ORIGINAL RESEARCH PAPER

Heterologous expression of *metK1-sp* and *afsR-sp* in *Streptomyces venezuelae* for the production of pikromycin

Sushila Maharjan · Tae-Jin Oh · Hei Chan Lee · Jae Kyung Sohng

Abstract Two regulator genes, *metK1-sp* and *afsR-sp*, from *Streptomyces peucetius* ATCC 27952 were heterologously expressed in *S. venezuelae* ATCC 15439, to produce 14-membered pikromycin antibiotics. The production of pikromycin was increased by 1.6-fold and 2.6-fold by the expression of *metK1-sp* and *afsR-sp*, respectively. The overexpression of *metK1-sp* and *afsR-sp* in *S. venezuelae* stimulated the expression of the pathway-specific regulatory genes, *pikD* and ketosynthase, as demonstrated by RT-PCR. The elevated transcripts of the *pikD* and ketosynthase genes were consistent with the enhanced production of pikromycin.

Keywords Global regulatory gene ·
Pikromycin · *S*-adenosylmethionine synthetase ·
Streptomyces venezuelae · Transcription

Introduction

Pikromycin (Fig. 1), a 14-membered macrolide antibiotic, is structurally related to the semi-synthetic ketolide antibiotics, which are active against multidrug-resistant respiratory pathogens (Agouridas et al. 1998). Due to the wide range of biological activities, the biosynthesis of the pikromycin series of antibiotics has attracted significant interest. Pikromycin was initially isolated from *Streptomyces venezuelae* ATCC 15439. However, low levels of pikromycin production have led to the development of a strain genetically engineered to produce higher levels of pikromycin. Many of the previous studies on the enhancement of secondary metabolites were focused on either the homologous or heterologous expression of positive regulators. Among the positive regulators, *S*-adenosylmethionine synthetase (MetK) and a global regulatory gene, *afsR*, were previously reported to be involved in the enhanced production of various secondary metabolites from different *Streptomyces* species (Lee et al. 2002; Kim et al. 2003; Okamoto et al. 2003; Wang et al. 2007).

MetK plays an important role in the conversion of ATP and L-methionine to *S*-adenosyl-L-methionine (SAM), which acts as a major methyl group donor for numerous transmethylation reactions. Furthermore, SAM regulates antibiotic biosynthesis in a manner independent of its role as a methyl donor, in which it acts as a direct intracellular signaling molecule for *Streptomyces* (Zhao et al. 2006). It also activates the transcriptional activators responsible for the induction of antibiotic synthetic genes, thereby increasing the production of antibiotics (Kim et al. 2003). Recent reports have suggested that SAM induces several ABC transporters in order to modulate

S. Maharjan · T.-J. Oh · H. C. Lee · J. K. Sohng (✉)
Institute of Biomolecule Reconstruction (iBR),
Department of Pharmaceutical Engineering,
SunMoon University, #100, Kalsan-ri, Tangjeong-myeon,
Asan-si, Chungnam 336-708, Republic of Korea
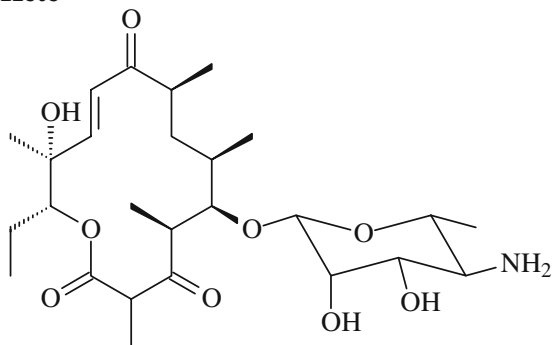e-mail: sohng@sunmoon.ac.kr

Hete

Fig. 1 The structure of pikromycin

secondary metabolism and morphological development in *S. coelicolor* (Shin et al. 2007).

On the other hand, *afsR* is a pleiotropic, global regulator that controls the production of secondary metabolites in *Streptomyces* species. AfsR is widely distributed as the AfsK-AfsR system in *Streptomyces* and influences the secondary metabolism and morphogenesis of the organism (Horinouchi 2003). As a transcriptional activator, AfsR greatly enhances its DNA-binding activity toward the respective promoter region, thereby enhancing the transcriptional activation for the biosynthesis of secondary metabolites in streptomyces (Tanaka et al. 2007).

On the basis of these findings, we heterologously expressed these two genes, *metK1-sp* (GenBank accession no. CAJ43278) and *afsR-sp* (GenBank accession no. AJ786384) from *S. peucetius* ATCC 27952 (Parajuli et al. 2005), individually in *S. venezuelae* ATCC 15439 in order to analyze the production of pikromycin. Reverse transcription-polymerase chain reaction (RT-PCR) was used to study the effects of the overexpression of these genes on the regulation of pikromycin biosynthetic gene clusters.

Materials and methods

Bacterial strains and growth conditions

E. coli XL1 Blue MRF′ (Stratagene) was used for DNA amplification. *E. coli* ET 12567 was used to propagate non-methylated DNA. *S. venezuelae* ATCC 15439 was the parent strain and was grown in R2YE media for the preparation of protoplasts and

isolation of plasmid DNA. Protoplast transformation was carried out according to the standard protocol (Kieser et al. 2000). To isolate pikromycin, wild type and transformant strains of *S. venezuelae* were cultured SCM liquid medium (15 g soluble starch, 20 g soytone, 1.5 g yeast extract, 0.1 g calcium chloride and 10.5 g MOPS/l distilled water), supplemented with trace amounts of $FeSO_4$ (12.5 mg/l) at 28°C for 60 h (Lee et al. 2006).

To study the growth rate, wild type and transformant strains of *S. venezuelae* were cultured in 50 ml SCM media at 28°C after 36 h of incubation in R2YE seed media. The cells were collected at intervals of 12 h by centrifuging 50 ml culture broth of each strain at $6000 \times g$. Cell pellets were washed with distilled water and dried at 72°C in vacuum oven to constant weight. Supernatants obtained after removing cell pellets were used to study the rate of pikromycin production. Values were the average of four determinations. For the isolation of total RNA, *S. venezuelae* and transformants were grown in APM medium (60 g glucose, 8 g yeast extract, 20 g malt extract, 2 g NaCl, 15 g MOPS and 10 ml trace elements/l distilled water).

Cloning and heterologous expression of *metK1-sp* and *afsR-sp*

The expression vector, pIBR25, under the control of the *ermE** promoter, which leads to the expression of DNA in *Streptomyces* species, was used for cloning (Basundhara et al. 2004). The *metK1-sp* gene was amplified by PCR of genomic DNA from *S. peucetius*, using metK1-F and metK1-R primers. The PCR product (1,209 bp) of the *metK1-sp* gene was cloned into the *Bam*HI and *Hin*dIII sites of pIBR25 to produce the recombinant plasmid pSIBR. Similarly, *afsR-sp* from *S. peucetius* was amplified using afsR-pF and afsR-pR primers. The PCR product (2,946 bp) was cloned into the *Eco*RI and *Hin*dIII sites of pIBR25 to produce the recombinant plasmid pGIBR (Parajuli et al. 2005). The primers used in this study are listed in Table 1. Recombinants pSIBR and pGIBR (Fig. 2a and b) were transformed into wild type *S. venezuelae* (SV) to obtain *S. venezuelae*/pSIBR (SVSIBR) and *S. venezuelae*/pGIBR (SVGIBR), respectively. The transformation was accomplished by following the standard protocol (Kieser et al. 2000).

HPLC analysis of pikromycin production

Each 50 ml culture of SV, SVSIBR and SVGIBR was centrifuged at 6000 × g for 15 min. The supernatants

Table 1 Primers used in this study

Primers	Sequences (5′–3′)
metK1-F	TTGGGGATCCATGTCCCGTC
metK1-R	CCGCAAGCTTCTGTGCACTCA
afsR-pF	TCCGGAATTCCCCGGCAGGGGGC
afsR-pR	CGAAGCTTCGGACCGAGCACGA
metK1-RTF	GCAAGACCCAGGTCACCATCGAGTACC
metK1-RTR	GTCGAAGACCTGGGTGATGGCCTTCTC
afsR-pRTF	TACCGGTACCACGACCTTGTGCGTCT
afsR-pRTR	TGTTCGTCGGCCTCGTCGTAACGGC
pikD-RTF	CATCTCAGGGCCGTTCTTGACGCATCC
pikD-RTR	CGTAGTAGTGGGCGAGTAACTGGCGTAC
16S rRNA-RTF	CCTTCGGGTTGTAAACCTCTTTCAGCA
16S rRNA-RTR	CAACACCTAGTTCCCAACGTTTACGGC
KS-RTF	CGAACGGTTTCGCGGAGCAGACTCATG
KS-RTR	GAAGTCGTGGTGCACCACGGTCACATG

were extracted with 2 vol ethyl acetate. The extract was dried in a rotary evaporator and was reconstituted by 1.5 ml methanol. A 15 µl of the extract was analyzed by HPLC using a reverse-phase C_{18} column with 80% acetonitrile in 5 mM ammonium acetate buffer containing 0.05% acetic acid, adjusted to pH 8 with NH_4OH at 1 ml/min. Detection was at 220 nm. The major peak corresponding to pikromycin was confirmed by LC/MS analysis. The production was averaged from four separate cultivations and extractions. The antibacterial activity of SV, SVSIBR, and SVGIBR was assayed against *Bacillus subtilis* ATCC 23857 (Lee et al. 2006).

RNA sample preparation and RT-PCR analysis

Each aliquot (5 ml) of the cultures grown for about 48 h was suspended in RNA Protect Bacteria Reagent (Qiagen) for 5 min in order to isolate total RNA. An RNeasy Mini kit (Qiagen) was used for RNA isolation according to the manufacturer's instructions. Contaminant DNA in the sample was eliminated by using RNase-free DNase (Qiagen) and verified by PCR analysis using the RNA as the

Fig. 2 Recombinant Plasmids. (**a**) pSIBR, *metK1-sp* from *S. peucetius* cloned into the pIBR25 vector under the control of the *ermE** promoter. (**b**) pGIBR, *afsR-sp* from *S. peucetius* cloned into the pIBR25 vector under the control of the *ermE** promoter. (**c**) Antibacterial activity assay of the isolated pikromycin against *B. subtilis*. W, isolates from SV; S, SVSIBR; and A, SVGIBR. (**d**) Comparison of pikromycin production from SV, SVSIBR and SVGIBR. Pikromycin was extracted from each strain, after being grown at 28°C for 60 h in SCM medium, and analyzed by HPLC and LC/MS analysis. The experiment was averaged from 4 different extractions
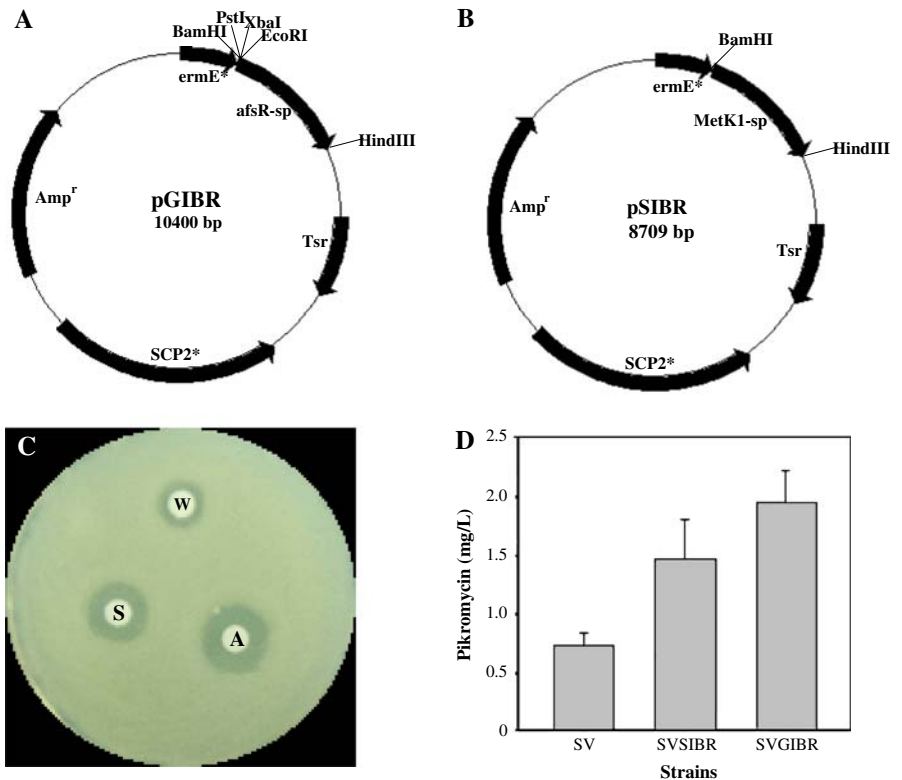
template. The total RNA concentration and purity were determined from the 260/280 nm ratio. RT-PCR was performed with a QuantiTech SYBER Green RT-PCR Kit (Qiagen). The primers used for RT-PCR are described in Table 1.

Equal amounts (5.54 µg) of RNA from each sample were used for RT-PCR analysis. The reaction conditions were as follows: first strand cDNA synthesis at 50°C for 30 min; initial denaturation at 95°C for 15 min; and 45 cycles of 1 min at 94°C, 1 min at 63°C and elongation at 72°C for 2 min. The RT-PCR products were electrophoresed on a 1% agarose gel and visualized using ethidium bromide staining. Negative controls were carried out with Taq DNA polymerase without reverse transcripts to confirm that the amplified products were not derived from chromosomal DNA, which could contaminate the RNA preparations. The 16S rRNA gene from *S. venezuelae* was used as a positive internal control.

Results and discussion

To enhance the production of pikromycin from *S. venezuelae* wild strain, we chose two genes, namely *metK1-sp* and *afsR-sp*, which encode for the MetK protein and global regulatory protein, respectively. Both *metK1-sp* (1.2 kb) and *afsR-sp* (2.9 kb) were amplified from *S. peucetius* using the metK1-F/metK1-R and afsR-F/afsR-R primers, respectively (data not shown). The PCR products of *metK1-sp* and *afsR-sp* were separately cloned into pIBR25 to construct recombinant plasmids pSIBR and pGIBR, respectively, as described in the Materials and methods section. The recombinants pSIBR and pGIBR were then transformed into SV to generate SVSIBR and SVGIBR, respectively. Pikromycin produced by strains SV, SVSIBR and SVGIBR is given in Figs. 2d and 3 and confirmed by LC/MS. Production of pikromycin was enhanced by 1.6-fold in SVSIBR and 2.6-fold in SVGIBR in comparison with pikromycin production by SV. Thus, the biological activities of the pikromycin obtained from SV, SVSIBR and SVGIBR were tested against *B. subtilis*, and the diameters of the inhibition zones in the two mutants showing the least growth were indicative of high levels of pikromycin rather than SV (Fig. 2c). These results revealed that *metK1-sp* and *afsR-sp* had a positive influence on the production of pikromycin.
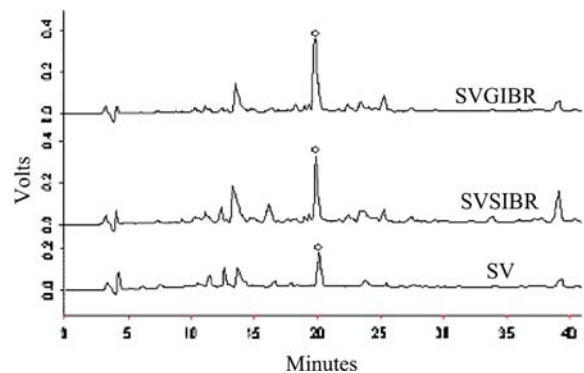

Fig. 3 Typical HPLC traces for pikromycin. Peak corresponding to pikromycin is indicated by a circle (RT = 20 min), and the pikromycin peaks in every case were verified by LC/MS analysis

SVSIBR and SVGIBR had higher growth yields than SV itself with. Maximum growth at 48–60 h (Fig. 4a). However, the influence of *afsR-sp* on the growth rate as well as the production of pikromycin was greater than that induced by *metK1-sp* (Fig. 4b). Previously, the overexpression of *metK* stimulated the expression of the pathway-specific regulatory gene *actII*-ORF4 for the production of actinorhodin in *S. coelicolor* A3 (2) (Okamoto et al. 2003). Furthermore, AfsR serves as a transcriptional activator of *afsS*, which activates the pathway-specific genes *actII*-ORF4, in the actinorhodin biosynthetic gene cluster (Lee et al. 2002). Similarly, strains overexpressing *metK1-sp* and *afsR-sp* were most likely to induce the biosynthetic pathway of pikromycin for the enhanced production of pikromycin. The influence of *metK1-sp* and *afsR-sp* on the expression levels of two genes, ketosynthase and *pikD*, involved in the production of pikromycin were assayed by RT-PCR analysis. Ketosynthase is a domain from PikAI that catalyses polyketide chain extension in modular multifunctional polyketide synthases (PKS) (Bisang et al .1999), whereas *pikD* operates as a pathway-specific activator of the pikromycin biosynthetic gene cluster (Wilson et al. 2001). The RT-PCR results showed that the transcription levels of both the ketosynthase and *pikD* genes were substantially increased in SVSIBR and SVGIBR when compared to SV (Fig. 5).

In *S. venezuelae*, *metK* is located downstream of *pikD*, which might help to provide the methyl group in desosamine synthesis (Xue et al. 1998). Therefore,
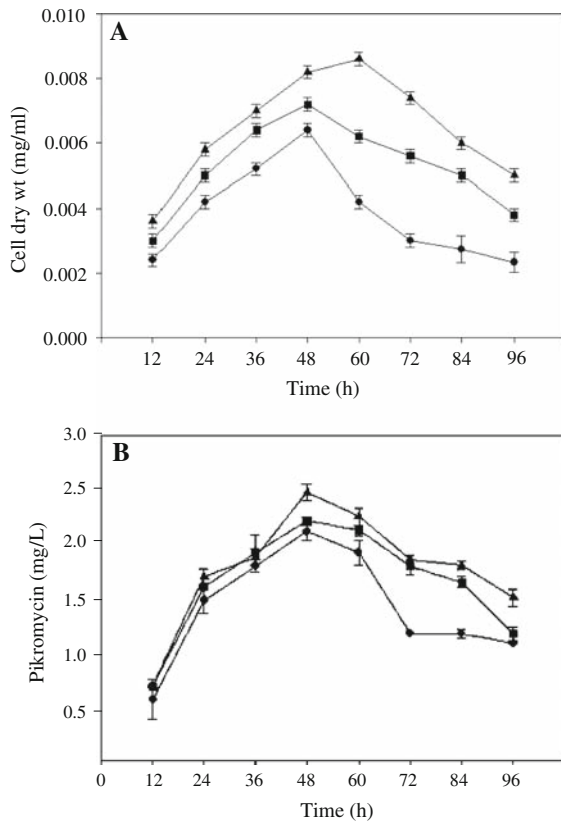
Fig. 4 Comparison of the growth rate (a) and production rate (b) of SV, SVSIBR and SVGIBR. (a) Cell pellets were collected at 12-h intervals, starting from 12 h to 96 h. (b) Isolation of the compound was carried out at the same intervals and quantification was performed via HPLC analysis. Closed circle, SV; closed square, SVSIBR; and closed triangle, SVGIBR

Fig. 5 Quantitative RT-PCR analysis of ketosynthase and *pikD* which involved in pikromycin biosynthetic pathway using equal amount (5.54 μg) of total RNA isolated from SVSIBR (a) and SVGIBR (b). 16S rRNA gene was used as internal control. M, DNA marker. The relative intensities of the transcriptional bands were measured using a PhosphoImager and were calculated with the Imagequant program. Lane 1, use total RNA from wild type (SV); lane 2, use total RNA from mutants (SVSIBR and SVGIBR)

the increased transcripts of *pikD* might correspond to the enhanced production of desosamine and pikromycin. Studies on the biosynthesis of desosamine have consistently established *DesVI* (*N,N*-dimethyltransferase) as a SAM-dependent methyltransferase in the pikromycin biosynthetic gene cluster (Chen et al. 2002). The increased level of SAM production would apparently aid in the overproduction of desosamine by *desVI* and thereby enhance the production of pikromycin. In addition, the enhanced expression of *metK*, which increases the intracellular level of SAM, resulted in the overproduction of actinorhodin from *S. lividans* (Kim et al. 2003). Although the mechanism underlying the increased production of antibiotics with the increased intracellular level of SAM remains unclear, it has been proposed that SAM activates various transcriptional
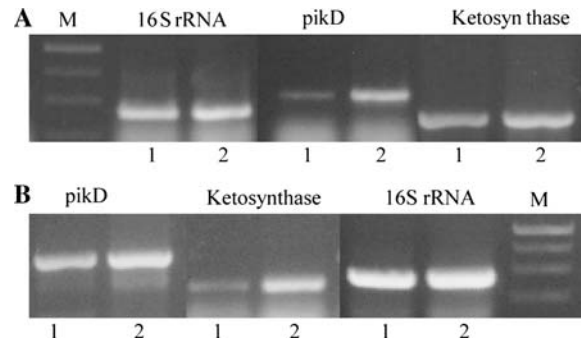
activators, which are responsible for the induction of antibiotic synthetic genes or serves as a methyl donor directly to the antibiotics.

Similarly, *afsR* is a global regulator gene that seems to control the secondary metabolism and morphological and physiological differentiation in *Streptomyces* (Umeyama et al. 2002). Moreover, *afsR-sp* functioned as a transcriptional activator in order to regulate the production of secondary metabolites in *Streptomyces* species (Parajuli et al. 2005). Heterologous expression of *afsR-sp* in SVGIBR enhanced the transcript levels of the ketosynthase and *pikD* pikromycin biosynthetic genes, resulting in the overproduction of pikromycin. Although the exact mechanism of the increased production of antibiotics should be further elucidated, we found that the *metK1-sp* and *afsR-sp* genes influenced the stimulation of the biosynthetic genes to overproduce pikromycin through transcriptional activation in *S. venezuelae*.

References

Agouridas C, Denis A, Auger JM et al (1998) Synthesis and antibacterial activity of ketolides (6-*O*-methyl-3-oxoery-thromycin derivatives) a new class of antibacterials highly

potent against macrolide-resistant and susceptible respiratory pathogens. J Med Chem 41:4080–4100

Basundhara S, Oh TJ, Lamichhane R et al (2004) Neocarzinostatin naphthoate synthase: an unique iterative type I PKS from neocarzinostatin producer *Streptomyces carzinostaticus*. FEBS Lett 566:201–206

Bisang C, Long PF, Cortés J et al (1999) A chain initiation factor common to both modular and aromatic polyketide synthases. Nature 401:502–505

Chen H, Yamase H, Murakami K et al (2002) Expression, purification, and characterization of two *N,N*-dimethyltransferases, TylM1 and DesVI, involved in the biosynthesis of mycaminose and desosamine. Biochemistry 41:9165–9183

Horinouchi S (2003) AfsR as an integrator of signals that are sensed by multiple serine/threonine kinases in *Streptomyces coelicolor* A3(2). J Ind Microbiol Biotechnol 30:462–467

Kieser T, Bibb MJ, Buttner MJ et al (2000) Practical Streptomyces genetics. The John Innes Foundation, Norwich

Kim DJ, Huh JH, Yang YY et al (2003) Accumulation of S-adenosyl-L-methionine enhances production of actinorhodin but inhibits sporulation in *Streptomyces lividans* TK23. J Bacteriol 185:592–600

Lee SK, Hong JSJ, Choi CY et al (2006) Enhanced production of hydroxylated macrolides from pikromycin pathway of *Streptomyces venezuelae*. Enzyme Microb Technol 39:778–782

Lee PC, Umeyama T, Horinouchi S (2002) *afsS* is a target of AfsR, a transcriptional factor with ATPase activity that globally controls secondary metabolism in *Streptomyces coelicolor* A3(2). Mol Microbiol 43:1413–1430

Okamoto S, Lezhava A, Hosaka T et al (2003) Enhanced expression of S-adenosylmethionine synthetase causes overproduction of actinorhodin in *Streptomyces coelicolor* A3 (2). J Bacteriol 185:601–609

Parajuli N, Hung TV, Ishida K et al (2005) Identification and characterization of the *afsR* homologue regulatory gene from *Streptomyces peucetius* ATCC 27952. Res Microbiol 156:707–712

Shin SK, Park HS, Kwon HJ et al (2007) Genetic characterization of two S-adenosylmethionine-induced ABC transporters reveals their roles in modulations of secondary metabolism and sporulation in *Streptomyces coelicolor* M145. J Microbiol Biotechnol 17:1818–1825

Tanaka A, Takano Y, Ohnishi Y et al (2007) AfsR recruits RNA polymerase to the *afsS* promoter: a model for transcriptional activation by SARPs. J Mol Biol 369:322–333

Umeyama T, Lee PC, Horinouchi S (2002) Protein serine/threonine kinases in signal transduction for secondary metabolism and morphogenesis in *Streptomyces*. Appl Microbiol Biotechnol 59:419–425

Wang Y, Boghigian BA, Pfeifer BA (2007) Improving heterologous polyketide production in *Escherichia coli* by overexpression of an S-adenosylmethionine synthetase gene. Appl Microbiol Biotechnol 77:367–373

Wilson DJ, Xue Y, Reynolds KA et al (2001) Characterization and analysis of the PikD regulatory factor in the pikromycin biosynthetic pathway of *Streptomyces venezuelae*. J Bacteriol 183:3468–3475

Xue Y, Zhao L, Liu HW et al (1998) A gene cluster for macrolide antibiotic biosynthesis in *Streptomyces venezuelae*: Architecture of metabolic diversity. Biochemistry 95:12111–12116

Zhao XQ, Jin YY, Kwon HJ et al (2006) S-Adenosylmethionine (SAM) regulates antibiotic biosynthesis in *Streptomyces* spp. in a mode independent of its role as a methyl donor. J Microbiol Biotechnol 16:927–932