Cloning of Separate Meilingmycin Biosynthesis Gene Clusters by Use of Acyltransferase-Ketoreductase Didomain PCR Amplification

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Five meilingmycins, A to E, with A as the major component, were isolated from Streptomyces nanchangensis NS3226. Through nuclear magnetic resonance (NMR) characterization, meilingmycins A to E proved to be identical to reported milbemycins α11, α13, α14, β1, and β9, respectively. Sequencing of a previously cloned 103-kb region identified three modular type I polyketide synthase genes putatively encoding the last 11 elongation steps, three modification proteins, and one transcriptional regulatory protein for meilingmycin biosynthesis. However, the expected loading module and the first two elongation modules were missing. In meilingmycin, the presence of a methyl group at C-24 and a hydroxyl group at C-25 suggests that the elongation module 1 contains a methylmalonyl-coenzyme A (CoA)-specific acyltransferase (ATp) domain and a ketoreductase (KR) domain. Based on the conserved motifs of the ATp and KR domains, a pair of primers was designed for PCR amplification, and a 1.40-kb expected fragment was amplified, whose sequence shows significant homology with the elongation module 1 of the aveAl-encoded enzyme AVES1. A polyketide synthase (PKS) gene encoding one loading and two elongation modules, with a downstream C-5-O-methyltransferase gene, meiD, was subsequently localized 55 kb apart from the previously sequenced region, and its deletion abolishes meilingmycin production. A series of deletions within the 55-kb intercluster region rules out its involvement in meilingmycin biosynthesis. Furthermore, gene deletion of meiD eliminates meilingmycins D and E, with methyls at C-5. Our work provides a more specific strategy for the cloning of modular type I PKS gene clusters. The cloning of the meilingmycin gene clusters paves the way for its pathway engineering.

Backbones of macrolides (e.g., erythromycin), polyenes (e.g., candicidin), polyethers (e.g., nanchangmycin), ansamycins (e.g., ansamitocin), and the polyketide portion of peptide-polyketide hybrids (e.g., oxazolomycin) are usually biosynthesized from simple carboxylic acids by the modular polyketide synthases (PKSs; type I PKSs) (6, 8, 30, 37, 38). Type I PKSs are multifunctional polypeptides and divided into modules, and each module is responsible for the incorporation of one carboxylic acid into the polyketide backbone. A minimal module is usually an ordered array of ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains, with the β-carboxyl group unreduced in its thioester products (16). KS catalyzes the carbon-carbon bond formation between the incoming extender unit and polyketide intermediate by the Claisen condensation, whereas ACP serves as the carrier for both the incoming extender units and the extended chains using a covalently bound phosphopantetheine arm. Selection and loading of extender acyl units is executed by acyltransferase (AT) domains, which contributes to the structural diversity of polyketides by recruiting different extender units such as acetate-derived malonyl coenzyme A (CoA), propionate-derived methylmalonyl-CoA, and glycerol-derived methoxymalonyl-ACP (5, 35). Deduced from sequence alignments of AT domains, the specificity for malonyl-CoA is usually determined by the presence of an HAFH motif in the active sites, whereas methylmalonyl-CoA is commonly selected by AT domains with a YASH motif in the active sites (7, 11, 24, 36). The fully extended polyketide chains bound to terminal enzymatic templates like acyl-ACP thioesters are usually released and cyclized by a type I thioesterase (TE) domain fused to the carboxyl terminus of the last elongation module.

Structure diversity of polyketides could also be attributed to the further modifications of β-ketothioester to hydroxythioester, α,β-unsaturated thioester, or the fully reduced β-carbon atom in the presence of ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains (27). KR domains usually contain a conserved motif, GGXGXXGXXA, for NADP(H) binding and conserved residues of K2664, S2686, Y2699, and N2703 for catalysis like that in KR6 of 6-deoxyerthronolide B synthase (DEBS) (25). Moreover, different γ-hydroxyl or γ-hydroxyl stereoisomers regarding the chiral β-carbon are determined by the presence or absence of an LDD motif in the KRs (4, 25).

Antibiotic biosynthetic genes are often clustered together, which suggests successful cloning of a complete gene cluster through the localization of any essential structural, regulatory, or resistant genes (20). Since the cloning of the erythromycin biosynthetic gene cluster, numerous type I PKSs gene clusters have been cloned either through Southern hybridization using the DEBS genes as probes (12) or through PCR amplifications with degenerate primers of the erythromycin KS domains (21).

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However, since nearly each actinomycete genome contains many type I PKS gene clusters, this strategy will result in the cloning of dozens of clones from a genomic library. Further identification of the target PKS gene cluster requires additional strategies or is quite time-consuming. Therefore, a more straightforward strategy is required for the efficient cloning of a PKS biosynthetic gene cluster of interest.

Meilingmycin A, a 16-membered macrolide and identical to milbemycin, is the major product of Streptomyces nanchangensis (31). It has aglycone similar to and anthelmintic activity comparable to those of avermectins (3). However, meilingmycin A has a 5-carbon side chain on the methyl group of C-4, a saturated C-22-O-C-23 bond, and no sugar moiety attached (Fig. 1B). A 103-kb genomic region of S. nanchangensis was identified through the PCR amplifications of homologous fragments of aveE, aveF, and aveTE (14, 19), and its involvement in meilingmycin biosynthesis was confirmed by the deletion of an internal 24-kb genomic fragment (32).

In this work, the previously cloned 103-kb region was sequenced and analyzed through gene deletions. However, the PKS genes encoding the loading and first two elongation steps for meilingmycin biosynthesis were not found in this region. Herein, a new strategy of acyltransferase-ketoreductase didomain PCR amplification was applied to localize this undefined segment, which was proved to be located 55 kb away from the previously identified gene cluster.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, culture conditions, and general techniques.** Bacterial strains and plasmids used in this study are listed in Table S2 in the supplemental material. S. nanchangensis NS3226, the wild-type producer for meilingmycin, was used for meilingmycin isolation and generation of mutant strains. DH10B (Gibco-BRL) and ET12567 (pUZ8002) (23) were used as Escherichia coli hosts for cloning and conjugation, respectively. Cosmid vector pHZ1358 (29) and fosmid vector pCC2FOS (Epicentre Biotechnologies) were used for constructing genomic library. Two pHZ1358-based cosmid genomic libraries, NS1 and NS2, and one pCC2FOS-based fosmid genomic library, NS3, had been constructed for this work. pJTU1278 and pJTU1289 derived from pHZ1358 were used for gene inactivation. pMD18-T (Takara) and pBlueScript II SK(+) (Stratagene) were used for DNA sequencing.

YMS solid medium (13) was used for S. nanchangensis sporulation, and tryptic soy broth (TSB) supplemented with 10.3% sucrose and 1% yeast extract was used for the growth of mycelia for the isolation of total DNA. SFM solid medium (17) was used for conjugation between E. coli and Streptomyces. Luria-Bertani medium was used for E. coli propagation. For Streptomyces, thioestrepton and apramycin were used at 12.5 μg/ml and 15 μg/ml, respectively, in solid and liquid media.

Recombinant DNA techniques used for E. coli were described by Sambrook and Russell (26), and those used for Streptomyces were described by Kieser et al. (17).

**Fermentation, purification, and analysis of meilingmycins.** One 8-day-old YMS agar plate of the wild-type strain or mutants was extracted with absolute ethanol, and the extract was concentrated under reduced pressure to yield an oily substance, which was further extracted with 1 ml methanol. The methanol extract was analyzed by liquid chromatography-mass spectrometry (LC-MS) with the Agilent 1100 series LC/MSD Trap system. A high-performance liquid chromatographer (HPLC) was operated at a flow rate of 0.2 ml/min, with methanol-water (80/20, vol/vol) on an Agilent SB-C18 (2.1- by 50-mm, 2-μm) column. The
ion trap mass spectrometer was operated with the electrospray ionization source in the positive-ion mode. The drying gas flow was at 8 liters/min, and nebulizer pressure was 30 lb/in². The drying gas temperature used was 325°C, and the fragmentation amplitude was varied from 1.0 to 1.8 V.

For structure elucidation, the 8-day-old culture broth of the wild type (12 liters) was extracted with an equal volume of ethanol three times. The ethanol extract was concentrated to approximately 500 ml under reduced pressure and extracted further with an equal volume of ethyl acetate five times. The ethyl acetate extract was concentrated under reduced pressure to yield 4.43 g of an oily substance, which was chromatographed through silica gel (30 g, 300 to 400 mesh) and eluted with chloroform. Fractions possibly containing meilingmycins were combined and concentrated to give 0.6 g of crude sample. Methanol extract of the crude sample was applied to the preparative HPLC (Shimadzu) using Shim-pack (Prep-ODS, 20 by 250 mm; Shimadzu) and eluted with methanol-water (90:10, vol/vol) as a mobile phase at 6 ml/min to yield 15 fractions. Detected by LC-MS, fractions 3, 4, and 6 probably containing meilingmycins were further purified by thin-layer chromatography (TLC) on HF254 silica gel with chloroform-methanol (10:1, vol/vol) as a solvent. Together with fractions 9 and 10, five different meilingmycins were purified by preparative HPLC for the second time with the same condition to yield meilingmycin D (4.7 mg), E (2.9 mg), A (12.4 mg), B (3.9 mg), and C (2.1 mg).

Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker AM 500 NMR spectrometer in CDCl₃ at 25°C, using the solvent as internal reference downfield of tetramethylsilane (TMS) at 0 ppm.

**Sequence and analysis of meilingmycin biosynthetic gene clusters.** Cosmids/contig assembly and base editing were performed with the Phred/Phrap/Consed package (9). The sequence data were analyzed with the FramePlot-3.0beta online program (15) and GeneMark.hmm for Prokaryotes (version 2.4) using Streptomyces avermitilis as the model organism (16). DNA and deduced protein sequence homology searching was performed using BLAST (1). The PKS region was analyzed with NRPS-PKS (2). Methylmalonyl-CoA-specific AT (ATp)-KR domain PCR amplification. A forward primer ATp1 (5'-CCGGTCGACTCGCCTTCCACTCGC-3') was designed, according to the conserved methylmalonyl-CoA-specific motif PVYDASHC of the AT domain. A degenerate reverse primer, KRp (5'-CCGGTCGCTGGCGGTA TsgAgFaGTTT-3') -“s” stands for C, G, or T; “g” stands for C or G; “y” stands for C or T), was designed for the NADP(H) binding motif T(V/I)LITGGTG of the KR domain. The resulting 1.4-kb amplified PCR products with primers ATp1 and KRp was cloned into the pMD18-T vector and sequenced. A pair of specific homologous primers, meiAT-P1 (5'-AAAGGTACCTCGAGGTCGACGGTATC-3') and meiAT-P2 (5'-ACCGGTCCCTGAGATGTTCC-3') was designed, according to the sequence results. Through PCR screening with this pair of primers, four cosmids (1F1, 14F7, 14G1, and 20G1) were cloned from the genomic library NS3, and a cosmids (1D8) was cloned from the genomic library NS2.

**Gene deletion of the region containing the amplified ATp-KR fragment.** Complete digestion of cosmid 10D8 DNA by KpnI and religation resulted in the construction of pJTU4459, in which an internal 18.6-kb region of 1D8 was deleted and the 3.5-kb and 4.5-kb flanking fragments were connected. Then, a 1.50-kb KpnI fragment containing aceJ/SIV was amplified from pHGF827 (6) with primers SK-P1 (5'-AACGACGTACCGCAGCGGCTGAT-3') and SK-P2 (5'-AAAGTGACCGGCCCTGTTAGAACJTAG-3'), with engineered KpnI underlined) and SK-P2 (5'-AAAGTGACCGGCCCTGTTAGAACJTAG-3', with engineered KpnI underlined), which was inserted into the two flanking fragments to generate pJTU4460 for subsequent conjugation, as previously described (30). Allelic replacement of the 18.6-kb region in the mutant was confirmed by Southern hybridization. A pair of primers, 10D8-C1 (5'-CCGGCCGATCTGCAGAAAAGC-3') and 10D8-C2 (5'-CTCGAGTGACACTTCGCGGTGTGTT-3'), was designed for amplifying the probe. Comparison between the wild-type and mutant HYL23 was conducted through fermentation, extraction, and LC-MS analysis.

**Gene deletion of meiD.** The 8.40-kb KpnI fragment containing meiD from cosm1 1G1 was firstly cloned into pBluescript SK (+) to generate pJTU4465a. Then, the 8.40-kb KpnI fragment from pJTU4465a was inserted into pJTU129 to generate pJTU4466. The orf1-aceJ/SIV cassette was amplified from pJT775 (10), with the primers MeiD-T1 (5'-ATGGCCTTGAACATCGCCATGGCGCCTCCGTGGTGTCTGTT-3') and MeiD-T2 (5'-TGATCAGC TCTTGCGACCGACATCCCAGGGGCTTGGCGGTatcggggaagttg-3') (pJT775 homologous sequences are in lowercase). The resulting PCR product was used to replace the internal 615-bp region of meiD in pJTU4466 to generate pJTU4466 through Red Direct Technology (10), which was used to generate mutant HYL28 through conjugation. Allelic replacement of meiD in the HYL28 mutant was confirmed by PCR amplification with primers meiD-C1 (5'-TGAAAA CGGACCGACCGAAGAC-3') and meiD-C2 (5'-GGCGAACAGGACCCGACGA-3'). Comparison between the wild-type and mutant HYL28 was performed through fermentation, extraction, and LC-MS analysis.

**Supplemental material.** Detailed constructions for deletion of the pks1-pks2 region, deletion of meiC, deletion of meiE, deletion of the 55-kb intercluster region, determination of the left and right boundaries, NMR data for structural elucidation of meilingmycins, and chromosomal walking were described in the supplemental material.

**Nucleotide sequence accession number.** The nucleotide sequence reported in this paper was deposited in GenBank under accession number FJ952082 (Fig. 2A).

**RESULTS**

**Structure elucidation of meilingmycins.** HPLC analysis of the fermentation broth of *S. nanchangensis* NS3226 identified five components of meilingmycins with similar UV absorbance, including the previously characterized meilingmycin A (Fig. 1A) (31). From a 12-liter liquid culture of the wild-type NS3226, meilingmycin A (12.4 mg, m/z 649.7 [M + Na]+), B (3.9 mg, m/z 663.7 [M + Na]+), C (2.1 mg, m/z 663.7 [M + Na]+), D (4.7 mg, m/z 567.8 [M + Na]+), and E (2.9 mg, m/z 583.7 [M + Na]+) were purified as amorphous powder. According to 1-H-NMR, 13C-NMR, heteronuclear multiple-bond correlation (HMBC), heteronuclear multiple-quantum coherence (HMQC), and 1H,1H correlated spectroscopy (1H,1H COSY) analyses, the structures of A to E were proved to be identical with those of milbemycin α11, α13, α14, β1, and β9, respectively (Fig. 1B) (see the supplemental material). Meilingmycin A/milbemycin α11 is the main product in *S. nanchangensis* NS3226, whereas milbemycin β1/milbemycin D is the major component in milbemycin producer *Streptomyces hygroscopicus* subsp. *aureolacinmosus* SANK 60286 (34).

**Sequence analysis of the previously cloned 103-kb region.** Analysis of the available DNA sequence (103,513 bp) (Fig. 2B, circled with dotted lines) led to identification of 37 open reading frames (ORFs), as shown in Fig. 2B and Table 1; see also Table S1 in the supplemental material. The overall gene organization between meilingmycin and avermectin biosynthetic gene clusters is different. However, homologous genes for aveC, aveE, aveF, and aveR were identified and named meiC (52% identity/66% similarity), meiE (62% identity/74% similarity), meiF (64% identity/78% similarity), and meiR (49% identity/60% similarity), respectively, in the meilingmycin biosynthetic gene cluster.

Five ORFs (pks1, pks2, meiA2, meiA3, and meiA4) encoding typical type I PKS subunits were identified, among which meiA2, meiA3, and meiA4 are highly homologous with aveA2, aveA3, and aveA4 of the avermectin biosynthetic gene cluster, respectively (Fig. 2B; Table 1). MeiA2 has the same domain organization as AVES2 (14), putatively encodes modules 1 to 6, and is responsible for the incorporation of C-13 to C-20 to the meilingmycin polyketide backbone. MeiA3 putatively contains modules 7 to 9 and is responsible for the incorporation of C-7 to C-12 to the backbone. There are KR, DH, and ER domains in module 7, which catalyze the full reduction of the carbonyl group on C-13. However, the corresponding module in AVES3 contains a dysfunctional DH domain and lacks an ER domain, which leads to the retention of a hydroxyl group on C-13, serving as the attachment site for the lean-
drose moiety (14). MeiA4 shows nearly the same domain organization as AVES4 for the incorporation and modification of C-1–O-C-6 (14), with the KR domain of module 10 (KR*) presumably active, as deduced from sequence analysis, but nonfunctional, as predicted from the meilingmycin structure. However, an inactive KR domain (KRI) is present within module 11 because it has a 16-residue deletion, including the required Lys residue, and also does not have the required Tyr and Asn residues (25).

**pks1 and pks2** were 22.0 kb away from meiA2 (Fig. 2B). Pks1 contains two modules (see Table S1 in the supplemental material). One is aberrant (KS1–AT–ACP), with a shorter 123-amino acid (aa) KS domain and an RAFH motif in the AT domain, and the other is a malonyl-CoA-specific extension module (KS–malonyl-CoA specific AT [ATa]–KR–ACP). Pks2 has a methyldmalonyl-CoA-specific extension module (KS–ATp–DH–KR–ACP) and a TE domain at the C terminus (see Table S1). A mutant with the pks2-orf15 region deleted maintains the same meilingmycin productivity as wild-type NS3226, as detected by LC-MS (see Fig. 4C), which clearly indicates that meiA2 is at the left boundary of the meilingmycin biosynthetic gene cluster. So far, the PKS portion responsible for the synthesis of C-21–O-C-26 was still missing, and new strategy was required to localize this portion.

**ATp-KR didomain amplification.** The presence of a methyl group at C-24 of meilingmycins predicts that the AT domain of the first extension module (module 1) is methylmalonyl-CoA specific (ATp), and the retention of a hydroxyl group at C-25 suggests the presence of the KR domain in the same module. Based on the canonical organization of PKS modules, module 1 was deduced to have a KS–ATp–KR–ACP assembly (Fig. 3A) (16). Therefore, a pair of degenerate primers was designed for amplifying the ATp-KR region using the total DNA of *S. nanchangensis* as template. Based on the alignment of available AT domains of MeiA2, MeiA3, and MeiA4, a forward primer ATp1 was designed, according to the conserved motif PVDA SHC (aa 190 to 197) of the ATp domain (Fig. 3B). Based on the alignments of available KR domains of MeiA2, MeiA3, and MeiA4, the reverse primer KRp was designed within the
conserved NADPH-binding motif T(V/I)LITGGTG of the KR domain (Fig. 3C).

Using the genomic DNA of *S. nanchangensis* NS3226 as the template, an expected 1.40-kb DNA fragment was amplified (Fig. 3D), cloned, and sequenced. The sequences of all the cloned 1.40-kb fragments are identical and show high homology with that of meiA1. Based on this newly obtained sequence, a pair of primers, meiAT-P1 and meiAT-P2, was designed and used to screen the whole genomic library. Four fosmids (1F1, 14E7, 14G1, and 20G1) and one cosmid (10D8) were identified using the genomic DNA of *S. nanchangensis* (Fig. 2A). Chromosome walking and sequence analysis of a newly cloned 1.40-kb region, chromosomally located downstream of meiAT, resulted in the cloning of a continuous 180-kb region (Fig. 2B). Moreover, a C-5–ketoreductase (orf69), and meilingmycin biosynthesis. Functional analysis of orf24-orf69

TABLE 1. Deduced functions of mei biosynthetic genes

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<th>Proposed function</th>
<th>Homolog/origin</th>
<th>% Identity/ % similarity</th>
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<tr>
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<td>Intracellular protease/amidase</td>
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<td>64/75</td>
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<tr>
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<td>LysR family transcriptional regulator</td>
<td>AbaB2/Saccharopolyspora erythraea NRRL 2338</td>
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* Gene products not involved in meilingmycin biosynthesis, as confirmed through gene deletions, are listed in Table S1 in the supplemental material.

* Gene products involved in meilingmycin biosynthesis, as confirmed through gene deletions or complementation to meiA1 mutant. KR*, inactive ketoreductase domain; KR, nonfunctional ketoreductase domain deduced from the meilingmycin structure.
18.6-kb ATp-KR-containing region in HYL23 covers orf60–meiA1 (Fig. 4C), and the exclusion of orf60–orf69 in the meilingmycin biosynthesis confirms the unambiguous involvement of meiA1 in meilingmycin biosynthesis.

Gene **meiD** encodes a methyltransferase for the biosynthesis of meilingmycins D and E. The involvement of meiD in meilingmycin production was proved through gene replacement. Using the ReDirect Technology (10), an internal 615-bp DNA region of meiD was replaced by the oriT-aac(3)IV cassette, which generated the mutant HYL28 (Fig. 5A). Allelic replacement of meiD in the HYL28 mutant was confirmed by PCR amplification with primers meiD-C1 and meiD-C2. An expected 1.90-kb product was amplified from the genomic DNA of HYL28, whereas a 1.20-kb expected product was amplified from the genomic DNA of wild-type NS3226 (Fig. 5B). Analyzed by LC-MS, the peaks of meilingmycins D and E disappeared in the fermentation extract of HYL28, while meilingmycins A, B, and C were still produced in this mutant (Fig. 5C). Thus, meiD proved to be responsible for the methylation of the hydroxyl group on C-5 of meilingmycins D and E.

Moreover, meiD also serves as the right boundary of the meilingmycin biosynthetic gene clusters, because the deletion of the 10 ORFs (orf70–orf79) downstream of meiD has no effect on the meilingmycin production in mutant HYL29 (Fig. 4C) (see the supplemental material).

**DISCUSSION**

Genes involved in the biosynthesis of single secondary metabolites are usually clustered, which could be considered a general principle and serves as the guideline for the cloning of new biosynthetic gene clusters. However, more and more biosynthetic genes are not found closely located, making it very difficult to clone all required genes and to perceive the complete biosynthetic mechanisms. It has been reported that the genes for ansamitocin (37), clavam (33), and moenomycin A (22) biosyntheses are separated into two or three parts on the chromosome. For example, cluster I and cluster II of the ansamitocin biosynthetic genes are separated by a 30-kb intercluster region. However, only three genes required for the synthesis of the 3-amino-5-hydroxy-benzonate (AHBA) starter unit are present in cluster II, and all other essential genes, including the PKS machinery, are located in cluster I (37). Through a series of large-fragment deletions, the meilingmycin biosynthetic genes, especially the PKSs, were proved to be divided into two clusters by a 30-kb intercluster region. However, only three genes required for the synthesis of the 3-amino-5-hydroxy-benzonate (AHBA) starter unit are present in cluster II, and all other essential genes, including the PKS machinery, are located in cluster I (37). Through a series of large-fragment deletions, the meilingmycin biosynthetic genes, especially the PKSs, were proved to be divided into two clusters by a 30-kb intercluster region. However, only three genes required for the synthesis of the 3-amino-5-hydroxy-benzonate (AHBA) starter unit are present in cluster II, and all other essential genes, including the PKS machinery, are located in cluster I (37). Through a series of large-fragment deletions, the meilingmycin biosynthetic genes, especially the PKSs, were proved to be divided into two clusters by a 30-kb intercluster region. 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synthetic gene cluster with different strategies had been tried. First, four of the eight previously identified PKS contigs were deleted, and none of the four mutants lost meilingmycin productivity (data not shown) (31, 32). Second, routine chromosome walking was also performed on both sides of the previously cloned meilingmycin biosynthetic genes, and subsequent deletions on both sides failed to abolish meilingmycins biosynthesis in the mutants. Third, based on the saturated C-22

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\text{O} \text{C-23 in meilingmycins, a pair of degenerate primers was designed, according to the conserved motifs of PKS ER domains. Unfortunately, too many positive cosmids were identified from the genomic library, making it impossible to identify the rest of the meilingmycin gene cluster through numerous deletions.}
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The strategy of amplifying the ATp-KR didomain through PCR utilizes the presence of a hydroxyl group at C-25, usually catalyzed by the KR domain of the downstream module, and the presence of a methyl group at C-24, reflecting the nature of the AT domain of the same module that recruits a methylmalonyl-CoA extender unit. Therefore, the first extension module

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\text{of mei PKS is supposed to have ATp and KR domains. According to the evolutionary hypothesis on PKS, the KR domain is the first reduction domain inserted between AT and ACP to form a KS-AT-KR-ACP assembly (16). The predicted length between either the YASH motif of ATa or the HAFH motif of KR is 1.40 kb (Fig. 4). For modules with DH domains, the DH domains are usually present between the AT and KR domains to form a KS-AT-DH-KR-ACP assembly, and the amplified lengths between the AT and KR should be increased to about 2.10 kb. Furthermore, for modules with complete reductions, the ER domains were generally inserted between DH and KR domains as a late event in PKS evolution to form a KS-AT-DH-ER-KR-ACP assembly, which is supposed to give an amplified 3.0-kb large fragment with the pair of AT-specific and KR-specific primers.}
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Through amplification of ATp-KR and ATp-DH-KR regions, the lasalocid A biosynthetic gene clusters was successfully cloned in our group (H. Wang and L. Bai, unpublished data).

Since most of the modules in one polyketide biosynthetic pathway have evolved from an ancestor module (16), the spec-
ificity for amplifying the correct ATP-KR region could be highly increased if the degenerate primers are designed based on available domains/modules from the same PKS machinery. In our experiment, a nondegenerate ATP-specific forward primer was designed according to the alignment of mei-AT6p, mei-AT7p, and mei-AT9p, and a degenerate KR-specific reverse primer was designed based on the alignment of mei-KR4 to mei-KR9 and mei-KR12 (Fig. 4).

Due to the presence of an atypical IPAH motif in the ATP domain of the loading module, the meilingmycin biosynthesis is initiated with either malonyl-CoA (meilingmycins A, B, D, and E) or methylmalonyl-CoA (meilingmycin C) and proceeded by condensation of seven malonyl-CoA and five methylmalonyl-CoA extender units (Fig. 6). In the biosynthesis of avermectin, AveC was required for the dehydration of C-22–C-23 to yield the double bond between C-22 and C-23 with an unknown mechanism (28). MeiC, the homolog of AveC in the mei cluster, was proved to be essential for meilingmycin biosynthesis through gene inactivation (see Fig. S2 in the supplemental material) and may be involved in the complete reduction of the keto group on C-23 to generate the saturated bond between C-22 and C-23.

After being released and cyclized by the TE domain of MeiA4, the polyketide chain of meilingmycin is probably oxidized by the cytochrome P450 hydroxylase MeiE to generate a furan ring between C-8 and C-6 (meilingmycins A, B, and C), a hydroxyl group on C-8 (meilingmycin D), or two hydroxyl groups on both C-8 and C-6 (meilingmycin E). meiE could restore avermectin productivity in the aveE mutant (J. Qiu and L. Bai, unpublished data). However, AveE from the avermectin cluster catalyzes only the formation of a furan ring between C-8 and C-6 (13). Subsequently, the C-5 keto group is reduced to hydroxyl by the ketoreductase MeiF, an AveF homolog, after which the meilingmycin biosynthetic pathway is presumably diverged by either methylation or hydroxylation/acylation. Surprisingly, the inactivation of meiF abolishes meilingmycin productivity, and so far, no new derivative has been detected from the mutant through LC-MS (see Fig. S3 in the supplemental material). For the intermediates without the furan ring, the hydroxyl on C-5 was methylated by the methyltransferase MeiD to generate minor components (meilingmycins D and E). On the other hand, through hydroxylation/acylation at C-27 still to be characterized, major components of meilingmycins carrying a 3-methylbut-2-enoyl (meilingmycins A and C) or a 3-methylpent-2-enoyl side chain (meilingmycin B) are formed. However, the putative genes for the synthesis of these two side chains have not been found in the 185.0-kb sequenced region,
which suggests that these genes are located in other regions of the chromosome.

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