbohydrate transporters to allow them to quickly adapt to and colonize changing surroundings (Jeckelmann and Erni 2020). Sugars are the most commonly used carbon sources for microorganisms. Sugar transporters act as critical gates to allow the import of external sugars into cells, and their activities are not only important to maintain the normal cellular processes, but also significant to efficient bioconversion of target chemicals within microbial producers (Jin et al. 2020; Lv et al.

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2016; Yan and Fong 2018). Therefore, sugar transporter engineering has become a popular approach to construct efficient microbial cell factories for diverse requirements, such as chemical production, pharmaceutical biosynthesis, and agro-waste utilization (Farwick et al. 2014; Jin et al. 2020; Kuanyshev et al. 2021; Yan and Fong 2018; Young et al. 2012; Zhu et al. 2017).

In genomes of sequenced bacteria, eukaryotes, and archaea, over 10% of the encoded proteins are annotated as transporters (Radi et al. 2022). As of June 13, 2023, the Transporter Classification Database contained 22,770 transporters, while only 7.04% of them (1,603 proteins) had corresponding crystal structures, demonstrating that majority of transporters are poorly characterized. Since understanding of transporter function and specificity are basis for sugar transporter engineering, current limited recognition of sugar transporters restricts construction of high-yield microbial cell factory by this approach. To address this issue, researchers have explored and characterized transporters through different approaches, including phenotypic characterization via gene knock-out and overexpression (Wang et al. 2017), sequence similarity-based function prediction (Darbani et al. 2018), ligand-responsive biosensor system-aided high-throughput mining (Genee et al. 2016), transportome-wide identification (Wang et al. 2021), and adaptive laboratory evolution (Radi et al. 2022). To date, a series of useful sugar transporters, which are capable of transporting single or multiple sugars, have been discovered and applied to promote substrate utilization or enhance production of desired chemicals in diverse microorganisms, such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Corynebacterium glutamicum* (Doshi et al. 2013; Hu et al. 2018; Kuanyshev et al. 2021; Liu et al. 2022).

Streptomyces is a well-known chassis for production of natural product pharmaceuticals. Generally, transporters account for over 10% of the proteome of *Streptomyces*, among which carbohydrate transporters are the most abundant with a portion of approximately 26.8% (Zhou et al. 2016). Several sugar transporters have been characterized in *Streptomyces*, such as the glucose transporter GlcP (Van Wezel et al. 2005), the maltose transporter MalEFG (Li et al. 2010), the xylan transporter BxlEFG (Tomoo et al. 2017), and the cellobiose and cellotriose transporter CebEFG (Bertram et al. 2004). Aided by bioinformatic analyses, TP2 and TP5 were screened from *Streptomyces bingchenggensis* and validated as useful sugar transporters in promoting sugar utilization including glucose, fructose, sucrose, and starch, as well as in boosting biosynthesis of natural products in our previous work *Streptomyces* (Jin et al. 2020). Despite this progress, transporter characterization still faces challenges posed by experimental and computational methods. The currently investigated sugar transporters represent only a small portion of the possible transporters which could be studied (Genee et al. 2016). Therefore, exploration and characterization of useful carbohydrate transporters for *Streptomyces* may be necessary for construction of high-yield producers of natural products.

Here, we report a novel ATP-binding cassette (ABC) sugar transporter TP6568 from *Streptomyces avermitilis*. This transporter was obtained through Tn5-based transposon mutagenesis, which allows target identification without a priori knowledge. TP6568 possesses a broad sugar substrate spectrum, which could promote uptake efficiency of different sugars as well as boost the production of natural products in different *Streptomyces*. TP6568 could be used as a general sugar transporter for *Streptomyces* species, highlighting its great potential in engineering high-yield *Streptomyces* microbial cell factories for desired natural product pharmaceuticals.

Materials and methods

Bacterial strains, culture conditions and chemicals

All strains used in this study are listed in Table S1. *Escherichia coli* JM109 and ET12567/pUZ8002 were used for plasmid cloning and conjugative transfer, respectively. *E. coli* TransforMax™ EC100D™ pir-116 was used to identify transposition target genes. *S. avermitilis* S0 is a derivative of *S. avermitilis* MA-4680 obtained by random mutagenesis, which has been deposited at the China General Microbiology Culture Collection Center (accession no. CGMCC 4.8011). *S. avermitilis* S45 obtained in this work is a mutant strain with higher avermectin B_{1a} production than its parental strain S0. Luria–Bertani (LB) agar/liquid medium was used for cultivations of *E. coli* by adding appropriate antibiotics. For conjugations, all *Streptomyces* strains were grown on mannitol-soya flour (MS) agar plates (Kieser et al. 2000). For spore collection, *S. avermitilis*, *Streptomyces bingchenggensis*, and *Streptomyces roseosporus* were grown on yeast-malt-soluble (YMS) (Ikeda et al. 1988), sucrose-skimmed milk-yeast-malt (SKYM) (Jin et al. 2020), and DA1 (Zhang et al. 2015) agar plates for 7, 9 and 7 days, respectively, while *Streptomyces coelicolor* and *Streptomyces caniferus* were grown on MS agar plates for 3 and 7 days, respectively. For fermentation of *S. coelicolor* M145 and its derivatives, spores were scraped off and inoculated into 50 mL supplemented liquid minimal medium (SMM) (Kieser et al. 2000) to a final concentration of 4×10^6 spores/mL, and cultivated for 5 days. For fermentations of *S. avermitilis*, *S. bingchenggensis*, *S. caniferus*, and their derivatives, seed cultures were prepared by inoculating one square centimeter of spores into 25 mL seed medium and cultivated for 40, 46, and 24 h, respectively. For *S. roseosporus* NRRL 11379 and its derivatives, one square centimeter of spores was scraped off and inoculated into 30 mL seed medium, and shaken at 200 rpm

at 30 ℃ for 24 h. After that, the seed cultures were transferred to the 25 mL fermentation medium with 6% inoculum, and further cultured for 10, 9, and 8 days, respectively. Fermentation of *S. roseosporus* NRRL 11379 and its derivatives were conducted by inoculating 5% seed cultures into 30 mL fermentation medium, and shaken at 200 rpm at 30 ℃ for 10 days. The seed medium and fermentation medium of *S. avermitilis* and *S. bingchenggensis* were described in previous studies (Gao et al. 2009; Zhang et al. 2016). The seed medium for *S. roseosporus* contains 0.5% glucose, 1.5% dextrin, 0.5% peptone, 0.5% yeast extract, 0.5% groundnut meal, 0.05% $K_2HPO_4{\cdot}3H_2O$, 0.05% $MgSO_4{\cdot}7H_2O$ and 0.02% $CaCO_3$, pH 7.5, while the fermentation medium contains 5% soluble starch, 1% glucose, 1% dextrin, 1% tryptone, 0.05% $K_2HPO_4{\cdot}3H_2O$, 0.05% $MgSO_4{\cdot}7H_2O$ and 0.02% $CaCO_3$, pH 7.5. The seed medium for *S. caniferus* contains 2% malt dextrin, 6% soybean meal, 2% sucrose and 0.3% $CaCO_3$, pH 7.0, while the fermentation medium contains 6% malt dextrin, 4% soybean meal, 1% sucrose and 0.4% $CaCO_3$, pH 7.0. Except for *S. roseosporus*, other *Streptomyces* strains were cultivated at 28 ℃ statically or shaken at 250 rpm.

Gene cloning and plasmid construction

Plasmids, primers and oligonucleotides used in this work are listed in Tables S1 and S2. Native temporal promoters used in this work are listed in Table S3. Genome of *S. avermitilis* S0 was used as template for amplification of genes of *S. avermitilis*. Plasmid pSET152, which can integrate into *Streptomyces* chromosome by site-specific recombination at the bacteriophage phiC31 attachment site *attB* (Paranthaman and Dharmalingam 2003). Fragment LpSET152 generated from the *Eco*RI and *Xba*I double digested plasmid pSET152 was used as a backbone to construct plasmids for overexpression of different genes in *Streptomyces*. Gene *GM006564* (F6-1), and fragment GM006565–GM006568 (FTP-1) were amplified using primer pairs K6564-F/K6564-R, and KTP-F/KTP-R, respectively. Promoter *kasO*p* was amplified from pDR4-K* using primer pair KasO-F/KasO-R (Wang et al. 2013). The backbone LpSET152 and the promoter *kasO*p** were assembled with F6-1 and FTP-1 to form plasmids p6564-pK and pTP-pK, respectively. The promoter fragments p3 (FP3-1), p5 (FP5-1), p8 and fragment GM006565–GM006568 (FTP-2) were amplified by PCR using the primer pairs P355-F/P355-R, P5847-F/P5847-R, P8731-F/P8731-R and TP-F/TP-R. And then three promoter fragments were digested by *Xba*I and *Kpn*I, the FTP-2 was digested by *Kpn*I and *Eco*RI. The *Xba*I-*Kpn*I double digested promoter fragments (p3, p5 and p8) and fragment FTP-2 were ligated with the backbone LpSET152, generating corresponding plasmids pTP-p3, pTP-p5 and pTP-p8. LpTP-p8-1 and LpTP-p8-2 were obtained by double digested by *Pac*I and *Xba*I, and single digested by *Kpn*I of plasmid pTP-p8, respectively. The promoter p3 and p5, gene *GM006564* (F6-2) were amplified by PCR using the primer pairs P355-6564-F/P355-6564-R, P5847-6564-F/P5847-6564-R and 6564-P-F/6564-P-R. And then Gibson assembly was used to assemble the backbone LpTP-p8-1, the gene fragment F6-2 and two promoter fragments, p3 (FP3-2) and p5 (FP5-2), separately into plasmids pTP-p8-6564-p3 and pTP-p8-6564-p5. The gene *GM006564* (F6-3) was amplified by PCR using the primer pairs 6564-P8731-F/6564-P8731-R. And then Gibson assembly was used to assemble the backbone LpTP-p8-2 and F6-3 into plasmids pTP-p8-6564-p8. The plasmid pSETddCpf1 was digested by *Nde*I and *Spe*I to generate LpSETddCpf1. The fragment D6564 containing the crRNA-6564 started at 4 bp upstream of *GM006564* (5'-CTCCATGGCAGCGAACCGCCGCC-3') was amplified from the plasmid pSETddCpf1 by PCR using the primer pairs D6564-F/D6564-R. The PCR products were subjected to *Nde*I-*Spe*I double digestion and ligated with LpSETddcpf1 to generate plasmid pd6564.

Transposon and target identification

The transposon plasmid pTNM was introduced into S0 by intergeneric conjugation. The ensuring exconjugants were transferred from MS agar plates to liquid YEME medium with apramycin (8 μg/mL) and cultivated for 2 days at 28 ℃ (Kieser et al. 2000). This transposition was then induced with thiostrepton (5 μg/mL) for 2 h. Subsequently, the cultivation temperature was increased to 37 ℃, causing the loss of pTNM harboring the $pSG5_{rep}$ replicon. To ensure the loss of pTNM, the culture was cultivated two times more without antibiotics at 37 ℃. Aliquots were plated on MS agar plates at 37 ℃ for 7 days, and spores were collected and diluted to 10^{-7} and plated out again. Single colonies were obtained and transferred to MS agar plates containing apramycin (8 μg/mL) and another MS agar plates containing hygromycin (80 μg/mL). The colonies showing resistance to apramycin and sensitive to hygromycin were the qualified mutants. To identify transposition loci, genomic DNA of mutants were digested by *Apa*I that is absent within pTNM transposon, generating DNA fragments with an average length of 1.3 kb. The digested DNA fragments were self-cyclized and introduced into *E. coli* TransforMax™ EC100D™ pir-116. Apramycin resistant clones containing Tn5 based insertion fragment with flanking chromosomal sequences were isolated on LB agar plates. The primers ME-F and ME-R were used for reverse amplification of the chromosomal sequence and identification of the transposition loci.

Bioinformatic analysis

Orthologues of GM006568 were explored from 269 *Streptomyces* species with complete genomes deposited in National

Center for Biotechnology Information (NCBI) (up to July, 2021, Table S4) by Basic Local Alignment Search Tool (BLAST + 2.6.0). The threshold of identity and coverage were set as 30% and 70%, respectively. Protein functional domains were predicted and analyzed using Simple Modular Architecture Research Tool (SMART) (http://www.smart.embl-heidelberg.de/). Operons were predicted and analyzed using Prokaryotic Operon DataBase (ProOpDB) (http://operons.ibt.unam.mx/OperonPredictor/).

Transcriptome data analysis and promoter selection

Cells of *S. avermitilis* S0 were harvested at the fourth and eighth day for transcriptome analysis. Sample treatment, RNA extraction, quality examination and synthesis of cDNA were described in our previous work (Zhang et al. 2016). Transcriptome data have been deposited in the Gene Expression Omnibus database with an accession number of GSE227240. Temporal promoters for fine-tuning the overexpression level of TP6568 were selected based on above-mentioned transcriptome data, and the promoter region was the 500-bp sequence upstream of the corresponding gene as we previously described (Chu et al. 2022).

Quantitative real-time PCR (qRT-PCR)

Cells of *S. avermitilis* S0 and its derivatives were harvested at the second, fourth and eighth day. After sample treatment and RNA extraction, cDNA samples were synthesized for qRT-PCR. Details for qRT-PCR experiments and data analysis were implemented as described previously (Chu et al. 2022). The 16S rRNA was employed as an internal control. All experiments were run in three independent biological repeats.

Homology modeling and molecular docking

The receptor structure of GM0068658 was constructed by SWISS-MODEL (https://swissmodel.expasy.org) with the crystal structure of RbsB from *Streptomyces agalactiae* (PDB ID: 7E7M) as template. The ligand structure of glucose, fructose, maltose, cellobiose, galactose, xylose, mannitol, and ribose were obtained from the PubChem database (PubChem ID: 5793, 2723872, 6255, 10712, 6036, 135191, 6251, and 10975657). Molecular docking was performed by AutoDock 4.2 using flexible-body docking algorithm. According to the ribose binding site of 7E7M, the grid boxes of GM006568 binding with different ligands were set as 70 × 70 × 70 grid points with a grid spacing of 0.353 Å. The docking modes of receptor-ligand combination were selected based on the binding energy (ΔG, kcal/mol). The docking files were visualized using UCSF Chimera X 1.4.

Analysis of target secondary metabolites of *Streptomyces*

Fermentation broth of *S. bingchenggensis* and *S. avermitilis* were extracted with ethanol (1:3, $v_{samples}/v_{ethanol}$), and methanol (1:4.8, $v_{samples}/v_{methanol}$), respectively. After that, the supernatant was filtered for further analysis by HPLC. Thereinto, the supernatant of *S. avermitilis* required further dilution with equal volume of methanol before analysis. The detailed analytical conditions of milbemycin and avermectin were described as previously (Jin et al. 2020). And the detect method of daptomycin was described as previously (Zhang et al. 2015). Determination of actinorhodin (ACT) and undecylprodigiosin (RED) of *S. coelicolor* were carried out as previous description (Kieser et al. 2000). To detect guvermectin, fermentation broth of *S. caniferus* was extracted with four volumes of ethanol, then the supernatant was analyzed by HPLC (Agilent 1260) equipped with an ultraviolet detector, and a Zorbax SB-Aq column (4.6 mm × 250 mm, 5 μm). Acetonitrile (11%, v/v) was used as mobile phase at a flow rate of 0.8 mL/min, and the detection wavelength was 260 nm.

Analysis of sugar concentration

The concentration of residual total sugar was determined by the total sugar content kit (Cominbio, China). The residual concentration of each sugar in fermentation broth was detected by HPLC. After centrifugation of fermentation broth, the supernatant was treated with twice volumes of methanol, and then used for detection by HPLC (Shimadzu LC-20AT) equipped with a refractive index detector and a carbohydrate column (Zorbax, 4.6 mm × 250 mm, 5 mm). Acetonitrile (80%, v/v) was used as mobile phase at a flow rate of 1 mL/min.

Results

Discovery of a previously unidentified carbohydrate transporter

Avermectin B_{1a} is the precursor for different semisynthetic derivatives such as emamectin benzoate, and ivermectin, which are useful insecticides and antiparasitic agents in humans and animals, respectively (Menchikov et al. 2022). Therefore, titer improvement of B_{1a} is quite important. To obtain high-yields of avermectin B_{1a}, non-replicative Tn5 transposon-aided strain screening was implemented in *S. avermitilis* S0. A mutant named SP152 showed the best B_{1a} production capacity, with a 35.22% B_{1a} titer enhancement and 18.44% residual sugar reduction (Fig. 1a–b). We further identified the mutation site occurred 12 bp upstream of gene *GM006568* (Fig. 1c). This

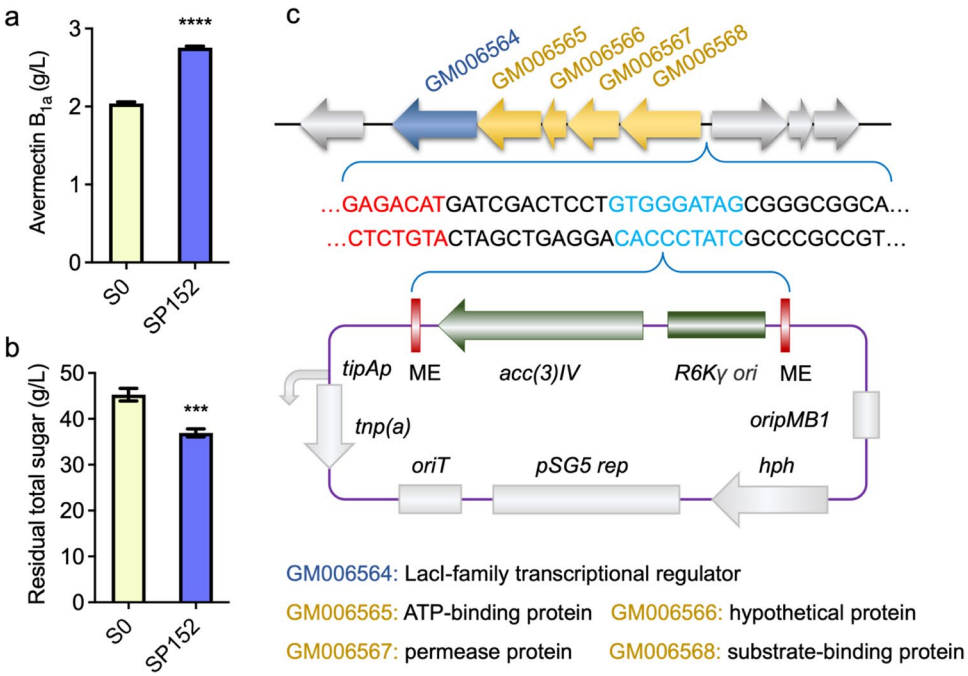

Fig. 1 Discovery of a sugar transporter TP6568 from *S. avermitilis*. **a** Comparison of avermectin B_{1a} production of transposon mutant SP152 and parent strain S0. **b** Comparison of residual total sugar in fermentation broth of SP152 and S0. **c** Identification of transposon insertion site in SP152. Data were obtained from three independent replicates. Differences were analyzed by Student's *t*-test, and $p < 0.05$ was considered statistically significant. The levels of significance are **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

gene encodes a substrate-binding protein containing a type 1 periplasmic binding protein (PBP1) superfamily domain, and members of this family generally transport various sugars in bacteria and archaea. We also discovered a permease protein (GM006567), a hypothetical protein (GM006566), and an ATP-binding protein (GM006565) in the downstream of GM006568. Bioinformatic analysis showed that GM006565 and GM006568 were identified to SAV_5702 and SAV_5704 of *S. avermitilis* MA-4680, respectively, while GM006566 and GM006567 were identified as two parts of SAV_5703, which might function together as an integrated permease in S0 (Fig. S1). The four proteins from GM006565 to GM006568 formed a putative ABC-type sugar transporter, named TP6568 in this work. Moreover, a transcription regulator encoded by gene *GM006564* was found in the downstream of TP6568, which is identical to SAV_5701 of MA-4680. This regulator belongs to the LacI family, which generally regulates the transcription of sugar utilization genes (Ravcheev et al. 2014). Operon prediction showed that gene *GM006564* to *GM006568* belonged to the same operon, moreover, transcription of *GM006564* showed similar profile to that of genes belonging to TP6568 (Fig. S2). We therefore speculated that *GM006564* might co-transcribe alongside TP6568, suggesting a closed relationship between this regulator and TP6568.

Influence of TP6568 on avermectin production in *S. avermitilis*

Transposon insertion destroyed the original promoter region of *GM006568* (Fig. 1c), thus we speculated that the increased titer might be attributed to the changed transcriptional level of this putative sugar transporter. Further analysis demonstrated that transcription level of genes encoding GM006564 and TP6568 in SP152 were all increased compared to S0 (Fig. 2a). Moreover, we compared the transcriptional level of these genes in different *S. avermitilis* strains with diverse avermectin B_{1a} production (SP66: 0.47 g/L; S0: 2.03 g/L; SP104: 2.69 g/L), and found a positive linear relationship between gene transcription level and avermectin production (Fig. 2b). These data indicated that TP6568 might be a useful transporter for titer improvement of avermectin B_{1a} in *S. avermitilis*. We overexpressed TP6568 under the control of a strong promoter *kasO*p* in S0, generating strain S0-TPK. Transcription level of genes belonging to TP6568 in S0-TPK were all significantly higher in comparison to that in S0 (Fig. S3). Correspondingly, the titer of avermectin B_{1a} of S0-TPK was 75.61% higher (Fig. 2c). Meanwhile, the residual total sugar was reduced by 36.16% (Fig. 2d). These data demonstrated that TP65658 might be a useful sugar transporter for avermectin titer improvement.

Characterization of the sugar transporter TP6568

Despite we assumed TP65658 might be a useful sugar transporter for *Streptomyces* engineering, detailed experimental confirmations were quite limited. To characterize TP6568, we first carried out orthologue analysis of the substrate-binding protein GM006568 in 269 *Streptomyces* strains with complete genomes (Table S4), and found 293 orthologues in 197 *Streptomyces* strains with high identity (Table S5).

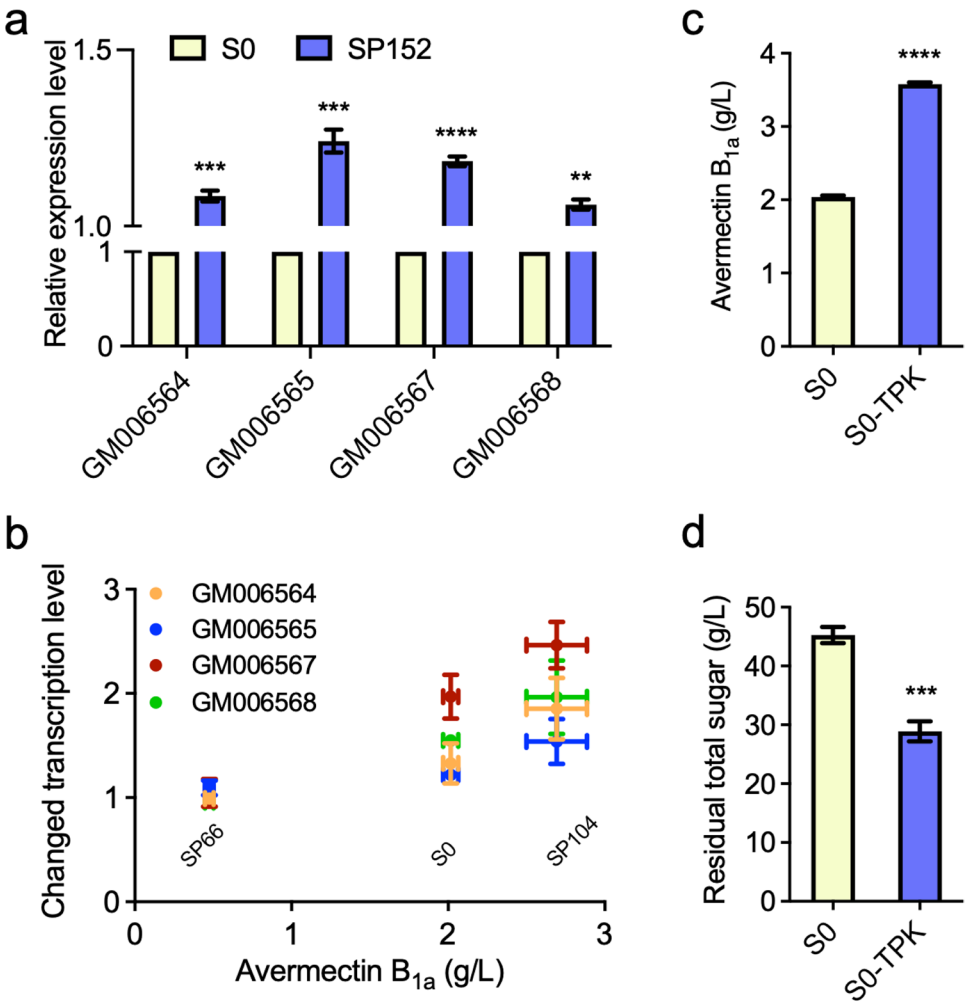

Fig. 2 Effect of TP6568 on avermectin biosynthesis in *S. avermitilis* S0. **a** The relative changed transcription level of genes from *GM006564* to *GM006568*. Data were obtained by normalizing the relative transcriptional level for each gene of SP152 to that of S0 collected at the eighth day. **b** Correlation between the transcription level of genes in TP6568 and avermectin B_{1a} production. The transcriptional level change for each gene was obtained by normalizing its transcriptional level of the fourth day to that of the eighth day. **c** Comparison of avermectin B_{1a} production between the TP6568 overexpression strain S0-TPK and the parent strain S0. **d** Comparison of residual total sugar in fermentation broth of S0-TPK and S0. Data were obtained from three independent replicates. Differences were analyzed by Student's *t*-test, and $p < 0.05$ was considered statistically significant. The levels of significance are **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

Protein SAV_5704 from *S. avermitilis* MA-4680 demonstrated the closest relationship with GM006568 and was annotated as a substrate-binding protein of fructose transporter, while other orthologues were annotated as different putative sugar binding proteins including ribose, xylose, and allose (Table S5), implying TP6568 harboring GM006568 might be a versatile transporter capable of promoting uptake of diverse sugars.

To confirm our assertions, we characterized TP6568 in the model organism *S. coelicolor*. Strains were grown on SMM agar plates or in liquid culture media, which used different sugars as the sole carbon source. Here we chose generally used or promising sustainable carbon sources for industrial fermentations, including glucose, fructose, xylose, galactose, mannitol, ribose, maltose, and cellobiose. We found that M145-TPK overexpressed TP6568 by *kasO*p* enhanced production of secondary metabolite biosynthesis (ACT or RED) on different tested agar plates except for that containing cellobiose (Fig. 3a). A similar phenomenon was also found in liquid fermentations (Fig. 3b). Correspondingly, TP6568 promoted uptake of different tested sugars except cellobiose (Fig. 3c). To further examine the sugar binding capacity of GM006568, we carried out in silico molecular docking. In addition, we compared the ligand-binding sites of GM006568 with other known sugar binding proteins, such as ribose binding protein (PDB ID: 7E7M), galactose binding protein (PDB ID: 1GLG), xylose binding protein (PDB ID: 3C6Q), glucose binding protein (PDB ID: 7X0H) and fructose binding protein (PDB ID: 2X7X). Molecular docking data indicated that GM006568 could bind all tested sugars (Fig. S4). The results also showed that the spatial locations of the binding sites were relatively conserved, especially of the highly conserved amino acid residues Arg189 and Asp264 (Figs. 3d, S5, and S6). These data suggested the broad substrate binding capability of GM006568, and TP6568 might be a promising multi-sugar transporter.

Application of TP6568 in engineering of different *Streptomyces*

TP6568 was capable of promoting uptake most tested sugars. These sugars are commonly used carbon sources, or hydrolysis products of industrially used carbon sources, such

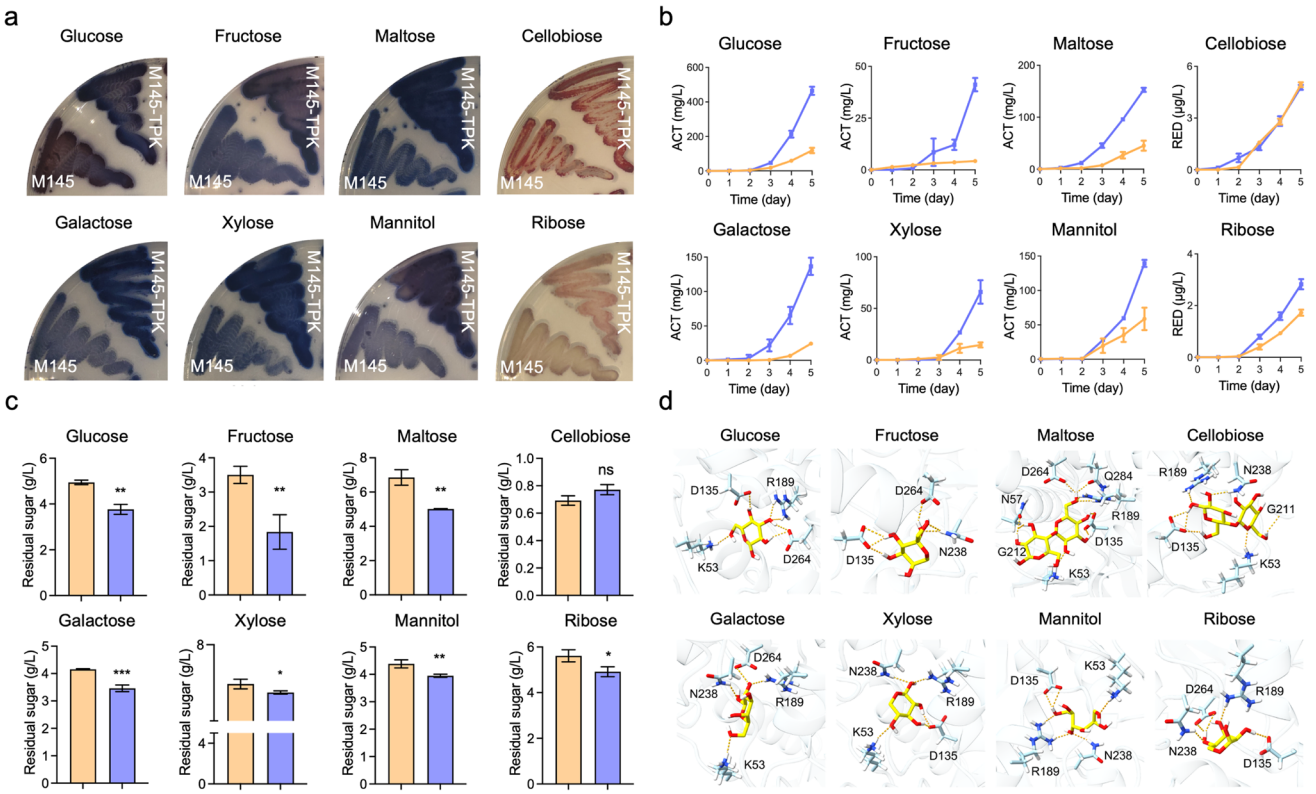

Fig. 3 Function characterization of TP6568 in *S. coelicolor* M145. **a** The phenotype of TP6568 overexpression strain M145-TPK and parent strain M145 on SMM agar plates. Each agar plate contains one tested sugar as the sole carbon source. **b** Comparison of production (ACT or RED) between M145 (orange) and M145-TPK (blue) cultivated in SMM liquid cultures containing different tested sugars as the sole carbon source. **c** Comparison of residual sugar between M145 (orange) and M145-TPK (blue) cultivated in SMM liquid cultures containing different tested sugars as the sole carbon source. Data were obtained from the fifth day. **d** In silico molecular modeling of GM006568 with eight tested sugars. For (**a**), (**b**) and (**c**), the liquid or solid SMM cultural media contained different tested sugars with the same carbon moles, the initial sugar concentration for glucose, fructose, galactose, xylose, and ribose are 10 g/L, mannitol is 10.1 g/L, maltose and cellobiose are 9.5 g/L, respectively. Data were obtained from three independent replicates

as starch, sucrose, and dextrin. Therefore, TP6568 might be a promising sugar transporter in engineering of high-yield *Streptomyces* for industrial production purpose. For that, we overexpressed TP6568 under the control of the strong promoter *kasO*p* in a high-yield avermectin-producing strain *S. avermitilis* S45, as well as other three industrial valuable *Streptomyces* producers utilizing different carbon sources, including a milbemycin producing strain *S. bingchenggensis* BC-101–4, a guvermectin producer *S. caniferus* NEAU6, and a daptomycin producer *S. roseosporus* NRRL 11379. According to fermentation data, it was found that the residual sugars from all engineered strains (S45-TPK, BC-TPK, N6-TPK, and NR-TPK) were significantly reduced (35.55%, 33.56%, 39.04%, and 30.21%) compared to their individual parent strains (Fig. 4a). TP6568 overexpression also significantly boosted the titer of milbemycin and guvermectin by 41.25% and 56.89% in BC-TPK and N6-TPK, respectively (Fig. 4b). Together, these results showed the great potential of TP6568 in industrial fermentation applications. Despite of that, we found that TP6568 showed little influence on titer improvement of daptomycin in NR-TPK and avermectin B_{1a} in S45-TPK (Fig. 4b). As sugar uptake in these strains were enhanced, we speculated that the lack of increased titer of products might be due to the mismatched expression patterns of TP6568 to metabolism of the producers. We found that biomass of NR-TPK and S45-TPK were 60.55% and 14.95% higher than their parent strains (Fig. 4c). These data implied that, although TP6568 promoted uptake of sugars, these sugars are probably used for biomass accumulation rather than product biosynthesis. Therefore, fine-tuning the expression pattern of TP6568 to coordinate the metabolism of hosts might be quite necessary to engineer efficient *Streptomyces* cell factories.

Construction of a high-yield B_{1a} producer by engineering of TP6568

An increase in biomass of S45-TPK may be due to constitutive expression of TP6568. This thereby enhanced sugar uptake at the beginning of fermentation and resulted

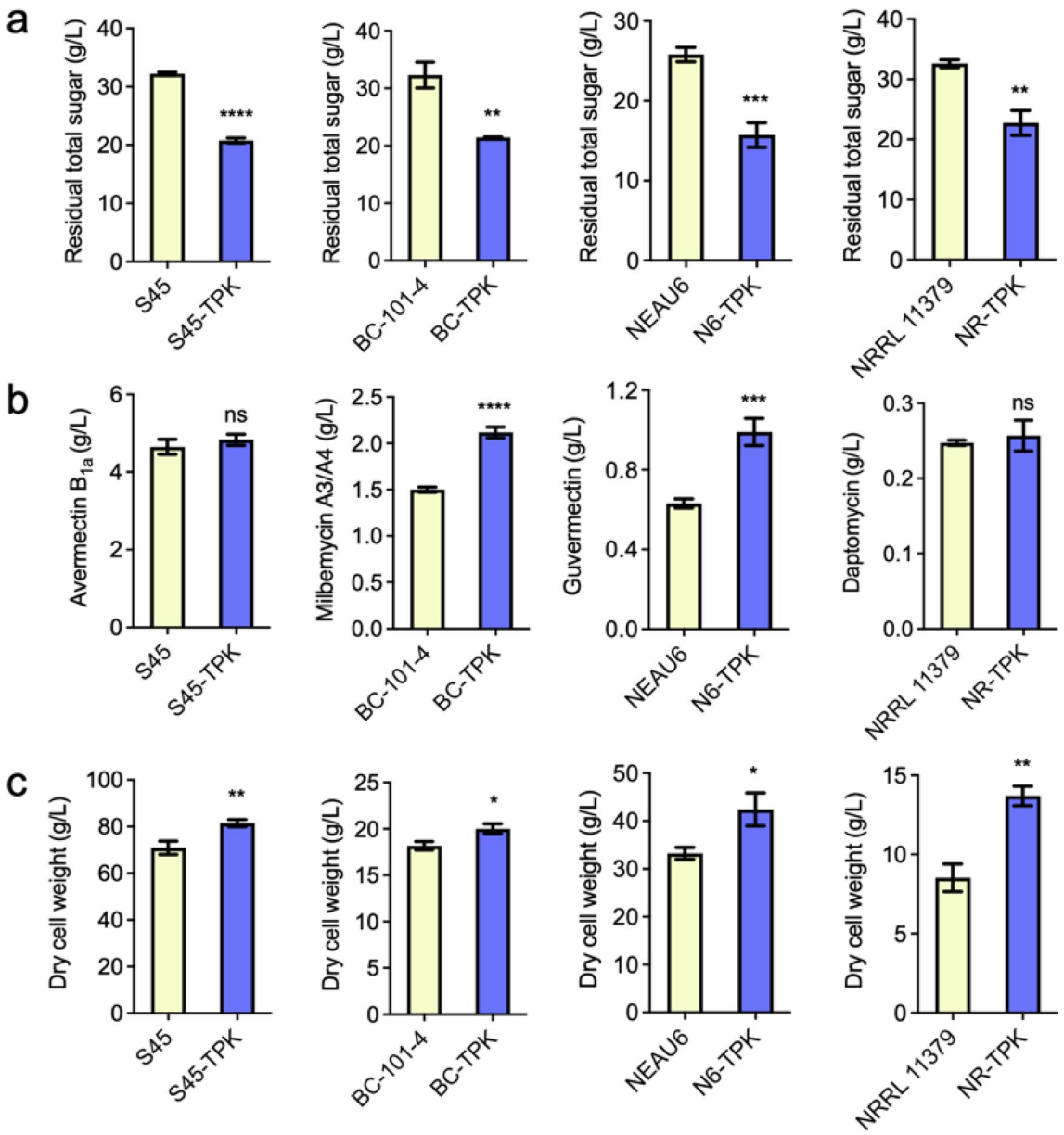

Fig. 4 Application of TP6568 in different *Streptomyces* producers. **a** Comparison of residual total sugar of TP6568 overexpressed strains and its individual parent strains. **b** Titer comparison of different products in TP6568 overexpressed strains and its individual parent strains. **c** Comparison of dry cell weight in TP6568 overexpressed strains and its individual parent strains. Differences were analyzed by Student's *t*-test, and $p < 0.05$ was considered statistically significant. The levels of significance are **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

in increased carbon flux towards biomass synthesis during primary metabolism as opposed to product biosynthesis occurring during secondary metabolism. It was found that the sugar uptake efficiency was significantly decreased after eight days in S45 (Fig. 5a). This indicates the necessity of replacing *kasO*p* with temporal promoters with enhanced strength after eight days to promote sugar uptake during the avermectin production stage. Therefore, three candidate promoters (p3, p5, and p8) were selected based upon transcriptome data which were used to optimize the overexpression level of TP6568 in S45 (Fig. S7 and Table S3). In comparison to S45-TPK, the amount of residual total sugar was reduced by 11.12%, 17.29%, and 25.73% in TP6568 temporally overexpressed strains S45-TP3, S45-TP5, and S45-TP8, respectively. The amount of residual total sugar from these three strains were 42.72%, 46.68%, and 52.14% lower than that of S45 (Fig. 5b). Biomass of the three TP6568 temporally

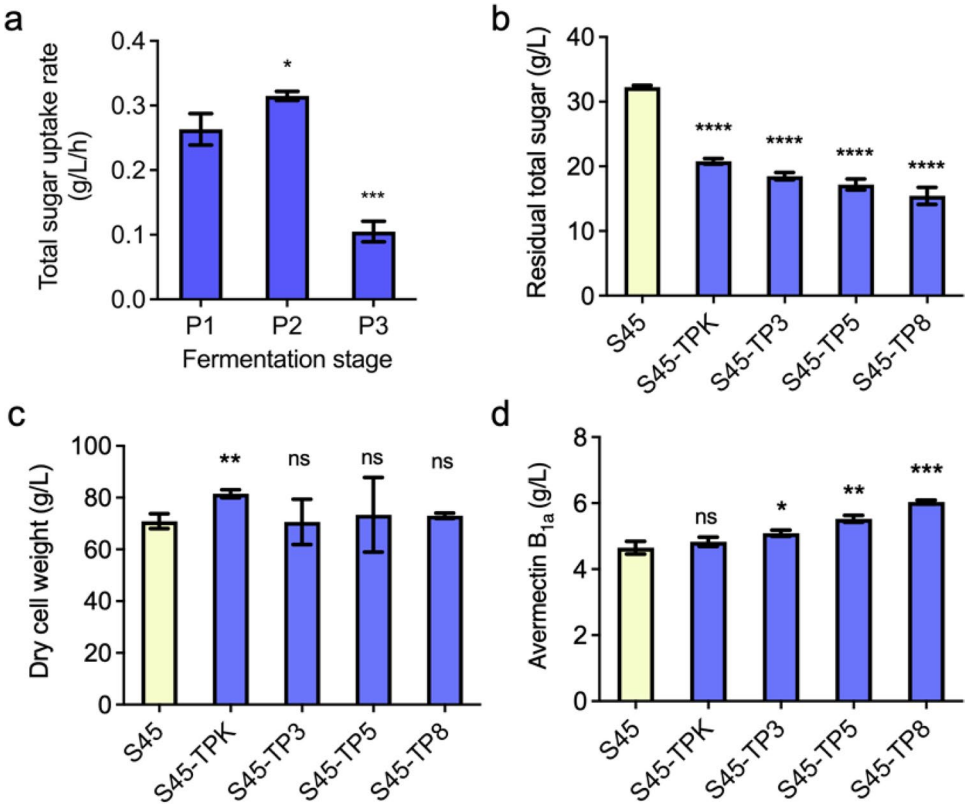

Fig. 5 Fine-tuning the overexpression of TP6568 in S45. **a** Total sugar absorption rate of S45 at different fermentation stages: P1: 1–4 d, P2: 4–8 d, P3: 8–10 d. **b** Comparison of the residual total sugar of strains with different expression level of TP6568. **c** Comparison of the dry cell weight of strains with different expression level of TP6568. **d** Comparison of the avermectin B_{1a} production of strains with different expression level of TP6568. Data were obtained from three independent replicates. Differences were analyzed by Student's *t*-test, and $p < 0.05$ was considered statistically significant. The levels of significance are **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

overexpressed strains were approximately 11.66% lower than S45-TPK while similar to that of S45 (Fig. 5c). Accordingly, the titer of avermectin B_{1a} of the three strains were 9.48%, 18.82%, and 29.79% higher than that of S45-TPK, respectively (Fig. 5d). These data indicate that more cellular carbon flux was redirected towards the avermectin biosynthetic pathway through fine-tuning of the expression level of TP6568.

There is a LacI family transcription regulator GM006564 in the downstream of TP6568. Previous studies have shown that most LacI transcriptional regulators are involved in transport and metabolism of carbon sources (Goda et al. 1998; Marushima et al. 2009; Schaffert et al. 2019). We therefore speculated that GM006564 might regulate the expression level of TP6568, and impact carbon utilization, avermectin production and yield. To confirm our speculation, we overexpressed GM006564 by *kasO*p* and suppressed *GM006564* by CRISPRi in S0, generating S0-6564 K and generated S0-d6564, respectively. Compared to S0, S0-6564 K increased the production of avermectin B_{1a} by 58.81% and decreased the residual total sugar amount by 53.96%, while S0-d6564 decreased production by 83.08% and increased the remaining concentration of total sugars by 23.83% (Fig. 6a). Thereafter, it was found that in comparison to S0, the transcription level of all genes belonging TP6568 in S0-6564 K were significantly enhanced by more than 12-fold, while these levels were greatly reduced by over than 95% in S0-d6564 across different fermentation stages (Fig. 6b). These data demonstrate that GM006564 acts as a crucial activator of TP6568, and engineering of this regulator may further promote utilization of residual sugars in fermentation and enhance production of avermectin B_{1a}. In light of these data, we further overexpressed GM006564 under control of different temporal promoters in S45-TP8, and discovered that strain S45-TP8-6p5 in which GM006564 controlled by p5 promoter resulted in the highest titer (7.02 g/L) and yield (0.05 $g/g_{corn\ starch}$) of avermectin B_{1a}, which were 16.32% higher than S45-TP8, and 50.97% higher than the parent strain S45, respectively (Fig. 6c).

Discussion

Sugar uptake plays significant roles in living cells, with soluble sugar serving as a source of carbon, transient energy storage, osmolytes, and signaling molecules (Chen et al. 2015). To adapt to diverse environments, cells have evolved a plethora of sugar transporters. In bacteria, archaea, and eukaryotic organisms, transporter accounts for over 10% of encoded proteins, and about one fifth of transport proteins are sugar transporters (Jin et al. 2020; Saier and Ren 2006). However, the reported and well-characterized sugar transporters are the tip of iceberg. Sugar transporter discovery and application have been

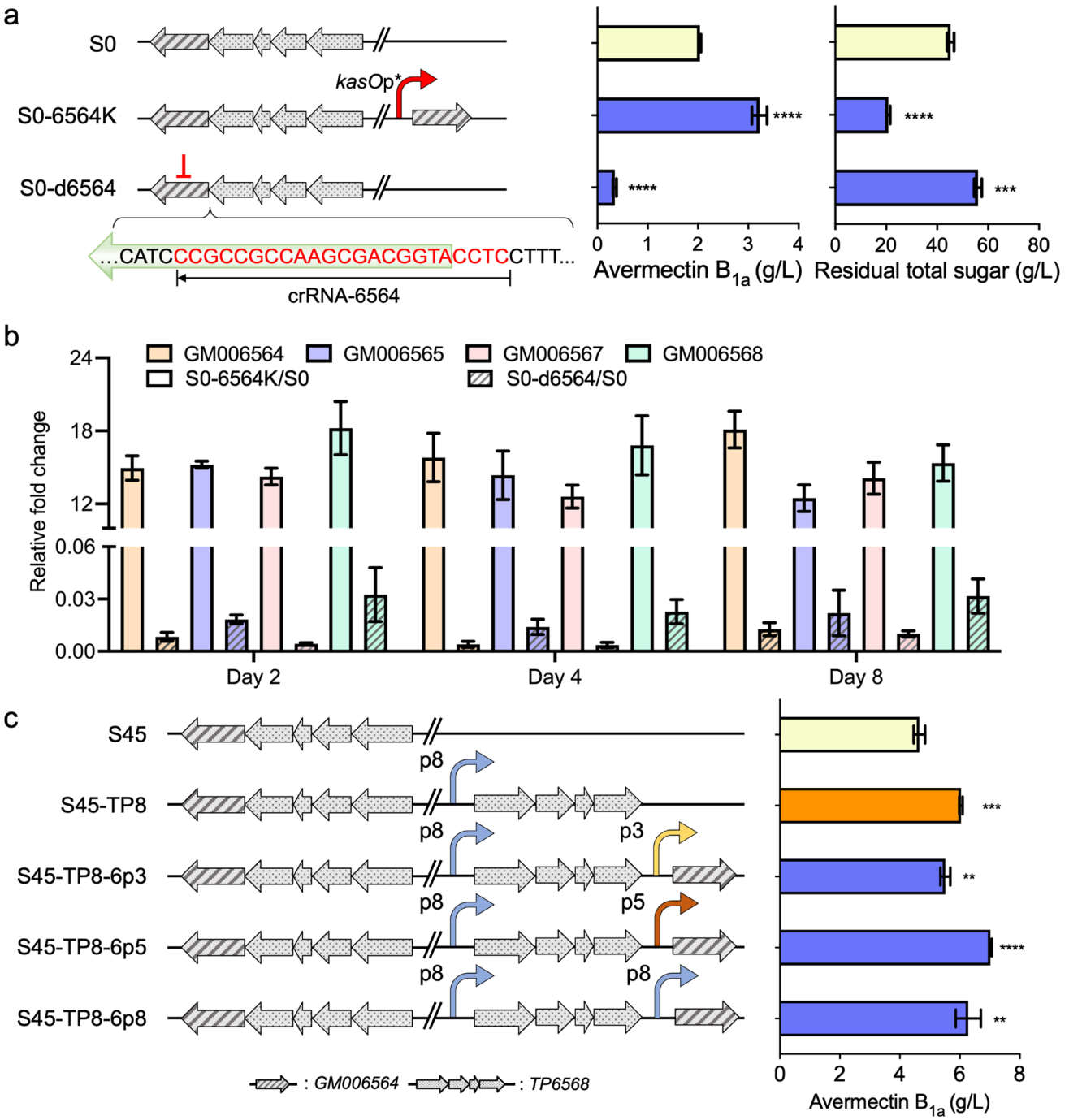

Fig. 6 Construction of a high-yield avermectin producer by co-expression of TP6568 and GM006564. **a** Influence of GM006564 on avermectin production and residual total sugar in S0. **b** Influence of GM006564 on transcription level of genes in TP6568. **c** Titer improvement of B_{1a} by co-expression and fine-tuning of GM006564 with TP6568. Data were obtained from three independent replicates. Differences were analyzed by Student's *t*-test, and $p < 0.05$ was considered statistically significant. The levels of significance are **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

attracting great attentions, especially in engineering of high-yield cell factories for diverse chemicals, including drugs and chemical platform compounds (Giacomini et al. 2010; Kuanyshev et al. 2021; Podolsky et al. 2021; Radi et al. 2022). In comparison to previous transporter mining strategies that generally rely on transporter database or omics data (Podolsky et al. 2021; Chu et al. 2022), here we discovered TP6568 by employing the Tn5 based transposon, which is an efficient tool for selection of targets in *Streptomyces* without a priori knowledge.

Characterization of novel transporters is necessary to enrich the synthetic toolbox of sugar transporters and promote their applications in strain engineering. Herein, we characterized TP6568 based on bioinformatic analysis, in silico molecular docking and genetic experiments. TP6568 is a previously unidentified sugar transporter, which is different from previously reported sugar transporters, such as MalEFG (Li et al. 2010) and TP2 and TP5 (Jin et al. 2020). Both protein sequence analysis and in silico molecular docking suggested that the substrate-binding protein GM006568 possesses conserved amino acid residues and a conserved substrate binding pocket, which could hold many different sugars (Figs. S5 and S6), and most of which were experimentally validated (Fig. 3). However, we also found the discrepancies between results from in silico molecular docking and experiments (Fig. 3). Molecular docking is quite useful in helping us to investigate the binding property of a protein to its targets, whereas experimental validation is indispensable to confirm the biological function of the protein. In other words, the substrate binding ability of the substrate binding protein of a given transporter does not mean the substrate transport ability of this transporter. The substrate binding ability is the basis for efficient transport of GM006568, while other factors, such as the conformational changes of transmembrane protein caused by ATP binding, are essential for efficient substrate transport, which has been validation in other investigations (Ma et al. 2022). TP6568 is promising in construction of high-yield *Streptomyces*, as it could influence the cellular carbon flux and production through regulation of the uptake efficiency of sugar substrates. TP6568 is a promising sugar transporter due to its broad substrate specificity, which enables engineered stains to use either commonly used sugars such as glucose, fructose, and maltose, or renewable carbon sources like xylose. Moreover, we established the protein model of GM0006568, which facilitates transporter improvement by directed evolution. Considering the limited investigation into sugar transporters in *Streptomyces*, TP6568 could serve as a useful genetic part to develop *Streptomyces* for titer improvement of desired chemicals.

Fine-tuning the expression level of sugar transporters during strain engineering is a significant undertaking. Transporters are membrane proteins, and transporter overexpression may result in overloading stress and cause cytotoxicity (Zhu et al. 2020). Moreover, the control of overexpression sugar transporters could promote sugar uptake over time, and therefore not only alleviate cytotoxicity but also balance cell growth and production, which are crucial for titer improvement (Steiger et al. 2019; Zhou et al. 2018). Employing different promoters to meet this demand may be a reasonable path forward, including use of temporal promoters, inducible promoters, and target intermediate responsive promoters. It has also been reported that co-expression of specific regulators alongside the target transporter could optimize the expression level of the transporter. Coincidently, the LacI family regulator GM006564 was found located downstream of TP6568 and was demonstrated to be an important activator of TP6568. Co-expression of this regulator and TP6568 indeed further enhanced avermectin B_{1a} production, which is aligned with previous investigations (Skretas et al. 2012). In addition to these approaches, transporter expression may also be optimized through host strain engineering, as well as co-expression of chaperone proteins (Skretas et al. 2012; Zhu et al. 2020).

In this work, we discovered and characterized a previously unidentified sugar transporter, TP6568. It is a sugar transporter with broad substrate specificity, which proved to be useful to promote sugar uptake of commonly used industrial sugars, including various monosaccharides, starch, sucrose, and dextrin, as well as cost-effective sugars like xylose. Engineering of TP6568 in various *Streptomyces* hosts demonstrate the high potential of TP6568 in development of industrial *Streptomyces* cell factories for titer enhancement of target natural products. In particular, we engineered TP6568 in a high-yield avermectin-producing *S. avermitilis* strain, which increased the titer by 50.97% compared to the parent strain. In summary, this work provides a useful sugar transporter to promote sugar substrate uptake in *Streptomyces*, which shows great potential in construction of high-yield *Streptomyces* microbial cell factories of desirable natural products.

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Author contributions SL, WX, and ZD conceived and designed the study. ZD carried out the main experiments. ZD, LL, and GD performed data collection and data analysis. LL performed in silico studies and docking analysis. SL, WX, ZD, YZ and XW wrote and 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 manuscript and its supplementary information files. Requests for any additional information can be made to the corresponding authors.

Declarations

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

Conflict of interest The authors declare no conflict of interest. We have filed a provisional patent for this work.

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