Rapid Cloning and Heterologous Expression of the Meridamycin Biosynthetic Gene Cluster Using a Versatile *Escherichia coli*–*Streptomyces* Artificial Chromosome Vector, pSBAC[⊥]

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Expression of biosynthetic pathways in heterologous hosts is an emerging approach to expedite production improvement and biosynthetic modification of natural products derived from microbial secondary metabolites. Herein we describe the development of a versatile *Escherichia coli*–*Streptomyces* shuttle Bacterial Artificial Chromosomal (BAC) conjugation vector, pSBAC, to facilitate the cloning, genetic manipulation, and heterologous expression of actinomycetes secondary metabolite biosynthetic gene clusters. The utility of pSBAC was demonstrated through the rapid cloning and heterologous expression of one of the largest polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) biosynthetic pathways: the meridamycin biosynthesis gene cluster (*mer*). The entire *mer* gene cluster (~90 kb) was captured in a single pSBAC clone through a straightforward restriction enzyme digestion and cloning approach and transferred into *Streptomyces lividans*. The production of meridamycin (**1**) in the heterologous host was achieved after replacement of the original promoter with an *ermE** promoter and was enhanced by feeding with a biosynthetic precursor. The success of heterologous expression of such a giant gene cluster demonstrates the versatility of BAC cloning technology and paves the road for future exploration of expression of the meridamycin biosynthetic pathway in various hosts, including strains that have been optimized for polyketide production.

Natural products derived from microbial secondary metabolites remain a unique source for privileged chemical scaffolds in drug discovery efforts.[1,2] However, development of natural products for therapeutic application is often hindered by the limited amount of material from native producing organisms. The speed at which current technologies enable the discovery and chemical modification of novel bioactive microbial metabolites has made it impractical to optimize each new microorganism by traditional strain improvement procedures. On the other hand, combinatorial biosynthesis, a technology of manipulating genes responsible for the biosynthesis of secondary metabolites, has emerged as a promising tool to generate novel natural product analogues that might not be accessible by semisynthesis. Such operations, unfortunately, are not easily applied to microbial metabolites for which the natural producers are genetically difficult to handle, slow growing, uncultivable, or even unknown. Consequently, there has been a growing interest in reconstituting the biosynthesis of bioactive natural products in genetically amenable, fast growing or fermentation-optimized strains.[3] By using a genetically amenable and fermentation friendly heterologous host, it is possible to enhance the titer of a target compound and eliminate the production of undesirable side products through a combination of traditional medium optimization processing and a direct genetic engineering approach. For those biologically active natural products that belong to either polyketides or nonribosomal peptides, biosynthesis is a relatively homogeneous process, and most of them share the same or closely related precursors, biosynthetic enzymes, and product export machinery. Therefore, it is possible to reuse productive strategies for overproduction in different cases, and neither the overproduction process nor the genetic manipulation system has to be individualized for each product or microorganism. Indeed, studies have shown that in at least two industrially utilized high-producer strains, *Saccharopolyspora erythrae* (erythromycin) and *Streptomyces fradiae* (tylosin), the overproduction characteristics are mainly due to mutations in non-PKS genes and therefore should operate on other PKSs.[4]

Since the genes responsible for the production of microbial secondary metabolites are located in clusters that are usually larger than 25 kb, development of vectors capable of cloning the entire gene clusters as well as shuffling these genetic segments between different hosts would be desirable to harness the biosynthetic potential from different sources. Recently, a number of *E. coli*–*Streptomyces* shuttle Bacterial Artificial Chromosomal (BAC) vectors have been built to meet this need.[5–7] These BAC vectors can maintain the stability of large DNA inserts due to the low-copy or even single-copy origin of plasmid replication. However, the low-copy state of BAC vectors is also at a disadvantage for various genetic manipulations due to very low levels of DNA recovery and consequently reduced purity of DNA with respect to host DNA. In addition, these vectors utilize the *attP-int* locus from phage ΦC31 to integrate the cloned DNA into the specific chromosomal *attB* site in various *Streptomyces* species. Despite their broad host range and high integration efficiency, detrimental effects on antibiotic production have been reported in some strains due to the integration of these vectors into the ΦC31 *attB* site.[8] For these reasons and also to accommodate the need for two compatible integrating vectors in the same organism, we have developed a new *E. coli*–*Streptomyces* shuttle BAC vector system that enables the convenient switch from single-copy to high-copy replication in *E. coli* and utilizes the phage ΦBT1 *attP-int* site-specific integration system, which is different from yet compatible with a ΦC31 *attP-int* system.[9]

Traditional methods used for cloning gene clusters from *Streptomyces* involve tedious construction and screening of cosmid or BAC libraries generated by partial digestion or random shearing of genomic DNA. Although small gene clusters can readily be included in a single cosmid or BAC clone in a genomic library, capturing larger pathways in a single *E. coli* clone remains challenging, and success appears achievable only after screening a vast number of clones.[10] To date, two rational strategies have been reported to address this problem. One is to construct several

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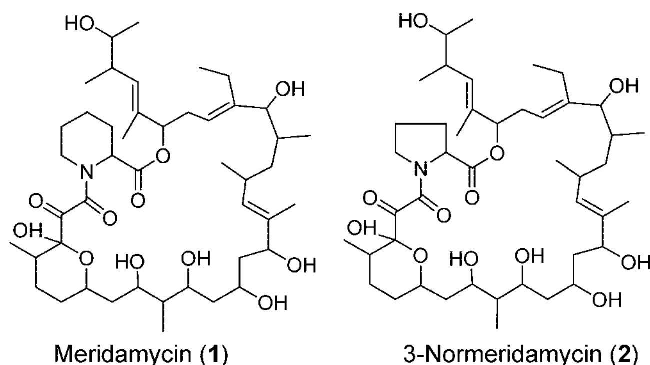
plasmids that harbor subsets of the biosynthesis genes followed by reconstituting the whole pathway through sequential introduction into a heterologous host.[11,12] Another approach is to employ Red/ET recombineering to reassemble the entire pathway by "stitching" several cosmids in *E. coli* prior to mobilizing the whole gene cluster into a heterologous host.[13] Here we report a more straightforward restriction enzyme digestion and cloning approach to capture the entire ~90 kb gene cluster for the neuroprotective polyketide meridamycin (**1**).[14] The production of **1** was detected successfully in *Streptomyces lividans* K4-114 after replacing the original *mer* promoter with an *ermE** promoter. These results demonstrate the value of BAC cloning technology for expressing large biosynthetic gene clusters for production and engineering of natural products in heterologous hosts.



Results and Discussion

Construction of an *E. coli*−*Streptomyces* Conjugative BAC Vector (pSBAC). Various BAC vectors have been used extensively in the present decade for the preparation of DNA libraries to facilitate physical genomic mapping and large-scale DNA sequencing efforts. However, only a few BAC vectors have been developed for the study of microbial secondary metabolite biosynthesis. It is advantageous to construct *E. coli*−*Streptomyces* shuttle BAC vectors that would streamline various genetic manipulations of *Streptomyces* secondary metabolites biosynthetic pathways, including cloning and sequencing of an entire gene cluster, genetic manipulations of the biosynthetic pathway, and heterologous expression of the native or engineered biosynthetic gene clusters in a host that is amenable to in vivo genetic manipulation or has been optimized for the high-yield production of certain types of secondary metabolites. Particular features desirable for such BAC vectors would include (1) capable of accepting and maintaining DNA segments greater than 40 kb; (2) allowing "on command" in vivo amplification of vectors and clones when high yields of DNA are required, such as preparing vector DNA for library construction or isolating cloned DNA for sequencing or genetic engineering; (3) permitting easy shuttle between *E. coli* and various *Streptomyces* strains; and (4) capable of maintaining the stability of the cloned large DNA fragment in the recipient *Streptomyces* cells. An *E. coli*−*Streptomyces* conjugative BAC vector, pSBAC (Figure 1), was constructed in the current work, which possesses all of these features.

Derived from the previously developed CopyControl BAC cloning vector (Epicenter, Madison, WI),[15] pSBAC contains two replication origins—*ori2* for initiation of single-copy replication and *oriV* for initiation of high-copy replication—and a partitioning system (ParA, ParB, and ParC) from *E. coli* F factor. Under normal growth conditions, pSBAC replicates autonomously in *E. coli* in single copy to maintain its stability, which is crucial when large DNA fragments were inserted. Multiple copies of pSBAC or its clone can be induced by L-arabinose to provide increased DNA yield. An origin of transfer (*oriT*) from an IncP transmissible plasmid[16] allows pSBAC to transfer from *E. coli* to various

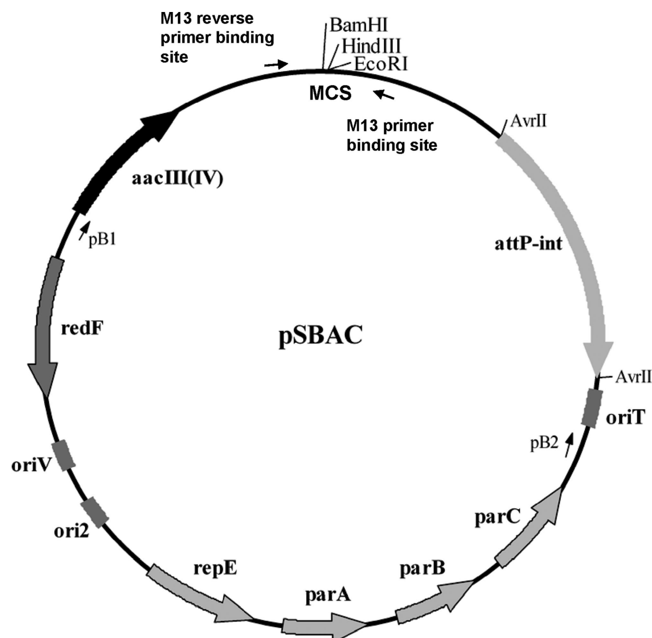


Figure 1. Map of pSBAC. Essential components of the vector are indicated: replication origins *ori2* and *oriV*, partitioning system (ParA−C), apramycin resistance gene *aacIII(IV)*, *oriT*, and ΦBT1 *attP-int*, and unique restriction enzyme recognition sites (*Bam*H I, *Hin*d III, and *Eco*R I).

Streptomyces strains through intergeneric conjugation, which would bypass the need to develop transformation protocols for protoplast formation and regeneration for different *Streptomyces* strains. The ΦBT1 *attP-int* system directs the integration of pSBAC or pSBAC derivatives into the recipient *Streptomyces* chromosome at a specific ΦBT1 *attB* site that is different from, yet compatible with, the commonly used ΦC31 *attB* locus. A single apramycin resistance gene *aacIII*(*IV*) confers selection in both *E. coli* and *Streptomyces*. pSBAC also possesses several features for convenient BAC cloning, including a multiple cloning site (MCS) with a number of unique restriction sites, easy blue-white screening of recombinant BAC clones on X-Gal plates, and direct end-sequencing of inserted DNA using the conventional pUC19 sequencing primers. The whole pSBAC vector has been completely sequenced to ensure that no detrimental mutation was introduced during the construction process. Comparison studies demonstrated that the transferring efficiency of pSBAC into *S. lividans* approached half of that of pSET152, a highly efficient *Streptomyces* integrating cloning vector utilizing the ΦC31 *attP-int* system. DNA fragments of varied length from 40 kb to over 100 kb have been successfully transferred between *E. coli* and *Streptomyces coelicolor* and *S. lividans* by pSBAC (data not shown).

Cloning of the Meridamycin Biosynthetic Gene Cluster into a Single *E. coli* Clone. Meridamycin (**1**) and its naturally occurring analogue 3-normeridamycin (**2**) are nonimmunosuppressive, FKBP12-binding macrocyclic polyketides with potent neuroprotective activity in dopaminergic neurons.[17] Efforts to develop **1** into a clinically useful neuroprotective agent proved challenging, mainly due to the difficulty to further improve its production in the native producing strain after reaching a certain level through a traditional medium optimization and strain improvement process. This presents a big hurdle to chemically derivatize **1** for superior analogues, which requires exponentially greater quantities of material. Generating analogues through genetic engineering of the biosynthetic pathway of **1** is also challenging, since such manipulations often result in significant reduction in production level. In fact, the yield of a genetically engineered analogue of **1** is so low that not enough materials can be generated for biological activity

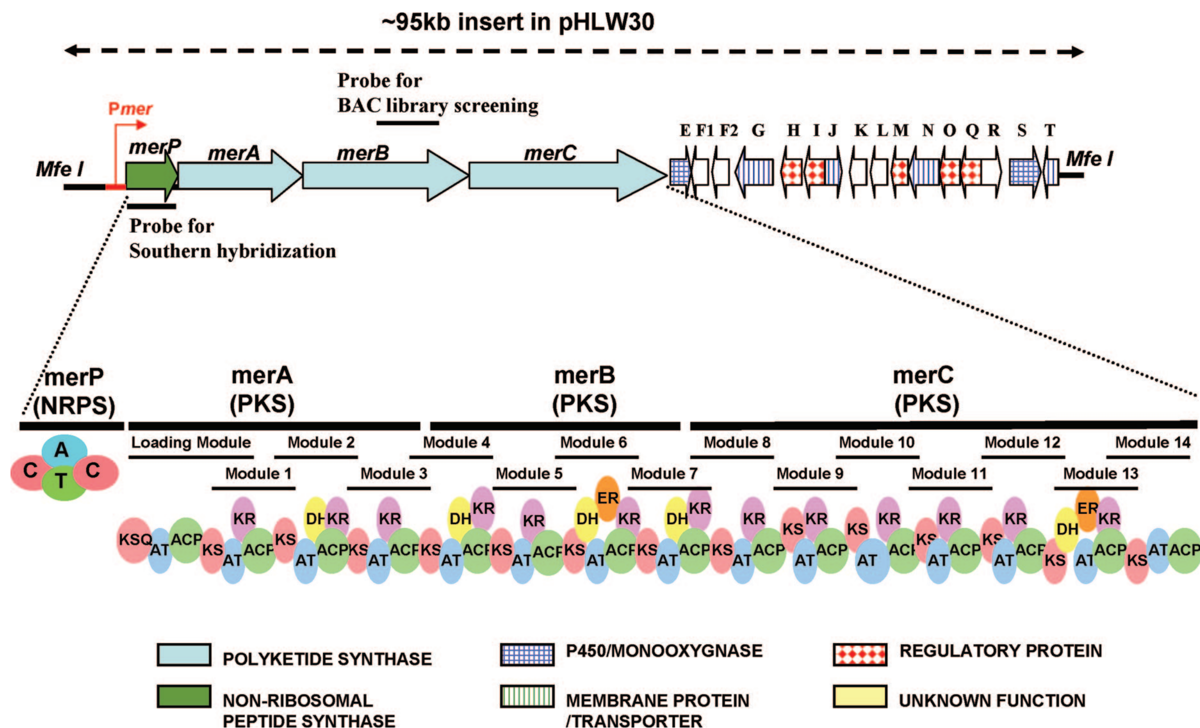


Figure 2. Schematic description of the insertion of pHLW30, featuring the revised gene organization of the *mer* biosynthesis cluster, two *Mfe* I restriction sites flanking the *mer* cluster, and the location of DNA probes used in BAC library screening and Southern hybridization. Also shown is the schematic representation of the modular organization of PKS and NRPS complexes encoded by *merA, B, C*, and *P* genes, with catalytic domains represented by colored circles.

evaluation.[14] Therefore, there is a great need to investigate a new host, either a species that is more amenable for strain improvement or one that has already been optimized for high-level production of similar metabolites, as an alternative producer for this medically interesting compound.

The biosynthetic gene cluster of meridamycin (**1**) has been cloned from *Streptomyces* sp. NRRL 30748 in a previous study and is located on several overlapping cosmids.[14] The entire ~90 kb *mer* cluster contains genes encoding type I polyketide synthase (MerA−D), nonribosomal peptide synthetase (MerP), cytochrome P450 monooxygenase (MerE), and several putative regulatory (MerH, MerI, MerM, MerO, and MerQ) and transportation proteins (MerG, MerJ, and MerN). The giant polyketide synthase complex with a total of 15 modules was previously found to comprise four large subunits designated as MerA, MerB, MerC, and MerD, respectively. However, recent efforts to resequence the DNA region between *merC* and *merD* led to the identification of a guanine nucleotide at position 88333 as a misread in the previous sequencing process. Elimination of this guanine nucleotide resulted in a contiguous single ORF (designated as the new *merC* gene) of 33 564 bp, which encodes a multifunctional polyketide synthase (MerC) of 11 188 amino acids (Figure 2). Comprised of 7 PKS extension modules and 29 catalytic domains, MerC represents one of the largest polyketide synthase subunits that have been identified to date. The revised organization of Mer PKS also agrees with that of a closely related cluster isolated from *Streptomyces* sp. DSM 4137.[18]

It was considered desirable to re-isolate the *mer* gene cluster in a single clone to facilitate the transferring and expression in heterologous hosts. Sequence analysis revealed two *Mfe* I restriction enzyme recognition sites flanking the entire *mer* gene cluster. We therefore decided to use a straightforward single restriction enzyme digestion and cloning approach to capture the whole *mer* gene cluster into pSBAC. This strategy would greatly increase the chances of identifying a single clone with the complete *mer* gene cluster. A pSBAC library was constructed with enriched DNA fragments of ~100 kb generated from *Mfe* I digestion of the genomic DNA of the NRRL 30748 strain. Screening of this library with a probe specific to the *mer* gene cluster identified a single clone, pHLW30, which contains a ~95 kb DNA fragment, starting from ~360 bp upstream of *merP* and ending at ~16 kb downstream of *merE*. This insert includes the whole *mer* gene cluster with an original promoter upstream of *merP*, as well as some putative regulatory and transporter genes (Figure 2). The presence of essential *mer* biosynthetic genes in pHLW30 has also been confirmed by PCR amplification analysis using primers specific to different regions of the *mer* cluster (data not shown).

Heterologous Expression of the *mer* Gene Cluster in *S. lividans*. *S. lividans* strains TK24 and K4-114[19] were chosen as the preliminary hosts for the heterologous expression of the *mer* gene cluster. The lack of endogenous production of macrolide metabolites in these two strains would provide a clean background for the detection of any new metabolite derived from *mer* genes. pHLW30 was transferred into TK24 and K4-114 via conjugation, and the resultant strains with the *mer* gene cluster integrated into the chromosomes were named HL30-2 and HL30-K3, respectively. Southern hybridization and PCR amplification analysis confirmed the presence of the full-length *mer* gene cluster in these two recombinant strains (data not shown). However, fermentation of both strains in several different media failed to generate detectable amounts of meridamycin (**1**) or any related compound. We suspected a lack of transcription efficiency of *mer* genes in *S. lividans* was likely the main reason for this failure of production, considering the essential genes for meridamycin biosynthesis (*merP*−*E*) might form a giant operon of ~78 kb, which may require strong transcriptional initiation.[14] Semiquantitative RT-PCR thus was used to analyze the transcripts of the *merP* gene in different hosts. The results revealed that the transcription level of *merP* in HL30-2 and HL30-K3 was much lower than that of the original producer, NRRL 30748 (Figure 3). Therefore, we were prompted to change the *mer* promoter with a constitutive *ermE** promoter,

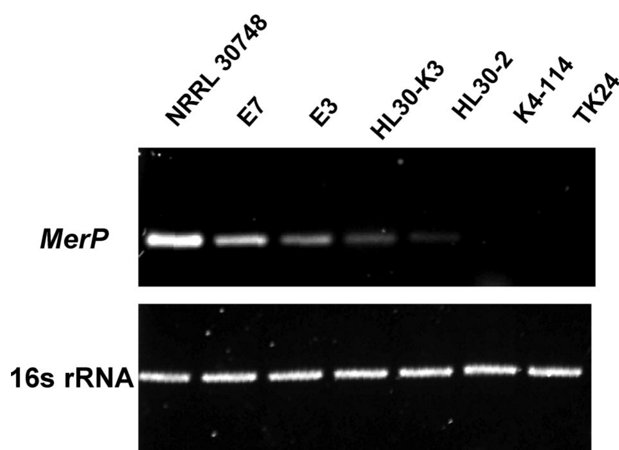


Figure 3. RT-PCR analysis to investigate the transcription of the *merP* gene in different strains (top panel) and the amplification of 16s rRNA from each sample as the internal control to ensure the equal amount of total RNA used in this analysis (bottom panel).

which had been proven to be able to drive the expression of heterologous genes efficiently in *S. lividans*.[20,21] Plasmid pHLW71, which has the *ermE** promoter in front of a 2 kb DNA fragment at the 5′-terminal of the *merP* gene, was used to introduce a single crossover homologous recombination in HL30-2 and HL30-K3, resulting in the generation of strains E3 and E7, respectively. As a result of this recombination, the *ermE** promoter was placed in front of *merP* to drive the transcription of *merP−E* genes (Figure 4). Semiquantitative RT-PCR demonstrated that this replacement indeed increased the transcription of the *merP* gene (Figure 3), though the level is still lower than that in the original producer NRRL 30748. Subsequently, production of **1** was successfully detected in the fermentation extract of E7 by LC-MS (Figure 5, panel C). However, the yield was very low (~100 μg/L).

Besides the commonly used building blocks for polyketide synthesis, malonyl-CoA and methylmalonyl-CoA, the biosynthesis of meridamycin (**1**) also requires ethylmalonyl-CoA as an extender unit, which is incorporated into the macrolide ring of **1** by an ethylmalonyl-specific acyltransferase domain in module 4 of Mer PKS. The supply of this precursor has been demonstrated to be a critical factor that limits the synthesis of some polyketides by heterologous hosts.[22] Although it has been shown that *S. lividans* could accommodate the production of a polyketide that requires ethylmalonyl-CoA,[23] the internal pool of this precursor might not be sufficient for an efficient synthesis of **1**. To address this question, strain E7 was cultured in FKA medium supplemented with diethyl malonate, which has been proven to be an effective precursor for ethylmalonyl-CoA.[22] This supplementation indeed has increased the production of **1** by about 2-fold (~240 μg/L). When both diethyl malonate and L-proline were supplemented in the fermentation, coproduction of 3-normeridamycin (**2**) was also detected by high-resolution mass spectrometry (HR-MS) (Figure 6), though the yield was very low (~10 μg/L). Interestingly, feeding strain E7 with pipecolic acid, another unique precursor for the biosynthesis of **1**, did not significantly increase the production. In fact, feeding pipecolic acid to the native producer, NRRL 30748, failed to enhance the production of **1** (data not shown). This might due to the fact that *Streptomyces* cells cannot take up exogenous pipecolic acid efficiently.

Despite repeated efforts, we could not detect the production of meridamycin (**1**) in strain E3. Since the only difference between strains E3 and E7 was the lack of the production of an aromatic polyketide, actinorhodin, in E7 due to the deletion of *act* genes in the parent strain K4-114, we speculate that recruitment competition of malonyl-CoA, a common biosynthesis precursor for both actinorhodin and **1**, likely had contributed to the failure of the production of **1** in E3. Nevertheless, the successful production of **1** and 3-normeridamycin (**2**) in E7 proved that pHLW30 certainly contains all the necessary genes for the heterologous biosynthesis of these 27-membered macrolides. Although the current production level of **1** in *S. lividans* is lower than that in the native producer, this work validated the BAC-based technology for expressing a giant polyketide biosynthetic gene cluster in the heterologous host. Future exploration of this approach, including extensive medium optimization and the placement of strong promoters at each of the key meridamycin biosynthesis genes, might significantly increase the yield of **1** in *S. lividans*. Mobilization of pHLW30 into industrially optimized strains for polyketide production, as well as metabolic engineering of host strain for enhanced precursor supply, is expected to further improve the heterologous production level of **1**, and therefore expedite the development of semisynthetic and biosynthetic analogues of this potent non-immunosuppressive immunophilin ligand.

Experimental Section

Strains, Plasmids, Reagents, and Media. Various strains and plasmids used in this study are summarized in Table 1. *E. coli* strains were grown in Luria−Bertani (LB) medium supplemented with either apramycin (50 μg/mL) or ampicillin (100 μg/mL). *S. coelicolor* and *S. lividans* strains were grown at 28 °C in MYM, R2YE,[24] or FKA media.[25] R6 medium[26] used for conjugation was supplemented with apramycin (50 μg/mL) and nalidixic acid (25 μg/mL). KOD Hot Start DNA polymerase (Novagen, San Diego, CA) was used for PCR amplification following the manufacturer's instructions. Genomic DNA of *Streptomycetes* was isolated using the procedure described previously.[26] Plasmid DNA was isolated using the Zappy plasmid miniprep kit (Zymo Research, Orange, CA). BAC clones with large inserts were isolated using the BACMAX DNA purification kit (Epicenter, Madison, WI). Transformation of plasmid into *E. coli* was performed using either NovaBlue competent cell or EPI300 electro competent cells. Conjugation experiments were performed as described previously.[14]

Construction of pSBAC. The backbone of the pSBAC vector was amplified from plasmid pCC1BAC (Epicenter) using primer set pCC1BACFor (5′-AGGGCTTCCCGGTATCAACAG-3′) and pCC1BACRev (5′-GGTTACTCCGTTCTA CAGGTTAC-3′). The origin of transfer region (*oriT*) and apramycin resistance gene, *aacIII*(IV), together with the multiple cloning site were amplified from plasmid pBWA2[26] using the primer set pB1 (5′-TCAGGCCTTCGCCACCTCTGACTTGAGC-3′) and pB2 (5′-ATAGGCCTCAGTGAGGCACCTATCTCAG-3′). The 6.5 kb PCR product amplified from the first primer set and the 4 kb PCR product amplified from the second primer set were ligated together to produce plasmid pHLW3. A 2 kb DNA fragment containing the *attP-int* of ΦBT1 was synthesized (Celtek-genes, Nashville, TN), digested with *Eco*R V, and then ligated into the unique *Sca* I site of pHLW3 to give the final construct pSBAC. pSBAC has been completely sequenced to ensure integrity (454 Life Science, Bradfort, CT).

Correction of the Original *mer* Biosynthesis Gene Cluster Sequence. Two PCR primers (forward: 5′-TGTGTCCTCGTTCGGGGT-CAGTG-3′, reverse: 5′-CTCCAAC AGTTCCAACGCCATTCC-3′) were used to amplify a DNA fragment flanking the 3′ of the *merC* gene and the 5′ of the *merD* gene in the originally reported meridamycin biosynthetic gene cluster. After being cloned into pUC19, this fragment was completely sequenced multiple times, and the data were used to identify potential mistakes in the previously reported *mer* gene cluster.

One-Step Cloning of the Meridamycin Biosynthesis Gene Cluster into pSBAC. Preparation of the genomic DNA plug of *Streptomyces* sp. NRRL 30748 was carried out following the instruction manual for CHEF genomic DNA plug kits (Bio-Rad, Hercules, CA). Briefly, the mycelium pellet was suspended in cell suspension buffer and then embedded into CleanCut agarose. The solidified agarose plugs were treated with lysozyme and subsequently with proteinase K. The plugs were washed twice with wash buffer before being treated with 1 mM PMSF to inactivate residual proteinase K. Finally, the plugs were washed thoroughly and stored in wash buffer at 4 °C until use. Pretreatment and restriction digestion of the DNA plugs were performed using the protocol described by Peterson et al.[27] The DNA plugs were then cut into small pieces and digested by restriction enzyme *Mfe* I. The digestion reaction was stopped by adding EDTA to the final concentration of 50 mM. The small pieces of the digested DNA plugs

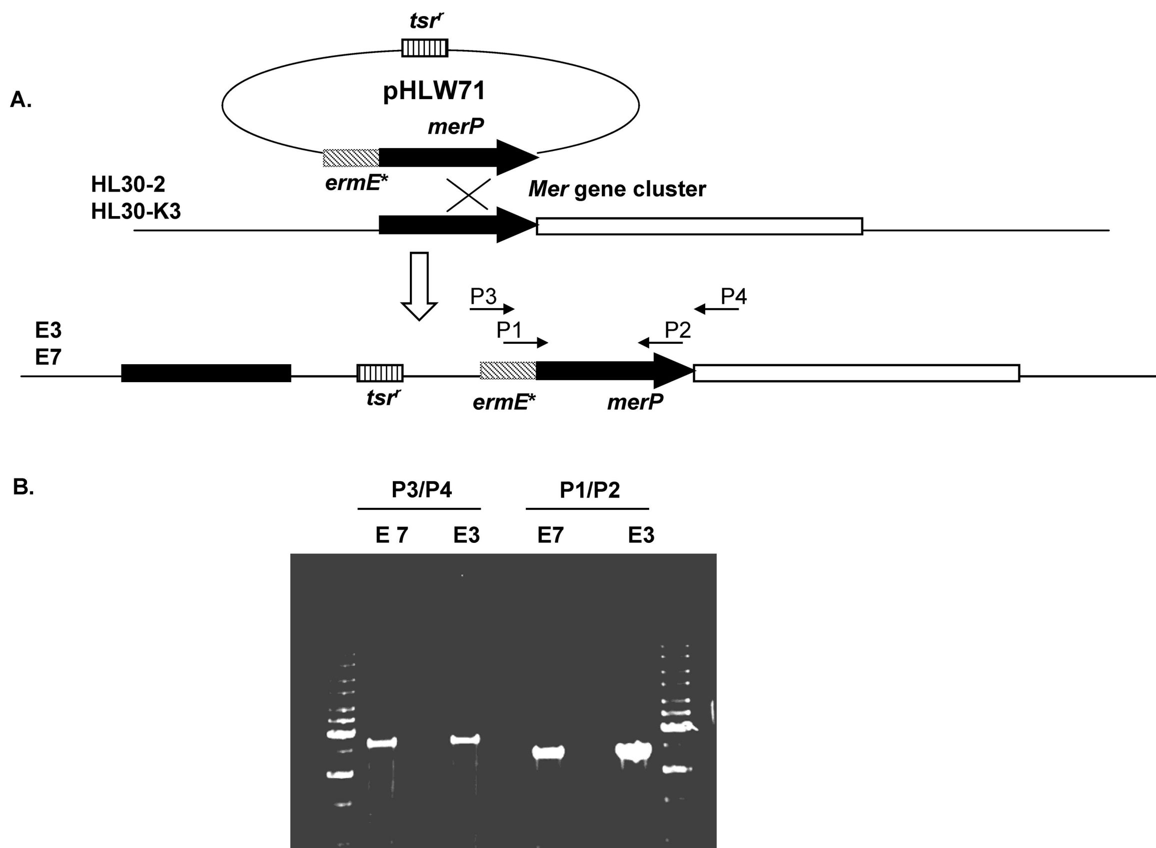


Figure 4. Schematic description of the strategy to replace the *merP* promoter with the *ermE** promoter (A) and the PCR amplification analysis to confirm the replacement (B). Locations of the PCR primers are indicated.

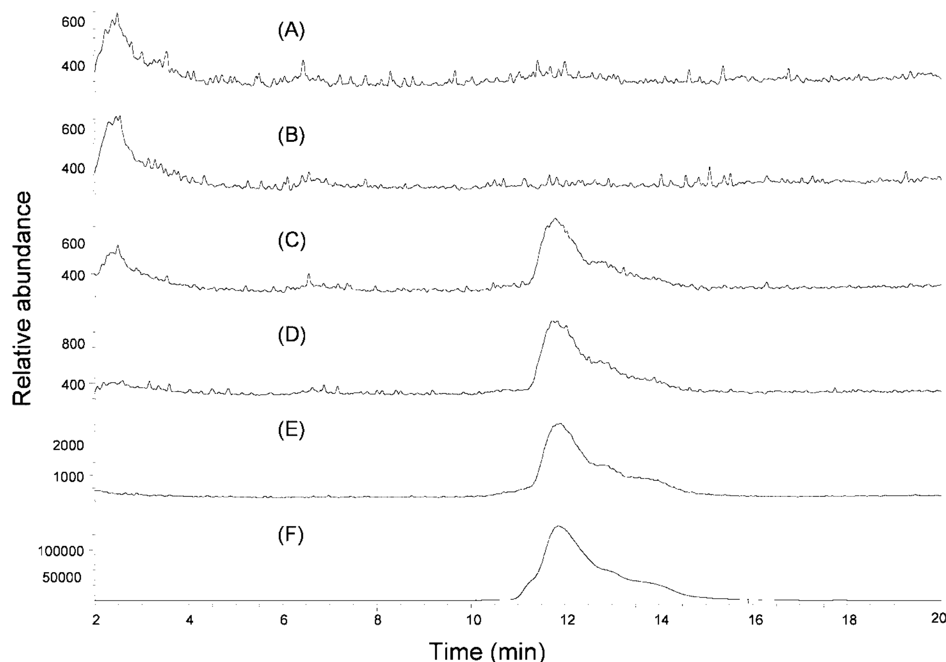


Figure 5. LC-MS analysis of meridamycin ($[M + Na]^+$ *m/z* 844) production from broth extracts of cultures grown in FKA medium. Ion trace of *m/z* 844 from (A) K4-114; (B) HL30-K3; (C) E7 without supplementation; (D) E7 with supplementation of 2 mM L-pipecolate; (E) E7 with supplementation of 10 mM diethylmalonate; (F) meridamycin standard.

were then subjected to pulsed field electrophoresis in 1% pulse field agarose (Bio-Rad). The DNA fraction corresponding to 90 to 110 kb was excised and eluted from the gel by electroelution. The eluted DNA was precipitated and concentrated before ligation into *Eco*R I-digested pSBAC vector using Fast-link DNA ligation kit (Epicenter). After desalting, the ligation mixture was used to electroporate *E. coli* EPI300 competent cells. Recombinant colonies were screened using the DNA probe corresponding to the end of the *merB* gene and resulted in the identification of pHLW30.

RT-PCR Analysis. Total RNA from different *Streptomycetes* strains was isolated using the method described by Van Dessel et al.[28] with modifications. Briefly, a 3 mL culture from 72 h growth was collected

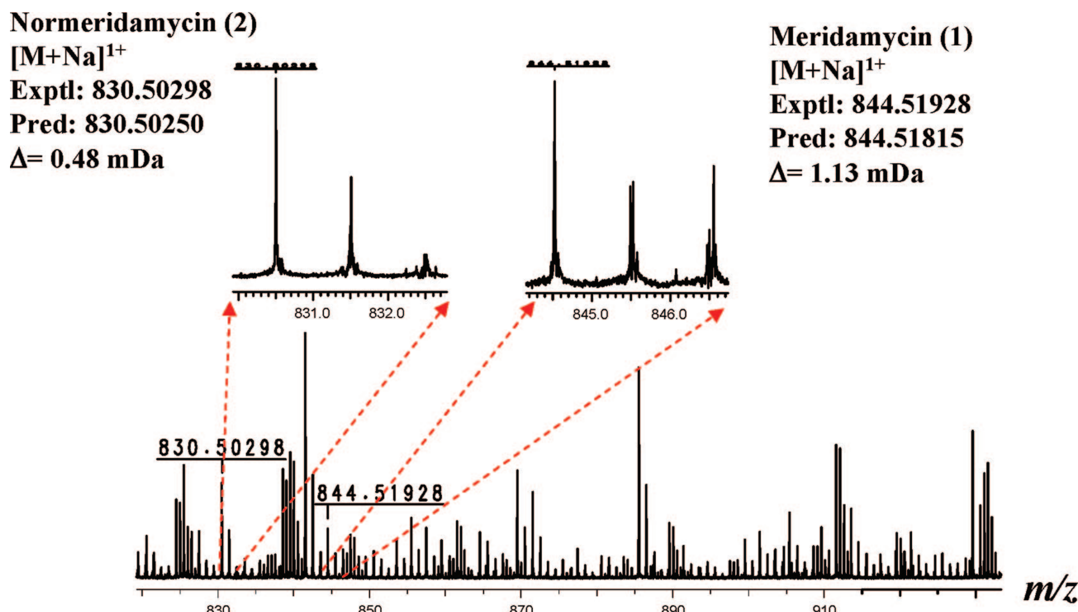


Figure 6. HR-MS analysis to confirm the production of meridamycin (**1**) and 3-normeridamycin (**2**) produced by strain E7 grown in FKA medium supplemented with 4% L-proline and 10 mM diethyl malonate.

Table 1. Bacterial Strains and Plasmids Used in This Study

strain/plasmid	relevant genotype/comments	source/reference
E. coli		
EPI300TM	F^- *mcrA*−*D*(*mrr-hsd*RMS-*mcrBC*) *trf*A host for cloning and amplification of various BAC vectors and constructs derived from it	Epicenter
S17-1	*E. coli* host for transferring various plasmids into *Streptomyces* via conjugation	32
ET12567(pUZ8002)	*E. coli* host for transferring various plasmids into *Streptomyces* via conjugation	33
S. lividans		
TK24	*Rps*L(Smr) Act^+Red^+	John Innes Centre, Norwich, UK
K4-114	*str*-6, SLP2-, SLP3-, Δ*act*::*ermE Streptomyces* host for the expression of the act gene cluster	19
HL30-2	*S. lividans* TK24 with *mer* gene cluster integrated into chromosome	present study
HL30-K3	*S. lividans* K4-114 with *mer* gene cluster integrated into chromosome	present study
E3	*S. lividans* TK24 with *mer* gene cluster under *erm*E* promoter integrated into chromosome	present study
E7	*S. lividans* K4-114 with *mer* gene cluster under *erm*E* promoter integrated into chromosome	present study
Streptomyces sp. NRRL30748	original meridamycin-producing strain	14
plasmids		
pCC1BAC	copy control BAC cloning vector	Epicenter
pSBAC	*aacIII*(IV), *oriT*, *attP-int*, backbone of pCC1BAC	present study
pHLW30	pSBAC with 97 kb DNA insert containing whole *mer* gene cluster	present study
pHLW70	pSE34 derivative containing the 2 kb 5′-end of *merP* following the *ermE** promoter	present study
pHLW71	pHLW70 with *oriT*	present study

and two volumes of RNA protect reagent (Qiagen, Hilden, Germany) were added immediately. The reaction mixture was allowed to stand at room temperature for 5 min and then centrifuged to obtain the mycelium pellets. The pellets were treated with 1 mL of 5 mg/mL lysozyme for 1 h at 37 °C, then extracted with phenol−chloroform (5:1; pH 4.5) and precipitated with 2 mL of ethanol, 250 μL of 1 M Tris (pH 8.0), and 100 μL of 5 M NaCl. The precipitated RNA was washed with 80% ethanol once and resuspended in 100 μL of RNA storage buffer (Ambion, Austin, TX). The primer sets used for RT-PCR were RT1 (5′-GCGCGGACCGAGCCCTACGAC-3′) and RT2 (5′-CCCCCGGCCCTCCAGCAGATG-3′) for amplification of the 5′-end of the *mer* gene cluster. Primers 16sFor (5′-GGTTACCTTGT-TACGACTT-3′) and 16sRev (5′-AGAGTTTGATCCTGGCTCAG-3′) were used as an internal control to ensure an equal amount of total RNA was present in each sample. Semiquantitative RT-PCR was conducted according to the method described previously,[29,30] except that a one-step RT-PCR kit (Qiagen) was used following the instruction manual. Cycle numbers and template amount were carefully calibrated to ensure that the RT-PCR was carried out within the exponential phase of amplification.

Replace the *mer* Promoter with the *ermE Promoter.** The 2 kb PCR product corresponding to the immediate downstream of the original *merP* promoter was amplified using primer set P1 (5′-GCTCTA-GAGTGGGGAATTCAGGCGCACCC-3′ (*Xba* I site is underlined)) and P2 (5′-AGCAAGCTTGGGGACTCCGGTGGAGCCGG A-3′ (*Hind* III site is underlined)). The PCR product was purified and digested with *Xba* I and *Hind* III, then cloned into the corresponding sites of plasmid pSE34[21,31] to produce plasmid pHLW70. A 1.2 kb

oriT DNA fragment was cloned into the blunted *Bst*B I site of pHLW70 to generate pHLW71. The plasmid was conjugated into HL30-K3. Colony PCR analyses were used to select the single cross-over recombinant strain that contains the *ermE** promoter in front of the *mer* gene cluster.

Metabolite Analysis. Meridamycin (**1**) and 3-normeridamycin (**2**) were detected by liquid chromatography−mass spectrometry (LC-MS) and high-resolution and accurate mass measurement (HR-MS), as previously described.[14,17]

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