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High Level of Spinosad Production in the Heterologous Host *Saccharopolyspora erythraea*

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ABSTRACT

Spinosad, a highly effective insecticide, has an excellent environmental and mammalian toxicological profile. Global market demand for spinosad is huge and growing. However, after much effort, there has been almost no improvement in the spinosad yield from the original producer, *Saccharopolyspora spinosa*. Here, we report the heterologous expression of spinosad using *Saccharopolyspora erythraea* as a host. The native erythromycin polyketide synthase (PKS) genes in *S. erythraea* were replaced by the assembled spinosad gene cluster through iterative recombination. The production of spinosad could be detected in the recombinant strains containing the whole biosynthesis gene cluster. Both metabolic engineering and UV mutagenesis were applied to further improve the yield of spinosad. The final strain, AT-ES04PS-3007, which could produce spinosad with a titer of 830 mg/liter, has significant potential in industrial applications.

IMPORTANCE

This work provides an innovative and promising way to improve the industrial production of spinosad. At the same time, it also describes a successful method of heterologous expression for target metabolites of interest by replacing large gene clusters.

Spinosad, a combination of spinosyn A and spinosyn D (Fig. 1), is a tetracyclic macrolide produced by fermentation of *Saccharopolyspora spinosa* (1). Spinosad is highly effective against target insects and has an excellent environmental and mammalian toxicological profile (2). Global launch of spinosad production at an industrial scale is expected to be achieved in the near future. However, the yield of spinosad so far is still too low to meet industrial production requirements.

During recent decades, great effort has been exerted to improve the yield of spinosad by such means as mutagenesis (3), fermentation process optimization (4–7), genome engineering (8, 9), and metabolic engineering (10–13). However, the titer of spinosad has still not reached a level sufficient for industrial production. A poorly understood bottleneck might occur in optimizing the original spinosad producer *S. spinosa* that neither traditional mutagenesis methods nor modern molecular approaches have relieved. Heterologous expression was used in an attempt to unlock this bottleneck.

Heterologous expression has been widely applied as an efficient approach for natural product discovery, optimization of product yield, functional elucidation of cryptic gene clusters, and generation of novel derivatives (14, 15). There are a number of potent actinomycete strains which can be used as heterologous expression hosts. Some are widely used laboratory strains that are easy to manipulate genetically, such as *Streptomyces coelicolor*, *Streptomyces lividans*, and *Streptomyces albus* J1074. Some other interesting hosts are producers of commercial products and produce very large quantities of specific compounds (16), such as the well-known antibiotic erythromycin, which is produced by *Saccharopolyspora erythraea*. In fact, *S. erythraea* was considered to be able to express heterologous macrolide biosynthetic pathways (17), and it was proven to be capable of heterologous expression of spinosyn polyketide synthases (PKSs) (18).

The spinosad gene cluster includes five large genes encoding type I PKS subunits and 14 genes involved in sugar biosynthesis and sugar attachment to the polyketide or in cross-bridging of the polyketide (19). Most of the genes involved are located in a cluster that spans 81 kb (GenBank accession number AY007564) of the *S. spinosa* genome, except for the four rhamnose biosynthetic genes, which are located outside the spinosad gene cluster.

Here, we report the heterologous expression of the spinosad gene cluster using *S. erythraea* as a host. The heterologous expression strain was constructed by replacing the native erythromycin PKS genes with the spinosad gene cluster. For this purpose, a special genomic library was constructed, and four cosmids containing fragments in the same orientation and spanning the whole spinosad gene cluster were selected. These four recombinant cosmids were separately modified and sequentially transformed into the host for homologous recombination to generate the heterologous expression strain AT-ES04. This strain was then subjected to metabolic engineering methods and UV mutagenesis to improve the yield of the target metabolites. The final strain, AT-ES04PS-3007, could produce spinosad with a titer of 830 mg/liter.

MATERIALS AND METHODS

Plasmids, strains, and culture conditions. Vector plasmids and strains used in this study are listed in Table 1. All strains utilized in this study will be made available upon request. *Escherichia coli* cells were cultured in LB

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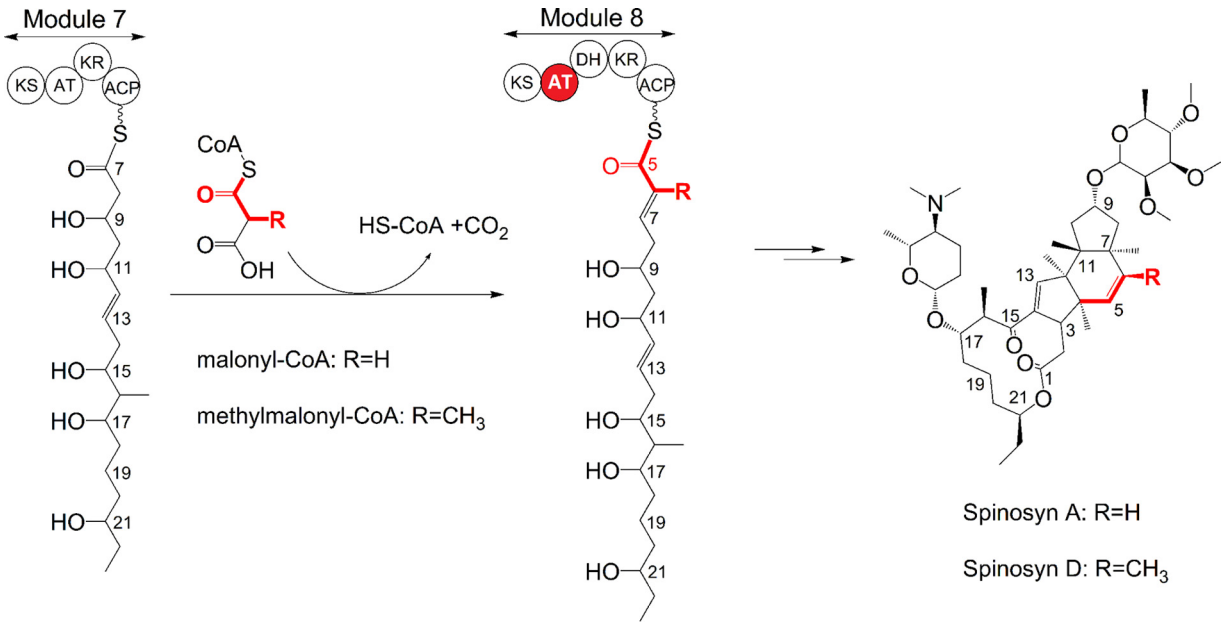

FIG 1 Putative mechanism of the structural difference between spinosyns A and D determined by differences (indicated by red atoms and bonds) in the acyltransferase domain 8 regions of the spinosad polyketide synthases. AT, acyl transferase; ACP, acyl carrier protein; KS, ketosynthase; KR, ketoreductase; DH, dehydratase.

medium (20). *Saccharopolyspora* strains were cultured in slant medium, described previously (13). Antibiotics were added at the following concentrations when needed: apramycin, 50 μg/ml; carbenicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 20 μg/ml.

Molecular biology techniques. Amplification of DNA fragments by PCR was performed with rTaq DNA polymerase (TaKaRa) or PrimeStar polymerase (TaKaRa), according to the manufacturer's instructions. Primers used in this study are listed in Table 2. Isolation of *Saccharopolyspora* genomic DNA was performed using standard protocols (21). Plas-

TABLE 1 Vector plasmids and strains used in this study

Plasmid or strain	Relevant description[a]	Source or reference
Plasmids		
pUC19	*colE1*, *bla*, *lacZ*(α)	TaKaRa, Inc.
pBluScript SK(+)	*colE1*, *bla*, *lacZ*(α)	Stratagene, Inc.
SuperCos 1	*colE1*, *bla*, *cos*	Stratagene, Inc.
pIJ773	*bla*, *aac(3)IV*, *oriT*	22
pUAmT14	*aac(3)IV*, *oriT*	37
SupAmT	*aac(3)IV*, *oriT*	38
Strains		
E. coli strains		
DH5α	Cloning host	22
ET12567	Donor strain for conjugation	22
Saccharopolyspora strains		
S. erythraea ATCC 40137	Wild-type erythromycin-producing strain	ATCC
S. erythraea HS067	High-erythromycin-yielding strain derived from ATCC 40137 by classical mutagenesis	Hisun[b]
S. spinosa S13-3	Spinosad-producing strain derived from ATCC 49460 by classical mutagenesis	Hisun
S. erythraea HS-ES14	Recombinant of HS067, whose native erythromycin PKS genes were replaced by the spinosad gene cluster	This study
S. erythraea AT-ES04	Recombinant of ATCC40137, whose native erythromycin PKS genes were replaced by the spinosad gene cluster	This study
S. erythraea AT-ES04P	Recombinant of AT-ES04, whose *spnF-spnG* genes were under the control of the bidirectional promoter *eryAB*	This study
S. erythraea AT-ES04P-sfp	AT-ES04-P1FG with the *ermE* gene replaced by the *sfp* gene from *Bacillus subtilis*	This study
S. erythraea AT-ES04P-sRHS	AT-ES04P-sfp with an additional copies of the rhamnose synthesis genes	This study
S. erythraea AT-ES04PS-3007	Spinosad-producing strain derived from AT-ES04P-sRHS by UV mutagenesis	This study

[a] *bla*, ampicillin resistance gene; *aac(3)IV*, apramycin resistance gene.
[b] Hisun Pharmaceutical Co., Ltd.

TABLE 2 Primers used in this study

Primer	Sequence (5′–3′)[a]	Introduced sequence[b]
euF	**TTCGCGGCCGC**AACCCGCCCTCGTACATCCCTGCTC	NotI
euR	**TTCGGATCC**ACGCGTCCCCCTACTCGACGACCAC	BamHI
edF	**TTCCTGCAG**CCACGACCGATCGCGCCGGG	PstI
edR	**TTCCTCGAGCGGCCGC**GCTCATCTCGCCGCCGACC	XhoI, NotI
spnRF	TCACTTTCGGAGTGGTGATCTTTGG	
spnRR	GCAACGTGATCAACCTGCACCA	
spnFF	AGAGATCAGGCATACCGGTGTTGC	
spnFR	AACGTCATCGAATTGCGGCG	
spnBF	AAGCTGGTGCTCACCATGCCTC	
spnBR	GGTGCTGGCCATGCCATGTT	
spnDF	GAGTGCCGAAGAGGATCTTGTTGC	
spnDR	ACATCAATGCATCTGCCTGGGC	
spnEF	TGCGTTGGGCTCGATGGAAC	
spnER	AGGAAAGTCCTGTCCAACTTCGCC	
aprHF	**ATCAAGCTT**TGTAGGCTGGAGCTGCTTC	HindIII
aprHR	**ATCAAGCTT**ATTCCGGGGATCCGTCGACC	HindIII
8BAL	**GCCGTTGTCCACCAAGGAGGGATTGCGGCTGTTCGATGC**ATTCCGGGGATCCGTCGACC	39-Homo
8BAR	**CCGGTGGTGTTATCTGGCAGCAGTGCCGTCGATAGTATG**TGTAGGCTGGAGCTGCTTC	39-Homo
9B5L	**TGCGACCGAGTCCGATCGGGTTGGGCGGATTTCCTCGAT**ATTCCGGGGATCCGTCGACC	39-Homo
10G3L	**CACCCGTATCGCCGGTCCTGTCGTTCCCGCGGATCCCGG**ATTCCGGGGATCCGTCGACC	39-Homo
ISUF	**CCCTCTAGATC**TGCTCCTCGGGTGCGAACAGGCG	XbaI^M
ISUR	**CCCTCTAGA**TGGAGAACCCGCACGAGCGACCG	XbaI
ISDF	**CCCAAGC**TTGTACGAGGCGGCGAAGATCGACGG	HindIII
ISDR	**CCCATTAA**TTCCAGACCGAGTTGCCCTTTCAGGGTC	AseI
gdhF	**GATTCTAGA**ACGTCGCGAAGAAGATCTCCAAGAAG	XbaI
gdhR	**GCCTCTAGA**TCGACGGTCCGCGATTTCAATGG	XbaI^M
epiF	**GATTCTAGA**GGGATCAACAACAACTTCACCAGCAGG	XbaI
epiR	**GCCTCTAGA**TCGGTACGTCGAGGGCGACAACAAC	XbaI^M
gttF	**GATTCTAGA**AGCACCAGCACGGACAGCAGCGC	XbaI
gttR	**GATTCTAGA**GCGTTCAGGGTTGCTTCGGCG	XbaI
SIF	**TCCTCTAGATC**AGGAAGCAGTGCAGGTGGC	XbaI^M
SGR	**TCCTCTAGA**TGCGCGTACTCGTCGTTCC	XbaI
SFF	**TCCTCTAGA**ACCGGTGTTGCCAGGTGGCGCAC	XbaI
SAR	**TCCTCTAGATC**AGCTCTCGGTCTGCGCACTCGGCCAG	XbaI^M
PA1F	**GACGAGTACGCGCAT**CGGAGCATTTGCTCGCTTTCC	15-Homo
PA1R	**GCCACCTGGCAACAC**GCGTCCCCCTACTCGACGAC	15-Homo
EEU1	**AACTCTAGATC**CGAGGAGCGGCGTCCGAAAAGTCC	XbaI^M
EEU2	**AACTCTAGA**CGCAATCGCGACGACGGACGAACC	XbaI
EED3	**AACTCTAGA**CGCTGGATCCTACCAACCGGCACG	XbaI
DED4	**AACTCTAGATC**AGCCAGCAGGGCGTCGATGATGC	XbaI^M
SpfF	**TGGTAGGATCCAGCG**ATGAAGATTTACGGAATTTATATGGACCG	15-Homo
SpfR	**GTCGTCGCGATTGCG**TTATAAAAGCTCTTCGTACGTTTTCATCTC	15-Homo

[a] Introduced sequences are in boldface; restriction sites are underlined.

[b] This column describes the characteristics of the introduced sequences. 15-Homo, the PCR product flanked by a 15-bp homologous sequence was used for recombination with a CloneEZ kit; 39-Homo, the PCR product flanked by a 39-bp homologous sequence was used for recombination by PCR targeting; XbaI^M, XbaI sequence followed by the bases TC at the 3′ end. Since the recombinants were extracted from DH5α, all of the A bases were methylated so that the XbaI^M site could not be further digested by XbaI.

mids were extracted from *E. coli* cells by alkaline lysis (20). The Inoue method (20) was used for preparation and transformation of competent *E. coli* cells. *S. erythraea* was transformed by conjugation (22). The strategy of PCR targeting was carried out as described previously (22). A CloneEZ kit (Genscript) was used for recombination according to the manufacturer's handbook (23).

Construction of a special genomic library and screening of spinosad gene cluster. Two 2.5-kb fragments, the upstream region (EU) and the downstream region (ED) flanking the erythromycin PKS gene cluster, were amplified from *S. erythraea* ATCC 40137 genomic DNA with primer pairs euF/euR and edF/edR, respectively. These two fragments were successively subcloned into cloning vector pBluScript SK(+), with EU as a NotI-BamHI fragment and ED as an XhoI-PstI fragment. Finally, the NotI-NotI fragment containing both EU and ED was recovered and applied to replace the NotI-NotI fragment of the cosmid SuperCos 1 to yield EryUD-Cos2 (Fig. 2).

The cosmid vector EryUD-Cos2 was used to construct a genomic library of *S. spinosa* ATCC 49460 according to the handbook of the SuperCos 1 cosmid vector kit (Stratagene) (24). Recombinant cosmids were screened by PCR amplification with the primer pairs spnRF/spnRR, spnFF/spnFR, spnBF/spnBR, spnDF/spnDR, and spnEF/spnER, whose binding sites were inside the genes *spnR*, *spnF*, *spnB*, *spnD*, and *spnE*, respectively. Four cosmids, 15D1, 8B8, 9B5, and 10G3 (Fig. 2), were screened, and sequencing was used to confirm that the inserted fragments were in the same directions and spanned the whole spinosad gene cluster (Fig. 3A). These four cosmids were selected for the construction of the heterologous expression strains.

Modification of cosmids 15D1, 8B8, 9B5, and 10G3. The apramycin resistance cassette (AmT) containing the apramycin resistance gene *aac(3)IV* and conjugative origin *oriT* was PCR amplified from pIJ773 with the primer pair aprHF/aprHR. The PCR product was digested by HindIII and inserted into the HindIII site of cosmid 15D1 to generate cosmid

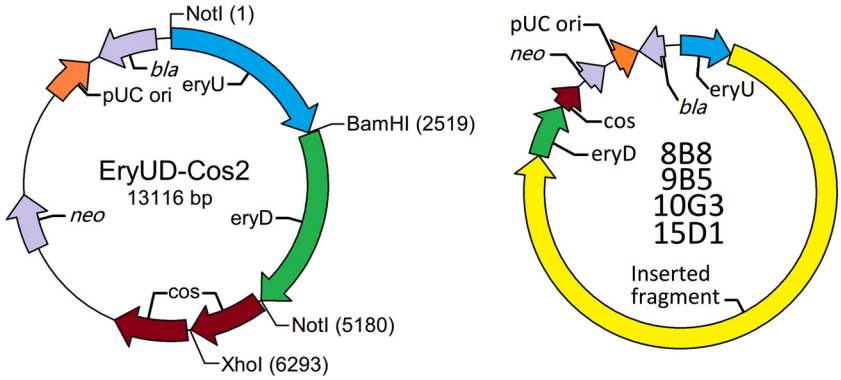

FIG 2 Maps of cosmid EryUD-Cos2 and its derivatives containing fragments of the spinosad gene cluster. eryU (EU), fragment upstream of the erythromycin gene cluster; eryD (ED), fragment downstream of the erythromycin gene cluster; *neo*, kanamycin resistance gene; *bla*, ampicillin resistance gene.

15D1-AmT. For cosmid 8B8, modification was achieved by using the strategy of PCR targeting. The AmT was PCR amplified from plasmid pIJ773 with primers 8BAL and 8BAR, which were designed according to the principle described by Sambrook and Russell (22). PCR targeting was carried out using the PCR product to give rise to cosmid 8B8-AmT, in which the ED and part of the sequence in the 3′ end of the fragment contained in 8B8 were replaced by AmT (Fig. 3A). The modification strategies for cosmids 9B5 and 10G3 were the same as those of 8B8, using primer pairs 9B5L/8BAR and 10G3L/8BAR, respectively. The modified cosmids were correspondingly named 9B5-AmT and 10G3-AmT (Fig. 3A).

Construction of plasmid pAT-DgegU for rhamnose biosynthesis genes duplication. Plasmid pAT-DgegU was constructed to introduce the four genes *gtt*, *gdh*, *epi*, and *kre* from *S. spinosa* ATCC 49460 into the host's genome by homologous recombination. Therefore, the four genes carried by pAT-DgegU should be flanked by two homologous arms from the host's genome. The two arms were PCR amplified from the "additional sequence" indicated in Fig. 3A, which was from *S. spinosa* and which also existed in the *S. erythraea* heterologous expression strains. The primer pairs used were ISUF/ISUR and ISDF/ISDR, and the template DNA was extracted from *S. spinosa* ATCC 49460. These two arms were then inserted into plasmid pUAmT14 in turn: the first one as an XbaI-XbaI fragment and the second one as an HindIII-AseI fragment. The generated plasmid pAT-UD was analyzed by restriction digestion to confirm the relative directions of these two arms to be in accord. The fragments containing *gdh* plus *kre*, *epi*, and *gtt* were amplified from *S. spinosa* ATCC 49460 with the primer pairs gdhF/gdhR, epiF/epiR, and gttF/gttR, respectively, followed by digestion with XbaI. The target plasmid pAT-DgegU was generated by inserting all of these digested fragments into the XbaI site of plasmid pAT-DU step by step.

Construction of plasmid pPSFGtoEAB. The sequence between the *spnG* open reading frame (ORF) and the *spnF* ORF was named PspnFG. The up- and downstream fragments from PspnGF, designated SpnF-U and SpnF-D (Fig. 4A), were PCR amplified with the primer pairs SIF/SGR and SFF/SAR, respectively. These two PCR products were digested by XbaI and successively subcloned into plasmid SupAmT to generate plasmid SAT-FUD (Fig. 4B). Finally, PeryAB (the sequence between *eryAI* and *eryBIV*) flanked with two 15-bp homologous sequences at both the 5′ and 3′ ends was generated by PCR amplification with the primer pair PA1F/PA1R. The PCR product was recombined with the XbaI-digested SAT-FUD by using the CloneEZ kit, yielding plasmid pPSFGtoEAB (Fig. 4B).

Construction of plasmid SAT-sfp for addition of the *sfp* gene. The *sfp* gene from *Bacillus subtilis* was recruited to replace the *ermE* ORF in the constructed heterologous expression strain. For this purpose, the up- and downstream fragments from the *ermE* ORF were PCR amplified with the primer pairs EEU1/EEU2 and EED3/EED4, respectively. These two fragments were successively subcloned into the XbaI site of SupAmT to generate plasmid SAT-EEUD. The *sfp* gene with two 15-bp homologous sequences at both the 5′ and 3′ ends was PCR amplified with the primer pair SpfF/SpfR and recombined with XbaI-digested SAT-EEUD to give rise to plasmid SAT-sfp.

Mutagenesis by UV light. Mutagenesis by UV light was carried out as described by Kieser et al. (21).

Fermentation and product detection. Strains were cultured in 25 ml of seed medium (0.5% glucose, 2.5% corn starch, 1% yeast extract, 1% whole-milk powder, 0.2% $MgSO_4 \cdot 7H_2O$, pH 7.2) at 28°C for 72 h on a rotary shaker at 250 rpm. Then 2 ml of seeds was cultured in 30 ml of fermentation medium (9% glucose, 2% whole-milk powder, 2.5% cottonseed cake powder, 0.2% yeast powder, 0.1% lactic acid, 0.4% trisodium citrate, 0.2% K_2HPO_4, pH 7.2) under the same conditions for 10 days. After fermentation, 1 ml of each culture was mixed with 4 ml of methyl for 1 h. Samples prepared by filtering the mixtures with filter paper were analyzed by high-performance liquid chromatography (HPLC). HPLC was performed with a Shimadzu LC-2010 CHT system (Shimadzu, Koyoto, Japan) by using a Nova-Pak C_{18} column (3.9 by 150 mm, 5-μm pore size; Waters, Milford, MA) at a flow rate of 1.0 ml/min and detected at 250 nm. The mobile phase was methyl–acetonitrile–0.05% sodium acetate with the volume ratio of 45:45:10. The yield in the supernatant was quantified by spiking original standards of spinosyns A and D.

RESULTS

The heterologous expression strains *S. erythraea* AT-ES04 and HS-ES14 were constructed via successive gene replacement. The fragments present on cosmids 15D1, 8B8, 9B5, and 10G3 (Fig. 3A) spanned the whole 81-kb spinosad gene cluster of *S. spinosa* ATCC 49460. In addition, there were additional sequences adjacent to the 3′ and 5′ ends of the spinosad gene cluster in cosmids 15D1 and 10G3, respectively (Fig. 3A). The construction strategy for the heterologous expression strains was based on the mode of iterative homologous recombination (Fig. 3B). Therefore, the four cosmids had to be modified prior to their sequential introduction into *S. erythraea* hosts by conjugation in order to make them conjugative. To do so, the AmT was added.

Cosmid 15D1 was used to replace the erythromycin PKS genes with the fragment it contained. Since the fragment included in 15D1 was already flanked by the two homologous arms EU and ED, the only needed modification was the insertion of the AmT into the vector backbone, generating cosmid 15D1-AmT. For the other three cosmids, EDs were no longer the homologous arms. The new arms coupling with EUs were the sequences located at the 3′ ends of the fragments contained, which overlapped the last cosmid (Fig. 3A). In addition, in order to balance the sizes of the

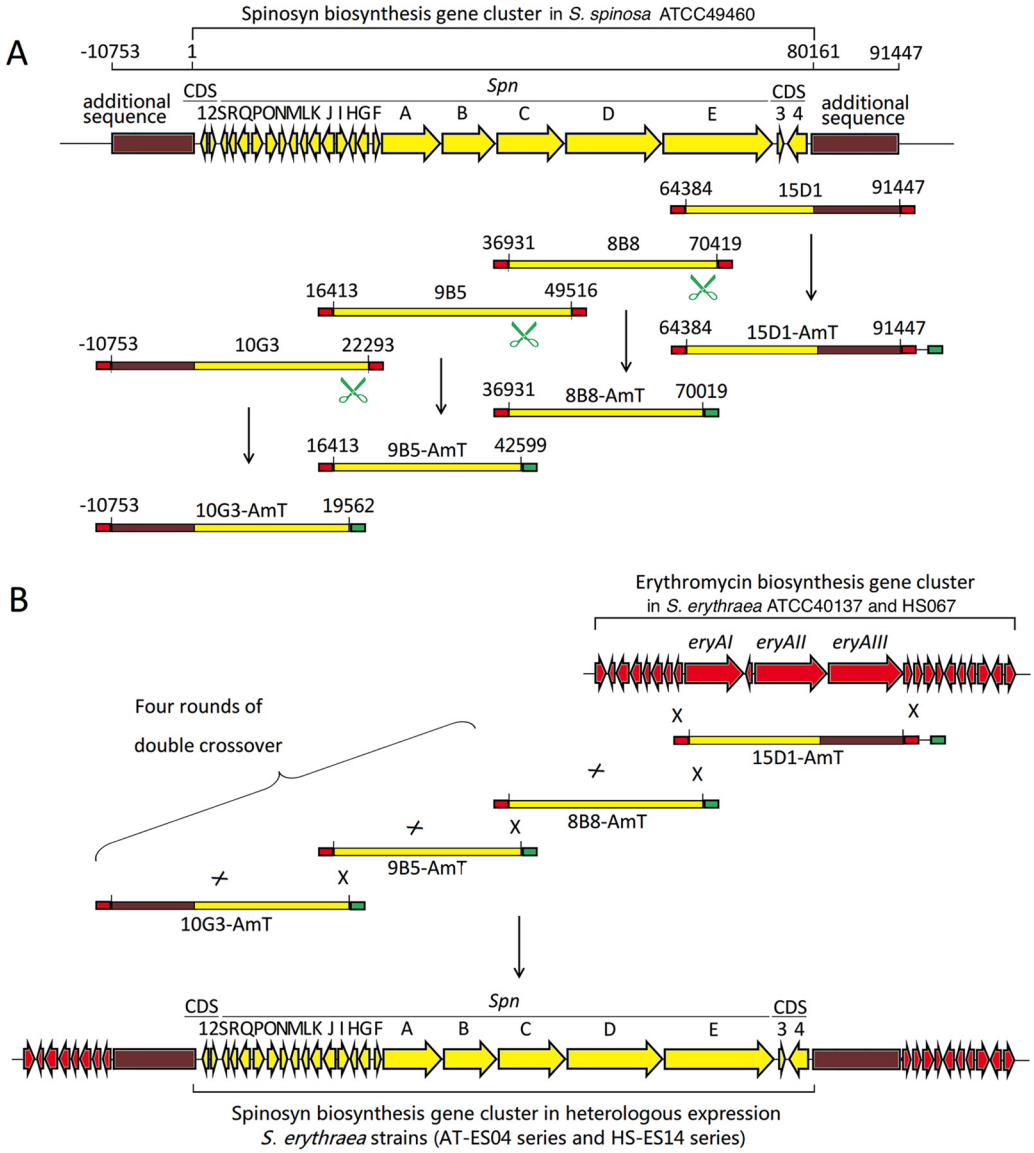

FIG 3 Scheme showing the construction of heterologous expression strains. (A) Fragments contained in the cosmids before and after editing. (B) Iterative recombination for the replacement of erythromycin PKS genes with the spinosad gene cluster. The yellow arrows and boxes represent DNA fragments of the spinosad gene cluster, while the red ones represent those of the erythromycin gene cluster. The blue and green boxes represent the upstream (EU) and downstream (ED) regions, respectively, of the erythromycin PKS genes which are PCR amplified from *S. erythraea*. The white boxes represent cassettes containing the apramycin resistance gene and conjugative origin (AmT). Green scissors in panel A represent replacements of the fragment containing ED and partial overlapping sequence with the PCR-amplified AmT via the method of PCR targeting. CDS, coding sequence.

two homologous arms, the overlapped sequences were truncated appropriately. Therefore, the EDs and parts of the overlapped sequences of these three cosmids were replaced by AmT using the strategy of PCR targeting, generating cosmids 8B8-AmT, 9B5-AmT, and 10G3-AmT, respectively (Fig. 3A).

Construction of the heterologous expression strain AT-ES04 consisted of four steps. First, cosmid 15D1-AmT was transformed into *S. erythraea* ATCC 40137. A double-crossover mutant, named AT-ES01, was selected for further manipulations. For the second to the fourth steps, cosmids 8B8-AmT, 9B5-AmT, and 10G3-AmT were successively transformed into the double-crossover mutants generated in the previous steps, giving rise to AT-ES02, AT-ES03, and AT-ES04, respectively. In the final strain, AT-ES04, the native erythromycin PKS genes were replaced by the whole spinosad gene cluster (Fig. 3B). In addition, using *S. erythraea* HS067, a high-yielding erythromycin-producing strain, as a parental strain, the same steps were performed to construct another heterologous expression strain, HS-ES14.

The heterologous expression strains AT-ES04 and HS-ES04 produce spinosyns A and D at different ratios. The two heterologous expression strains HS-ES14 and AT-ES04 together with their parental strains HS067 and ATCC 40137 and the natural spinosad-producing strain *S. spinosa* S13-3 were cultured for fermentation tests. The mycelial extracts were analyzed by HPLC (Fig. 5). Both HS-ES14 and AT-ES04 produced two novel compounds (Fig. 5B and H) that were not present in the parental

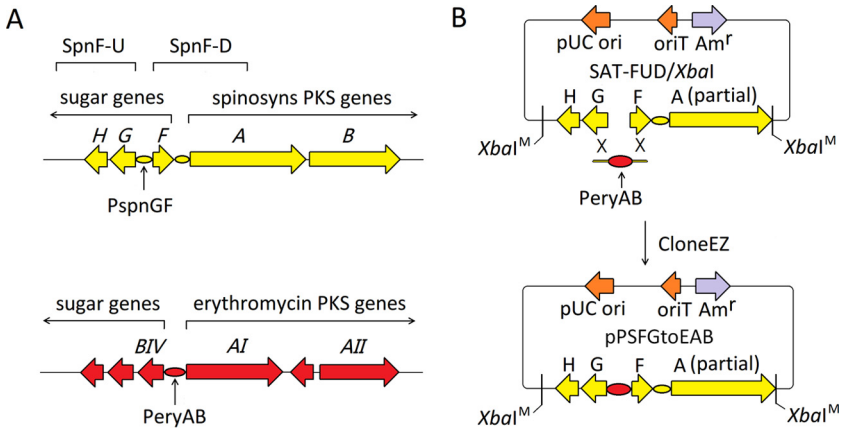

FIG 4 Construction of plasmid pPSFGtoEAB. (A) Positions of relevant promoters and homologous arms. (B) Construction of plasmid pPSFGtoEAB by homologous recombination. Yellow arrows, genes for spinosad biosynthesis (*spn*) from *S. spinosa*; red arrows, genes for erythromycin biosynthesis (*ery*) from *S. erythraea*. Am^r, apramycin resistance gene *aac(3)IV*.

strains (Fig. 5A and G). The retention times of these two compounds seemed to be identical to those of spinosyns A and D (Fig. 5D and J). However, since their yields were too low to accurately compare their retention times, we added comparable amounts of the spinosyn A and D standards to the sample of HS-ES14. No additional peaks appeared (Fig. 5C), indicating the coincidence of the peaks. In fact, after yield improvement in strain AT-ES04P-sfp (see below), the two novel compounds were structurally identified by HPLC-mass spectrometry (MS) and nuclear magnetic resonance (NMR) as spinosyns A and D, as anticipated (see Fig. S1 to S16 in the supplemental material for the NMR and HPLC-MS diagrams). Surprisingly, the amount of total spinosyns produced in HS-ES14 (which was derived from the high-yielding erythromycin producer) was about the same as that produced in AT-ES04 (which was derived from the ATCC strain) (Fig. 6A). Another surprising result was that the ratios of spinosyn A to spinosyn D differed greatly in the two heterologous expression strains (Fig. 6A). In HS-ES14, the amount of spinosyn A was notably smaller than that of spinosyn D, while in AT-ES04, the amount of spinosyn A much larger than that of spinosyn D, as in the natural spinosad-producing strain *S. spinosa* S13-3 (Fig. 5I and 6C). Spinosyn A structurally differs from spinosyn D in the C-6 site: in spinosyn A, a hydrogen atom is present, whereas in spinosyn D, a methyl group is present in this position (Fig. 1). According to the biosynthesis pathway of spinosad (19), the structure of C-5–C-6 is determined by module 8 of the spinosad PKS. Based on what is

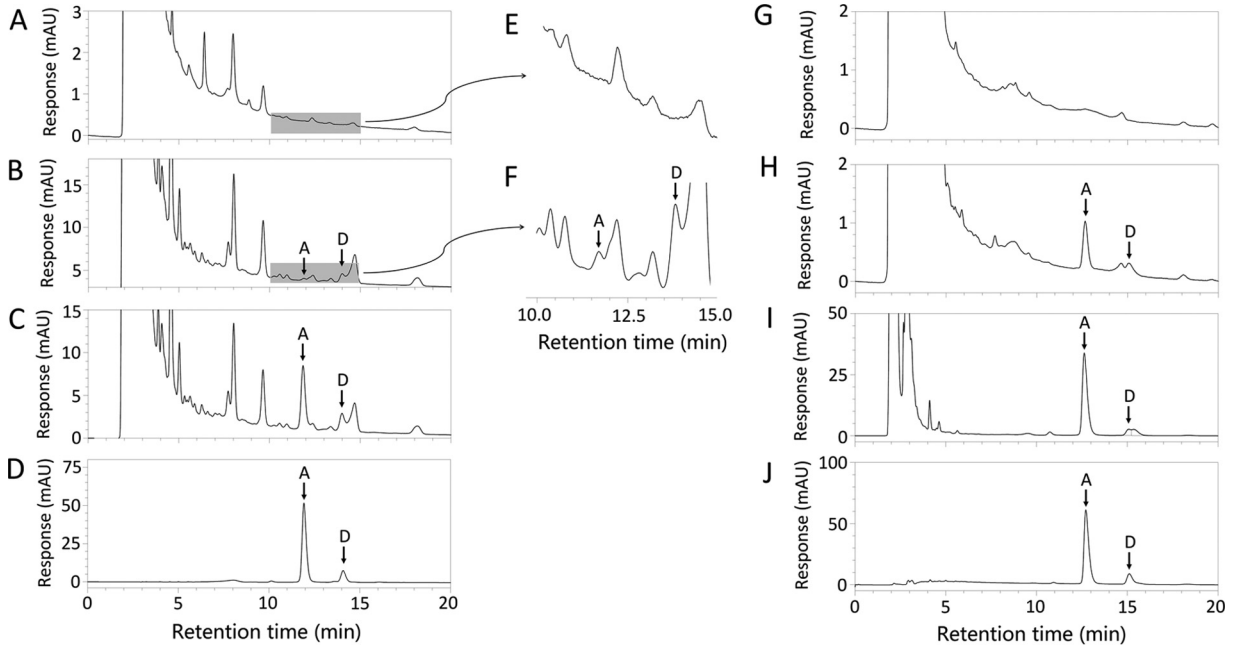

FIG 5 HPLC analysis of the mycelial extracts from the parent and mutant strains. (A) *S. erythraea* HS067. (B) *S. erythraea* HS-ES14. (C) *S. erythraea* HS-ES14 plus spinosyn A and D standards. (D and J) Spinosyn A and D standards. (E) Enlarged view of the shaded part of panel A. (F) Enlarged view of the shaded part of panel B. (G) *S. erythraea* ATCC 40137. (H) *S. erythraea* ES04. (I) *S. spinosa* S-13. A and D, spinosyns A and D, respectively; AU, arbitrary units.

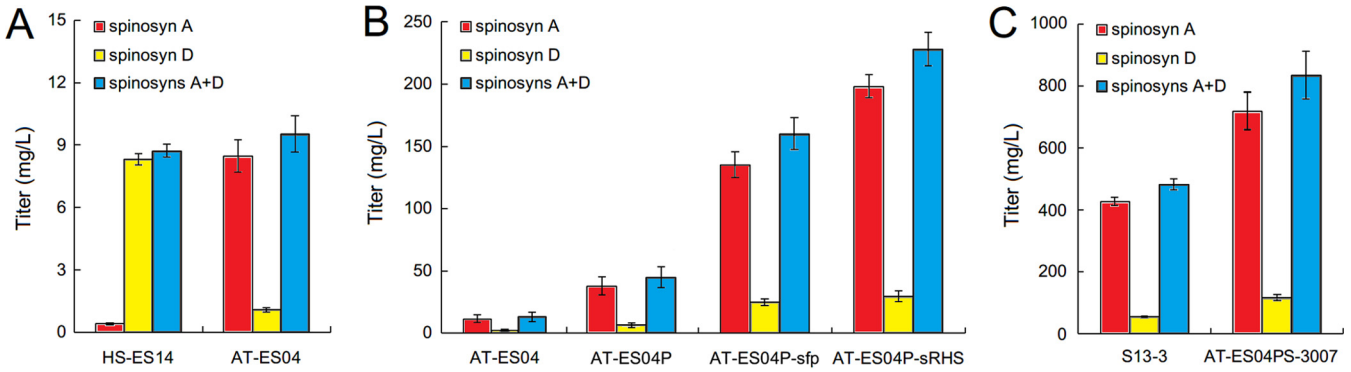

FIG 6 Spinosyn accumulation in natural and heterologous expression strains producing spinosad.

known about PKS activity (25), the acyltransferase domain of module 8 (AT8) should utilize both malonyl-coenzyme A (CoA) and methylmalonyl-CoA as substrates to finally generate spinosyns A and D, respectively (Fig. 1). To our knowledge, erythromycin PKSs utilize one propionyl-CoA as a starting unit and six methylmalonyl-CoAs as extenders to generate 6-deoxyerythronolide B, the intermediate of erythromycin B (26). In contrast, the numbers of the two extenders in spinosyn synthesis, methylmalonyl-CoA and malonyl-CoA, are 1 and 9, respectively (for spinosyn A), or 2 and 8, respectively (for spinosyn D). Therefore, in a high-erythromycin-yielding strain, more methylmalonyl-CoA than malonyl-CoA could be produced so that it benefits the biosynthesis of spinosyn D rather than that of spinosyn A. Thus, it is not surprising that spinosyn D was more abundant than spinosyn A in HS-ES14. However, since, in contrast to HS-ES14, the spinosyn A/spinosyn D ratio in AT-ES04 is similar to that in the commercial product spinosad, the former was selected as the parental strain for further engineering.

Overexpression of the rhamnosyltransferase and/or enzyme for the transannular [4 + 2] cycloaddition has a positive impact on the yield of spinosad. Spinosyn structurally consists of an aglycone (AGL), a modified rhamnose, and a forosamine. The AGL is constructed first by a type I PKS, followed by proposed postmodification events. One such event is a transannular [4 + 2] cycloaddition, which is catalyzed by SpnF, forming the cyclohexene ring in spinosad (27). Rhamnose is the first sugar attached to the AGL and is tri-O-methylated to generate the intermediate pseudoaglycone. The attachment is catalyzed by a rhamnosyltransferase encoded by *spnG* (28).

We transformed plasmid pPSFGtoEAB into AT-ES04 to substitute PeryAB for PspnFG, the promoter between *spnF* and *spnG*. In erythromycin biosynthesis, PeryAB is a bidirectional promoter that simultaneously controls two genes in opposite orientations, *eryAI* and *eryBIV*. Similar to PeryAB, PspnFH simultaneously controls *spnF* and *spnG* genes that are divergently orientated in spinosad biosynthesis. The results showed that when PspnFG was replaced by PeryAB to generate strain AT-ES04P, the titer of spinosad was enhanced about 3-fold (Fig. 6B). This enhancement is thought to be due to the overexpression of the *spnF* and/or *spnG* gene even if evidence is lacking that the PeryAB promoter has greater strength than the *spnF* and/or *spnG* promoter.

Introduction of the *sfp* gene notably improves the yield of spinosad. PKSs require a posttranslational modification by a phosphopantetheinyl transferase (PPTase). Spinosad PKS is a type I PKS, which is activated by an Sfp-type PPTase. The utility of Sfp in metabolic engineering strategies has been widely recognized (29).

In this study, the *sfp* gene of *B. subtilis* was used to replace the *ermE* ORF of strain AT-ES04P to generate strain AT-ES04P-sfp. This was achieved by transforming the plasmid SAT-sfp into AT-ES04P for homologous recombination. The fermentation results showed that the yield of spinosad in AT-ES04P-sfp improved approximately 2- and 10-fold in comparison with that of AT-ES04P and AT-ES04, respectively (Fig. 6B).

Duplication of rhamnose biosynthesis genes does not influence the yield of spinosad in low-producing strains but improves the yield in relatively high-producing strains. Previous studies (30) revealed that the biosynthesis of rhamnose was one of the key steps of the biosynthesis of spinosad in *S. spinosa*. Duplication of rhamnose biosynthesis genes improved the yield of spinosad 2-fold. We transformed plasmid pAT-DgegU into AT-ES04 to introduce additional copies of each of these four genes. However, the titer of spinosad was not influenced in the generated strain ES04-RHS (data not shown). In contrast, when the four genes were introduced in the relatively high-yielding strain AT-ES04P-sfp, the titer of spinosad in the resulting strain AT-ES04P-sRHS was further improved (Fig. 6B).

UV mutagenesis improves the yield of spinosad. AT-ES04P-sRHS was selected as the parental strain for UV mutagenesis. After three rounds of mutagenesis, a mutant, named AT-ES04PS-3007, was selected from a total of 600 mutants (200 mutants per round). The total titer of spinosyns A and D reached 830 mg/liter (Fig. 6C), which was 12-fold higher than that of the parental strain AT-ES04P-sfp. We also used HS-ES14, the heterologous expression strain derived from the erythromycin industrial producer, for mutagenesis, but the yield was only marginally improved (data not shown) after two rounds of UV mutagenesis.

DISCUSSION

S. erythraea is a natural and potent producer of erythromycin. It was subjected to heterologous expression of spinosad based on the following hypotheses: (i) *S. erythraea* and *S. spinosa* belong to the same genus, which suggested to us that the gene clusters from one of them could be expressed in the other; (ii) *S. erythraea* is a very good producer of erythromycin. Such characteristics might be beneficial for spinosad production. Furthermore, the genetic background of *S. erythraea* is well known so that it will be convenient to use this organism for further genetic manipulations. In

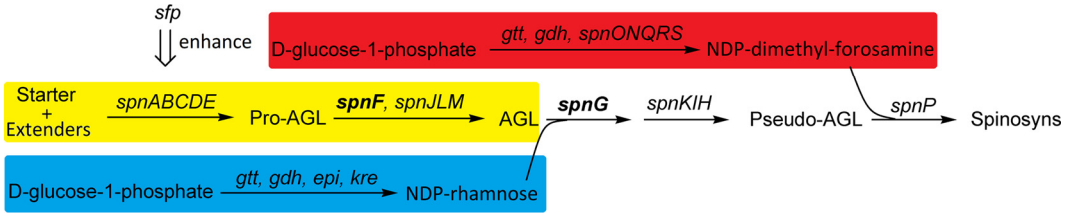

FIG 7 Sketch map indicating biosynthesis and modification of rhamnose, forosamine, and the aglycone (AGL) to generate spinosyn.

the present study, both an ATCC strain and an industrial strain of *S. erythraea* were used as heterologous hosts. Since the industrial strain HS067 was subjected to numerous rounds of mutagenesis to increase the yield of erythromycin, the cell environment of this strain was optimized to express the erythromycin gene cluster. However, the two heterologous expression strains AT-ES04 and HS-ES14, though derived from parents with dramatically different abilities for erythromycin production, produced almost the same low levels of target compounds, indicating that an optimized genetic environment for erythromycin production did not show a clear positive impact on spinosad production.

The biosynthesis of spinosad should be a complicated interactional process which involves the synthesis of the AGL, the generation and methylation of the two sugars, and their transfer on the AGL (Fig. 7). For instance, the introduction of the four rhamnose synthesis genes did not improve the yield of spinosad in AT-ES04 whereas overexpression of *spnF* or/and *spnG* in AT-ES04P did. And when the *sfp* gene, whose final effect is to enhance the activity of PKS, was introduced into AT-ES04P to generate AT-ES04P-sfp, the titer of spinosad was further increased. These results suggest that in AT-ES04, the rhamnose generated by the native genes of *S. erythraea* is not limiting for spinosad synthesis. Unexpectedly, the introduction of the four rhamnose genes into AT-ES04P-sfp to generate AT-ES04P-sRHS further increased the yield of spinosad. This can be explained by the following: since the activity of the PKS was enhanced upon introduction of the *sfp* gene, the amount of rhamnose became limiting, and so more rhamnose gave rise to more spinosad. Therefore, how to tune the stoichiometry of these reactions might be one of the key elements of yield improvement.

Although a great number of attempts have been made for metabolic engineering of spinosad biosynthesis, there are only a few reports concerning the regulation of the expression of the spinosad gene cluster (31–33). In fact, there is as yet no report of yield improvement through manipulation of regulatory genes. It is possible that the regulators beneficial for spinosad production are located outside the gene cluster and remain to be characterized. Indeed, UV mutagenesis of AT-ES04P-sRHS substantially improved the yield. This partially supports the hypothesis and indicates that the heterologous expression strain derived from the ATCC strain is very sensitive to traditional culturing methods. On the other hand, this strain is still so "natural" that it can be further optimized by traditional methods, providing sufficient opportunity for further yield improvement.

As a result of the great efforts of our coworkers in Hisun, which took about 5 years, the yield of spinosad was improved from 40 mg/liter in the wild *S. spinosa* strain ATCC 49460 to 480 mg/liter in the improved strain S13-3. However, an unknown roadblock limits further improvement of spinosad production in this strain, and such a roadblock might also be encountered in the heterologous expression strain. Fortunately, since in a relatively short time we have greatly improved the yield of spinosad in the heterologous expression strain, with a yield that is higher than that in the improved strain S13-3, it seems that such a risk could be ignored.

Many elements should be considered in the construction of a heterologous expression strain. Even when these key elements are carefully considered, it is not certain that the heterologous enzymes will be expressed. Indeed, the amount of product obtained in the heterologous host is usually very low (34). Here, we constructed heterologous expression strains for spinosad production, using an *S. erythraea* strain from ATCC as the host. Metabolic engineering methods improved the yield 23-fold, and simple mutagenesis further improved it 2.7-fold. The final heterologous expression strain produced spinosad with a titer of 830 mg/liter, and there is still potential for further improving the yield by traditional culturing methods and metabolic engineering methods. To date, there are only a small number of successful examples of heterologous expression which produce high levels of target secondary metabolites (14, 35, 36). Our work represents one such successful example and will greatly benefit the industrialization of spinosad. Finally, the mode of construction using gene cluster replacement can also be used in heterologous expression for other target metabolites of interest.

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