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ORIGINAL PAPER

Overexpression of *metK* shows different effects on avermectin production in various *Streptomyces avermitilis* strains

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Abstract The *S*-adenosylmethionine synthetase gene (*metK*) from *Streptomyces avermitilis* was cloned into multi-copy vector pIJ653 and integrative vector pSET152 yielding two *metK* expression plasmids pYJ02 and pYJ03, respectively. When wild-type strain ATCC31267 was transformed with these two plasmids, avermectin production was increased about 2.0-fold and 5.5-fold, respectively. The introduction of integrative expression plasmid pYJ03 into the engineered strain GB-165, which produces only avermectin B, promoted the production of avermectin approximately 2.0-fold. However, introduction of pYJ02 did not influence avermectin accumulation in GB-165. Moreover, transformation of the avermectin-overproducing industry strain 76-05 with these two plasmids did not stimulate avermectin production. These results showed that there were different effects of *metK* expression levels on avermectin production in various *S. avermitilis* strains. Additionally, the transcript levels of *metK*, *aveR* (the avermectin pathway-specific regulatory gene) and *aveA1* (one avermectin biosynthesis gene) meet the expectation of fermentation levels of avermectin in wild-type strain and its recombinant strains. The gene expression levels of *metK*, *aveR* and *aveA1* in GB-165 and 76-05 were much higher then those in wild-type strain, which probably limited the increasement of avermectin by overexpression of *metK*.

Keywords *S*-adenosylmethionine synthetase · *metK* · Avermectin · *Streptomyces avermitilis*

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Introduction

Avermectins, which are produced by *Streptomyces avermitilis*, are widely used in the fields of veterinary medicine and agriculture as effective agricultural pesticides and antiparasitic agents. They are a series of 16-membered macrocyclic lactones composed of A1a, A1b, A2a, A2b, B1a, B1b, B2a and B2b. The eight compounds have structural differences at C5, C22-C23, and C26 (Alber-Schönberg et al. 1981; Burg et al. 1979; Miller et al. 1979). Among these components, the B1 fractions (a mixture containing more than 80 % of B1a and less than 20 % of B1b) have the most effective antiparasitic activities. For decades the primary approach to improve industrial organisms has been classic strain improvement such as mutagenesis and selection. In recent years, genetic engineering strategies have been used to improve the production of secondary metabolites in many industrial organisms. Overexpression *avtAB* operon encoding the ABC transporter AvtAB and maltose ABC transporter system encoding gene (*malEGFa*) of *Streptomyces avermitilis* have been demonstrated to result in avermectin

overproduction (Qiu et al. 2011; Li et al. 2010b). Increasing the intracellular concentration of SAM by overexpression of *S*-adenosylmethionine synthetase gene (*metK*) was also found to stimulate the production of antibiotics in different Actinomycetes strains (Kim et al. 2003; Maharjan et al. 2008; Okamoto et al. 2003; Wang et al. 2007; Zhang et al. 2008). *S*-adenosylmethionine (SAM) is synthesized from methionine and adenosinetriphosphate by *S*-adenosylmethionine synthetase (MetK) (Lu 2000). It has been proposed that SAM activated the transcriptional activators responsible for antibiotic biosynthesis or served as a methyl donor in the primary and in secondary metabolism (Chiang et al. 1996; Kim et al. 2003). Although earlier work reported that the production of avermectin B1a was increased two-fold by introducing multiple copies of *metK* gene under the control of its own promoter in wild-type strain of *S. avermitilis* (Yoon et al. 2006), it has not been studied extensively in avermectin overproducing strains. Here we investigated the role of SAM on avermectin production through overexpression of *metK* with different copy numbers under the control of the strong constitutive *ermE** promoter in various *S. avermitilis* strains, including engineered strain GB-165 and industry strain 76-05. To quantitatively investigate the relationship between *metK* level, SAM titer, as well as the avermectin biosynthesis, the transcriptional levels of *metK*, avermectin biosynthesis gene *aveA1*, as well as the avermectin biosynthesis regulation gene *aveR* in studied strains were analyzed by qRT-PCR. In addition, the intracellular levels of SAM of these strains were also presented.

Materials

Bacterial strains and culture conditions

All bacterial strains used are listed in Table 1. *E. coli* strains carrying plasmids were grown in Luria–Bertani (LB) medium at 37 °C, and were selected with ampicillin (100 μg/ml) or apramycin (100 μg/ml). *S. avermitilis* was

Table 1 Bacterial strains and plasmids used in this study

Strains or plasmids	Description	Source/reference
E. coli		
DH 5α	General cloning host, *hsdR*17(rk^-, mk^+)	Sambrook et al. (1989)
ET12567	Demethylation host, *dam*$^-$ *dcm*$^-$ *hsd*$^-$	MacNeil and Klapko (1987)
S. avermitilis		
ATCC31267	Wild- type strain, avermectin producer	ATCC
653W	31267 harboring multi-copy vector pIJ653	This work
152W	31267 harboring integrative vector pSET152	This work
653SW	31267 harboring multi-copy vector pYJ02	This work
152SW	31267 harboring integrative vector pYJ03	This work
GB-165	An engineered strain constructed from green spore producing mutant, producing only avermectin B by deletion of *aveD* gene	This lab
653G	GB-165 harboring multi-copy vector pIJ653	This work
152G	GB-165 harboring integrative vector pSET152	This work
653SG	GB-165 harboring multi-copy vector pYJ02	This work
152SG	GB-165 harboring integrative vector pYJ03	This work
76-05	Avermectin-overproducing industrial strain, derived from ATCC31267 by continuous mutagenesis	This lab
653H	76-05 harboring multi-copy vector pIJ653	This work
152H	76-05 harboring integrative vector pSET152	This work
653SH	76-05 harboring multi-copy vector pYJ02	This work
152SH	76-05 harboring integrative vector pYJ03	This work
Plasmids		
pJL117	pIJ2925 derivative containing the *Streptomyces* strong constitutive promoter *ermE** from pIJ4090, AmpR	This lab
pSET152	*E. coli-Streptomyces* shuttle vector, *attB*, *lacZα*, $Apra^R$	Bierman et al. (1992)
pIJ653	*E. coli-Streptomyces* shuttle vector, tsr	Hu et al. (1994)
pYJ01	pJL117 derivative containing the *metK* gene under the control of *ermE** promoter	This work
pYJ02	pIJ653 containing the *metK* gene under the control of *ermE** promoter	This work
pYJ03	pSET152 containing the *metK* gene under the control of *ermE** promoter	This work

grown at 28 °C on YMS agar (Ikeda et al. 1988) or in modified YEME liquid medium (MacNeil and Klapko 1987). RM14 medium was used to regenerate protoplasts of *S. avermitilis* (MacNeil and Klapko 1987). If necessary, apramycin was added with a concentration of 10 μg/ml for YMS and 5 μg/ml for YEME. Thiostrepton was added at 10 μg/ml for YMS and 3 μg/ml for YEME.

To study the growth rate, *S. avermitilis* and its transformants were grown in soluble fermentation medium II (50 g soluble starch, 12 g yeast extract, 0.5 g $MgSO_4 \cdot 7H_2O$, 4 g KCl, 5 mg $CoCl_2 \cdot 6H_2O$ and 1 g of $CaCO_3$ per liter deionized water). For avermectin fermentation experiments, *S. avermitilis* wild type and its transformants were first grown in seed medium, which composed of 30 g of soluble starch, 4 g of yeast extract, 2 g of soya peptone and 5 mg of $CoCl_2 \cdot 6H_2O$ per liter deionized water and were then grown in the fermentation medium A (70 g corn starch, 15 g soybean meal, 10 g yeast meal, 0.5 g $K_2HPO_4 \cdot 3H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 5 mg $CoCl_2 \cdot 6H_2O$ and 1 g of $CaCO_3$ per liter deionized water). GB-165 and its transformants were cultured in fermentation medium G (95 g corn starch, 25 g peanut protein powder, 7 g cottonseed protein, 3 g maltose, 0.3 g $MgSO_4 \cdot 7H_2O$, 7.5 mg $CoCl_2 \cdot 6H_2O$, 0.1 g $(NH_4)_2SO_4$, 0.3 g $K_2HPO_4 \cdot 3H_2O$ and 1 g of $CaCO_3$ per liter deionized water). 76-05 and its transformants were cultured in fermentation medium H (120 g corn starch, 20 g soybean meal, 0.3 g $MgSO_4 \cdot 7H_2O$, 5 mg $CoCl_2 \cdot 6H_2O$, 0.2 g $(NH_4)_2SO_4$, 0.3 g $K_2HPO_4 \cdot 3H_2O$ and 1 g of $CaCO_3$ per liter deionized water). For avermectin fermentation, strains were incubated on YMS plates to allow for abundant sporulation. Spore suspensions were first cultured in 50 ml seed culture medium in 250 ml baffled flasks for 24 h at 28 °C with shaking at 180 rpm, then 2.5 ml of the seed culture broths was inoculated into 250 ml baffled flasks with 50 ml fermentation medium and the incubation was carried out on a rotary shaker (250 rpm) at 28 °C for 8–10 days.

Table 2 Synthetic oligonucleotides used in this study

Primer name	Oligonucleotides sequence (5′–3′)
metK1	GCAT*GGATCC*TGATCAGCCAGCAGCCGC (*Bam*HI)
metK2	CATG*AAGCTT*GAGGGAATGCCGGGCTCC (*Hin*dIII)
apr1	GATGTCATCAGCGGTGGAGTGCAATGTC
apr2	CAACGTCATCTCGTTCTCCGCTCATGAG
HrdB-F	CCAAGGGCTACAAGTTCTCC
HrdB-R	TTGATGACCTCGACCATGTG
metK-F	ACACCATTCTTGATGCGCTT
metK-R	ATCTCCAGGATCTTGTCGCG
aveR-F	CAGAAGAACTCACGCTCGTC
aveR-R	ACTCTTTCCACAGCCCATTC
aveA1-F	CGGACAGGACTACGCACTTC
aveA1-R	ACGAGATACGACCGGAGATG

Construction of recombinant plasmids and recombinant strains

All plasmids used are listed in Table 1. The *metK* gene was amplified by PCR with genomic DNA of *S. avermitilis* as template using primers metK1 and metK2. The primers used in this study are listed in Table 2. The PCR product (1.3 kb) digested with *Bam*HI and *Hin*dIII was ligated into pJL117 treated with the same enzymes, resulting in pYJ01. The *metK* with *ermE** promoter was digested from the pYJ01 by *Bgl*II, and cloned into the *Bam*HI site of pIJ653 and pSET152, respectively. The resulting recombinant plasmids pYJ02 and pYJ03, as well as the empty vectors pIJ653 and pSET152, were separately introduced into ATCC31267, GB-165 and 76-05 following the protocol previously described (MacNeil and Klapko 1987). The resulting transformants were designated 653W, 152W, 653SW, 152SW, 653G, 152G, 653SG, 152SG, 653H, 152H, 653SH and 152SH (Table 1). All above transformants were obtained by selection with thiostrepton or apramycin. After transformation, pYJ02 vector remained multi-copy in the cell, while pYJ03 was integrated into the chromosome confirmed by PCR using primers apr1 and apr2 for apramycin resistant gene *aac(3)IV* fragment. The expected 0.8 kb DNA fragment was obtained from transformants containing pYJ03 while there was no PCR product from original strains.

RNA extraction and quantitative real-time PCR

Mycelium of *S. avermitilis* grown in soluble fermentation medium II was collected at day 5. RNA isolation was carried out with Trizol (Invitrogen) according to the manufacturer's instructions. Contaminant DNA in the sample was eliminated by digestion with DNase I (Takara). Total RNA (1 μg) was subjected to reverse transcription using the PrimeScript™1st Strand cDNA Synthesis Kit (Takara). qRT-PCR assays were performed using the SYBR Premix Ex Taq™ (Takara). Primers sequences for qRT-PCR are listed in Table 2. Detection was performed using an Applied Biosystems 7300 Real-Time PCR System. All data are presented as a relative quantification, based on the relative expression of target genes versus *hrdB* as reference gene.

Determination of biomass, intracellular SAM concentration and avermectin production in fermentation

For estimation of dry cell weight (biomass), samples (30 ml) of the fermentation broth were centrifuged at 5,000 rpm for 10 min. Cell pellets were washed twice with distilled water and dried at 80 °C to constant weight. Intracellular SAM concentration was determined as follows: Samples of the

fermentation broth were centrifuged at 5,000 rpm for 10 min. After removal of the supernatant, intracellular SAM was extracted with 500 µl of 1 M formic acid at 4 °C for 1 h (Okamoto et al. 2003). The extract was centrifuged at 12,000 rpm for 15 min, and the SAM concentration was analyzed by HPLC with C18 reverse-phase column (10 µm; 4.6 × 150 i.d. mm^2). The mobile phase consisting of 20 % (v/v) methanol in 40 mM $NH_4H_2PO_4$ and 8 mM Sodium 1-heptanesulfonate was set at a flow rate of 1 ml/min. Analysis of avermectin production by HPLC was carried out as follows: 1 ml of fermentation broth was extracted with 4.0 ml of methanol for 30 min and centrifuged at 5,000 rpm for 10 min. The supernatant was directly analyzed by HPLC with C18 reverse-phase column (10 µm; 4.6 × 150 i.d. mm^2). The mobile phase containing 85 % methanol was set at a flow rate of 1 ml/min.

Results and discussion

The recombinant strains carrying pYJ02 or pYJ03, as well as the strains carrying the empty vector were cultured separately in fermentation medium for 10 days. The avermectin productivity of each culture was quantitatively measured by HPLC analysis. As shown in Fig. 1a, *S. avermitilis* ATCC31267 transformants 653W containing empty vector pIJ653 and 152W containing empty vector pSET152 produced avermectin almost as same as that of parental strain ATCC31267, while avermectin production in the strain 653SW and 152SW was increased by approximately 2.0-fold and 5.5-fold compared with that in the control strains. The cell growth and avermectin production rate of the 152SW and 152W were monitored and shown in Fig. 1b, c. Results showed 152SW carrying pYJ03 exhibited a profound increase in avermectin production than 152W, while their biomass was almost identical during the growth stages. The kinetic study revealed that avermectin overproduction in recombinant strain was not caused by the change of cell growth rate (Fig. 1b, c).

Compared with the wild-type strain, there were the modest increases or no increase at all by overexpression of *metK* in GB-165. The recombinant strain 152SG containing pYJ03 showed a 2-fold increase of avermectin B, whereas the recombinant strain 653SG containing pYJ02 did not show a stimulatory effect on avermectin production (Fig. 1d). For industry strain 76-05, the attempt to

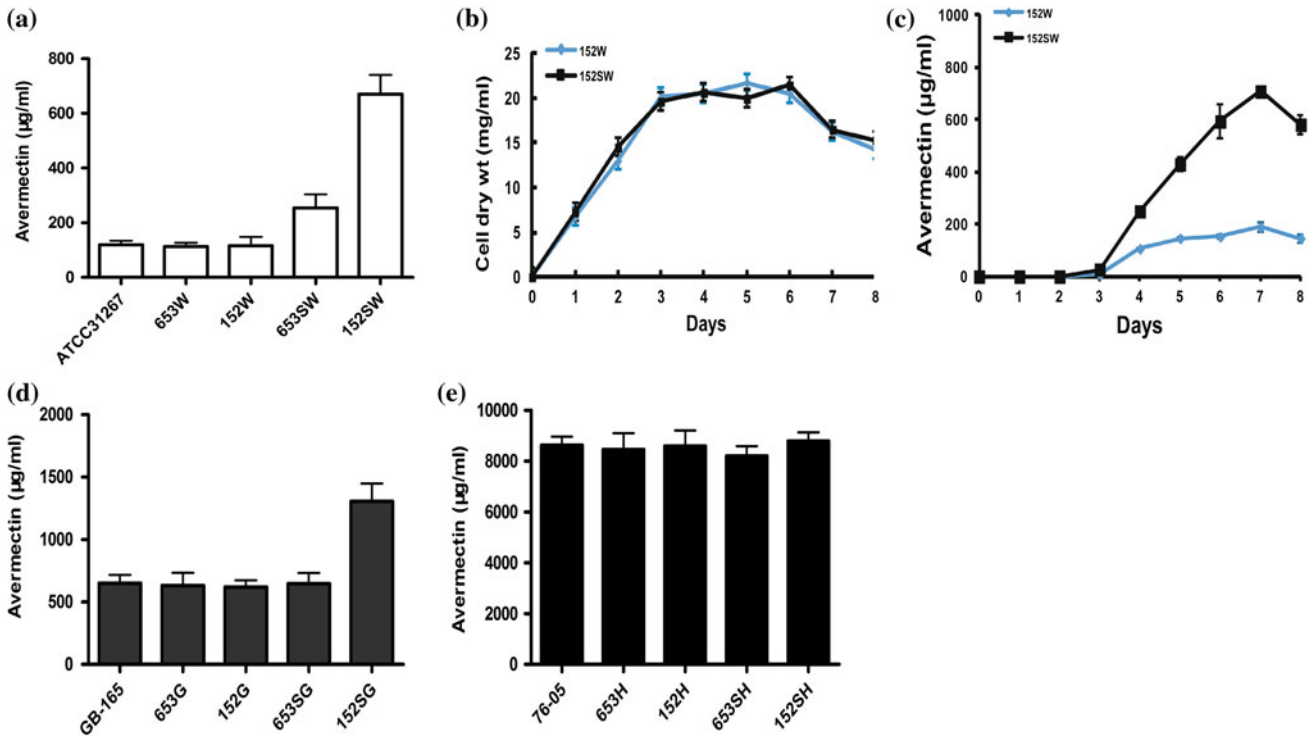

Fig. 1 Effect of overexpression of *metK* on avermectin production in wild-type strain ATCC31267, engineered strain GB-165 and industry strain 76-05. **a** avermectin production of ATCC31267 and its recombinant strains 653W(pIJ653), 152W(pSET152), 653SW(pYJ02) and 152SW(pYJ03) in fermentation medium A (*white*). Comparison of the growth rate (**b**) and avermectin production rate (**c**) in 152SW(pYJ03) and 152W(pSET152) in soluble fermentation medium II. **d** avermectin production of GB-165 and its recombinant strains 653G(pIJ653), 152G(pSET152), 653SG(pYJ02) and 152SG(pYJ03) in fermentation medium G (*gray*). **e** avermectin production of 76-05 and its recombinant strains 653H(pIJ653), 152H(pSET152), 653SH(pYJ02) and 152SH(pYJ03) in fermentation medium H (*black*)

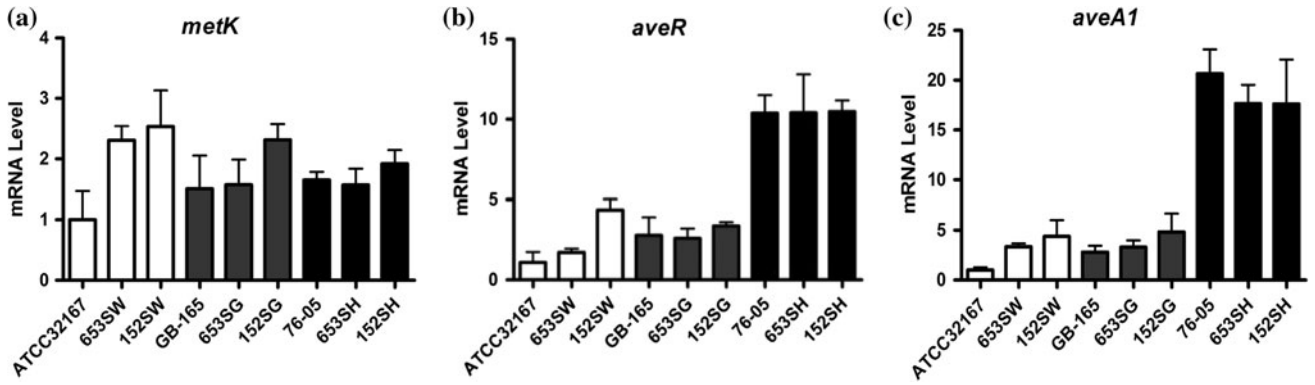

Fig. 2 Gene expression analysis of **a** *metK*, **b** *aveR* and **c** *aveA1* in various *S. avermitilis* strains. Samples were collected from soluble fermentation medium II at day 5 at 28 °C. *hrdB* was used as the internal control

overexpress the SAM biosynthesis gene failed to achieve a pronounced increase of antibiotic production (Fig. 1e).

To investigate the interrelation between overexpression of *metK* and avermectin production levels in various strains. qRT-PCR was performed to compare the transcriptional levels of *metK*, *aveR* and *aveA1* between these recombinants and their ancestral strains after 5 days fermentation. Compared to the original strain, ATCC31267, the two recombinants, 653SW and 152SW, with a higher yield of avermectin production, also exhibited the increased transcription levels of *metK* (2.3-fold and 2.5-fold), *aveR* (1.7-fold and 4.4-fold) and *aveA1* (3.4-fold and 4.4-fold) in all parallel assays (Fig. 2). In addition, we checked the intracellular level of SAM to analyze the correlation between the transcriptional level of the *metK* and SAM biosynthesis. As expected, the levels of intracellular SAM led to 1.5-fold and 3.3-fold increases in 653SW and 152SW than that in wild-type stain (Fig. 3). Therefore, it can be conclude that increasing of *metK* gene copies results in elevations of *metK* mRNA level and SAM concentration, and further upregulates the pathway-specific regulatory gene *aveR*, which then activates transcription of biosynthetic gene *aveA1* and thus increases avermectin production. This finding is consistent with the previous study, which reported that SAM activated transcription factors for antibiotic production in addition to as a methyl group donor (Kim et al. 2003; Okamoto et al. 2003). Compared to the original strain GB-165, the recombinant 152SG, with about twice yield of avermetin production, also exhibited increased transcription levels of *metK*, *aveR* and *aveA1* (1.5-fold, 1.2-fold and 1.7-fold) (Fig. 2) and SAM concentration (1.5-fold) (Fig. 3), whereas introduction of muti-copy *metK* did not have any observed effects on the levels of *metK* mRNA and SAM in 653SG, and failed to stimulate the transcripts of *aveR* and *aveA1* (Fig. 2). Compared to the original strain 76-05, the transcription levels of *metK*, *aveR* and *aveA1* as well as SAM level in 653SH were similar to those of the parental strain 76-05 (Figs. 2, 3). The overexpression of the *metK* in 152SH strain yielded 1.2-fold and 1.5-fold increases in *metK* mRNA and SAM concentration compared to the levels present in the 76-05 strain (Fig. 2a, 3), however, the mRNA levels of *aveR* and *aveA1* were not distinct when compared to 76-05 (Fig. 2b, c), and the three strains(76-05, 653SH, and 152SH)showed parallel ability of avermectin production (Fig. 1e). Thus, the results indicate that there may be a maximum concentration of SAM for the production of avermectin in *S. avermitilis*, over which it has no any effect on the antibiotic production.

As presented in Fig. 1, the results obtained revealed that an extra copy (pYJ03) achieved higher avermectin yield than multi-copy expression (pYJ02) in wild-type and GB-165 recombinant strains, and the improvement of avermectin is correlated with higher transcript levels of *metK* gene and SAM concentrations in recombinant strains harboring pYJ03 plasmid than that in the recombinant strains with pYJ02 (Fig. 2a, 3). It indicates that the increase in antibiotic production was not based on copy number effect. One possibility is that the muti-copy plasmid (pYJ03) in recombinant stains was not stable during fermentation process. Compared with the integration of the insert in the host's chromosome, plasmid loss is significant over the post exponential growth phase due to the existence of plasmid-free segregants even when selective pressure is existed as a result of the loss of the selective gene product into the fermentation media (Xu et al. 2006; Williams et al. 1998).

In recent years, gene overexpression has been shown to be an effective approach to improve antibiotic production. However, in some cases, gene overexpression has no effect or a negative effect on antibiotic production. For example, overexpression of the avermectin pathway-specific regulatory gene *aveR* either from multi-copy plasmid or from integrated plasmid resulted in a substantial decrease or complete loss of avermectin production (Kitani et al. 2009). Overexpression of SAM biosynthesis gene failed to

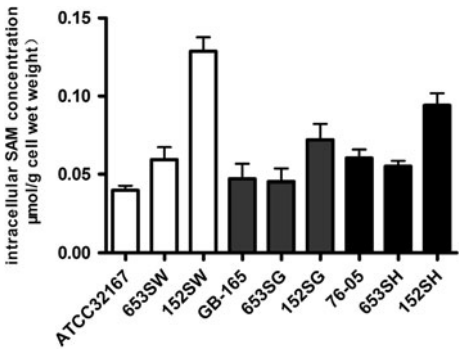

Fig. 3 Comparison of intracellular SAM levels in various *S. avermitilis* strains. Samples were collected from soluble fermentation medium II at day 5 at 28 °C. Intracellular concentrations of SAM were determined by HPLC

achieve a pronounced increase of novobiocin production in *Streptomyces cinnamonensis* (Zhao et al. 2010). Our attempt to achieve a higher level of predictability in industry strain has not gained encouragement result by overexpressing *metK* gene. In agreement with this observation, introduction of the wild-type *hrdB* into the avermectin high-producing strain 3-115 had no significant influence on avermectin production (Zhuo et al. 2010). Li et al. (2010a) reported that multiple frr copies and one extra frr copy in industrial strain on avermectin stimulation effect were not sharply marked as in wild-type strain. Secondary metabolism of microorganisms is regulated by a variety of different factors. The engineered strain and industry strain used in our experiment, which had a notably higher avermectin production than the wild-type, derived from random mutagenesis. The enhanced yield of avermectin in the mutants might be the result of co-regulation of many factors, including the downregulation of negative stimulatory factors that suppress avermectin production, and the upregulation of positive factors that promote avemectin production. As shown in Fig. 2, the results indicate that *metK* and *aveR* were up-regulated in GB-165 and 76-05 than ATCC31267. The two strains showed 1.5-fold and 1.6-fold higher *metK* expression compared to that of the wild-type strain. And the *aveR* mRNA levels in the two strains were 2.7-fold and 10-fold higher than that of wild-type strain. The stimulation of avermectin production by overexpression of *metK* gene in GB-165 and 76-05 was less obvious or no positive stimulation, probably because of the different expression levels of many regulators that controlled the antibiotic biosynthesis including *metK* and *aveR*, and this limited potential for further improvement of avermectin yield. In this concern, achieving a higher level of predictability in industry strain requires a more basic and applied research relating to the regulatory networks that govern antibiotic production. So the approach of whole-cell engineering through the selection and identification of key genes responsible for improving antibiotics would be necessary.

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