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Effects of *S*-adenosylmethionine on production of secondary metabolites in *Streptomyces diastatochromogenes* 1628

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Streptomyces are famous for their ability to synthesize a large number of bioactive compounds as secondary metabolites containing antibiotics, enzyme inhibitors, and other small molecules with potential physiological activity (Niu et al., 2016; Song et al., 2019; Yin et al., 2019). Secondary metabolites are produced by a multi-step reaction of a primary metabolite as a precursor (Liu et al., 2013; Li et al., 2021). Therefore, it is of great research significance to increase the overall synthesis level of antibiotics by increasing the amount of synthesis of precursors.

S-adenosylmethionine (SAM), which is synthesized by a synthase (MetK) from L-methionine and adenosine triphosphate, is the major donor of methyl groups (Zhao XQ et al., 2010) and plays an important role in primary and secondary metabolisms (Liu et al., 2020). In the last few years, some research groups have shown that exogenous addition of SAM and/or overexpression of the *metK* gene are effective strategies to increase secondary metabolite production in *Streptomyces* strains (Okamoto et al., 2003; Wang et al., 2012; Gu et al., 2016); examples of such strains are actinorhodin (Kim et al., 2003), pristinamycin (Huh et al., 2004), avermectin (Tian et al., 2017), nosiheptide (Zhang et al., 2008), pikromycin (Maharjan et al., 2008), and doxorubicin (Oh et al., 2010). However, although the SAM in *Streptomyces* species has been studied extensively, no studies have yet been conducted on the antagonistic strain *Streptomyces diastatochromogenes* 1628.

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S. diastatochromogenes 1628 was isolated and deposited in the China General Microbiological Culture Collection Center (CGMCC, No. 2060) and exhibits good bioactivity against a variety of phytopathogenic fungi (Ma et al., 2014a). In our previous study, toyocamycin (TM) was identified in the fermentation broth of *S. diastatochromogenes* 1628 (Ma et al., 2014a). TM, a member of the nucleoside antibiotics family, is considered to be a promising fungicide in the plant protection field (Battaglia et al., 2011). Therefore, some efforts have been made to improve the TM production of *S. diastatochromogenes* 1628 (Ma et al., 2014b, 2016, 2020; Xu et al., 2019). In addition, recent studies have demonstrated that the fermentation products of *S. diastatochromogenes* 1628 contain three other tetraene macrolides (tetramycin A, tetramycin P, and tetrin B) (Fan et al., 2020). Tetramycin, a 26-member tetraene antibiotic, has been applied to treat some plant-pathogenic fungi diseases in China (Zhao XH et al., 2010) and to control the growth of the brown planthopper, which is one of the most destructive insect pests in rice ecosystems (Shentu et al., 2016). Therefore, we developed an interest in improving the tetraene macrolide production of *S. diastatochromogenes* 1628. Improvement in production of these three tetraene macrolides has been accomplished by introducing cumulative drug-resistance mutation (Fan et al., 2020). However, how SAM affects the biosynthesis of TM and tetraene macrolides in *S. diastatochromogenes* 1628 remains unknown.

The objective of this study was to determine the effects of SAM on production of secondary metabolites in *S. diastatochromogenes* 1628. First, we investigated the effect of SAM on TM and on the production

of three tetraene macrolides (tetramycin A, tetramycin P, and tetrin B) by exogenous supplementation of SAM. Subsequently, we further investigated the relationship between SAM and secondary metabolite biosynthesis in *S. diastatochromogenes* 1628 by experiments involving $metK_{sd}$ overexpression, disruption, and complementation. In addition, we determined the effects of SAM on transcriptional levels of key genes involved in secondary metabolite biosynthesis.

We initially investigated the effect of external addition of SAM on biosynthesis of secondary metabolites in *S. diastatochromogenes* 1628. The strain was cultured in the fermentation medium with varying concentrations of SAM (2, 10, 25, and 50 μmol/L), and the levels of tetraene macrolides (tetramycin A, tetramycin P, and tetrin B) and TM were measured. As shown in Fig. 1, production of tetraene macrolides was not increased with any experimental concentration of SAM (Fig. 1a). Similarly, under the final concentrations of SAM from 2 to 50 μmol/L in the fermentation medium, the TM production of *S. diastatochromogenes* 1628 showed no significant change (Fig. 1a). In addition, we found that extracellular SAM concentrations generally remained constant during the whole fermentation process (Fig. 1b). The intracellular level of SAM did not vary depending on the amount of added SAM (Fig. 1c).

We cloned the $metK_{sd}$ gene which encodes SAM synthetase of *S. diastatochromogenes* 1628 by polymerase chain reaction (PCR). The nucleotide sequence was submitted to the GenBank database under accession No. KX689260. To increase intracellular concentration of SAM in *S. diastatochromogenes* 1628, the obtained 1209-bp $metK_{sd}$ was placed under the control of promoter $ermE^*$ in plasmid pIB139 to create pIB139-$metK_{sd}$ (Fig. S1). Then, the plasmid pIB139-$metK_{sd}$ was introduced into *S. diastatochromogenes* 1628 by intergeneric conjugation to generate recombinant strain 1628-MetK. Both the control strain (1628) and the recombinant strain (1628-MetK) were cultured in fermentation medium as described in the supplementary materials.

As shown in Fig. 2a, we observed no significant differences in cell growth between the control strain and recombinant strain. As expected, the SAM synthetase activity of recombinant strain 1628-MetK was found to be markedly increased compared to that of the wild-type strain. Compared with the control strain, recombinant strain 1628-MetK successively exhibited a 140.0%, 63.3%, 57.3%, and 96.2% increase in intracellular SAM during the fermentation periods of 24, 48, 72, and 96 h, respectively (Table 1). Consistent with the increase of intracellular SAM, recombinant strain 1628-MetK showed higher production levels of tetramycin A (201.3 mg/L vs. 135.8 mg/L), tetramycin P (155.5 mg/L vs. 108.6 mg/L), and tetrin B (57.2 mg/L vs. 40.3 mg/L) (Fig. 2b). In contrast, overexpression of $metK_{sd}$ had no obvious stimulating effect on TM production (138.4 mg/L vs. 131.7 mg/L) (Fig. 2b). Since the SAM synthetase level slightly affected the growth of the transformants, the specific production per mg dry cell weight (DCW) was also compared between recombinant strain 1628-MetK and wild-type strain 1628. The experimental data were as follows: tetramycin A (1628-MetK vs. 1628: 63.3 mg/g vs. 46.1 mg/g), tetramycin P (1628-MetK vs. 1628: 48.9 mg/g vs. 36.8 mg/g), tetrin B (1628-MetK

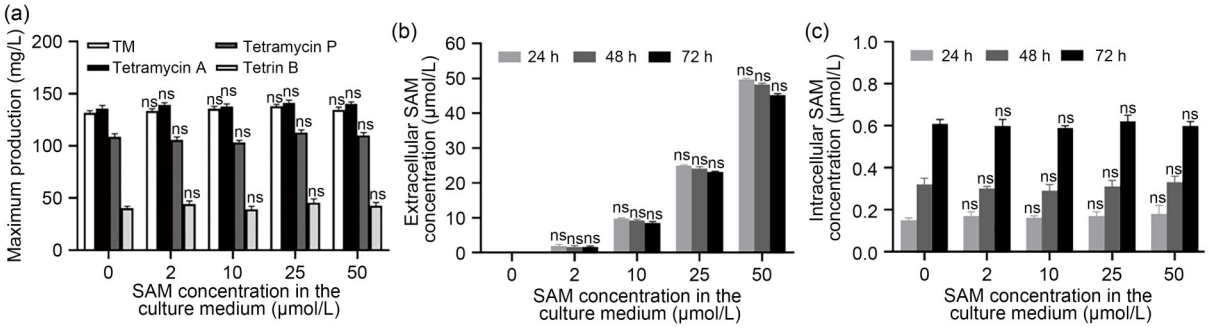

Fig. 1 Effect of exogenous SAM addition on production of secondary metabolites in *Streptomyces diastatochromogenes* 1628. (a) Secondary metabolite production after adding SAM with different final concentrations (0, 2, 10, 25, and 50 μmol/L). Determination of extracellular (b) and intracellular (c) SAM concentrations with different amounts of exogenous addition of SAM after 1, 2, and 3 d of incubation. Data are expressed as mean±standard deviation (SD), *n*=3. ns: no statistically significant results (*P*>0.05) compared with 0 μmol/L SAM (a, c) or compared with each time-point at every concentration of SAM (b). SAM: *S*-adenosylmethionine.

Table 1 Detection and comparison of SAM synthetase activity and intracellular level of SAM in *Streptomyces diastatochromogenes* 1628, mutant 1628-ΔmetK$_{sd}$, and recombinant strain 1628-MetK

Time (h)	SAM synthetase activity[a] (mU/mg protein)			Intracellular level of SAM[b] (pmol/mg wet weight)		
	1628	1628-ΔmetK$_{sd}$	1628-MetK	1628	1628-ΔmetK$_{sd}$	1628-MetK
24	1.00±0.06	0.20±0.01	3.1±0.3	10.1±1.4	3.6±0.5	24.4±2.2
48	2.30±0.15	0.40±0.03	6.7±0.4	69.2±6.2	12.3±1.6	113.3±8.3
72	1.50±0.08	0.20±0.02	5.3±0.4	76.4±6.8	8.3±1.1	120.2±7.6
96	1.30±0.07	0.10±0.02	4.3±0.2	55.1±5.4	7.5±0.7	108.1±6.2

[a] One unit of activity is defined as the amount of enzyme that produced a change in optical density at 340 nm (Okamoto et al., 2003). [b] Strains were cultured in fermentation medium. Samples were taken at the indicated time points, and SAM synthetase activity and intracellular levels were determined as described in Materials and methods (Okamoto et al., 2003). SAM: *S*-adenosylmethionine.

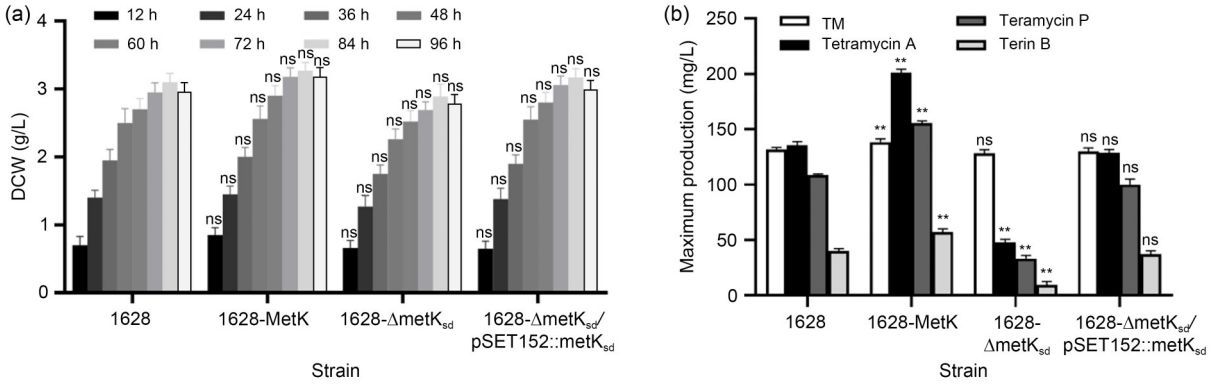

Fig. 2 Detection and comparison of cell growth (a) and maximum production of secondary metabolites (b) in the strains *Streptomyces diastatochromogenes* 1628, 1628-MetK, 1628-ΔmetK$_{sd}$, and 1628-ΔmetK$_{sd}$/pSET152::metK$_{sd}$ in shake-flask fermentation. Data are expressed as mean±standard deviation (SD), n=3. ** P<0.01, compared with control strain 1628. ns: no statistically significant results (P>0.05). DCW: dry cell weight; TM: toyocamycin.

vs. 1628: 18.0 mg/g vs. 13.7 mg/g), and TM (1628-MetK vs. 1628: 44.5 mg/g vs. 44.6 mg/g), suggesting that the effect on production and specific production per mg DCW was very similar.

To further determine whether SAM was involved in tetraene macrolide biosynthesis in *S. diastatochromogenes* 1628, knock-out plasmid pKC1132-Δ*metK$_{sd}$* was constructed as described in the supplementary materials (Fig. 3a). We disrupted the *metK$_{sd}$* gene in the *S. diastatochromogenes* 1628 chromosome via double-crossover homologous recombination (Fig. 3a) to generate mutant *S. diastatochromogenes* 1628-ΔmetK$_{sd}$. As shown in Fig. 3b, a 1209-bp *metK$_{sd}$* gene could be amplified from control strain 1628, but not from 1628-ΔmetK$_{sd}$. In addition, amplicons of 5.4 kb and 4.2 kb represented products of the control strain and the mutant strain, respectively, suggesting that a double-crossover homologous recombinant event had occurred and that the *metK$_{sd}$* gene was disrupted in the mutant 1628-ΔmetK$_{sd}$. Subsequently, we determined the effect of disrupting the *metK$_{sd}$* gene on secondary metabolite production of *S. diastatochromogenes* 1628. The results showed that intracellular concentrations of both SAM (Table 1) and tetraene macrolides (Fig. 2b) in mutant 1628-ΔmetK$_{sd}$ were decreased sharply compared to those in control strain 1628. Complementation of 1628-ΔmetK$_{sd}$ with pSET152::*metK$_{sd}$* recovered SAM biosynthesis and tetraene macrolide production. Moreover, the results showed that disruption or complementation of *metK$_{sd}$* in *S. diastatochromogenes* 1628 had no effect on TM production (Fig. 2b) or on cell growth (Fig. 2a).

SAM has been found to act as an intracellular signal molecule for regulating the secondary metabolites in Streptomyces species. It was reported that the ttmRI gene, belonging to the large adenosine triphosphate (ATP)-binding regulator of LuxR (LAL) family, is involved in the biosynthetic pathway of tetraene macrolides in *Streptomyces hygrospinosus* (Cui et al., 2016). To test whether overexpression of *metK$_{sd}$* could stimulate the transcriptional level of *ttmRI* (GenBank accession No. MW880243), we performed quantitative

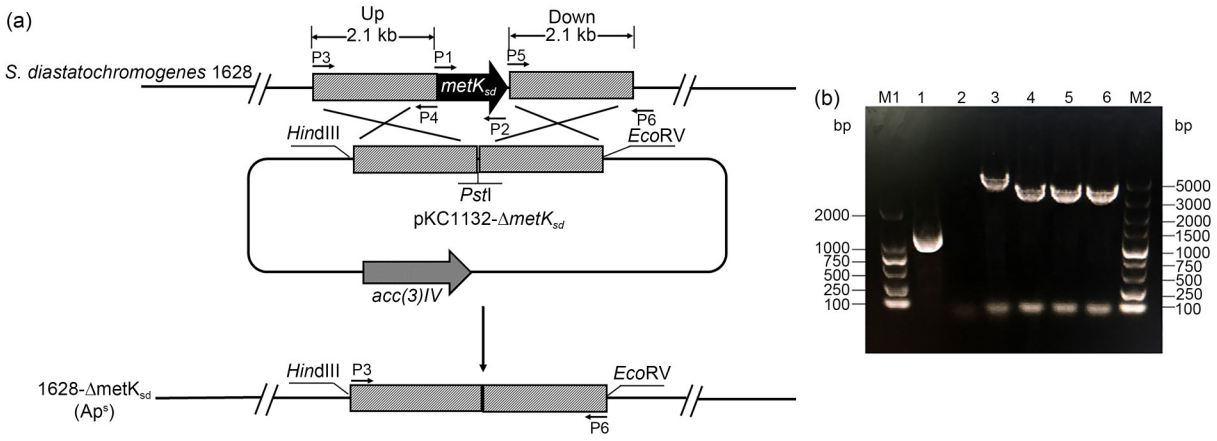

Fig. 3 Construction and identification of mutant *Streptomyces diastatochromogenes* 1628-ΔmetK$_{sd}$. (a) Schematic representation of the strategy used for deletion of the *metK$_{sd}$* gene. Disruption of *metK$_{sd}$* was performed by gene replacement via homologous recombination. (b) Polymerase chain reaction (PCR) verification of 1628-ΔmetK$_{sd}$. M1, DL2000 DNA marker; Lane 1, PCR products of *metK$_{sd}$* from the control strain *S. diastatochromogenes* 1628; Lane 2, PCR products of *metK$_{sd}$* from mutant *S. diastatochromogenes* 1628-ΔmetK$_{sd}$; Lane 3, PCR product amplified from the control strain *S. diastatochromogenes* 1628 using the primers P3/P6; Lanes 4–6, PCR product amplified from three randomly mutant *S. diastatochromogenes* 1628-ΔmetK$_{sd}$ using the primers P3/P6; M2, 5000 DNA marker. Ap^s: apramycin sensitivity.

real-time PCR (qRT-PCR) analysis. The result is shown in Fig. 4. As compared with the control strain, the transcriptional level of *ttmRI* was greatly decreased in the mutant 1628-ΔmetK$_{sd}$. In contrast, in recombinant strain *S. diastatochromogenes* 1628-MetK, the transcriptional level of *ttmRI* was obviously increased. This result demonstrated that overexpression of *metK$_{sd}$* enhanced the transcriptional level of *ttmRI* and further improved tetramycin A and tetramycin P production. Moreover, the results also indicated that the disruption or overexpression of *metK$_{sd}$* had no obvious effect on the transcriptional levels of *toy* genes (GenBank accession No. KY022432) (Fig. 4).

SAM, the major methyl donor in diverse biological processes, was found to be involved in the secondary metabolism in many *Streptomyces* species (Park et al., 2005). The major secondary metabolites of the antagonistic strain *S. diastatochromogenes* 1628 contain TM and three tetraene macrolides (tetramycin A, tetramycin P, and tetrin B).

Overexpression of *metK$_{sd}$* significantly promoted production of the three tetraene macrolides, whereas production of the three tetraene macrolides in mutant 1628-ΔmetK$_{sd}$ drastically decreased due to disruption of the *metK$_{sd}$* gene. Production of the three tetraene macrolides in the complemented strain 1628-ΔmetK$_{sd}$/pSET152::*metK$_{sd}$* was restored to a level comparable to that in the wild-type strain *S. diastatochromogenes* 1628. Therefore, increased SAM appears to promote overproduction of these three tetraene macrolides in *S. diastatochromogenes* 1628. In addition, it is worth mentioning that the slight special activity of SAM synthetase and concentration of intracellular SAM could still be detected in mutant 1628-ΔmetK$_{sd}$. This suggests that strain cell may have an SAM synthetase isoenzyme that is involved in SAM biosynthesis. In addition, neither overexpression nor disruption of the *metK$_{sd}$* gene has any obvious influence on cell growth of *S. diastatochromogenes* 1628. This observation is not consistent with report that SAM usually acts as a factor affecting morphological differentiation in *Streptomyces* (Park et al., 2005).

On the other hand, TM biosynthesis was not significantly affected by the level of intracellular SAM. This conclusion can be supported by our experimental fermentation data on TM, whether in the ΔmetK$_{sd}$ mutant strain or in the recombinant strain harboring overexpression of *metK$_{sd}$*.

It has been reported that SAM donates a methyl group to metabolites to affect the production of various types of secondary metabolites (Gu et al., 2016). So far, some studies have reported that overexpression of *metK* could enhance the production of polyketides (Wang et al., 2007) or macrolides (Wang et al., 2012), whereas the successful enhancement of nucleoside antibiotic production through exogenous addition of SAM or overexpression of *metK* has yet to be reported. TM is a pyrrolopyrimidine nucleoside antibiotic, and

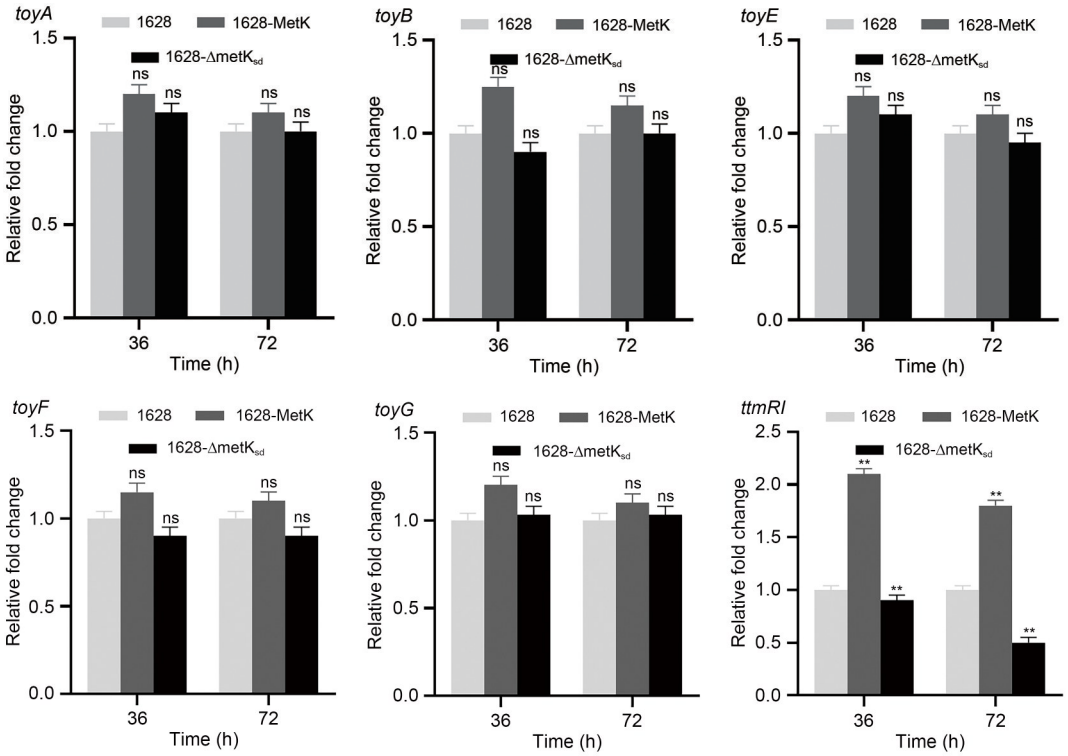

Fig. 4 Comparison of the transcriptional levels of *toy* genes and regulatory *ttmRI* in *Streptomyces diastatochromogenes* 1628, 1628-ΔmetK$_{sd}$, and 1628-MetK. Data are expressed as mean±standard deviation (SD), *n*=3. ** *P*<0.01, compared with control strain 1628. ns: no statistically significant results (*P*>0.05). The functions of *toy* genes: *toyA*, LuxR transcriptional regulator; *toyB*, 6-pyruvoyltetrahydropterin synthase; *toyE*, GMP reductase; *toyF*, adenylosuccinate lyase; *toyG*, adenylosuccinate synthetase.

its biosynthetic pathway in *Streptomyces rimosus* was reported by McCarty and Bandarian (2008), who used guanosine triphosphate (GTP) as a precursor. This could explain why SAM also has no significant effect on the biosynthesis of TM.

Tetramycin is a 26-member tetraene antibiotic whose biosynthetic pathway in *S. hygrospinosus* var. *beijingensis* was reported by Cao et al. (2012) and Sheng et al. (2020). The extension and maturation of tetraenes backbone were catalyzed by multifunctional type I polyketide synthases (PKSs) with ten malonates, an acetate, and a methylmalonate; and glycosylation and oxidation of PKS backbone were accomplished with a post-PKS tailoring step to generate tetramycin. It seems that methionine is not a precursor of tetramycin.

It has been reported that SAM can act as a signal factor to regulate the transcription of genes involved in secondary metabolite biosynthesis and further affect their production (Huh et al., 2004; Xu et al., 2018). The tetramycin gene cluster has been reported from the genome of *S. hygrospinosus* var. *beijingensis* ACCC40068 (Cao et al., 2012) and *Streptomyces ahygroscopicus* CGMCC 4.7082 (Ren et al., 2014; Cui et al., 2015). Cui et al. (2016) revealed that *ttmRI* is a positive regulator that activates transcription of the tetramycin biosynthetic gene cluster. Therefore, we employed qRT-PCR to analyze the expression level of *ttmRI* in the wild-type strain, mutant strain, and recombinant strain. In accord with the production of tetraene macrolides, the recombinant strain 1628-MetK and mutant 1628-ΔmetK$_{sd}$, respectively, showed remarkably higher and lower expression levels of *ttmRI* compared with the corresponding value of wild-type strain 1628, indicating that increased SAM promotes transcriptional expression of *ttmRI*. However, there were no significant differences in the transcriptional level of *toy* genes involved in TM biosynthesis among the three strains. Therefore, we speculate that SAM acts as a signal molecule to stimulate production of tetramycin, but not of TM. The greatest limitation of this study was that we did not study the regulatory mechanism of TTMRI on tetramycin biosynthesis in depth.

In conclusion, our study shows that overexpression of the *metK*$_{sd}$ gene in *S. diastatochromogenes* 1628 leads to increased SAM and promotes the production of three tetraene macrolides, but that it has no effect on TM production.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

Acknowledgments

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Author contributions

Yefeng HU, Juan WANG, and Jie XU conducted experiments. Yefeng HU wrote the original draft. Zheng MA designed the experiments and wrote this article. Andreas BECHTHOLD and Xiaoping YU gave some good suggestions on the revision of the manuscript, polished English and checked the final version. All authors have read and approved the final manuscript and, therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Yefeng HU, Juan WANG, Jie XU, Zheng MA, Andreas BECHTHOLD, and Xiaoping YU declare that they have no conflict of interest.

This article does not involve any studies with human or animal subjects.

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Supplementary information

Materials and methods; Fig. S1