

Article

Influence of Cluster-Situated Regulator PteF in Filipin Biosynthetic Cluster on Avermectin Biosynthesis in *Streptomyces avermitilis*

Guozhong Du¹, Xue Yang¹, Zhengxiong Wu¹, Minghui Pan^{1,2}, Zhuoxu Dong^{1,2}, Yanyan Zhang¹, Wensheng Xiang^{1,2,*} and Shanshan Li^{1,*}

¹ State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, No. 2 Yuanmingyuan West Road, Haidian District, Beijing 100193, China; dgz1005@163.com (G.D.); 82101211024@caas.cn (X.Y.); 17513232046@163.com (Z.W.); mhlu@163.com (M.P.); d15945317995@163.com (Z.D.); yyzhang@ippcaas.cn (Y.Z.)

² School of Life Science, Northeast Agricultural University, No. 59 Mucai Street, Xiangfang District, Harbin 150030, China

* Correspondence: xiangwensheng@neau.edu.cn (W.X.); lishanshan01@caas.cn (S.L.)

Simple Summary: Crosstalk regulation is a common regulatory phenomenon in *Streptomyces*, typically mediated by regulatory factors within gene clusters. Utilizing these regulatory factors to enhance the production of target compounds represents an important approach. This study focuses on two regulatory factors, PteF and PteR, within the filipin gene cluster in *Streptomyces avermitilis*, investigating their effects on both avermectin production and overall cellular metabolism. The findings provide theoretical groundwork for constructing high-yielding engineered strains of avermectins and provide new insights into the role of PteF in the biosynthesis of avermectins and its impact on cellular metabolic processes.

Abstract: Crosstalk regulation is widespread in *Streptomyces* species. Elucidating the influence of a specific regulator on target biosynthetic gene clusters (BGCs) and cell metabolism is crucial for strain improvement through regulatory protein engineering. PteF and PteR are two regulators that control the biosynthesis of filipin, which competes for building blocks with avermectins in *Streptomyces avermitilis*. However, little is known about the effects of PteF and PteR on avermectin biosynthesis. In this study, we investigated their impact on avermectin biosynthesis and global cell metabolism. The deletion of *pteF* resulted in a 55.49% avermectin titer improvement, which was 23.08% higher than that observed from *pteR* deletion, suggesting that PteF plays a more significant role in regulating avermectin biosynthesis, while PteF hardly influences the transcription level of genes in avermectin and other polyketide BGCs. Transcriptome data revealed that PteF exhibited a global regulatory effect. Avermectin production enhancement could be attributed to the repression of the tricarboxylic acid cycle and fatty acid biosynthetic pathway, as well as the enhancement of pathways supplying acyl-CoA precursors. These findings provide new insights into the role of PteF on avermectin biosynthesis and cell metabolism, offering important clues for designing and building efficient metabolic pathways to develop high-yield avermectin-producing strains.

Keywords: avermectin; filipin; regulator; *Streptomyces avermitilis*; transcriptome



Citation: Du, G.; Yang, X.; Wu, Z.; Pan, M.; Dong, Z.; Zhang, Y.; Xiang, W.; Li, S. Influence of Cluster-Situated Regulator PteF in Filipin Biosynthetic Cluster on Avermectin Biosynthesis in *Streptomyces avermitilis*. *Biology* **2024**, *13*, 344. <https://doi.org/10.3390/biology13050344>

Academic Editor: Tingyi Wen

Received: 24 April 2024

Revised: 9 May 2024

Accepted: 13 May 2024

Published: 15 May 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Avermectins are a class of 16-membered ring macrolide compounds, which currently represent the largest group of green natural-product pesticides produced and utilized globally owing to their outstanding properties, such as low dosage, long action time, low toxicity to mammals and easy decomposition [1]. *Streptomyces avermitilis* is the most significant producer of avermectins [2,3]. The titer of avermectin B_{1a} was improved from

0.009 g/L to 0.5 g/L by Merck using random mutation techniques, such as ultraviolet, methyl methanesulfonate and N-methyl-N'-nitro-N-nitrosoguanidine [4]. Further, with the rapid development of omics analytical technologies, high-yield avermectin producers have been engineered via different metabolic engineering strategies. For instance, the titer of avermectin B_{1a} of *S. avermitilis* has been enhanced through the manipulation of various transcription regulators, such as TetR family regulators SAV151 [5] and AveT [6], the MarR family regulator SAV4189 [7] and the sigma factor σ^{HrdB} [8]. Titer improvement could also be achieved by engineering transporters for carbon substrate uptake (e.g., *malEFG*, TP2, TP5 and TP6568) and putative product efflux (e.g., *AvtAB* and *MiltAB2*) [9–13]. Moreover, the titer of avermectin B_{1a} was boosted by approximately 50% to 9.31 g/L using a triacylglycerol dynamic degradation strategy in our previous work [14], and Hao et al. enhanced the titer of avermectin B_{1a} to 9.61 g/L through a combination engineering strategy [15]. To date, the titer of avermectin B_{1a} has been increased over 1000-fold since its first discovery. Nevertheless, understanding the influence of various regulatory proteins on avermectin biosynthesis in *S. avermitilis* is still the tip of the iceberg, which impedes us from unlocking the new targets for further strain improvement and titer enhancement.

The genome of *Streptomyces* generally contains more than 20 secondary metabolite biosynthetic gene clusters (BGCs), including polyketides, non-ribosomal peptides, terpenes, ribosomally synthesized and post-translationally modified peptides, and so on [2]. The biosynthesis of secondary metabolites is tightly restricted by complex regulatory and metabolic networks, including cross-regulation and metabolic flux competition [16,17]. The production enhancement of target chemicals could be achieved via the inactivation of competitive BGCs [18], whereas its effectiveness is not always guaranteed due to cross-regulation. For example, we discovered that the *Streptomyces* antibiotic regulatory protein (SARP) family regulator protein KelR controls the biosynthesis of a yellow polyketide compound, KEL, as well as our target chemicals, milbemycins, in *Streptomyces bingchengensis* [19]. Similarly, TtnQ is also an essential regulator that activates the biosynthesis of both tautomycin and spirotoamide in *Streptomyces griseochromogenes* [20]. Therefore, it is important to clarify whether or not a given regulator of the competitive BGC effectively possesses cross-regulatory effects on the target chemicals prior to regulatory protein engineering.

The wild-type strain *S. avermitilis* MA-4680 contains 38 BGCs in total, including 7 putative type I polyketide BGCs [21]. Avermectin, filipin and oligomycin can be detected among these compounds under conventional fermentation conditions [2]. Since the three BGCs share the same biosynthetic extender units of malonyl-CoA and methylmalonyl-CoA, they undergo metabolic competition while being simultaneously cross-regulated by different regulatory proteins. The cluster-situated regulator (CSR) AveR activates avermectin biosynthesis while inhibiting oligomycin biosynthesis [22], and another CSR, PteF, positively regulates the biosynthesis of both filipin and oligomycin [23]. However, apart from investigations into PteF's impact on the transcription level of avermectin BGC (*ave*) [24], few studies have focused on the relationship between filipin BGC (*pte*) and *ave*, as well as the influence of both CSR proteins PteF and PteR on avermectin production in *S. avermitilis*. Therefore, an elucidation of the influence of PteF and PteR on avermectin biosynthesis might be promising for guiding the rational engineering of high-yield avermectin producers via regulatory engineering.

In this work, we investigate the influence of PteF and PteR on avermectin production in *S. avermitilis* S0 with two copies of *ave* and *pte*, and elucidate the cross-regulatory effect of PteF on *pte* and *ave*. We further elucidate the mechanism of PteF deletion on avermectin production enhancement based on transcriptome data analysis. This work expands our understanding of the influence of PteF on avermectin biosynthesis, as well as on cell metabolism in *S. avermitilis*, providing new insights and targets for further strain engineering for higher avermectin production.

2. Materials and Methods

2.1. Strains, Plasmids and Culture Conditions

All strains and plasmids used in this study are listed in Table S1. *Escherichia coli* JM109 was used for plasmid cloning; *E. coli* ET12567/pUZ8002 served as a donor in intergeneric conjugations. *S. avermitilis* S0 is a derivative of *S. avermitilis* MA-4680 obtained via random mutagenesis, which has been deposited at the China General Microbiology Culture Collection Center (accession no. CGMCC 4.8011). *E. coli* was grown on Luria Bertani (LB) medium supplemented with antibiotics as required at 37 °C. For conjugations, *S. avermitilis* strains were grown on mannitol soya flour (MS) medium at 28 °C for 7 days [25]. For fermentations of *S. avermitilis* and their derivatives, seed cultures were prepared by inoculating one square centimeter of spores into 250 mL Erlenmeyer flasks, containing 25 mL of seed medium and cultivated at 28 °C for 40 h with a speed of 250 rpm. After that, 1.5 mL of seed culture was transferred into 250 mL Erlenmeyer flasks containing 25 mL of fermentation medium, and further cultured for 10 days. The seed medium and fermentation medium of *S. avermitilis* were described in previous studies [26].

2.2. Construction of In-Frame Deletion Mutant Strains

Primers used in this work are listed in Table S2. For the construction of the *pteF* (gene ID: GM000692) and *pteR* (gene ID: GM000694) disruption mutant, two homologous fragments, Δ *pteF*-L (2034 bp) and Δ *pteF*-R (2076 bp), flanking the *pteF* operon were amplified from the genomic DNA of S0 via PCR using Δ *pteF*-LF/R and Δ *pteF*-RF/R as primer pairs; two homologous fragments, Δ *pteR*-L (2070 bp) and Δ *pteR*-R (1927 bp), flanking the *pteR* operon were amplified from the genomic DNA of S0 via PCR using Δ *pteR*-LF/R and Δ *pteR*-RF/R as primer pairs. The Δ *pteF*-L and Δ *pteF*-R PCR products were cloned into pKC1139 digested by EcoRI/XbaI using Gibson assembly to obtain pKC1139:: Δ *pteF*; the Δ *pteR*-L and Δ *pteR*-R PCR products were cloned into pKC1139 digested by EcoRI/XbaI using Gibson assembly to obtain pKC1139:: Δ *pteR*. Then, the resultant plasmid was introduced into strain S0 via conjugation to construct the *pteF* null mutant (sav Δ *pteF*) and *pteR* null mutant (sav Δ *pteR*) via homologous recombination as previously described [14]. The mutant strains sav Δ *pteF* and sav Δ *pteR* were confirmed via PCR with the primer pairs Δ *pteF*Y-F/R and Δ *pteF*LY-F/R, as well as Δ *pteR*Y-F/R and Δ *pteR*LY-F/R, respectively.

2.3. Transcriptome Analysis

S. avermitilis S0 and sav Δ *pteF* were grown in liquid fermentation medium and collected at day 2 and 6 for RNA isolation. Triplicate samples of total RNA were extracted and purified for RNA library preparation and sequencing. Sequencing libraries were prepared using NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (NEB, Ipswich, MA, USA) and TruSeq PECluster Kit v3-cBot-HS (Illumina, Inc., San Diego, CA, USA) following the manufacturer's recommendations before being sequenced on an Illumina HiSeq X-ten instrument in a paired-end (150 × 150 bp) approach (Novogene, Inc., Beijing, China). The high-quality reads were mapped using Bowtie/2.2.3 against the *S. avermitilis* S0 genome (GenBank accession code: GCA_029277425.1). HTSeq v0.6.1 was used to count the read numbers mapped to each gene. The fragment per kilobase of exon per million fragments mapped (FPKM) of each gene was calculated based on the length of the gene and the read count mapped to this gene, and statistically significant differences in gene expression were detected using the DESeq R package (1.18.0) in accordance with the criteria $|\log_2(\text{fold change})| > 1.0$ and $p\text{-value} < 0.05$. The technical and biological reproducibility of RNA-seq results was evaluated to be good via three independently fermented cDNA samples. Pearson correlation coefficients of the sequencing results were evaluated to be higher than 0.95 based on the log-transformed FPKM. Kyoto encyclopedia of genes and genomes (KEGGs) is a database resource for understanding high-level functions and utilities of the biological system (<http://www.genome.jp/kegg/>, accessed on 2023). ClusterProfiler (Version 3.8.1) software was used to test the statistical enrichment of differential expression genes in KEGG.

2.4. Extraction and High-Performance Liquid Chromatography (HPLC) Analysis of Avermectin B_{1a} and Filipin III

Avermectin B_{1a} was extracted and analyzed as described previously [11]. The procedure for the extraction of filipin III was the same as the method for avermectin B_{1a}, and the concentration of filipin III was determined as previously described [27].

2.5. Genomic Synteny Analysis

Genomic alignment between the *S. avermitilis* S0 genome and *S. avermitilis* MA-4680 genome were performed using the MUMmer (Version 3.23) [28] and LASTZ (Version 1.03.54) [29] tools. Genomic synteny was analyzed based on the alignment results.

2.6. Statistical Analysis

All experiments were performed independently in at least three biological triplicates, and data were presented as mean values \pm standard deviation (s.d.). Student's *t*-test (two-tail) was used to demonstrate the statistical significance, with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and "ns" meaning not significant.

3. Results

3.1. Analysis of Avermectin and Filipin Biosynthesis in *S. avermitilis* S0

S. avermitilis S0 is a high-yield avermectin-producing strain obtained via random mutation from *S. avermitilis* MA-4680 [11]. Genome analysis showed that there are two long inverted repeat segments in the flanking regions (left arm: 1 bp to 963,064 bp; right arm: 8,668,384 bp to 9,631,365 bp) in the S0 strain (Figure 1a). Each repeated segment contains six BGCs according to comparative genomic analysis between MA-4680 and S0, including three polyketide BGCs, *ave*, *pte* and *pks11* (Table 1), and they compete with acyl-CoA precursors while the known clusters *pte* and *ave* have same direct precursors, malonyl-CoA and methylmalonyl-CoA (Figure 1b). These data suggested that avermectin biosynthesis might significantly influenced by *pte* and *pks11* because they doubled in copy number. We first analyzed the time course transcription level of *pte* and *pks11* based on the transcriptome data on S0. It was shown that genes involved in *pte* were constantly transcribed at a level similar to that of genes in *ave* during the whole fermentation process (Figure 1c). Also, we detected filipin III, the major component of the filipin complex [30], in the liquid fermentation broth of S0 (Figure 1d). Given the fact that filipin causes competition between essential precursors and reducing power with avermectins, we thought it might be helpful to improve avermectin production in S0 by inhibiting filipin biosynthesis.

Table 1. BGCs involved in each repeated segment.

Category	Cluster	Length	Product
terpene	<i>ams</i>	1008 bp	Avermitilol, avermitilone
polyketide	<i>pks11</i>	5227 bp	Polyketide ¹
other	<i>mcj1</i>	3368 bp	Microcin ¹
polyketide	<i>pte</i>	80,344 bp	Filipin
polyketide	<i>ave</i>	80,849 bp	Avermectin
terpene	<i>crt</i>	8718 bp	Isorenieratene

¹ Putative products.

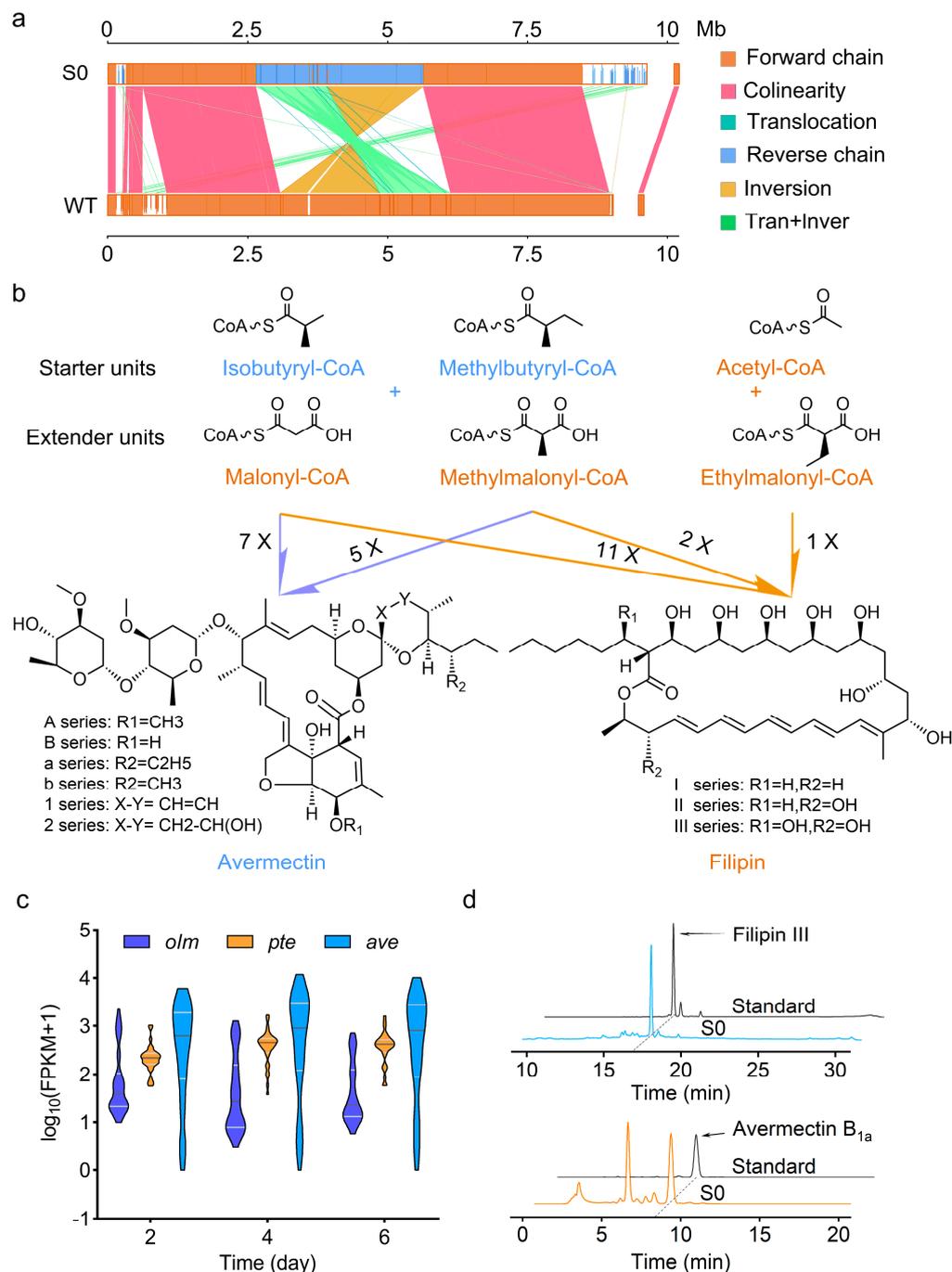


Figure 1. Analysis of avermectin and filipin biosynthesis in *S. avermitilis* S0. (a) Synteny analysis of S0 and MA-4680. (b) Acyl-CoA precursors for avermectin and filipin biosynthesis. Shared precursors are highlighted in orange. (c) Transcription level analysis of genes in *ave*, *olm* and *pte*. Data were obtained from transcriptome data on S0 at 2, 4, and 6 days from three independent repeats. *pte*, *olm* and *ave* indicate BGCs for filipin, oligomycin, and avermectin, respectively. (d) Analysis of avermectin and filipin of S0.

3.2. Influence of PteF and PteR on Avermectin and Filipin Production

Generally, deleting the activator of target BGCs is an effective approach to blocking the biosynthesis of corresponding metabolites. Therefore, in-frame deletion was carried out to obtain *pteF* and *pteR* deletion mutants *savΔpteF* and *savΔpteR*. Compared with the parent strain S0, the titer of filipin III in *savΔpteF* and *savΔpteR* decreased by 63.5% and 9.1%, respectively (Figure 2a), demonstrating that PteF plays a significant active role

in filipin biosynthesis in S0, which agrees with previous investigations obtained from other *Streptomyces* [24,27]. We also found that titers of avermectin B_{1a} in *savΔpteF* and *savΔpteR* were, respectively, 55.49% and 26.12% higher than those of the parent strain, S00 (Figure 2b). Moreover, the deletion of *pteF* boosted avermectin biosynthesis during the whole production stage (day 4–10) (Figure 2a,b). These data demonstrate that PteF might regulate the biosynthesis of filipin and avermectin simultaneously. Although many investigations have demonstrated the regulatory mechanism of PteF on filipin biosynthesis, to date, it remains unclear how PteF influences avermectin biosynthesis.

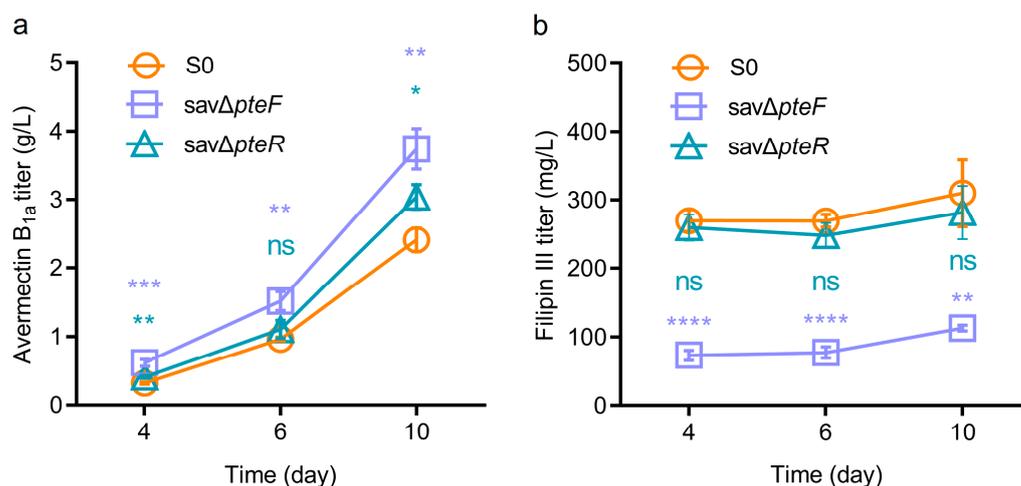


Figure 2. Effect of *pteF* and *pteR* deletion on avermectin and filipin production. (a) Filipin III titer of mutant strain *savΔpteF* and *savΔpteR* under different fermentation times. (b) Avermectin B_{1a} titer of mutant strain *savΔpteF* and *savΔpteR* under different fermentation times. Data were obtained from three independent replicates. Student's *t*-test (two-tail) was used to demonstrate the statistical significance, with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and "ns" meaning not significant.

3.3. Influence of *pteF* Deletion on Transcription of Genes Involved in Polyketide Synthase (PKS) BGCs

To comprehensively explore the influence of *pteF* deletion on avermectin biosynthesis, we carried out transcriptome data analysis on S0 and *savΔpteF* in the exponential phase (day 2) and mid-production phase (day 6). Since many CSRs are involved in orchestrated regulatory networks controlling the biosynthesis of more than one secondary metabolite [19], we first focused on the transcription level changes in genes in putative BGCs encoding polyketides that might compete for precursors and reducing power with avermectins. We found that the deletion of *pteF* significantly reduced the transcription level of genes in *pte* on day 2 and 6 (Figure 3a,b), while *pteF* deletion had almost no effect on the transcription level of genes involved in other PKS BGCs on day 2 and 6 (Figure 3c,d). Despite that, we also found that the transcription levels of genes in *ave* were slightly increased (Figure 3c,d). Moreover, the FPKM value of genes in *ave* quite high compared with those in the other PKS BGCs, increasing by 4- to 500-fold and from 4- to 750-fold on day 2 and 6 in S0, respectively (Figure 3c,d and Table S3). These data imply that, on one hand, PteF might not directly regulate the transcription of genes in PKS BGCs; on the other hand, the enhanced titer of avermectin B_{1a} might be due to a slight increase in the gene transcription level of *ave*, as well as the availability of more precursors and reducing power, which might be caused by the indirect effect of *pteF* deletion.

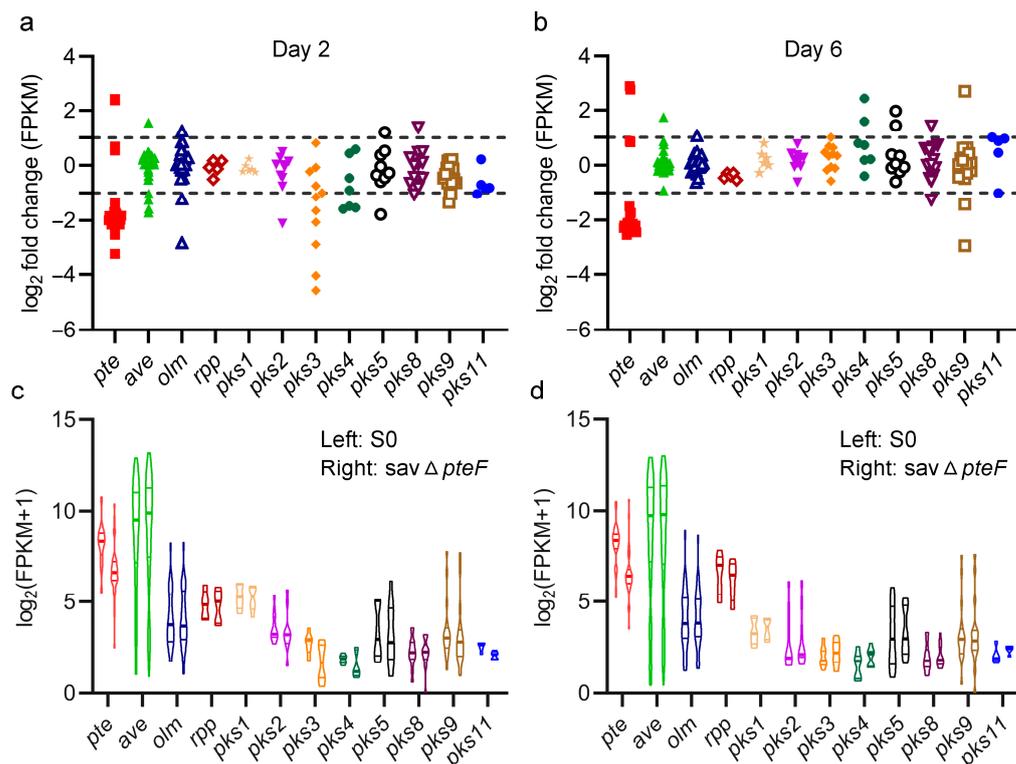


Figure 3. Effect of *pteF* deletion on transcription level changes in genes in different polyketide BGCs. Relative changes in transcriptional level of genes in polyketide BGCs on day 2 (a,c) and day 6 (b,d). The points in (a,b) represent the \log_2 -transformed fold change of FPKM for each gene, indicating the variation in expression levels of individual genes within the PKS gene cluster. The violin plots in (c,d) show the \log_2 -transformed of (FPKM + 1) for all genes within the cluster, representing the transcriptional level differences across the entire gene cluster between the S0 and *sav* Δ *pteF* strains. The dashed lines in (a,b) indicate the threshold of the genes with significant changes.

3.4. Influence of *pteF* Deletion on Global Regulatory Network

To further explore the indirect effect of *pteF* deletion on avermectin production, we conducted a comprehensive investigation of the transcriptome data. Considering that many CSRs are also global regulatory proteins, we further investigated the global influence of *pteF* deletion on the regulatory network. According to the transcriptome data, *pteF* deletion significantly influenced the transcription level of 141 genes on day 2, including 17 up-regulated and 124 down-regulated genes (Figure 4a and Table S4), as well as 100 genes on day 6, including 38 up-regulated and 62 down-regulated genes in different metabolic pathways (Figure 4b and Table S5). The data suggest that PteF not only tightly regulates filipin biosynthesis, but also exhibits a global regulatory effect on cell metabolism. We first focused on changed regulatory genes. *pteF* deletion resulted in 12 and 5 putative regulatory genes with significant transcription level changes on day 2 and 6, respectively (Figure 4b, Tables 2 and 3). These genes encode sigma/anti-sigma factors and transcriptional regulators from various families. Among them, *GM003939* and *GM003574*, encoding putative anti-sigma regulatory factors, showed significant transcription level changes at both day 2 and 6. Transcription levels of *GM003939* were significantly increased, while those of *GM003574* were decreased (Tables 2 and 3). Furthermore, we also found that *GM007052*, which encodes for a putative RNA polymerase extracytoplasmic function (ECF) subfamily sigma factor, showed significantly decreased gene transcription levels (Table 2). Since the expression of either sigma factors or anti-sigma factors influences the transcription of their potential target genes, we speculated that *pteF* deletion might have led to significant metabolic changes that would have favored avermectin biosynthesis.

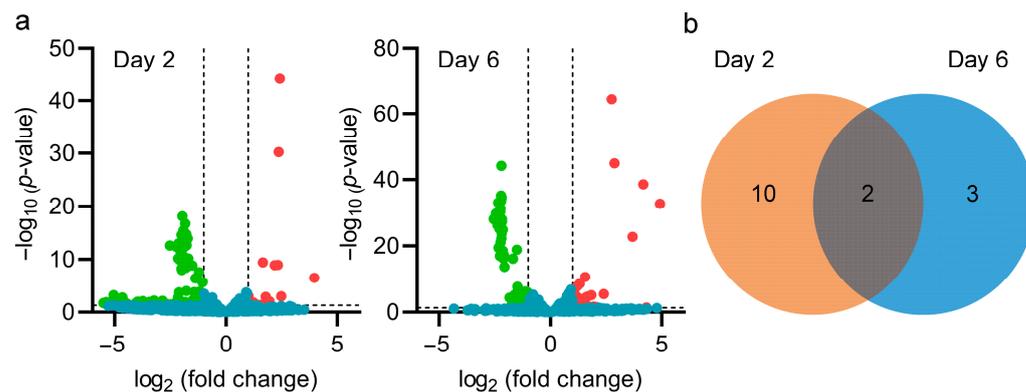


Figure 4. Global influence of *pteF* deletion on gene transcription level in S0. (a) Volcano plots of significantly different genes on day 2 and 6. The red, green, and blue point represent genes with significantly up-regulated ($\log_2(\text{fold change}) > 1$, $p\text{-value} < 0.05$), significantly down-regulated ($\log_2(\text{fold change}) < -1$, $p\text{-value} < 0.05$), and scarcely changed profiles, respectively. (b) Venn diagram of significantly changed regulator genes on day 2 and 6.

Table 2. Significantly changed regulator genes on day 2.

Gene ID	Functional Description	$\log_2(\text{Fold Change})$	$p\text{-Value}$
GM007052	putative RNA polymerase ECF subfamily sigma factor	−5.05	0.001
GM006412	putative IclR family transcriptional regulator	−2.29	0.020
GM003939	putative anti-sigma regulatory factor	2.19	1.42×10^{-9}
GM000222	putative TetR family transcriptional regulator	−1.68	0.022
GM007217	putative two-component system response regulator	1.57	0.042
GM003574	putative anti-sigma regulatory factor	−1.51	1.46×10^{-9}
GM003618	AsnC family transcriptional regulator	−1.47	0.044
GM001189	putative RNA polymerase ECF subfamily sigma factor	−1.30	0.037
GM000932	putative LacI family transcriptional regulator	−1.24	0.049
GM005356	putative two-component system sensor kinase	−1.06	0.001
GM007707	putative MerR family transcriptional regulator	1.02	0.036
GM001883	putative ArsR family transcriptional regulator	−1.00	0.030

Table 3. Significantly changed regulator genes on day 6.

Gene ID	Functional Description	$\log_2(\text{Fold Change})$	$p\text{-Value}$
GM003939	putative anti-sigma regulatory factor	4.17	2.51×10^{-39}
GM003307	putative regulatory protein	1.95	0.024
GM008359	LytR family transcriptional regulator	1.23	0.037
GM003574	putative anti-sigma regulatory factor	−1.13	6.45×10^{-7}
GM002997	TetR family transcriptional regulator	−1.01	3.05×10^{-4}

3.5. Influence of *pteF* Deletion on Cell Metabolism

To explore the effect of *pteF* deletion on changes in metabolic pathways, KEGG enrichment analysis was carried out to explore the global changes between *sav* Δ *pteF* and S0. Data showed that there were four and seven metabolic pathways with significant changes (gene ratio > 0.05 , $p\text{-value} < 0.05$) on day 2 and 6, respectively (Table S6). Among these genes, we first noted three metabolic pathways: glyoxylate and dicarboxylate metabolism, glycerolipid metabolism and propanoate metabolism pathway. These primary metabolic pathways are linked together by central carbon metabolism, and participate in the metabolism of different acyl-CoAs required for avermectin biosynthesis (Figure 5). Nine genes with transcription level changes were involved in three pathways. The transcription level of gene *acsA4* (GM002244), encoding putative acetyl-CoA synthetase, was increased on day 2 and 6

(Figure 5 and Table S6), and that of *accA1* (GM006049), encoding putative acetyl/propionyl-CoA carboxylase alpha subunit, was also up-regulated on day 2. Such a change might favor the biosynthesis of acetyl-CoA, propionyl-CoA, malonyl-CoA, and methylmalonyl-CoA, which are required for avermectin biosynthesis (Figure 5). Moreover, we also found that *tgs* (GM008560) and *plsC4* (GM007927), which encode for putative triacylglycerol synthase and 1-acylglycerol-3-phosphate O-acyltransferase, respectively, and participate in triacylglycerol biosynthesis, showed down-regulated transcription levels after *pteF* deletion on day 6. These data imply that *pteF* deletion might reduce carbon flux towards triacylglycerols that compete with acyl-CoA precursors for avermectin production [14]. Meanwhile, we also found that *pteF* deletion led to a decreased transcription level of *acnA* (GM002508), which encodes an aconitate hydratase in the tricarboxylic acid (TCA) cycle on day 6 (Figure 5 and Table S6). Since the repression of the TCA cycle has been proven to decrease carbon flux competition with polyketide biosynthesis [14], we speculated that the reduced expression level of TCA cycle caused by *pteF* deletion might also have been a possible reason for the improved avermectin production. In addition to genes in primary metabolic pathways, KEGG pathway enrichment analysis also showed genes involved in secondary metabolic pathways, such as *pteB* (crotonyl-CoA reductase) and *pteD* (cytochrome P450 hydroxylase) in *pte* (Table S6). All of these data indicate that *pteF* deletion rearranged cell metabolism, possibly redirecting more carbon flux from primary metabolism to the avermectin biosynthetic pathway.

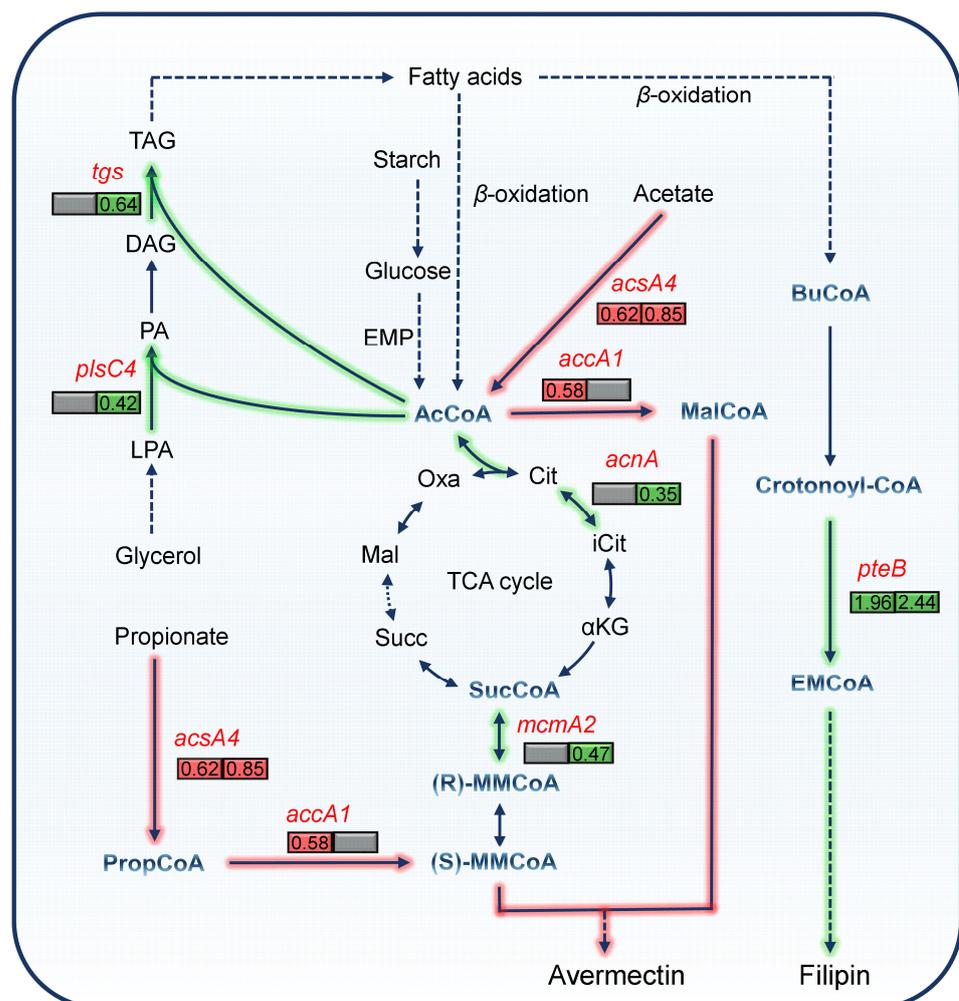


Figure 5. Effect of *pteF* deletion on cell metabolism. Genes enriched in significantly different metabolic pathways were mapped to a precursor supply metabolic pathway of avermectin biosynthesis. The

red, green, and gray colors represent genes with up-regulated, down-regulated, and scarcely changed transcription levels at day 2 (left) and day 6 (right), respectively. The data in the frame represent the absolute values of the \log_2 -transformed value (fold change) for the corresponding genes. Solid arrows represent a one-step reaction, while dashed arrows represent multi-step reactions. The red and green arrow indicate the pathway with enhanced and decreased transcription level, respectively. Abbreviations: AcCoA, acetyl-CoA; MalCoA, malonyl-CoA; PropCoA, propionyl-CoA; MMCoA, methylmalonyl-CoA; SucCoA, succinyl-CoA; BuCoA, butyryl-CoA; EMCoA, ethylmalonyl-CoA; TAG, Triacylglycerol; DAG, 1,2-Diacylglycerol; PA, Phosphatidic acid; LPA, Lysophosphatidic acid; Oxa, Oxaloacetate; Cit, Citrate; iCit, Isocitrate; α KG, 2-Oxoglutarate; Succ, Succinate; Mal, Malate.

4. Discussion

Regulator engineering is a convenient strategy to increase the production of valuable natural products. Given the fact that natural product biosynthesis is tightly controlled by complicated cellular regulatory networks, it is necessary to elucidate the influence of key regulators on target chemical production prior to strain engineering [31]. Avermectins, as valuable pharmaceuticals, possess diverse applications. Although the industrialization of avermectin has been highly developed, its production still needs to be improved for lower application costs. In the avermectin-producing strain *S. avermitilis*, oligomycin and filipin are generally biosynthesized along with avermectins. Moreover, CSRs such as OlmR I and OlmR II in *olm*, AveR in *ave*, and PteF, and PteR in *pte*, showed cross-regulation relationships between oligomycin and filipin, as well as oligomycin and avermectin [22,32]. However, the influence of PteF and PteR in *pte* on avermectin biosynthesis has been scarcely investigated. Moreover, many CSRs also possess global regulatory functions; thus, we also aimed to comprehensively explore the influence of PteF and PteR. Uncovering the influences of these two regulators would have facilitated the strain engineering of avermectin producers for higher yields.

Gene inactivation experiments showed that PteF plays a more important role than does PteR in regulating the biosynthesis of both filipin and avermectin B_{1a}. Therefore, we further focused on the influence of PteF in *S. avermitilis* S0. Based on time course transcriptome data analyses, we found that PteF showed a small influence on putative PKS BGCs including *ave*, whereas *pteF* deletion resulted in significant changes in many proteins closely related to gene transcription, including transcription regulators and sigma/anti-sigma factors. Among them, GM007052 and GM003939, with the most significant transcription level changes on day 2 and 6, encoded a sigma factor and an anti-sigma factor, respectively. The sigma factor, as an essential component of bacterial RNA polymerases, determines promoter specificity and regulates transcription as well as translation, while anti-sigma factors are responsible for inhibiting sigma factor function. In *Streptomyces*, sigma factors generally play essential roles in regulating signal transduction and regulatory networks, as well as secondary metabolite biosynthesis to assist their survival in complex environments [33]. For instance, the housekeeping σ factor σ^{HrdD} regulates the biosynthesis of actinohordin and undecylprodigiosin in *Streptomyces coelicolor* [34], and promotes avermectin biosynthesis in *S. avermitilis* [8]. In addition to σ^{HrdB} , σ^6 [35], σ^{25} [36], and σ^8 [37] in *S. avermitilis* have been reported as negative regulators of avermectin biosynthesis. Here, we found that 6 sigma/anti-sigma factors and 11 regulators in total were significantly influenced, implying that PteF might also act as a global regulator influencing cellular regulatory networks and favoring avermectin biosynthesis. However, the function of these regulatory proteins has never been characterized before. Therefore, further investigations on these regulators might favor regulatory network engineering for a higher yield of avermectins.

Changes in the regulatory network will generally influence cellular metabolic flux changes. Data analysis demonstrate that improved avermectin production might be due to the inhibition of filipin biosynthesis, repression of the TCA cycle, and improved supply of avermectin biosynthetic precursors. In addition to these findings, we further speculated that PteF might influence nitrogen metabolism and metabolic flux from primary metabolism to secondary metabolism. KEGG pathway enrichment analysis showed that

pteF deletion led to a down-regulation of nitrogen metabolism in S0 on day 2 (Table S6). Coincidentally, several proteins that are closely related to nitrogen metabolism were transcriptionally repressed on day 2, including the putative ECF sigma factor GM001189 as well as its upstream putative urea ABC transporter (GM001184-GM001187), a putative nitrate extrusion protein NarK (GM005862), a putative nitrite reductase (NAD(P)H) large subunit NirB (GM006513), a putative glutamine synthetase GlnA2 (GM006947), the urease gamma subunit UreA (GM008344) and beta subunit UreC1 (GM008348) (Tables S4 and S6). Since nitrogen sources are essential for the biosynthesis of protein and nucleic acid required for biomass, the repressed nitrogen metabolism on day 2 caused by *pteF* deletion might have reduced cell growth and saved cellular energy for other physiological activities, thus redirecting more carbon flux toward secondary metabolism, which is also in line with previous findings that nitrogen metabolism influences production of secondary metabolites in *Streptomyces* [38].

5. Conclusions

Our work investigated the relationship between two important BGCs, *pte* and *ave*, with two copies in *S. avermitilis* S0. Moreover, we comprehensively explored the influence of PteF on avermectin biosynthesis, as well as its influence on global regulatory and metabolic networks. We discovered many candidate targets with potential engineering value based on transcriptome data. As most of them were previously unreported, more experimental validations are required for detailed characterization. Despite that, our work gave new insight into regulatory networks regulating avermectin biosynthesis, which might provide more guidance on the further strain engineering of *S. avermitilis* for obtaining a high yield of avermectin.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biology13050344/s1>, Table S1: Strains and plasmids used in this work; Table S2: Primers used in this work; Table S3: Genes significantly differentially expressed in PKS BGCs; Table S4: Genes significantly differentially expressed at day 2; Table S5: Genes significantly differentially expressed at day 6; Table S6: Genes enriched in metabolic pathways with significant changes after *pteF* deletion. References [25,39] are cited in the supplementary materials.

Author Contributions: Conceptualization, S.L. and G.D.; methodology, G.D.; software, M.P. and Z.D.; validation, S.L., G.D. and X.Y.; formal analysis, Y.Z. and Z.W.; writing—original draft preparation, G.D.; writing—review and editing, S.L. and X.Y.; visualization, G.D.; supervision, S.L. and W.X.; project administration, S.L. and W.X.; funding acquisition, S.L. and Y.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Natural Science Foundation of China, grant numbers 32172502, 31972348 and 31872936, and Agricultural Science and Technology Innovation Program of Chinese Academy of Agricultural Sciences, grant number CAAS-CSCB-202401.

Institutional Review Board Statement: This article does not contain any studies on human participants or animals.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are included in this manuscript and its Supplementary Materials. Requests for any additional information can be made to the corresponding authors.

Acknowledgments: We thank Novogene (Beijing, China) for the transcriptome data collection.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Li, S.; Yang, B.; Tan, G.-Y.; Ouyang, L.-M.; Qiu, S.; Wang, W.; Xiang, W.; Zhang, L. Polyketide pesticides from actinomycetes. *Curr. Opin. Biotechnol.* **2021**, *69*, 299–307. [CrossRef]

2. Ikeda, H.; Ishikawa, J.; Hanamoto, A.; Shinose, M.; Kikuchi, H.; Shiba, T.; Sakaki, Y.; Hattori, M.; Omura, S. Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. *Nat. Biotechnol.* **2003**, *21*, 526–531. [[CrossRef](#)]
3. Komatsua, M.; Uchiyama, T.; Omura, S.; Cane, D.E.; Ikeda, H. Genome-minimized *Streptomyces* host for the heterologous expression of secondary metabolism. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 2646–2651. [[CrossRef](#)]
4. Siddique, S.; Syed, Q.; Adnan, A.; Nadeem, M.; Irfan, M.; Qureshi, F.A. Production of avermectin B_{1b} from *Streptomyces avermitilis* 41445 by batch submerged fermentation. *Jundishapur. J. Microbiol.* **2013**, *6*, e7198. [[CrossRef](#)]
5. He, F.; Liu, W.; Sun, D.; Luo, S.; Chen, Z.; Wen, Y.; Li, J. Engineering of the TetR family transcriptional regulator SAV151 and its target genes increases avermectin production in *Streptomyces avermitilis*. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 399–409. [[CrossRef](#)]
6. Liu, W.; Zhang, Q.; Guo, J.; Chen, Z.; Li, J.; Wen, Y. Increasing avermectin production in *Streptomyces avermitilis* by manipulating the expression of a novel TetR-Family regulator and its target gene product. *Appl. Environ. Microbiol.* **2015**, *81*, 5157–5173. [[CrossRef](#)]
7. Guo, J.; Zhang, X.; Lu, X.; Liu, W.; Chen, Z.; Li, J.; Deng, L.; Wen, Y. SAV4189, a MarR-Family regulator in *Streptomyces avermitilis*, activates avermectin biosynthesis. *Front. Microbiol.* **2018**, *9*, 1358. [[CrossRef](#)]
8. Zhuo, Y.; Zhang, W.; Chen, D.; Gao, H.; Tao, J.; Liu, M.; Gou, Z.; Zhou, X.; Ye, B.C.; Zhang, Q.; et al. Reverse biological engineering of *hrdB* to enhance the production of avermectins in an industrial strain of *Streptomyces avermitilis*. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 11250–11254. [[CrossRef](#)]
9. Li, M.; Chen, Z.; Zhang, X.; Song, Y.; Wen, Y.; Li, J. Enhancement of avermectin and ivermectin production by overexpression of the maltose ATP-binding cassette transporter in *Streptomyces avermitilis*. *Bioresour. Technol.* **2010**, *101*, 9228–9235. [[CrossRef](#)] [[PubMed](#)]
10. Jin, P.; Li, S.; Zhang, Y.; Chu, L.; He, H.; Dong, Z.; Xiang, W. Mining and fine-tuning sugar uptake system for titer improvement of milbemycins in *Streptomyces bingchenggensis*. *Synth. Syst. Biotechnol.* **2020**, *5*, 214–221. [[CrossRef](#)] [[PubMed](#)]
11. Dong, Z.; Li, L.; Du, G.; Zhang, Y.; Wang, X.; Li, S.; Xiang, W. A previously unidentified sugar transporter for engineering of high-yield *Streptomyces*. *Appl. Microbiol. Biotechnol.* **2024**, *108*, 72. [[CrossRef](#)]
12. Qiu, J.; Zhuo, Y.; Zhu, D.; Zhou, X.; Zhang, L.; Bai, L.; Deng, Z. Overexpression of the ABC transporter AvtAB increases avermectin production in *Streptomyces avermitilis*. *Appl. Microbiol. Biotechnol.* **2011**, *92*, 337–345. [[CrossRef](#)]
13. Chu, L.; Li, S.; Dong, Z.; Zhang, Y.; Jin, P.; Ye, L.; Wang, X.; Xiang, W. Mining and engineering exporters for titer improvement of macrolide biopesticides in *Streptomyces*. *Microb. Biotechnol.* **2022**, *15*, 1120–1132. [[CrossRef](#)]
14. Wang, W.; Li, S.; Li, Z.; Zhang, J.; Fan, K.; Tan, G.; Ai, G.; Lam, S.M.; Shui, G.; Yang, Z.; et al. Harnessing the intracellular triacylglycerols for titer improvement of polyketides in *Streptomyces*. *Nat. Biotechnol.* **2020**, *38*, 76–83. [[CrossRef](#)]
15. Hao, Y.; You, Y.; Chen, Z.; Li, J.; Liu, G.; Wen, Y. Avermectin B_{1a} production in *Streptomyces avermitilis* is enhanced by engineering *aveC* and precursor supply genes. *Appl. Microbiol. Biotechnol.* **2022**, *106*, 2191–2205. [[CrossRef](#)]
16. Culp, E.J.; Yim, G.; Waglechner, N.; Wang, W.; Pawlowski, A.C.; Wright, G.D. Hidden antibiotics in actinomycetes can be identified by inactivation of gene clusters for common antibiotics. *Nat. Biotechnol.* **2019**, *37*, 1149–1154. [[CrossRef](#)]
17. Niu, G.; Chater, K.F.; Tian, Y.; Zhang, J.; Tan, H. Specialised metabolites regulating antibiotic biosynthesis in *Streptomyces* spp. *FEMS Microbiol. Rev.* **2016**, *40*, 554–573. [[CrossRef](#)]
18. Lu, C.; Zhang, X.; Jiang, M.; Bai, L. Enhanced salinomycin production by adjusting the supply of polyketide extender units in *Streptomyces albus*. *Metab. Eng.* **2016**, *35*, 129–137. [[CrossRef](#)]
19. Wang, H.; Liu, Y.; Cheng, X.; Zhang, Y.; Li, S.; Wang, X.; Xiang, W. Titer improvement of milbemycins via coordinating metabolic competition and transcriptional co-activation controlled by *Streptomyces* antibiotic regulatory protein family regulator in *Streptomyces bingchenggensis*. *Biotechnol. Bioeng.* **2022**, *119*, 1252–1263. [[CrossRef](#)] [[PubMed](#)]
20. Yang, D.; Rateb, M.E.; Wang, N.; Shen, B. Competition and co-regulation of spirotoamide and tautomycin biosynthesis in *Streptomyces griseochromogenes*, and isolation and structural elucidation of spirotoamide C and D. *J. Antibiot.* **2017**, *70*, 710–714. [[CrossRef](#)] [[PubMed](#)]
21. Omura, S.; Ikeda, H.; Ishikawa, J.; Hanamoto, A.; Takahashi, C.; Shinose, M. Genome sequence of an industrial microorganism *Streptomyces avermitilis*: Deducing the ability of producing secondary metabolites. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 12215–12220. [[CrossRef](#)] [[PubMed](#)]
22. Guo, J.; Zhao, J.; Li, L.; Chen, Z.; Wen, Y.; Li, J. The pathway-specific regulator AveR from *Streptomyces avermitilis* positively regulates avermectin production while it negatively affects oligomycin biosynthesis. *Mol. Genet. and Genom.* **2010**, *283*, 123–133. [[CrossRef](#)]
23. Vicente, C.M.; Payero, T.D.; Santos-Aberturas, J.; Barreales, E.G.; de Pedro, A.; Aparicio, J.F. Pathway-specific regulation revisited: Cross-regulation of multiple disparate gene clusters by PAS-LuxR transcriptional regulators. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 5123–5135. [[CrossRef](#)]
24. Vicente, C.M.; Payero, T.D.; Rodriguez-Garcia, A.; Barreales, E.G.; de Pedro, A.; Santos-Beneit, F.; Aparicio, J.F. Modulation of multiple gene clusters' expression by the PAS-LuxR transcriptional regulator PteF. *Antibiotics* **2022**, *11*, 994. [[CrossRef](#)]
25. Kieser, T.; Bibb, M.J.; Buttner, M.J.; Chater, K.F.; Hopwood, D.A. *Practical Streptomyces Genetics*; John Innes Foundation: Norwich, UK, 2000; Volume 291.

26. Gao, H.; Liu, M.; Liu, J.; Dai, H.; Zhou, X.; Liu, X.; Zhuo, Y.; Zhang, W.; Zhang, L. Medium optimization for the production of avermectin B_{1a} by *Streptomyces avermitilis* 14-12A using response surface methodology. *Bioresour. Technol.* **2009**, *100*, 4012–4016. [[CrossRef](#)]
27. Vicente, C.M.; Santos-Aberturas, J.; Payero, T.D.; Barreales, E.G.; de Pedro, A.; Aparicio, J.F. PAS-LuxR transcriptional control of filipin biosynthesis in *S. avermitilis*. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 9311–9324. [[CrossRef](#)]
28. Kurtz, S.; Phillippy, A.; Delcher, A.L.; Smoot, M.; Shumway, M.; Antonescu, C.; Salzberg, S.L. Versatile and open software for comparing large genomes. *Genome. Biol.* **2004**, *5*, R12. [[CrossRef](#)]
29. Chiaromonte, F.; Yap, V.B.; Miller, W. Scoring pairwise genomic sequence alignments. *Pac. Symp. Biocomput.* **2002**, *7*, 115–126.
30. Bergy, M.E.; Eble, T.E. The filipin complex. *Biochem.* **1968**, *7*, 653–659. [[CrossRef](#)] [[PubMed](#)]
31. van der Heul, H.U.; Bilyk, B.L.; McDowall, K.J.; Seipke, R.F.; van Wezel, G.P. Regulation of antibiotic production in Actinobacteria: New perspectives from the post-genomic era. *Nat. Prod. Rep.* **2018**, *35*, 575–604. [[CrossRef](#)] [[PubMed](#)]
32. Yu, Q.; Bai, L.; Zhou, X.; Deng, Z. Inactivation of the positive LuxR-type oligomycin biosynthesis regulators OlmRI and OlmRII increases avermectin production in *Streptomyces avermitilis*. *Chin. Sci. Bull.* **2011**, *57*, 869–876. [[CrossRef](#)]
33. Sun, D.; Liu, C.; Zhu, J.; Liu, W. Connecting metabolic pathways: Sigma factors in *Streptomyces* spp. *Front. Microbiol.* **2017**, *8*, 2546. [[CrossRef](#)]
34. Fujii, T.; Gramajo, H.C.; Takano, E.; Bibb, M.J. *redD* and *actII-ORF4*, pathway-specific regulatory genes for antibiotic production in *Streptomyces coelicolor* A3(2), are transcribed *in vitro* by an RNA polymerase holoenzyme containing σ^{HrdD} . *J. Bacteriol.* **1996**, *178*, 3402–3405. [[CrossRef](#)]
35. Jiang, L.; Liu, Y.; Wang, P.; Wen, Y.; Song, Y.; Chen, Z.; Li, J. Inactivation of the extracytoplasmic function sigma factor Sig6 stimulates avermectin production in *Streptomyces avermitilis*. *Biotechnol. Lett.* **2011**, *33*, 1955–1961. [[CrossRef](#)]
36. Luo, S.; Sun, D.; Zhu, J.Y.; Chen, Z.; Wen, Y.; Li, J.L. An extracytoplasmic function sigma factor, σ^{25} , differentially regulates avermectin and oligomycin biosynthesis in *Streptomyces avermitilis*. *Appl. Microbiol. Biot.* **2014**, *98*, 7097–7112. [[CrossRef](#)]
37. Sun, D.; Wang, Q.; Chen, Z.; Li, J.; Wen, Y. An alternative σ factor, σ^8 , controls avermectin production and multiple stress responses in *Streptomyces avermitilis*. *Front. Microbiol.* **2017**, *8*, 736. [[CrossRef](#)]
38. Sivapragasam, S.; Grove, A. The link between purine metabolism and production of antibiotics in *Streptomyces*. *Antibiotics* **2019**, *8*, 76. [[CrossRef](#)]
39. Bierman, M.; Logan, R.; O'Brien, K.; Seno, E.T.; Rao, R.N.; Schonher, B.E. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* **1992**, *116*, 43–49. [[CrossRef](#)]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.