

# Cloning and Characterization of the Polyether Salinomycin Biosynthesis Gene Cluster of *Streptomyces albus* XM211

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Salinomycin is widely used in animal husbandry as a food additive due to its antibacterial and anticoccidial activities. However, its biosynthesis had only been studied by feeding experiments with isotope-labeled precursors. A strategy with degenerate primers based on the polyether-specific epoxidase sequences was successfully developed to clone the salinomycin gene cluster. Using this strategy, a putative epoxidase gene, *slnC*, was cloned from the salinomycin producer *Streptomyces albus* XM211. The targeted replacement of *slnC* and subsequent *trans*-complementation proved its involvement in salinomycin biosynthesis. A 127-kb DNA region containing *slnC* was sequenced, including genes for polyketide assembly and release, oxidative cyclization, modification, export, and regulation. In order to gain insight into the salinomycin biosynthesis mechanism, 13 gene replacements and deletions were conducted. Including *slnC*, 7 genes were identified as essential for salinomycin biosynthesis and putatively responsible for polyketide chain release, oxidative cyclization, modification, and regulation. Moreover, 6 genes were found to be relevant to salinomycin biosynthesis and possibly involved in precursor supply, removal of aberrant extender units, and regulation. Sequence analysis and a series of gene replacements suggest a proposed pathway for the biosynthesis of salinomycin. The information presented here expands the understanding of polyether biosynthesis mechanisms and paves the way for targeted engineering of salinomycin activity and productivity.

Polyether antibiotics (Fig. 1), such as monensin, nanchangmycin, lasalocid, and salinomycin, are widely used as anticoccidial drugs and growth promoters in animal husbandry (11). They have the ability to chelate metal ions selectively within a hydrophobic matrix and then transport the ions across the cell membrane, which destroys the physiological ion gradients of the cells and results in the death of *Eimeria* sp., the causative agent of coccidiosis (31). Depending on the arrangement of the first four biogenetic units of acetate (A) or propionate (P), polyethers are divided into two stereochemical prototypes (5). The APPA type is exemplified by monensin, nanchangmycin, and nigericin (Fig. 1A), whereas the PAPA type comprises salinomycin, lasalocid, and norbitomycin (Fig. 1B).

So far, five polyether biosynthesis gene clusters have been identified and characterized for the biosynthesis of monensin (27), nanchangmycin (37), lasalocid (35), nigericin (15), and tetronomycin (9). Based on *in silico* and experimental analysis, the polyene backbones, assembled by a type I polyketide synthase (PKS), could undergo oxidative cyclization catalyzed by an epoxidase and one or more epoxide hydrolases to generate the characteristic pattern of ether bonds. Accordingly, genes for the putative epoxidase and epoxide hydrolases have been identified in all five reported gene clusters. The epoxidase MonCI in the monensin cluster was proved to perform epoxidation by heterologous expression in *Streptomyces coelicolor*, using the unsaturated terpene (+/-)-linalool as a substrate (27). Moreover, the *E,E,E*-triene lactone isolated from the *monCI* mutant of *Streptomyces cinnamonensis* strongly implied that the *E*-configured double bonds are targets for MonCI to initiate oxidative cyclization (3). On the other hand, deletion of either or both of the epoxide hydrolase genes *monBI* and *monBII* abolished the production of monensin A and B (10). Recently, *in vitro* experiments directly demonstrated that the epoxide hydrolase Lsd19 from the lasalocid cluster could recognize the internal epoxide of the substrate and catalyze epoxide-opening

and concomitant polyether ring formation cascades (24, 26, 33). Although both epoxidases and epoxide hydrolases are key enzymes in polyether biosynthesis, epoxidases are more conserved and show higher homology with each other. Moreover, epoxidases involved in polyether biosynthesis are distinct from epoxidases for nonpolyether antibiotic biosynthesis. Therefore, the existence of such an epoxidase gene might be considered a sign of polyether-producing potential (21).

Salinomycin is an important commercial polyether comprised of a unique tricyclic spiroketal ring system and an unsaturated six-membered ring (Fig. 1B). It has been used for 30 years as an anticoccidial drug and to improve nutrient absorption and feed efficiency in ruminants and swine. Recently, salinomycin has also been identified as an agent to kill epithelial cancer stem cells (12). Previous isotope-labeled feeding experiments clearly revealed that the backbone of salinomycin is assembled from six acetate, six propionate, and three butyrate units (32). In a proposed pathway for salinomycin biosynthesis (5) (Fig. 1C), unsaturated acyclic diene intermediates initially undergo oxidation to form a diepoxide, followed by an epoxide-opening cascade reaction catalyzed by epoxide hydrolases, to generate the characteristic ether ring system. However, details of the biosynthesis of salinomycin have been elusive.

In 2003, cloning of the salinomycin biosynthesis gene cluster in *Streptomyces albus* ATCC 21838 was first claimed through PCR

Received 28 August 2011 Accepted 27 November 2011

Published ahead of print 9 December 2011

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Supplemental material for this article may be found at <http://aem.asm.org>.

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doi:10.1128/AEM.06701-11

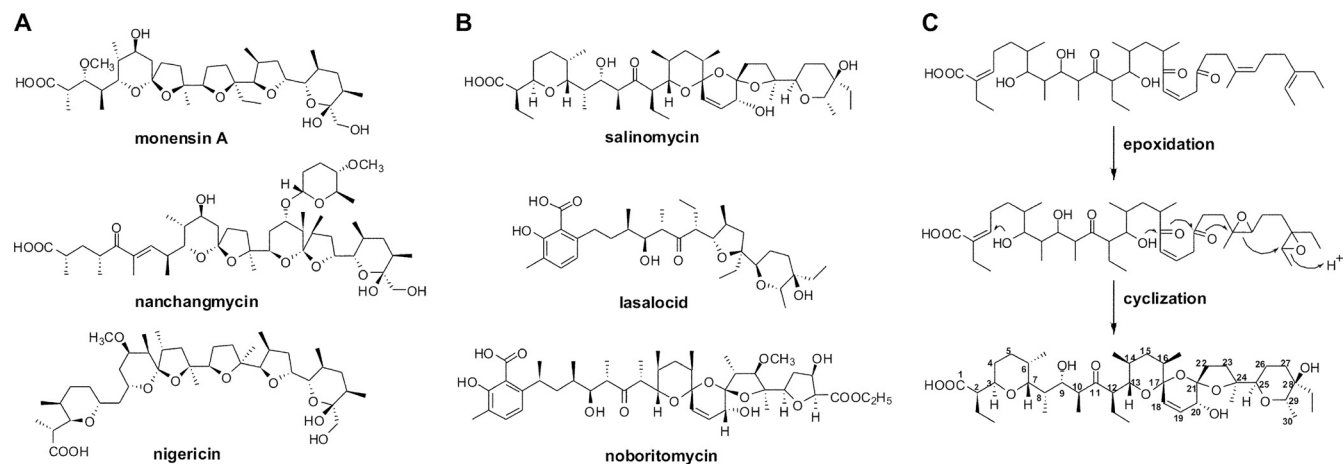


FIG 1 Structures of polyether antibiotics and proposed pathway for salinomycin biosynthesis. (A) APPA-type polyether antibiotics. (B) PAPA-type polyether antibiotics. (C) Hypothetical ether bond formation in salinomycin biosynthesis.

amplification using degenerate ketosynthase (KS) primers. The involvement of this 4.5-kb region in salinomycin biosynthesis was further suggested through a single-crossover gene disruption (17). In 2007, another group cloned a 25.81-kb region using the same strategy from the salinomycin producer *S. albus* CCM 4719, which contained the previously identified 4.5-kb DNA fragment. However, gene disruption was not conducted in this strain (19).

Here, a homologous region was first cloned according to the published sequence in our salinomycin producer, *S. albus* XM211. However, the polyketide backbone deduced from the sequences on polyketide synthases did not fit with any portion of the salinomycin structure. Alternatively, the strategy of PCR amplification with degenerate epoxidase-specific primers was designed, and another genomic region was cloned and then confirmed as the salinomycin biosynthesis gene cluster through gene replacement and complementation.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, culture conditions, and general techniques.** The bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material.

*S. albus* XM211, here called XM211, is the producer of salinomycin and was isolated from a mangrove forest in Xiamen, China. For sporulation, *S. albus* XM211 and its derivatives were grown at 30°C on ISP 4 agar plates (BD) (34) for 8 to 10 days. ISP 4 medium was also used for conjugation between *Escherichia coli* and *Streptomyces*. For salinomycin isolation, the seed culture was grown in a 250-ml flask containing 50 ml medium (4% glucose, 3% soy flour, 1% yeast extract, 0.2% calcium carbonate) on a rotary shaker at 200 rpm and 32°C for 26 to 32 h. Then, 5 ml of the seed culture was used to inoculate 50 ml fermentation medium (3% glucose, 1% casein hydrolysate, 0.2% sodium chloride, 0.2% potassium chloride, 0.5% ammonium sulfate, 0.02% dipotassium hydrogen phosphate, 0.01% magnesium sulfate, 0.01% calcium chloride, 0.5% calcium carbonate). The fermentation was continued at 32°C for 5 to 6 days. For isolation of the total DNA, *S. albus* XM211 was cultivated in Trypticase soy broth (TSB) medium (18) supplemented with 10.3% sucrose and 1% yeast extract.

*E. coli* DH10B (Invitrogen) was used as cloning host, and *E. coli* ET12567(pUZ8002) (28) was used as a conjugation host. The *E. coli* strains were grown at 37°C in LB broth and LB agar. Plasmid pMD18-T was used for cloning and sequencing of PCR-amplified products. pJTU824, a derivative of pSET152, was used for gene complementation in the mutants (40).

Conjugation between *E. coli* and *S. albus* XM211 was performed as described by Kieser et al. (18). DNA fragments were recovered from agarose gels using a Gel Recovery Kit (Omega).

**Isolation and analysis of salinomycin.** Liquid fermentation cultures of the wild-type strain and mutants were extracted twice with 2 volumes of ethyl acetate. The organic fraction was evaporated *in vacuo*, and the residue was redissolved in methanol for analysis. Samples were applied to liquid chromatography-tandem mass spectrometry (LC-MS-MS) (Agilent 1100 series LC/MSD Trap system) after passing through 0.2- $\mu$ m filters. The liquid chromatograph was operated at a flow rate of 0.3 ml/min with an Agilent Eclipse TC-C<sub>18</sub> column (4.6 by 250 mm; particle size, 5  $\mu$ m) with UV detection at 210 nm (4). The mobile phase was acetonitrile-2% acetic acid (92:8 [vol/vol]). The ion-trap mass spectrometer was operated with an electrospray ionization source in positive ion mode. The drying gas flow was 10 liters/min with a temperature of 325°C, and the nebulizer pressure was 30 lb/in<sup>2</sup>.

**PCR amplification with degenerate epoxidase-specific primers.** The degenerate primers Epo-110-F-2/Epo-1030-R-1, designed according to conserved domains of reported epoxidases, are listed in Table S4 in the supplemental material. GC buffer (Takara Biotechnology, Dalian, China) was used for amplification with templates of *Streptomyces* DNA. DNA fragments of the desired sizes were cloned into the pMD18-T vector and sequenced. A pair of specific primers, sln1-F/sln1-R (see Table S4 in the supplemental material), was then designed to screen the XM211 genomic library.

**Sequencing and analysis of the salinomycin biosynthesis gene cluster.** The XM211 genomic library was first screened by PCR amplification using sln1-F/sln1-R. For subsequent chromosome walking, three pairs of specific primers based on sequenced fosmids were used, i.e., sln2-F/sln2-R, sln3-F/sln3-R, and sln4-F/sln4-R (see Table S4 in the supplemental material). Fosmids 12H8, 25E8, 10B8, and 6G11 and 3.58-kb and 7.93-kb BamHI fragments of 19H1 were sequenced at Majorbio Biotech Co., Ltd. The raw sequence data were analyzed with FramePlot 4.0beta (16) and Glimmer (7) online programs. Annotation analysis was performed using the BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (1). The assembly of the PKS region was analyzed with the online NRPS-PKS program (2). Sequence alignments were performed with BioEdit Sequence Alignment Editor.

**Gene replacement and trans-complementation of *slnC*.** Gene replacement of *slnC* was carried out by Redirect Technology (13). Using the gel-purified 1,384-bp EcoRI/HindIII fragments from pIJ773 as templates, 1,449-bp products carrying the apramycin-resistant gene *aac(3)IV* and *oriT* were amplified by PCR with primers 12H8tar-F and 12H8tar-R (see Table S4 in the supplemental material). This amplified fragment was used

to replace the 1,416-bp region of *slnC* in pJTU5359 cloned with a 9.0-kb *SpeI*/*BglIII* DNA fragment from fosmid 12H8, giving rise to pJTU5360. Subsequent transfer of the 9.1-kb *BglIII* fragment from pJTU5360 to pJTU412 (36) resulted in the construction of pJTU5361 for *slnC* inactivation in XM211.

Then, pJTU5361 was transferred into strain XM211 by conjugation from *E. coli* ET12567(pUZ8002). The thiostrepton-sensitive and apramycin-resistant (Thio<sup>s</sup> Apr<sup>r</sup>) recombinants were selected from the initial Apr<sup>r</sup> exconjugants after several rounds of nonselective growth. For further verification, the mutant candidates were verified by PCR amplification with primers JCY16-F and JCY16-R (see Table S4 in the supplemental material) and *SacI* digestion of the amplified products.

To complement the *slnC* mutant JCY16, a pJTU824-derived construct, pJTU5376, was used. This plasmid contained the complete *slnC* gene under the control of the *ermE*<sup>\*</sup> promoter (an upregulated variant of the *ermE* promoter.). The *slnC* gene was amplified from fosmid 12H8 using the primers epoxidase-F and epoxidase-R (see Table S4 in the supplemental material). The 1.45-kb PCR product was purified, cloned into the pMD18-T vector, and sequenced. The *slnC* gene was transferred to pJTU824 digested with *NdeI* and *EcoRI*, and an integrative plasmid, pJTU5376, with the intact *slnC* gene under the control of the *ermE*<sup>\*</sup> promoter was constructed. Plasmid pJTU5376 was introduced into JCY16 through conjugation, and apramycin-resistant and thiostrepton-resistant (Thio<sup>r</sup> Apr<sup>r</sup>) exconjugants were selected.

Replacements of other genes in this study are described in the supplemental material.

**Nucleotide sequence accession number.** The sequence of the salinomycin biosynthesis gene cluster was deposited in GenBank with accession number [JN033543](#).

## RESULTS AND DISCUSSION

**Attempted cloning of the salinomycin biosynthesis gene cluster based on the previously reported sequences.** Based on previously published sequences (GenBank accession number [AB087998](#)), a pair of primers, SalPrimerF1/SalPrimerR1 (see Table S4 in the supplemental material), for amplifying the acyl carrier protein (ACP)-KS region were designed for screening an *S. albus* XM211 genomic library. The sequence of the amplified 0.20-kb fragment matches the predicted sequence. Finally, fosmid 37F1 was chosen to be sequenced, and the previously reported 4.50-kb DNA region was shown to be included in its sequence (17).

Sequence analysis identified 11 potential open reading frames (ORFs), as shown in Table S2 and Fig. S2 in the supplemental material. Three large ORFs, designated *samK1* to *samK3*, encode typical multifunctional type I PKSs. *SamK1* consists of extension module K1-1 (KS-ATp [methylmalonyl-coenzyme A {CoA}-specific acyltransferase]-KR [ketoreductase]-ACP), extension module K1-2 (KS-ATa [malonyl-CoA-specific acyltransferase]-DH [dehydratase]-KR<sup>a</sup>-ACP, where <sup>a</sup> represents an inactive domain), and one integrated thioesterase (TE) domain. *SamK2* contains extension module K2-1 (KS-ATa-DH<sup>a</sup>-KR<sup>a</sup>-ACP) and module K2-2 (KS-ATa-KR-ACP), and *SamK3* represents extension modules K3-1 (KS-ATa-KR<sup>a</sup>-ACP) and K3-2 (KS-ATa-KR-ACP). In fact, however, two sets of (CO-CH<sub>2</sub>)(CHOH-CH<sub>2</sub>) units are not present anywhere within the salinomycin backbone, suggesting these three PKS genes are not involved in salinomycin biosynthesis. However, an attempted deletion of this region to verify its involvement in salinomycin biosynthesis failed. Therefore, alternative strategies should be considered to clone the salinomycin biosynthesis gene cluster.

**Design of degenerate epoxidase-specific PCR primers and amplification of the epoxidase gene in salinomycin producers.** The formation of the characteristic ether bonds in polyethers are

catalyzed by epoxidases and cognate epoxide hydrolases, and the epoxidases are found to be conserved and could be used to clone homologous genes (21, 39). In order to design an efficient pair of primers for the amplification of putative epoxidase genes, the amino acid sequences of four available epoxidases, i.e., MonCI (27), NanO (37), NigCI (15), and LasC (35), were aligned to identify highly conserved blocks (Fig. 2). Several sets of degenerate primers were designed based on conserved blocks using the PaBaLiS (29) program. To validate the efficiency of these primers, the detection of epoxidase genes was first conducted with DNA isolated from polyether producer strains of monensin, nanchangmycin, nigericin, and lasalocid. PCR amplification with the primers Epo-110-F-2/Epo-1030-R-1, designed according to two conserved blocks, VTVV (E/D) RD and YGHGMS (Fig. 2), gave a 0.90-kb product with genomic DNA from each of the four strains. All the amplified products were confirmed by sequencing to be polyether-specific epoxidase genes (see Fig. S3 in the supplemental material).

The successful application of the degenerate primers Epo-110-F-2/Epo-1030-R-1 encouraged us to clone the salinomycin-specific epoxidase gene using the same strategy. PCR amplification of *S. albus* XM211 genomic DNA gave a 0.90-kb product (see Fig. S3 in the supplemental material), which was then cloned and sequenced. This portion was named *slnC*, and its product showed 54% and 52% identities with epoxidase LasC, encoded in the lasalocid gene cluster, and MonCI, encoded in the monensin gene cluster, respectively. Based on this newly obtained sequence, a pair of primers, *sln1*-F and *sln1*-R, were designed and used to screen the whole XM211 genomic library. Twelve overlapping fosmids, including 12H8, were found to give the expected 0.6-kb PCR products (data not shown).

Additionally, two 0.90-kb expected PCR products were also obtained from the BL580Δ (see Fig. S1 in the supplemental material) producer *Streptomyces* sp. NRRL 8180 and the mutalomycin (see Fig. S1 in the supplemental material) producer *Streptomyces* sp. NRRL 8088 (see Fig. S3 in the supplemental material). Both products were confirmed to be polyether-epoxidase genes through sequencing. They are therefore likely to be involved in the biosynthesis of the corresponding polyethers.

Phylogenetic analysis of the available epoxidase sequences (see Fig. S7 in the supplemental material) revealed that the distribution of epoxidases can reflect the structural differences of polyether antibiotics. *SlnC* and *LasC*, whose corresponding polyether antibiotics are both categorized as PAPA type, form one clade. On the other hand, *MonCI*, *NanO*, *NigCI*, and deduced epoxidases in mutalomycin and BL580Δ biosynthesis form another clade for APPA-type polyethers. This indicates that the sequences of epoxidases are correlated with the structures of the parental polyketides of polyethers.

**The epoxidase gene *slnC* is involved in the biosynthesis of salinomycin.** The involvement of *slnC* in salinomycin production was proved through gene replacement. A 1,416-bp DNA fragment of the epoxidase gene in the sequenced 12H8 fosmid was replaced by the apramycin-resistant gene *aac(3)IV* to generate the mutant JCY16 (Fig. 3A). Allelic replacement of *slnC* in JCY16 was confirmed by PCR amplification with primers JCY16-F/JCY16-R and *SacI* digestion. The 1.50-kb PCR product amplified from the mutant JCY16 could be digested into 0.62-kb, 0.75-kb, and 0.13-kb fragments, whereas a 1.55-kb expected product from wild-type XM211 was digested into 0.99-kb and 0.55-kb fragments (Fig. 3B).

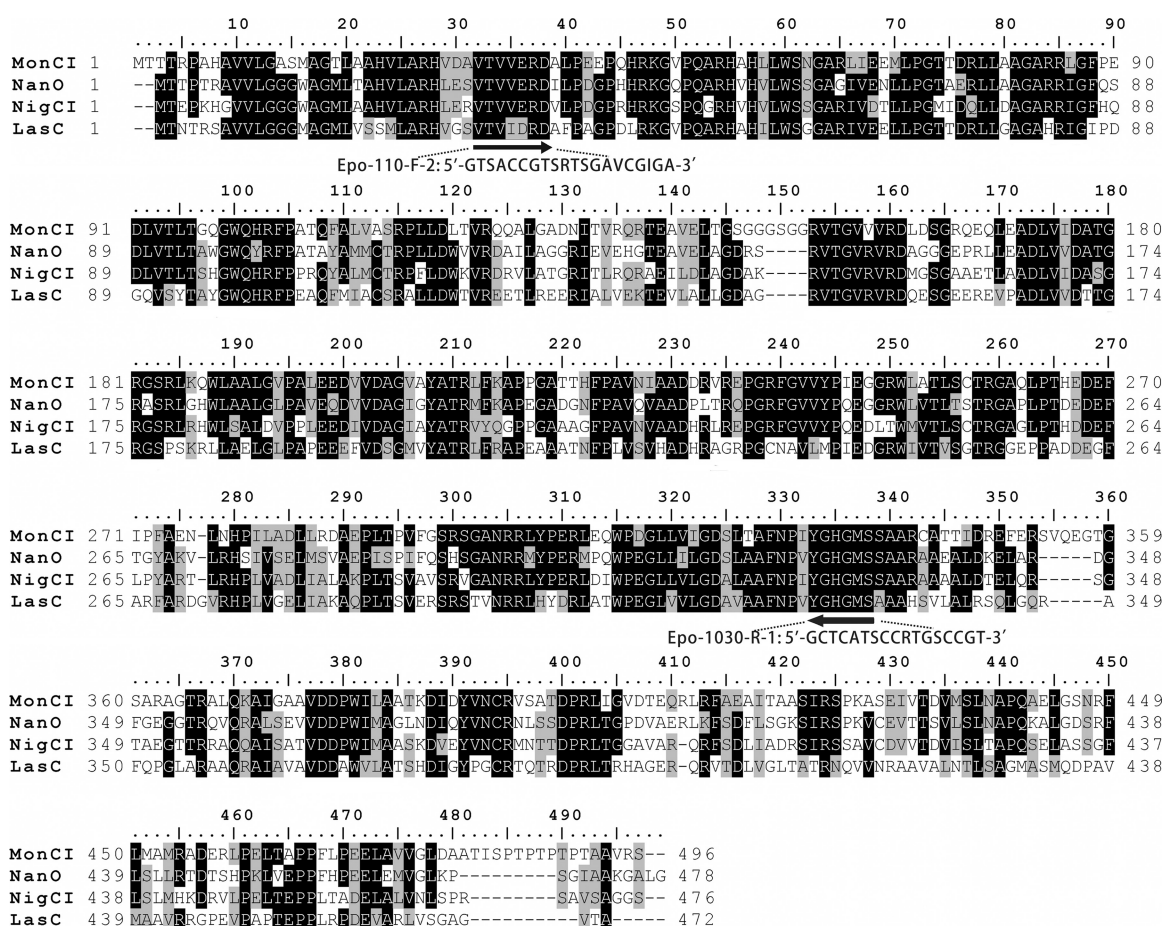


FIG 2 Multiple alignments of amino acid sequences of known epoxidases from polyether biosynthesis gene clusters. The arrows indicate the regions selected for the design of primers Epo-110-F-2 and Epo-1030-R-1. Black shading indicates identical amino acids, and gray shading indicates similar amino acids.

LC-MS analysis of the extracts from the mutant JCY16 displayed a complete loss of salinomycin production (Fig. 3C), suggesting that this epoxidase gene is involved in salinomycin biosynthesis.

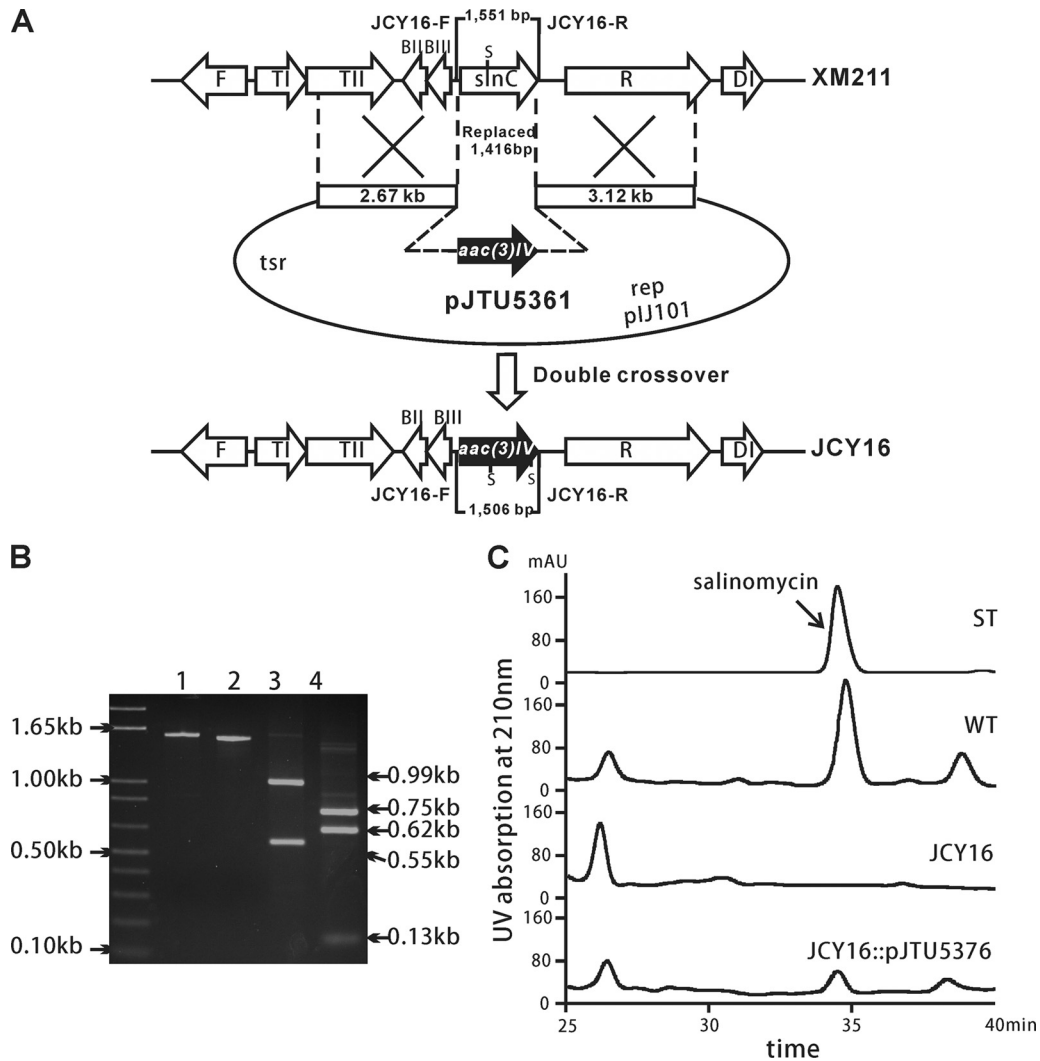
To confirm that the loss of salinomycin productivity was caused by the replacement of *slnC*, a *trans*-complementation experiment using pJTU5376 was performed. This plasmid, harboring the intact epoxidase gene *slnC* under the control of the *ermE*\* promoter, was introduced into JCY16 by conjugation. The high-performance liquid chromatography (HPLC) profile of the extract of the complemented strain revealed the restoration of salinomycin, albeit at a reduced level (~20%) compared to the wild-type strain (Fig. 3C; see Fig. S4 in the supplemental material). Thus, *slnC* was proved to be related to salinomycin biosynthesis.

**Cloning and sequence analysis of the salinomycin biosynthesis gene cluster.** DNA sequence analysis of fosmid 12H8 shows that the epoxidase gene *slnC* is located at the left end, and most ORFs in the right side seem not to be related to salinomycin biosynthesis. In order to clone the entire gene cluster, sequential chromosome walking was initiated from the left end of 12H8, revealing a continuous ~127-kb DNA region (Fig. 4A). Computer-assisted analysis of the cloned sequence led to the identification of the constituent genes (Fig. 4B). The putative functions of these genes are listed in Table 1 and in Table S3 in the supplemental material, including genes encoding a type I PKS, oxidative cy-

clization, hydroxylation, regulation, and export. Including the replacement of *slnC*, a total of 14 gene replacements were performed to investigate boundaries and gene functions of the salinomycin biosynthesis gene cluster (Fig. 4B).

**Determining the boundaries of the salinomycin biosynthesis gene cluster.** To the left of the PKS gene *slnA1*, two genes, *orf11* and *orf12*, are divergently transcribed (Fig. 4B) and annotated as 3-hydroxybutyryl-CoA dehydrogenase and 3-oxoacyl-(acyl carrier protein) synthase III genes, respectively. Genes *orf11* and *orf12* are thought to be involved in the dedicated supply of the butyrate extender unit for salinomycin biosynthesis (25, 35). Genes *orf11* and *orf12* were individually replaced with *aac(3)IV-oriT*, and the salinomycin yields of the *orf11* mutant JCY39 and the *orf12* mutant JCY28 were found to be 10% and 36% of the wild type, respectively (Fig. 5; see Fig. S5 in the supplemental material), suggesting the involvement of *orf11* and *orf12* in salinomycin biosynthesis, possibly through the supply of a butyrate extender units.

To the right of gene *slnDII*, gene *orf13*, putatively encoding a peptide synthetase, was first replaced by *aac(3)IV*. The salinomycin productivity was not changed, as expected, in the mutant JCY25 (Fig. 5). Further deletion of *orf14* to *orf17*, however, resulted in a moderate drop in salinomycin production in the mutant JCY26 to 45% of the wild type (Fig. 5; see Fig. S5 in the



**FIG 3** Replacement and complementation of epoxidase gene *slnC*. (A) Schematic representation of the replacement of *slnC*. A 1,416-bp region of *slnC* was replaced by the 1,371-bp *aac(3)IV* gene through double crossover. S, *SacI*. (B) Confirmation of the mutant JCY16 through PCR amplification and *SacI* digestion. An approximately 1.50-kb product was amplified using primers JCY16-F/JCY16-R. After *SacI* digestion, the electrophoresis gel revealed 0.62-kb, 0.75-kb, and 0.13-kb bands using the mutant genomic DNA as a template (lane 4) instead of the 0.55-kb and 0.99-kb bands of the wild type (lane 3). (C) HPLC analysis of the wild type (WT), *slnC* mutant JCY16, and complementation strain JCY16::pJTU5376. ST, salinomycin standard; AU, absorbance units.

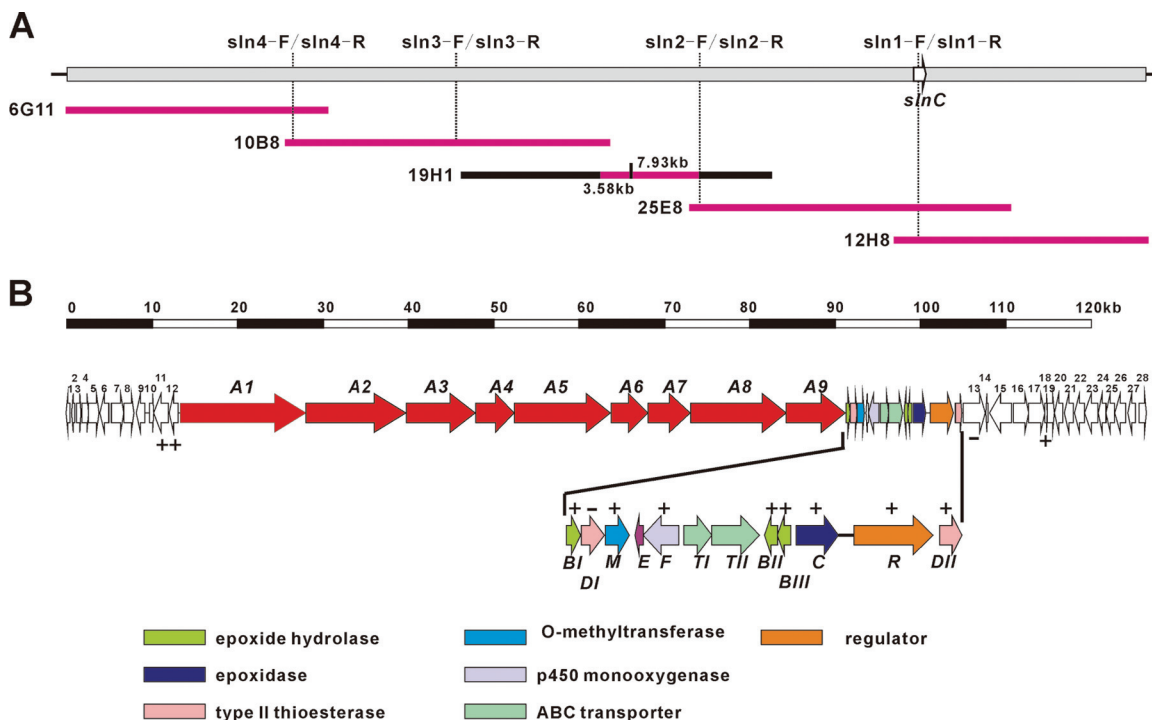
supplemental material). Among these four genes, the deletion of the SARP family transcriptional regulator gene *orf15* probably reduced the transcription of salinomycin biosynthesis genes.

**PKS genes.** Nine large adjacent genes (*slnA1* to *slnA9*) are collinearly arranged in the cluster (Fig. 4B). Four of these (*slnA4*, *slnA6*, *slnA7*, and *slnA9*) each encode a single extension module, while the other five (*slnA1*, *slnA2*, *slnA3*, *slnA5*, and *slnA8*) harbor two extension modules, in agreement with the 14 condensation steps required for the biosynthesis of the salinomycin polyketide skeleton. The linear polyketide is synthesized by the loading of an acetate starter unit and incorporation of 5 acetate, 6 propionate, and 3 butyrate units (see Fig. 7).

*SlnA1* contains a loading module (KSQ-ATa-ACP [where KSQ is the KS domain in the loading module with active-site cysteine replaced by glutamine]) for the initiation of salinomycin polyketide synthesis and modules 1 and 2 for polyketide chain extension from C-25 to C-30. The AT domains of the loading module

and module 2 have the characteristic motif HAFH for recognizing acetate (malonyl-CoA) extender units (ATa) (8, 38). The AT domain of module 1 is predicted to incorporate a butyrate (ethylmalonyl-CoA) unit due to the presence of a VASH motif (25, 35). *SlnA2* putatively encodes modules 3 and 4 and is responsible for the extension of C-21 to C-24 through incorporation of one propionate (methylmalonyl-CoA) unit (8, 38) and one acetate unit.

*SlnA3* putatively encodes modules 5 and 6 and is responsible for the incorporation of C-17 to C-20. According to the hypothetical salinomycin biosynthesis pathway (5), an unsaturated double bond at C-18 and C-19 could be formed during polyketide elongation. Surprisingly, module 6 has a KS-ATa-KR-ACP domain structure but lacks a DH domain for the required dehydration. *SlnA4* contains only module 7, presumably for the incorporation of C-15 and C-16. KR7 has four changes in the conserved NADP(H) binding motif GXGXXGXGXXA and in the essential res-



**FIG 4** Gene organization of the salinomycin biosynthesis gene cluster. (A) Overlapping fosmids encompassing the sequenced region. Primers *sln1-F* and *sln1-R* were used to screen for the salinomycin gene cluster from the *S. albus* XM211 genomic library. The other primers were used for chromosome walking. The pink lines represent the sequenced region, including 12H8, 25E8, 10B8, 6G11, and 3.58-kb and 7.93-kb *Bam*HI fragments from 19H1. (B) Gene organization of the salinomycin biosynthesis gene cluster. -, gene products not involved in salinomycin biosynthesis, as confirmed through gene replacement; +, gene products involved in salinomycin biosynthesis, as confirmed through gene replacement and/or *trans*-complementation to the corresponding mutants.

idues K-S-Y-N (30) and thus appears to be inactive. This is in full agreement with the presence of a keto group at C-17 in the polyketide chain. *SlnA5*, -A6, -A7, and -A8 putatively correspond to modules 8 to 13 for the incorporation of C-3 to C-14.

The last ORF, *SlnA9*, contains module 14 for the incorporation of C-1 and C-2; an apparently intact C-terminal KS domain, *SlnKSX*; and an inactive AT domain (137 amino acids [aa] shorter than AT14). Similar to module 6, module 14 has a KS-ATb [ethylmalonyl-CoA-specific acyltransferase]-KR-ACP domain assembly, which does not match the predicted double bond at C-2-C-3 required for the formation of the tetrahydrofuran. This discrepancy suggests the possibility that the required dehydration reactions may take place after polyketide chain assembly catalyzed by a discrete dehydratase. There is no putative dehydratase, however, in the cloned region, and these genes might be located in another region of the chromosome.

**Genes possibly involved in polyketide chain elongation and release.** Due to the absence of a conventional TE domain within the PKS or a NanE-like thioesterase (22, 23), there are three candidates for the requisite thioesterase activity in the salinomycin biosynthesis gene cluster. Genes *slnDI* and *slnDII*, encoding discrete type II TEs, display high similarity to *monAIX* and *monAX* in the monensin gene cluster (14). The *slnDI* disruption mutant JCY23 produced salinomycin at a level similar to that of wild-type XM211 (Fig. 6A), whereas the *slnDII* disruption mutant JCY21 had a 40 to 50% drop in salinomycin production (Fig. 6A). *SlnDI* and *SlnDII* are therefore predicted to serve a conventional editing function with the ability to remove nonelongateable residues from

unprocessed PKS proteins, thus ensuring the efficiency of polyketide biosynthesis.

In the pathway to monensin, the discrete MonKSX is proposed to be capable of catalyzing the transfer of the linear polyketide chain to the MonACPX, on which oxidative cyclization occurs (15). The extra *SlnKSX* domain fused downstream of ACP14 in the last module of the salinomycin PKS may catalyze the transfer (Fig. 7). However, the required discrete ACP gene is not present in an adjacent region. In the whole sequenced region, only *orf18* is predicted to encode a peptidyl carrier protein to potentially perform such a function. Targeted inactivation of *orf18* was therefore carried out to create a mutant JCY29. Interestingly, analysis of the mutant revealed a 50 to 60% drop in salinomycin production compared to wild-type XM211 (Fig. 6A). Thus, this result implies that *Orf18* may be relevant to salinomycin biosynthesis but not essential to the polyketide chain release.

Immediately downstream of the last PKS gene, *slnA9*, lies a putative epoxide hydrolase gene, *slnBI*. However, sequence alignments (see Fig. S6 in the supplemental material) reveal that *SlnBI* has an isoleucine in place of the conserved glutamic acid residue in an acidic amino acid pair (D38-E65), which was proven to play a critical role in stepwise epoxide opening and subsequent cyclization (26). Although *SlnBI* is much smaller than NanE-like enzymes and shows no significant similarity to them, its relationship with the chain release was investigated through gene replacement. In the *slnBI* mutant JCY22, the yield of salinomycin was dramatically decreased to less than 5% of that of the wild type (Fig. 6A). Since no other convincing candidate enzyme was found for this

TABLE 1 Deduced functions of ORFs in the salinomycin biosynthesis gene cluster

ORF	No. of amino acids	Proposed function <sup>a</sup>	Sequence similarity (protein, origin)	% identity/similarity	Accession no.
Orf11	572	3-Hydroxybutyryl-CoA dehydrogenase	StrviDRAFT_8389, <i>Streptomyces violaceusniger</i> Tü 4113	61/70	ZP07610704
Orf12	342	3-Oxoacyl-(acyl-carrier-protein) synthase III	SSNG_07461, <i>S. treptomyces</i> sp. C	77/86	ZP07291840
SlnA1	4,925	PKS	LasA1, <i>Streptomyces lasaliensis</i>	56/68	CAQ64686
Loading		KSQ-ATa-ACP			
Module 1		KS-ATb-DH-KR-ACP			
Module 2		KS-ATa-DH-ER-KR-ACP			
SlnA2	3,917	PKS	NanA5, <i>Streptomyces nanchangensis</i> NS3226	51/63	AAP42859
Module 3		KS-ATp-DH-KR-ACP			
Module 4		KS-ATa-DH-ER-KR-ACP			
SlnA3	2,692	PKS	MeiA2, <i>S. nanchangensis</i> NS3226	52/64	ADC45534
Module 5		KS-ATa-ACP			
Module 6		KS-ATa-KR-ACP			
SlnA4	1,642	PKS	NigAVI, <i>S. violaceusniger</i> DSM4137	51/62	DQ354110
Module 7		KS-ATp-KR <sup>a</sup> - ACP			
SlnA5	3,750	PKS	LasAII, <i>S. lasaliensis</i>	56/68	CAQ64687
Module 8		KS-ATp-DH-ER-KR-ACP			
Module 9		KS-ATb-KR-ACP			
SlnA6	1,435	PKS	LasAVI, <i>S. lasaliensis</i>	58/71	CAQ64691
Module 10		KS-ATp-DH <sup>*</sup> - ACP			
SlnA7	1,644	PKS	LasAIV, <i>S. lasaliensis</i>	56/68	CAQ64689
Module 11		KS-ATp-KR-ACP			
SlnA8	3,724	PKS	MeiA1, <i>S. nanchangensis</i> NS3226	52/64	ADC45586
Module 12		KS-ATp-KR-ACP			
Module 13		KS-ATa-DH-ER-KR-ACP			
SlnA9	2,312	PKS	AmphI, <i>Streptomyces nodosus</i>	52/66	AAK73501
Module 14		KS-ATb-KR-ACP			
KS		KS			
SlnBI	128	Epoxide hydrolase	NigBI, <i>S. violaceusniger</i> DSM4137	32/50	ABC84467
SlnDI	265	Thioesterase	MonAX, <i>S. cinnamomensis</i>	50/65	AA065810
SlnM	271	O-Methyltransferase	TcmP, <i>Streptomyces glaucescens</i>	39/59	AAA67510
SlnE	93	Ferredoxin	Tcs_55098_011, <i>Streptomyces</i> sp. ATCC 55098	53/70	ADU56364
SlnF	407	Cytochrome P450	NigD, <i>S. violaceusniger</i> DSM4137	35/52	ABC84463
SlnTI	319	ABC transporter ATP-binding protein	TnrB2, <i>S. longisporoflavus</i>	64/77	CAA52012
SlnTII	546	ABC transporter efflux pump	TnrB3, <i>S. longisporoflavus</i>	39/53	CAA52013
SlnBII	149	Epoxide hydrolase	NigBI, <i>S. violaceusniger</i> DSM4137	47/64	ABC84467
SlnBIII	153	Epoxide hydrolase	NigBI, <i>S. violaceusniger</i> DSM4137	52/72	ABC84467
SlnC	484	Epoxidase	NigCI, <i>S. violaceusniger</i> DSM4137	51/67	ABC84466
SlnR	906	LuxR family transcriptional regulator	Las4, <i>S. lasaliensis</i>	42/52	CAQ64683
SlnDII	253	Thioesterase	MonAX, <i>S. cinnamomensis</i>	40/54	AA065810
Orf13	862	Peptide synthetase	AcmC, <i>Streptomyces anulatus</i>	48/61	ADG27359
Orf14	82	Hypothetical protein	SSNG_00262, <i>Streptomyces</i> sp. C	74/80	ZP07284641
Orf15	855	SARP family transcriptional regulator	Sros_3791, <i>Streptomyces roseum</i> DSM 43021	33/45	YP003339454
Orf16	597	Acyl-CoA synthetase	PRK09192, <i>Streptomyces viridochromogenes</i>	43/54	AEF16021
Orf17	572	Acyl-CoA dehydrogenase	SSCG_02872, <i>Streptomyces clavuligerus</i> ATCC 27064	40/52	ZP05005545
Orf18	96	Peptidyl carrier protein	SSCG_02873, <i>S. clavuligerus</i> ATCC 27064	58/75	ZP05005546
Orf19	279	4'-Phosphopantetheinyl transferase	SCLAV_0457, <i>S. clavuligerus</i> ATCC 27064	50/61	ZP06769934

<sup>a</sup> \*, redundant domain; <sup>a</sup>, inactive domain; ER, enoyl reductase.

role in the cloned region, SlnBI merits consideration as a potential enzyme with thioesterase activity.

**Genes involved in epoxide-opening cascades.** Genes *slnBII* and *slnBIII*, encoding polypeptides of 149 aa and 153 aa, respectively, are putative epoxide hydrolase genes. *slnBII* and *slnBIII* overlap for 8 bp and are significantly similar to *nigBI* in the nigericin gene cluster, with 47% and 42% identity, respectively. In order to study the roles of these two genes in the biosynthesis of salinomycin, corresponding mutants were created. When ana-

lyzed by LC-MS, no trace of salinomycin was detected in either the *slnBII* mutant JCY19 or the *slnBIII* mutant JCY18, confirming their involvement in salinomycin biosynthesis (Fig. 6B). Moreover, LC-MS revealed a new peak with a shorter retention time (in both JCY18 and JCY19;  $m/z$  734.5 [M+H]<sup>+</sup>) and another new peak with a longer retention time (in JCY18 only;  $m/z$  735.5 [M+H]<sup>+</sup>) than salinomycin (data not shown). The chemical structures of these two novel salinomycin derivatives need to be further confirmed.

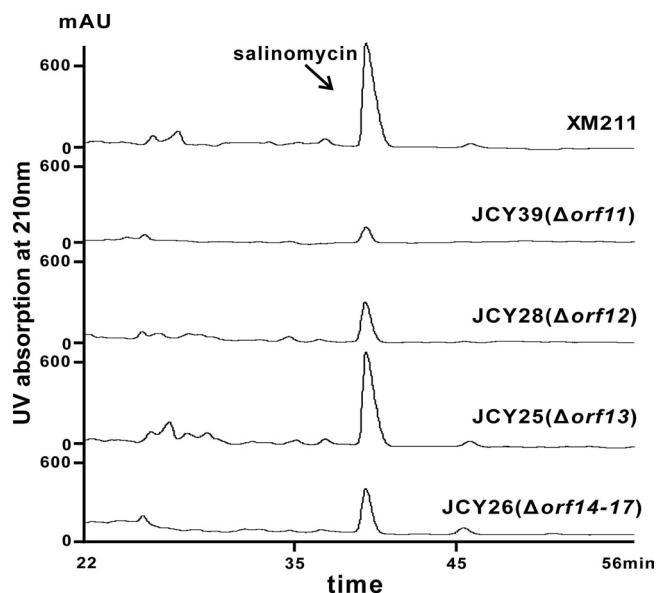


FIG 5 HPLC analysis of mutants of *S. albus* XM211 for boundary determination.

**Other ancillary genes.** The P450 monooxygenase SlnF, homologous to NigD in the nigericin biosynthesis gene cluster with 35% identity and 52% similarity, and the cognate ferredoxin SlnE are likely to accomplish the hydroxylation at C-20. However, gene replacement of *slnF* resulted in a mutant JCY24, which completely lost salinomycin productivity (Fig. 6B), suggesting that the hydroxylation by SlnF may take place prior to the release of the mature polyketide chain from ACP.

SlnM is predicted to be an *O*-methyltransferase homologous to

TcmP in tetracenomycin biosynthesis (37% identity, 59% similarity), which catalyzes methylation of a carboxyl group (6). Although methylated salinomycin was not detectable in XM211 fermentation extracts through LC-MS analysis, the function of *slnM* was preliminarily investigated through gene replacement. Surprisingly, the peak of salinomycin disappeared and a new peak emerged ( $m/z$  791.5  $[M+Na]^+$ ) in the *slnM* mutant JCY38 (Fig. 6B). The chemical structure of this novel derivative needs to be further confirmed, which may shed new light on its involvement in salinomycin biosynthesis.

The encoded product of *slnR* is similar to Las4 (42% identity, 52% similarity) in the lasalocid gene cluster and belongs to the LuxR family of transcriptional regulators (25, 35), most of which are positive regulators of antibiotic biosynthesis. Inactivation of *slnR*, through gene replacement with the *aac(3)IV* gene, abolished the production of salinomycin in the resulting mutant JCY20, which clearly indicated that SlnR is a positive pathway-specific regulator of salinomycin biosynthesis (Fig. 6B).

The gene products of *slnTI* and *slnTII* are significantly similar to TnrB2 and TnrB3 of the tetronecin biosynthesis gene cluster, with 64% and 39% identities, respectively. The ATP-binding protein TnrB2 and the transmembrane protein TnrB3 are presumed to form an ATP-dependent efflux system for polyether tetronecin self-resistance in *Streptomyces longisporoflavus* (20). SlnTI and SlnTII probably confer salinomycin resistance on the producer, *S. albus* XM211.

**Proposed salinomycin biosynthesis pathway.** Taking all this information into account, we have proposed a mechanism for salinomycin biosynthesis (Fig. 7). Loading of the malonyl-CoA onto SlnA1 is followed by condensation steps with 5 malonyl-CoA, 6 methylmalonyl-CoA, and 3 ethylmalonyl-CoA extender units. Dehydration at C-2–C-3 and C-18–C-19 is speculated to

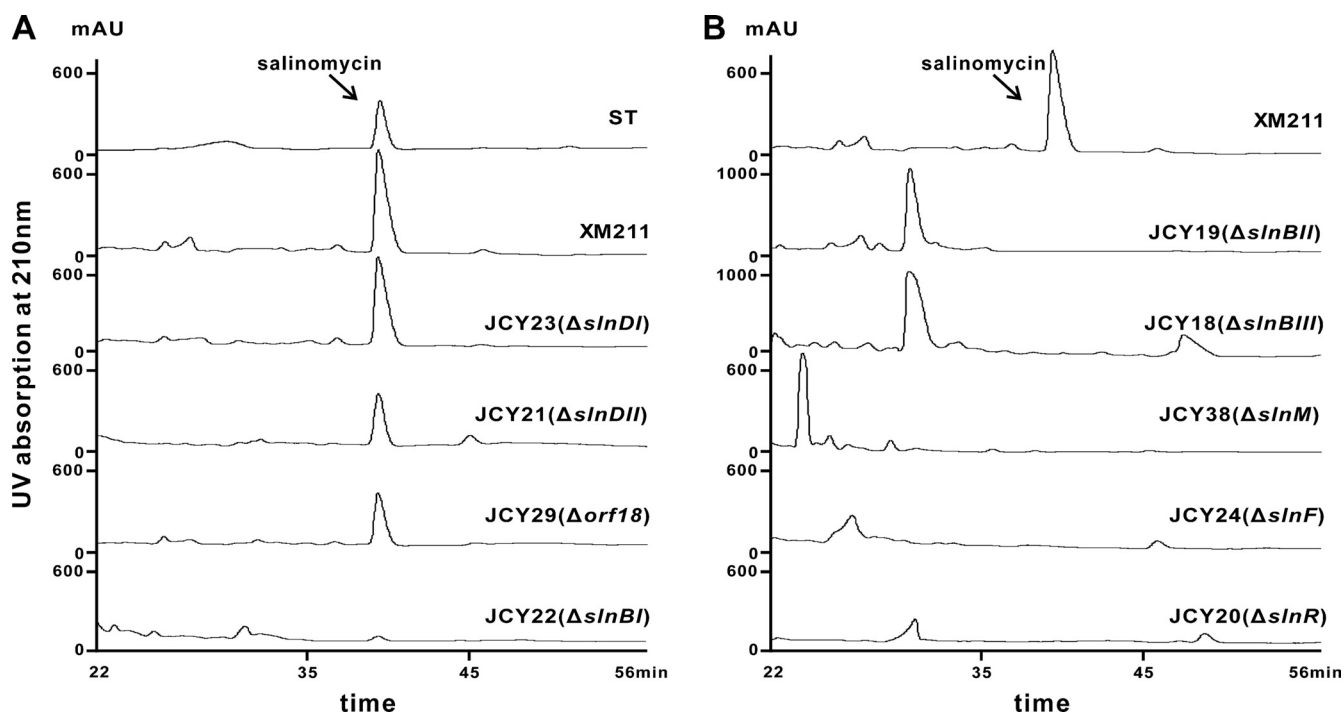


FIG 6 Comparison of the *S. albus* XM211 wild type and its various mutants by HPLC. ST, salinomycin standard purchased from Sigma.



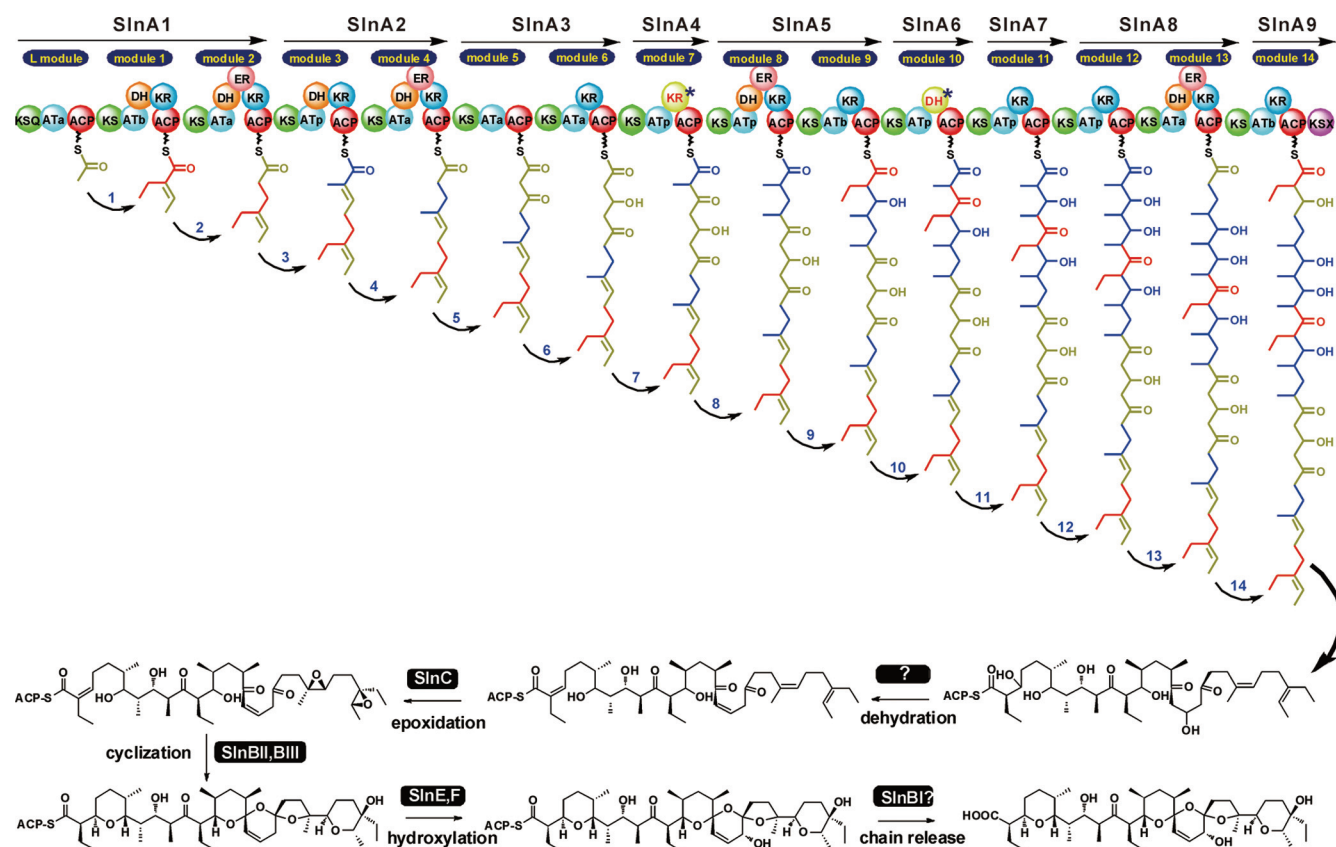


FIG 7 Proposed biosynthesis pathway for salinomycin.

occur on the mature polyketide chain. While still bound to the ACP, the diene intermediate is thought to be converted to the diepoxide through the action of SlnC, which serves as the substrate for SlnBII and SlnBIII for the subsequent epoxide-opening and ring closure reactions. Afterward, SlnF probably carries out the hydroxylation at C-20 when the polyketide chain is still enzyme bound. The final release of the fully modified polyether chain from the PKS is probably accomplished by SlnBI or other, uncharacterized enzymes (Fig. 7).

## ACKNOWLEDGMENTS

This work received financial support from the National Natural Science Foundation of China, the Ministry of Science and Technology (973 and 863 Programs), the Ministry of Education, and the Shanghai Municipal Council of Science and Technology.

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