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Cloning of Modular Type I Polyketide Synthase Genes from Salinomycin Producing Strain of *Streptomyces albus*

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Abstract—Cloning of polyether polyketide synthase (PKS) genes for salinomycin biosynthesis was attempted from *Streptomyces albus*. Seven β-ketoacyl synthase (KS) core regions were obtained by PCR amplification using primers designed based on the conserved KS domains of type I PKSs. Using the KS fragment as a probe, screening of an *S. albus* genomic DNA library was carried out by colony hybridization. From the positive cosmid clone isolated, a 4.5-kb*Bam*HI fragment was subcloned and sequenced. It showed high homology with bacterial type I PKSs and was deduced to code for KS, malonyl transferase, and ketoreductase motifs. By gene disruption with this 4.5-kb *Bam*HI fragment, the cloned gene was shown to be a part of the salinomycin biosynthetic gene cluster of *S. albus*.
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Introduction

Salinomycin is a member of the spiroketal class of polyether antibiotics produced by *Streptomyces albus*.[1,2] Like other polyether antibiotics, salinomycin has ionophoric properties and is known to mediate the transport of various metal ions, especially sodium and potassium, across biological membranes.[3] Due to its antibacterial and anticoccidial activity, salinomycin is widely used as an effective veterinary drug.

Feeding experiments using ^{13}C-labeled precursors have shown that salinomycin is biosynthesized from six acetate, six propionate and three butyrate units.[4] From our ^{18}O incorporation studies, C20-OH, C25-O-C29 and C28-OH oxygens were exclusively labeled by $^{18}O_2$. None of these oxygens was labeled by $H_2{}^{18}O$.[5] As proposed for polyether biosynthesis,[6] an acyclic diene intermediate should be subjected to epoxidation to form a di-epoxide. This is followed by epoxide ring opening, with concomitant sequential formation of the four ether rings, as shown in Figure 1. However, the biosynthetic scheme for polyethers has not been confirmed experimentally.

Active investigations of polyketide synthase (PKS) genes have been carried out since the pioneering work on actinorhodin biosynthesis gene cloning from *Streptomyces coelicolor* by Malpartida and Hopwood.[7] It has been established that biosynthesis of complex reduced polyketides is catalyzed in actinomycetes by large multifunctional enzymes, the modular type I PKSs.[8] However, most of our current knowledge of such systems stems from the studies on a restricted number of macrolide-synthesizing enzymes. To the best of our knowledge, only two reports on the biosynthesis genes for polyether antibiotics have been published. Cloning of the genes for the biosynthesis of monensin A, a typical polyether ionophore polyketide, was reported and an attractive mechanism was proposed for oxidative cyclization in monensin biosynthesis based on the assumed functions of genes in the cluster.[9] Polyketide gene cluster for the insecticidal polyether nanchangmycin was also reported.[10] However, the genes for polyether biosynthesis have remained largely unidentified. Thus, we attempted to clone biosynthetic genes for salinomycin from *S. albus* to contribute to the mechanistic analysis of polyether biosynthesis in actinomycetes.

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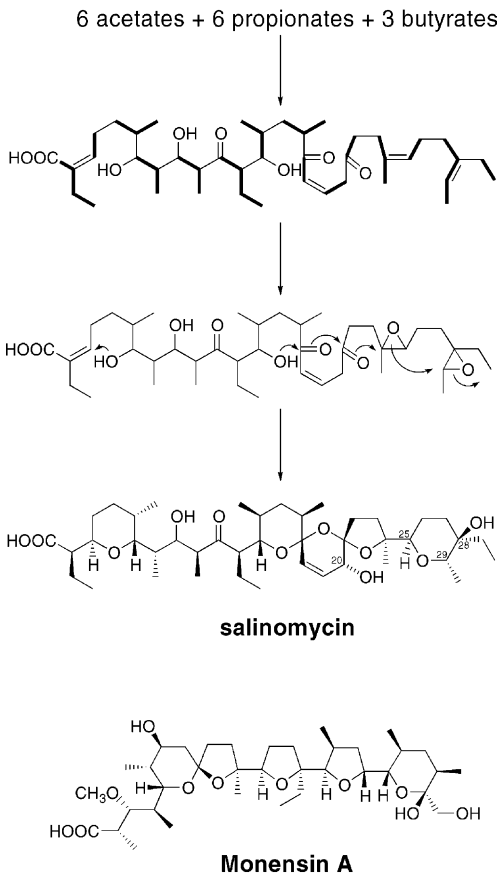

Figure 1. Hypothetical biosynthesis of salinomycin and structure of monensin A.

Results and Discussion

Amplification of β-ketoacyl synthase domain from *S. albus*

To clone the PKS genes for the salinomycin biosynthesis, a pair of degenerate primers were designed from sequences of bacterial modular type I PKS genes deposited in a DNA databank. Amino acid sequence alignment of available macrolide PKSs indicated a highly conserved region around the KS domains. The conserved amino acid sequences LAMDPQQ and VEAHGTGT located upstream and downstream, respectively, from the KS active-site cysteine were chosen for the KSMA-F and KSMB-R primers. Although few polyether PKS genes were cloned, similar conserved amino acid sequences in the KS domain were expected in polyether PKSs. The PCR amplification of *S. albus* chromosomal DNA gave an approximately 700-bp long fragment on agarose gel electrophoresis, which was subsequently cloned into pT7-Blue T vector. From the clones sequenced, seven different KS sequences (SA-KS1–SA-KS7) were obtained. These fragments preserved KS active-site motif (-DTACSS-) and showed high homology with KS domains of type I PKS genes such as *eryA*[11,12] and *rapA*.[13] Thus, these fragments were assumed to be parts of PKS genes possibly involved in salinomycin biosynthesis. When *S. albus* genomic DNA was digested with *Bam*HI and probed with the SA-KS1 fragment, multiple hybridizing bands were detected, which might show other KS domains of salinomycin PKS genes.

Cloning and sequencing of the PKS gene from *S. albus*

The *S. albus* genomic DNA library was constructed in cosmid pOJ446 by ligating with partial *Sau*3AI digest of genomic DNA. The library of approximately 1×10^4 clones was screened by colony hybridization with SA-KS1 probe. Out of 22 positive clones, 14 were selected by PCR amplification of the KS region. Upon Southern hybridization with SA-KS1 probe, 7 clones gave strong hybridizing signals. Clone pSA14, contained a 4.5-kb *Bam*HI fragment which strongly hybridized with the SA-KS1 probe. Since the same size *Bam*HI fragment of *S. albus* genomic DNA hybridized with the probe, the 4.5-kb *Bam*HI fragment of pSA14 was subcloned into pBluescript II SK^+ vector to construct pSA14-1B and was subjected to further analysis. Nucleotide sequencing of the 4.5-kb *Bam*HI fragment revealed that it consisted of 4458 bp and had a high G+C content of 71.9%. Comparison of this sequence with the available nucleotide sequences from a DNA data bank revealed significant similarities with type I PKS genes from actinomycetes such as *Streptomyces avermitilis* avermectin PKS genes,[14] *Streptomyces natalensis* pimaricin PKS genes,[15] *Streptomyces antibioticus* 8,8a-deoxyoleandolide PKS genes,[16] and so on. The fragment contained the conserved sequences used to design degenerate primers for KS domain amplification, although the KS sequence of the 4.5-kb *Bam*HI fragment was not identical to SA-KS1 but was to SA-KS6.

To characterize the gene further, the nucleotide sequence of the 4.5-kb *Bam*HI fragment was analyzed for open reading frames (ORFs) using the program FramePlot.[17] The result clearly revealed that the entire fragment represents one protein-coding region which is part of a large ORF (Fig. 2). The deduced amino acid sequence of the fragment also showed significant similarities with type I PKSs and a BLAST search delineated the enzymatic motifs, KS, acyltransferase (AT), and ketoreductase (KR). Comparison of the active-site sequence of AT domain with the known ATs for malonyl-CoA and methylmalonyl-CoA indicated that the AT domain in the 4.5-kb *Bam*HI fragment could be involved in malonyl-CoA acyltransferase activity (Fig. 3).

Gene disruption of the salinomycin PKS gene

A gene disruption experiment was performed with *S. albus* to demonstrate that the 4.5-kb *Bam*HI PKS gene fragment cloned was involved in salinomycin biosynthesis. The *Mlu*I-*Sph*I region in the 4.5-kb *Bam*HI fragment in pSA14-1B was replaced with a streptomycin resistance *aad* gene. The resulting 6.3-kb *Bam*HI fragment was introduced into pBS-oriT plasmid containing the *Eco*RI-*Dra*I fragment of the pOJ446 *ori*T region for conjugation transfer[18] (Fig. 4). The targeting plasmid pBS-oriT-1B-aad thus constructed was used to transform *S. albus*. The classical protoplast transformation assisted with polyethylene glycol was not successful.

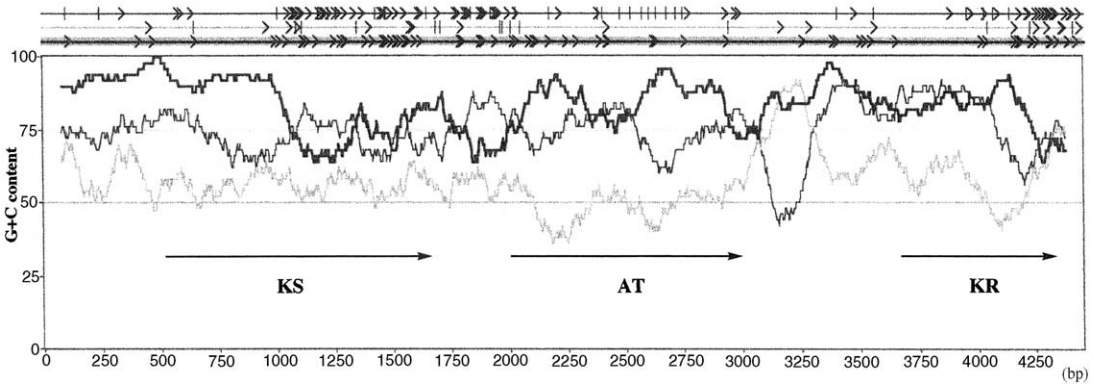

Figure 2. Frame analysis of the 4.5-kb *Bam*HI fragment cloned from *S. albus*: —— frame 1; —— frame 2; —— frame 3.

Malonyl-CoA acyltransferase

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Sal1   LRRTEHAQPALFAFEVALFRLLESFGVRPDFVAGHSVGEI
Rif2   LDQTMYTQGALFAVETALFRLFESWGVRPGLLAGHSIGEL
Rif9   LNQTVFTQAGLFAVESALFRLAESWGVRPDVVLGHSIGEL
Rap5   VNETGYAQPALFALQVALFGLLESWGVRPDAVVGHSVGEL
Rap14  VDDTLYAQAGIFAMEAALFGLLEDWGVRPDFVAGHSIGEA
          *              * *
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Methylmalonyl-CoA acyltransferase

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Rif1   LGRVDVLQPACFAVMVGLAAVWESVGVRPDAVVGHSQGEI
Rif3   LDRVDVVQPASFAMMVGLAAVWTSLGVTPDAVLGHSQGEI
Ery3   LDRVDVVQPVLFAVMVSLAELWRSYGVEPAAVVGHSQGEI
Ery5   LDRVDVVQPALFAVMVSLAALWRSHGVEPAAVVGHSQGEI
          ***         *   *  *              *
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Figure 3. Comparison of the active-site sequence of acyltransferase domains from type I PKSs. Conserved amino acids in both malonyl-CoA acyltransferases and methylmalonyl-CoA transferases are shown in bold face. Specifically conserved amino acids in malonyl-CoA acyltransferases or methylmalonyl-CoA transferases are shown with asterisk below.

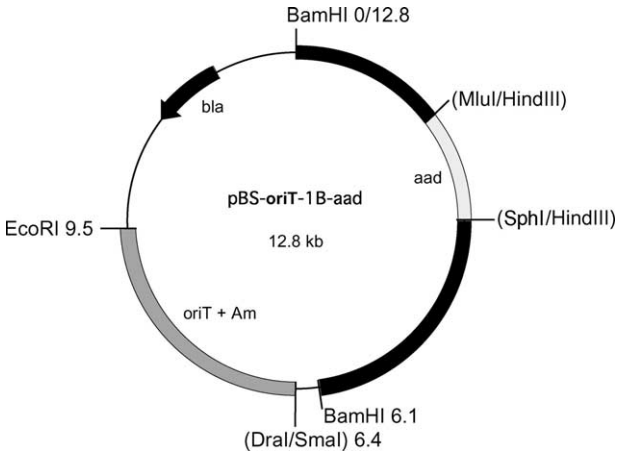

Figure 4. Gene disruption targeting plasmid pBS-oriT-1B-aad.

Conjugation with *Escherichia coli* was also not successful. No transformant showing streptomycin resistance was obtained by these transformation methods. However, electroporation of lysozyme-treated *S. albus* cells yielded the desired transformant. HPLC analysis of the transformant showed that it had lost the ability to produce salinomycin (Fig. 5).

The results of gene disruption experiments indicated that the cloned 4.5-kb *Bam*HI fragment is involved in salinomycin biosynthesis, though there might be other possibility that production of salinomycin was abolished by the disruption of not its biosynthesis gene but other gene which indirectly affected the biosynthesis. Although the entire nucleotide sequence for salinomycin biosynthetic gene cluster in *S. albus* has not been determined, the sequenced fragment possibly codes for one module of salinomycin PKS with KS, malonyl AT, and KR. Six other highly homologous KS domains were also obtained by PCR amplification of *S. albus* genomic DNA. Seven more KS domains may be involved in the biosynthesis of the putative acyclic precursor of salinomycin. Although cloning of type II PKS genes from *S. albus* was already reported,[19,20] this is the first cloning of modular type I PKS genes from *S. albus* and successful transformation and gene disruption of *S. albus*. This investigation was only the first step determining the entire gene sequence for polyether biosynthesis of salinomycin, and sequencing of the contiguous region is now underway.

Experimental

Bacterial strains and plasmids

Salinomycin producing *S. albus* strain ATCC 21838 was obtained from the American Type Culture Collection. The cosmid pOJ446 was described previously.[18] *E. coli* XL-1-Blue MRF' and pBluescript II SK$^+$ were purchased from Stratagene. *E. coli* DH5α was from Clontech. pT7-Blue T vector was from Novagen.

Preparation of *S. albus* genomic DNA

S. albus ATCC 21838 was shake-cultured at 200 rpm in YEME medium[21] at 28 °C. Harvested mycelia (3.6 g) were suspended in TSE buffer (10 mL; 25 mM Tris–HCl, pH 8, 0.3 M sucrose, 25 mM EDTA). After adding lysozyme (50 mg) and achromopeptidase (Wako Chemical Co.; 50 mg), the suspension was shaken at 37 °C for 1 h. Then, 0.25 M EDTA (6.66 mL, pH 8), 20% SDS (420 μL), and pronase (167 μL; 20 mg/mL) were added. The mixture was incubated at 37 °C for 1 h and then extracted with phenol–$CHCl_3$ followed by $CHCl_3$. After

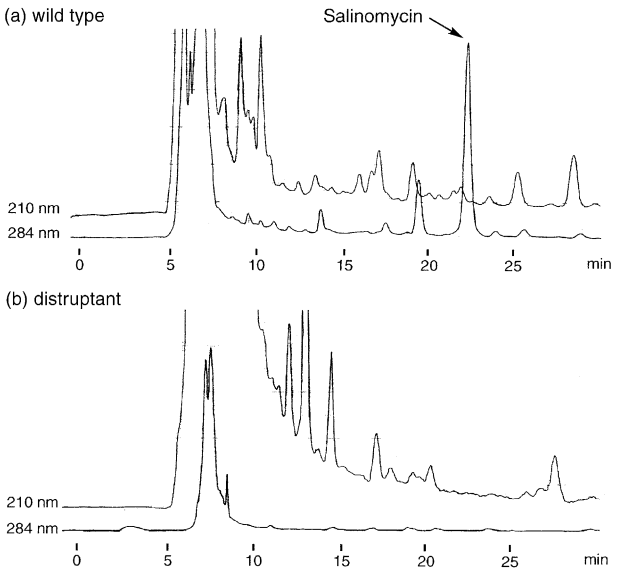

Figure 5. HPLC analysis of salinomycin production in wild type *S. albus* and gene disruption transformant: (A) *S. albus* wild type; (B) *S. albus* gene disruptant in the 4.5-kb *Bam*HI region. HPLC condition was described in Experimental.

RNase digestion at 37 °C for 1 h, genomic DNA was precipitated with ethanol. The genomic DNA obtained was dissolved in TE buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA) 1 mL.

Construction of an *S. albus* genomic DNA library

S. albus genomic DNA was partially digested with *Sau*3AI and fragments in the 30–40-kb size range were cloned into *Bam*HI/*Hpa*I digested cosmid vector pOJ446 as described by Bierman et al.[18]

PCR amplification of *S. albus* KS domain

Amplification of the β-ketoacyl synthase (KS) domains of *S. albus* was performed using the degenerate primers KSMA-F (5′-TS GCS ATG GAC CCS CAG CAG-3′) and KSMB-R (5′-CC SGT SCC GTG SGC CTC SAC-3′) designed from the conserved region of KS domains of bacterial modular type I PKS genes. The PCR mixture contained *S. albus* genomic DNA (0.5 μg), 100 pmol each of the primers KSMA-F and KSMB-R, AmpliTaq Gold buffer (Perkin–Elmer), 0.2 mM deoxynucleoside triphosphate mixture, 1.5 mM $MgCl_2$, DMSO (5%), and 2.5 U of AmpliTaq Gold polymerase (Perkin-Elmer). The mixture was heated at 95 °C for 12 min and then cycled three times at 94 °C for 30 s, 70 °C for 30 s, 72 °C for 1 min, three times at 94 °C for 30 s, 66 °C for 30 s, 72 °C for 1 min, three times at 94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min, three times at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, three times at 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min, then 40 times at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, followed by a final incubation at 72 °C for 5 min. The reaction generated an approximately 700-bp DNA fragment that was gel purified from a 1% low melting-point agarose gel using a PCR prep kit (Promega) and subcloned into pT7-Blue T vector.

Library screening

The *S. albus* genomic DNA library colonies (total ca. 1×10^4) were transferred onto Nytran membranes (Schleicher & Schuell) and fixed for hybridization following the instructions in the manufacturer's manual. The membranes were hybridized with digoxigenin-labeled *S. albus* KS domain probe, and positive signals were detected by DIG Color Detection kit (Roche).

DNA sequencing

Cosmids that hybridized to the *S. albus* KS domain probe were digested with *Bam*HI. The strongly hybridized 4.5-kb *Bam*HI fragment was subcloned into pBluescript II SK^+ vector. Sequence primer binding sites were randomly inserted into the target plasmid using the GPS-1 Genome Priming System (New England Biolabs) and resulting plasmids were sequenced by LIC-4000L sequencer (LI-COR) using a ThermoSequenase cycle-sequencing kit (Amersham).

S. albus transformation by electroporation

S. albus transformation for gene disruption was carried out by electroporation.[22] After shake-culturing in YEME medium 100 mL at 28 °C for 3 days, *S. albus* cells were harvested by centrifugation and washed three times with sterile water. Then, the mycelia were suspended in 25 mL of cold electroporation buffer (sucrose 10%, glycerol 15%, 3 mM sodium phosphate buffer pH 7.4) and supplemented with lysozyme (0.5 mg/mL). After 20-min incubation at 37 °C, cells were collected by centrifugation at 8000 rpm at 4 °C for 15 min, and then resuspended in 1 mL of chilled electroporation buffer. Fifty microliters of cell suspension was mixed with 5 μL plasmid solution and placed in an electroporation cuvette. A single electric pulse (2.38 kV; 5.14 ms) was applied using an Electro cell manipulator 600 (BTX Inc.). Then cells were diluted with 400 μL of cold SOC solution and incubated at 37 °C for 3 h. After collection by centrifugation, cells were spread on YEME agar plates and incubated at 30 °C for 1 day. Then antibiotic solution (streptomycin 25 μg/mL) was spread on the plate and incubated at 30 °C for 4 days.

HPLC analysis of salinomycin

S. albus mycelia were extracted with 80% aqueous acetone. The concentrated aqueous suspension was adjusted to pH 3 with 1 M HCl and extracted with ethyl acetate. The organic layer was dissolved in CH_3CN/H_2O/acetic acid (90:10:0.2) and passed through a small ODS column (ca. 0.5 mL) with the same solvent mixture. Salinomycin was analyzed by HPLC on an ODS column (YMC-pack ODS AM 120 AS-5, 10×250 mm) at a flow-rate of 2 mL/min. Salinomycin free acid was eluted at around 22 min when monitored at 284 nm.

Nucleotide sequence accession number

The DDBJ/GenBank/EMBL accession number for the salinomycin PKS gene is AB087998.

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