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Genetic Localization and Molecular Characterization of Two Key Genes (*mitAB*) Required for Biosynthesis of the Antitumor Antibiotic Mitomycin C

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Mitomycin C (MC) is an antitumor antibiotic derived biosynthetically from 3-amino-5-hydroxybenzoic acid (AHBA), D-glucosamine, and carbamoyl phosphate. A gene (*mitA*) involved in synthesis of AHBA has been identified and found to be linked to the MC resistance locus, *mrd*, in *Streptomyces lavendulae*. Nucleotide sequence analysis showed that *mitA* encodes a 388-amino-acid protein that has 71% identity (80% similarity) with the rifamycin AHBA synthase from *Amycolatopsis mediterranei*, as well as with two additional AHBA synthases from related ansamycin antibiotic-producing microorganisms. Gene disruption and site-directed mutagenesis of the *S. lavendulae* chromosomal copy of *mitA* completely blocked the production of MC. The function of *mitA* was confirmed by complementation of an *S. lavendulae* strain containing a K191A mutation in MitA with AHBA. A second gene (*mitB*) encoding a 272-amino-acid protein (related to a group of glycosyltransferases) was identified immediately downstream of *mitA* that upon disruption resulted in abrogation of MC synthesis. This work has localized a cluster of key genes that mediate assembly of the unique mitosane class of natural products.

Streptomyces spp. are filamentous gram-positive soil bacteria with a nucleotide base composition greater than 70 mol% G+C (53). They produce a wide array of biologically active compounds, including over two-thirds of the commercially important natural-product metabolites (1, 10). Genetic information accumulated over the past 15 years has demonstrated that genes encoding enzymes for natural product assembly are clustered on the *Streptomyces* genome (38). In addition, one or more pathway-specific transcriptional regulatory genes and at least one resistance gene are typically found within the antibiotic biosynthetic gene cluster (14). Among the strategies for cloning antibiotic biosynthetic genes, heterologous hybridization with gene probes based on highly conserved biosynthetic-enzyme amino acid sequences has been very effective (25, 49, 56).

Streptomyces lavendulae produces the clinically important antitumor antibiotic mitomycin C (MC) (22). MC has become one of the most effective drugs against non-small-cell lung carcinoma, as well as other soft tumors (24). The molecule has an unusual structure, comprised of aziridine, pyrrolizidine, pyrrolo-(1,2a)-indole, and amino-methylbenzoquinone rings to give the mitosane nucleus (58). A significant amount of information on the biosynthesis of MC has accumulated since 1970. The mitosane core was shown to be derived from the junction of an amino-methylbenzoquinone (mC_7N unit) and hexosamine (C_6N unit) (27) (Fig. 1). The C_6N unit consists of carbons 1, 2, 3, 9, 9a, and 10, with the aziridine nitrogen derived intact from D-glucosamine (29).

The mC_7N unit in MC and the ansamycins is derived from 3-amino-5-hydroxybenzoic acid (AHBA) (8, 33). AHBA was first shown to be incorporated into the ansamycin antibiotic actamycin (32). Subsequently, it was confirmed as an efficient precursor for rifamycin (21), geldanamycin (46), ansamitocin (23), ansatrienin (59), streptovaricin (54), and naphthomycin A (37). Anderson et al. demonstrated that [carboxy-^{13}C]AHBA could be efficiently and specifically incorporated into the C-6 methyl group of porfiromycin, which contains the same mitosane core as MC (3). ^{14}C-labeled precursor feeding studies with D-glucose, pyruvate, and D-erythrose indicated that de novo biosynthesis of AHBA resulted directly from the shikimate pathway. However, no incorporation into the mC_7N unit of either MC (27) or the ansamycin antibiotics (15) was found from labeling studies with shikimic acid, the shikimate precursor 3-dehydroquinic acid, or the shikimate-derived amino acids. These results led to the hypothesis of a modified shikimate pathway, in which a 3-deoxy-D-arabino-heptulosonic acid-7-phosphate (DAHP) synthase-like enzyme catalyzes the conversion to 3,4-dideoxy-4-amino-D-arabino-heptulosonic acid-7-phosphate (aminoDAHP) to give the ammoniated shikimate pathway (34). Kim et al. provided strong support for this new variant of the shikimate pathway by showing that aminoDAHP, 5-deoxy-5-amino-3-dehydroquinic acid (aminoDHQ), and 5-deoxy-5-amino-3-dehydroshikimic acid (aminoDHS) could be efficiently converted into AHBA by a cell extract of *Amycolatopsis mediterranei* (the rifamycin producer), in contrast to the normal shikimate pathway intermediate DAHP, which was not converted (34, 35). Recently, the AHBA synthase gene (*rifK*) from *A. mediterranei* has been cloned, sequenced, and functionally characterized (36).

Since AHBA is a biosynthetic precursor for MC, we decided to use *rifK* as a probe to identify a corresponding gene from *S. lavendulae* that may be linked with one of the previously characterized MC resistance genes (4, 50). A 3.8-kb *Bam*HI fragment from the *S. lavendulae* genome was identified, and its nucleotide sequence revealed three open reading frames (ORFs). One ORF (*mitA*) showed high similarity to previously identified AHBA synthase genes (36), while a second (*mitB*) showed sequence similarity to several procaryotic and eucaryotic glycosyltransferases. The involvement of both of these

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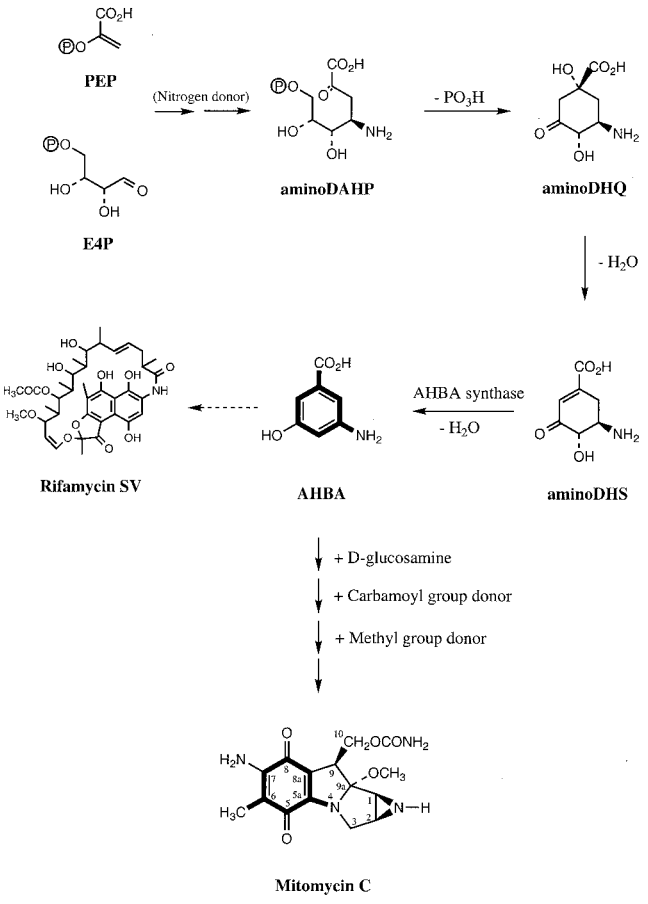

FIG. 1. Proposed biosynthetic pathway leading to mitomycins.

genes in MC biosynthesis was demonstrated by gene disruption, site-directed mutagenesis, and subsequent isolation of mutants blocked in antibiotic biosynthesis. MC production was restored when the *mitA* mutant strain was cultured in the presence of exogenous AHBA.

MATERIALS AND METHODS

Strains and culture conditions. *Escherichia coli* DH5α was grown in either Luria broth or tryptic soy broth (TSB) (Difco) as liquid medium or agar plates. *E. coli* DH5αF′, the host for harvesting single-stranded DNA, was grown at 37°C on TBG (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 55 mM K_2HPO_4, and 20 mM glucose). *E. coli* S17-1 (39), used for conjugation, was grown in TSB with 10 μg of streptomycin/ml. *S. lavendulae* was grown in TSB or on R5T plates (containing [grams per liter] sucrose, 121.2; K_2SO_4, 0.3; $MgCl_2 \cdot 6H_2O$, 11.92; glucose, 11.8; yeast extract, 5.89; Casamino Acids, 0.12; agar, 25.9; and 2.35 ml of trace elements [26]; after the mixture was autoclaved, 0.5% KH_2PO_4 [11.8 ml], 5 M $CaCl_2$ [4.71 ml], and 1 N NaOH [8.25 ml] were added). For MC production, *S. lavendulae* was grown in Nishikohri medium (containing [grams per liter] glucose, 15; soluble starch, 5; NaCl, 5; $CaCO_3$, 3; and yeast extract, 5) for 72 h from a 1% (vol/vol) inoculum of frozen mycelia. Pulse feeding of AHBA to the disruption mutant, MV100, and the site-directed mutant, MV102, was done with feedings of 2.5 mg of a 20-mg/ml solution of the sodium salt of AHBA (pH 7.1) in three pulses at 24, 43, and 57 h of growth of a culture that was harvested at 76 h.

DNA preparation and amplification. Isolation and purification of DNA was performed by standard methods (47). *S. lavendulae* NRRL 2564 genomic DNA was isolated by using the modified Chater protocol (26). Plasmid DNA was isolated from *E. coli* by using the alkaline-sodium dodecyl sulfate method.

pDHS2002 was constructed as follows. The 1.1-kb thiostrepton resistance gene (*tsr*) fragment was removed from pDHS5000 by *Sma*I-*Bam*HI digestion, blunt ended with the large fragment of DNA polymerase (Gibco BRL), and ligated to *Msc*I restriction enzyme-digested pDHS7601 to yield pDHS2001. *Msc*I digestion of pDHS7601 resulted in the removal of 155 nucleotides at the C terminus of the *mitA* gene, and ligation of the blunt-ended *Bam*HI site of the *tsr* adjacent to the *Msc*I site of pDHS7601 resulted in regeneration of the *Bam*HI site in pDHS2001. The 4.9-kb *Eco*RI-*Hin*dIII fragment from pDHS2001 containing the *tsr*-disrupted *mitA* gene was removed and ligated into *Eco*RI-*Hin*dIII-digested pKC1139 to yield pDHS2002.

Primer-mediated site-directed mutagenesis was employed to construct pDHS2015 containing a K191A mutation in *mitA*. Primer 1 (5′-GGCAAGGC ATGCGAGGGTCGC-3′) and primer 2 (5′-TTCCAGAACGGCGCCCTGAT GACCGCCGGC-3′) were used to amplify the 691-bp fragment of the 5′ end of *mitA*. The 3′ end of *mitA* was amplified with primer 3 (5′-GCCGGCGGTCAT CAGGGCGCCGTTCTGGAA-3′) and primer 4 (5′-TCAGAATTCGGATCCG AGGGCCGGAGT-3′) to generate a 1,151-bp band. A second round of PCR was performed, with the overlapping 691- and 1,151-bp units as the initial templates, with primer 1 and primer 4 to afford a 1.8-kb fragment. The final product, containing mutagenized *mitA*, was digested with *Eco*RI-*Sph*I and ligated to the 2.1-kb *Hin*dIII-*Sph*I fragment from pDSH2004 and the *Eco*RI-*Hin*dIII-digested pKC1139 to yield pDSH2015. The site-directed mutation of MitA K191A in pDHS2015 was confirmed by sequencing with forward primer (5′-ACCTACTG CCTCGATGCC-3′) and reverse primer (5′-CTGATCCTTCAAGCG-3′).

The *mitB* disruption vector pDHS7702 was constructed as follows. pDHS7601 was digested with *Bst*BI, blunt ended, and ligated with the 1.4-kb neomycin resistance gene fragment from pFD666 (17) (*Apa*L1-*Hin*dIII digestion; blunt ended). The 5.2-kb *Eco*RI-*Hin*dIII fragment from the resulting construct, pDHS7701, was subcloned into pKC1139 to create pDHS7702.

DNA library construction and screening. *S. lavendulae* NRRL 2564 genomic DNA was partially digested with *Sau*3AI, and a fraction containing 30- to 50-kb fragments was recovered by sucrose gradient centrifugation and ligated into the calf intestinal alkaline phosphatase-treated *Bgl*II site of the *E. coli-Streptomyces* shuttle vector pNJ1 (55) and then packaged with the Packagene Lambda DNA packaging system (Promega). The cosmid library was constructed by transfecting *E. coli* DH5α, and colonies that appeared on the Luria broth plates containing 100 μg of ampicillin/ml were transferred to a BioTrace NT nitrocellulose blotting membrane (Gelman Sciences). Colony hybridization was performed as specified by the manufacturer. A PCR-amplified 0.7-kb DNA fragment from plasmid pKN108 (Table 1) was used to screen the library. The primers used for PCR were 5′-GCGTCCGTGCTGCGCGCGCA-3′ and 5′-TGCGCGCGCAGCACGGAC GC-3′. The cosmids from the positive colonies were confirmed by Southern blot hybridization, and a 1.7-kb *Afl*III-*Bam*HI fragment from pDHS3001 containing the mitomycin resistance determinant (*mrd*) (50) was used as a probe to establish genetic linkage.

DNA sequencing and analysis. Deletion subclones from pDHS7601 were made with the exonuclease III Erase-a-Base system (Promega). Sequencing was accomplished with the PRISM dye terminator cycle-sequencing ready reaction kit (Applied Biosystems) and analyzed on an Applied Biosystems 377 DNA sequencer at the University of Minnesota Advanced Genetic Analysis Center. For generating single-stranded DNA, deletion subclones in pUC119 were transformed into *E. coli* DH5αF′, and M13K07 helper phage (Gibco BRL) was used. Nucleotide sequence data were analyzed with Wisconsin Genetics Computer Group software (version 9.0) (18) and GeneWorks software version 2.51 (Oxford Molecular Group).

Conjugation from *E. coli* S17-1 to *S. lavendulae*. To transfer plasmid from *E. coli* S17-1 to *S. lavendulae*, the procedure of Bierman et al. (12) was used with the following modification. A single colony of *E. coli* S17-1/pDHS2002 was used to inoculate 2 ml of TSB containing 100 μg of apramycin/ml and 10 μg of streptomycin/ml. Following overnight incubation at 37°C, a 1:100 inoculation was made into TSB with 100 μg of apramycin/ml and 10 μg of streptomycin/ml. This culture was grown for 3 h at 37°C, and the cells were washed twice with TSB and resuspended in 2 ml of TSB to provide the donor *E. coli* culture. The recipient *S. lavendulae* culture was generated by inoculating 9 ml of TSB with 1 ml of frozen wild-type culture. Following overnight (16-h) incubation at 29°C, the culture was homogenized by sonication, and 2 ml of this culture was used to inoculate 18 ml of TSB. Following overnight growth at 29°C and sonication treatment to homogenize the culture, a 1-ml inoculum was placed in 9 ml of TSB. This culture was grown for 3 h, and the mycelia were washed with TSB and resuspended in 2 ml of TSB to provide the stock recipient culture.

The donor and recipient cultures were mixed together in 9:1, 1:1, and 1:10 donor/recipient ratios, and 100 μl of the cell mixture was spread on AS1 plates (5). The plates were incubated overnight at 29°C and overlaid with 1 ml of water containing a suspension of 500 μg each of thiostrepton, apramycin, and nalidixic acid/ml. For the pKC1139 control, only apramycin and nalidixic acid were overlaid, while for pDHS7702, 500 μg of kanamycin/ml was used instead of thiostrepton. *S. lavendulae* exconjugants appeared in approximately 11 to 13 days at a frequency ranging from 10^{-7} to 10^{-5}. pKC1139 has a temperature-sensitive *Streptomyces* replication origin, which is unable to replicate at temperatures above 34°C (41), while the *S. lavendulae* host grows well at 42°C. Thus, after the conjugants were propagated at 39°C for several generations, double-crossover mutants were readily generated. The presence of the plasmid was determined by transformation of *E. coli* DH5α with plasmid extracts from *S. lavendulae* transconjugants.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
E. coli		
DH5α	F⁻ *recA* ϕ80 d*lacZ*ΔM15	Gibco BRL
DH5αF′	F′ ϕ80 d*lacZ*ΔM15	Gibco BRL
S17-1	Contains RP4 integrated into the chromosome; Str^r	39
A. mediterranei ATCC 27643	Rifamycin producer	ATCC
S. lavendulae		
NRRL 2564	MC producer	ATCC
MV100[b]	*mitA* insertional disruption mutant of NRRL 2564	This study
MV102[b]	*mitA* site-directed mutant (K191A) of NRRL 2564	This study
MV103	MV100 with plasmid pDHS2003	This study
MM101[b]	*mitB* insertional disruption mutant of NRRL 2564	This study
Plasmids		
pNJ1	Th^r Ap^r bifunctional *E. coli* and *Streptomyces* shuttle vector	55
pUC119	Ap^r *lacZ*α MCS; *E. coli* cloning vector	61
pKC1139	Am^r *lacZ*α MCS *oriT rep*(Ts)	12
pDHS3001	pIJ702 with 4.1-kb *Bcl*I DNA insert; contains *mrd* locus	50
pKN108	Contains rifamycin AHBA synthase gene	34, 35
pFD666	Neo^r bifunctional *E. coli* and *Streptomyces* shuttle cosmid	17
pSL301	Ap^r *lacZ*α MCS; *E. coli* cloning vector	Invitrogen
pDHS7529	pNJ1 with 37-kb inserted fragment from *S. lavendulae*; contains *mrd* and *mitABC* locus	This study
pDHS7601	pUC119 with 3.8-kb *Bam*HI subclone from pDHS7529; contains *mitABC* locus	This study
pDHS5000	pUC119 with 1.1-kb *Sma*I-*Pst*I fragment of *tsr* gene from pNJ1; blunt ended and subcloned in *Sma*I site	This study
pDHS2001	*mitA* disruption construct; 1.1-kb *Sma*I-*Bam*HI (*tsr*) fragment from pDHS5000 was blunt ended and inserted into the two *Msc*I sites of pDHS7601	This study
pDHS2002	*mitA* disruption vector; 4.9-kb *Eco*RI-*Hin*dIII insert from pDHS2001 was subcloned into pKC1139	This study
pDHS2003	pKC1139 with 3.8-kb *Eco*RI-*Hin*dIII insert from pDHS7601	This study
pDHS2004	pSL301 vector with 3.8-kb *Pst*I-*Eco*RI fragment from pDHS7601 inserted. Makes *Sph*I site in *mitABC* unique.	This study
pDHS2015	*mitA* site-directed disruption vector with lysine 191 replaced by alanine	This study
pDHS7701	*mitB* disruption construct; 1.4-kb *neo*-resistant fragment *Apa*L1-*Hin*dIII was blunt ended and subcloned into the *Bst*BI-digested, blunt ended pDHS7601	This study
pDHS7702	*mitB* disruption vector; 5.2-kb *Eco*RI-*Hin*dIII insert from pDHS7701 subcloned into pKC1139	This study

[a] Am^r, apramycin resistance; Ap^r, ampicillin resistance; MCS, multiple cloning site; Neo^r, neomycin resistance; *rep*(Ts), temperature-sensitive replicon for *Streptomyces*; Str^r, streptomycin resistance; Th^r, thiostrepton resistance; *tsr*, thiostrepton resistance gene.
[b] See Materials and Methods.

Double-crossover selection procedure. A single colony of *S. lavendulae*/pDHS2002 grown on R5T plates (50 μg [each] of thiostrepton and apramycin/ml) was used to inoculate TSB broth containing 20 μg of thiostrepton/ml. After 72 h of incubation at 39°C, 10^{-4}, 10^{-5}, and 10^{-6} diluted aliquots were used to inoculate R5T plates containing 50 μg of thiostrepton/ml. Following 48 h of growth at 39°C, 84 colonies were picked randomly and each colony was patched out on separate R5T plates containing 50 μg of thiostrepton/ml and 50 μg of apramycin/ml. One of the 84 colonies displayed the double-crossover phenotype of thiostrepton resistance and apramycin sensitivity. The integration of the *tsr*-disrupted *mitA* gene and the loss of plasmid pDHS2002 were confirmed by Southern hybridization analysis.

MitA K191A site-directed mutants (MV102) were selected by propagating MV100/pDHS2015 on R5T plates for two generations at 37°C. The colonies were replicated to plates containing 50 μg of thiostrepton/ml and plates without antibiotics. Of the 108 colonies replicated in the first round, one had the correct (thiostrepton-sensitive) phenotype. To confirm the K191A mutation, the *mitA* gene was amplifed from the chromosome with primers 1 and 4. Mutation of the conserved lysine codon (AAG) to an alanine codon (GCC) was verified with the same sequencing primers employed to confirm the correct construction of pDHS2015. The alanine codon was observed in both the forward and reverse sequence data.

Mutants for *mitB* (MM101) were selected as follows: *S. lavendulae*/pDHS7702 was propagated on R5T plates for five generations at 39°C before single colonies were replicated on R5T plates as described above. Of the 300 colonies tested, 12 clones displayed the correct phenotype (kanamycin resistance and apramycin sensitivity). The genotypes of selected *mitB* mutants were confirmed by Southern blot hybridization of *S. lavendulae* genomic DNA.

Analysis of MC production. All cultures intended for MC extraction were grown in Nishikohri medium (42) for a period of 72 h. In all cases, a wild-type *S. lavendulae* culture was grown concurrently with the mutant cultures to provide MC production reference points. A 72-h, 50-ml culture (250-ml flask) of the MitA K191A MV102 mutant strain was supplemented with 125 μl of a 20-mg/ml solution of the sodium salt of AHBA (pH. 7.05) at 24, 43, and 55 h. In each case, the culture broth was separated from mycelia by centrifugation and then extracted three times with equal volumes of ethyl acetate. The ethyl acetate extracts were pooled, and the solvent was removed by vacuum to provide the crude broth extract. The preliminary screen for MC production involved thin-layer chromatography (TLC) on silica gel plates (Whatman K6) eluted with 9:1 chloroform-methanol. Production of MC was monitored by high-performance liquid chromatography (HPLC) (C_{18} reverse-phase column) with a gradient of 80% 50 mM Tris buffer (pH 7.2)–20% methanol to 40% 50 mM Tris buffer (pH 7.2)–60% methanol with the UV detector set to 363 nm.

Bioassay detection of MC was performed by loading a 1-cm disk with fractions eluting at the mitomycin retention time from HPLC injections of wild-type, MV100, pKC1139 vector control crude extracts and MC standards. The disks were placed on antibiotic medium no. 2 agar plates (Difco) with *Bacillus subtilis* spores added directly to the medium. The plates were incubated overnight at 29°C and examined for zones of inhibition. To confirm the production of MC by MV102 in the presence of exogenous AHBA, the fraction eluting at the MC retention time was collected, dried down, desalted, and submitted for desorption ionization mass spectrometric analysis on a Bio-Ion 20R DS-MS instrument (Applied Biosystems). The MC (molecular weight, 334)-sodium (molecular weight, 23) adduct peak, $[M+Na]^+$, of 357 was diagnostic for the presence of MC in the AHBA-supplemented culture.

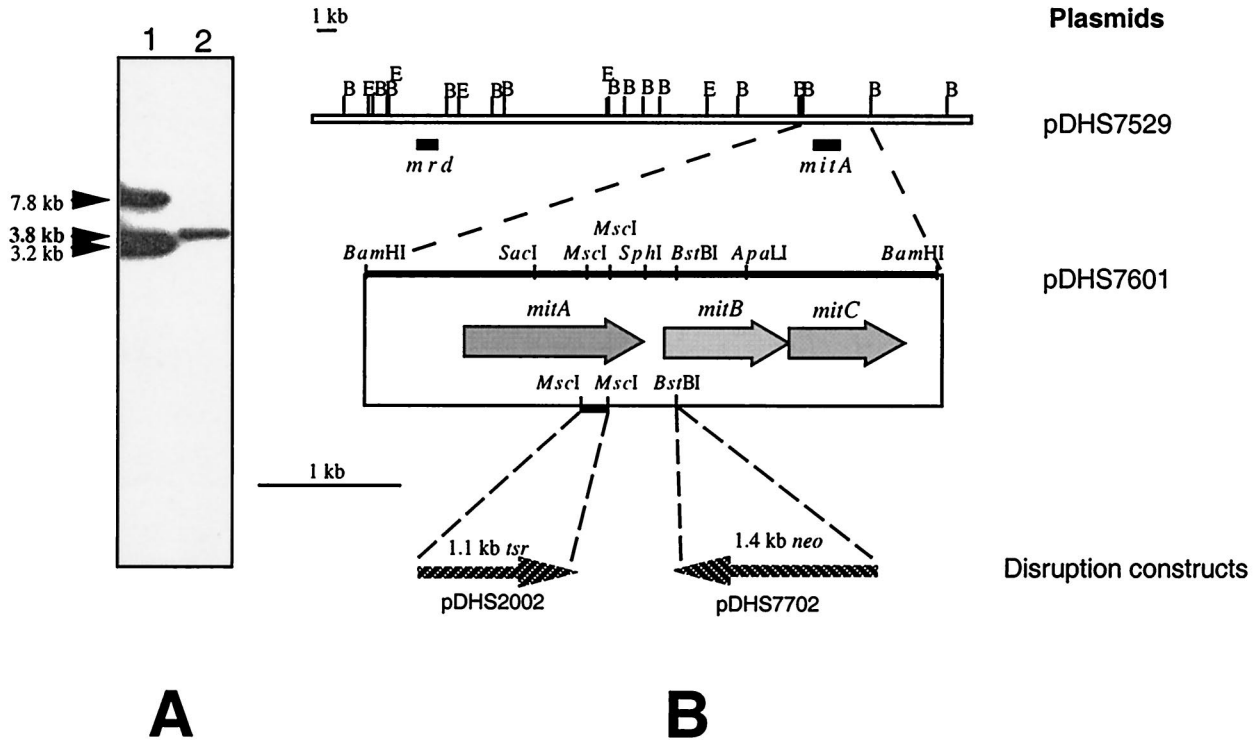

FIG. 2. Southern hybridization and restriction enzyme map of the *mrd* and *rifK* hybridizing regions from *S. lavendulae*. (A) Southern hybridization with the *rifK* gene probe (36). Lane 1, *A. mediterranei* ATCC 27643 genomic DNA digested with *Bam*HI; lane 2, *S. lavendulae* NRRL 2564 genomic DNA digested with *Bam*HI revealed a 3.8-kb hybridization band. (B) Physical map showing the *mitA*, *mitB*, and *mitC* genes. The locations of *mrd* and *rifK* hybridizing genes in cosmid pDHS7529 are indicated by solid bars. *E*, *Eco*RI; *B*, *Bam*HI. The sequenced 3.8-kb *Bam*HI fragment containing *mitA*, *mitB*, and *mitC* is enlarged (wide arrows). The thin arrows below show sites of resistance gene integration for disruption experiments.

Nucleotide sequence accession number. The GenBank accession number for *mitABC* is AF115779.

RESULTS

The *mrd* and *ahbas* genes are linked in the *S. lavendulae* genome. Southern blot analysis with the *A. mediterranei* AHBA synthase gene (*rifK*) probe (36) showed a single 3.8-kb band that hybridized with *Bam*HI-digested *S. lavendulae* genomic DNA (Fig. 2). Subsequently, a *S. lavendulae* genomic DNA library was constructed with the *E. coli-Streptomyces* shuttle cosmid pNJ1 (55). Of the 5,000 colonies screened, 21 positive clones were identified, with 6 of them hybridizing with the *mrd* gene probe (none hybridized with the *mcr* gene probe [reference 4 and data not shown]). Restriction enzyme mapping and reciprocal hybridization of the cosmid clones established that the *mrd* and *S. mediterranei* AHBA synthase homologous genes were ~20 kb apart in the *S. lavendulae* genome. The 3.8-kb *Bam*HI fragment bearing a putative *S. lavendulae* AHBA synthase gene was subcloned, and its nucleotide sequence was determined.

Three ORFs identified within the 3.8-kb *Bam*HI fragment. Three ORFs (*mitA*, *mitB*, and *mitC*) were identified within the sequenced 3.8-kb *Bam*HI fragment (Fig. 2 and 3). *mitA* comprises 1,164 nucleotides and starts from an ATG (position 579 of the sequenced fragment) that is preceded by a potential ribosome binding site (RBS), GAAAGG. The deduced product of the *mitA* gene encodes a hydrophilic protein of 388 amino acids with a predicted mass of 41,949 Da and a calculated pI of 5.62. A BLAST (2) search showed that the predicted MitA protein has high sequence similarity (~71% identity and 80% similarity) to AHBA synthases, from the rifamycin producer, *A. mediterranei* (36), and other ansamycin-producing actinomycetes, including *Actinosynnema pretiosum* (ansamitocin) and *Streptomyces collinus* (naphthomycin A and ansatrienin) (Fig. 4). A conserved pyridoxal phosphate (PLP) coenzyme binding motif ($GX_3DX_7AX_8EDX_{14}GX_{13}\underline{\mathbf{K}}X_{4-5}$ $geGGX_{19}G$) including the conserved lysine residue (boldface and underlined) can also be found in these four proteins (45).

The *mitB* gene is predicted to start at a GTG (position 1879) that is preceded by a presumed RBS (GGAACG). This gene encodes a 272-amino-acid protein with a deduced mass of 28,648 Da and a deduced pI of 6.06. Database sequence homology searches revealed that the protein product of *mitB* shows local sequence similarity to a group of *O*-glycosyltransferases involved in polysaccharide biosynthesis. One segment of 70 amino acid residues at the N terminus of MitB has 43% similarity (36% identity) to the two glycosyltransferases SpsL and SpsQ from *Sphingomonas* sp. strain S88 and ExoO from *Rhizobium meliloti*, involved in polysaccharide (S88) and succinoglycan biosynthesis, respectively (7, 60). Another 60 amino acid residues located at the C terminus displayed 30% identity with UDP-GalNAc–polypeptide *N*-acetylgalactosaminyltransferase from *Mus musculus* and *Homo sapiens* (9).

The third ORF, *mitC*, starts from the ATG at position 2694, which is coupled to the stop codon, TGA, of *mitB*, and encodes a putative protein of 260 amino acids with a predicted molecular mass of 27,817 Da and a pI of 10.45. Database searches with the deduced protein product showed significant similarity over the first 90 amino acids (38% identity and 40% similarity) to the *lmbE* gene product (unknown function) from *Mycobacterium leprae* (U15183).

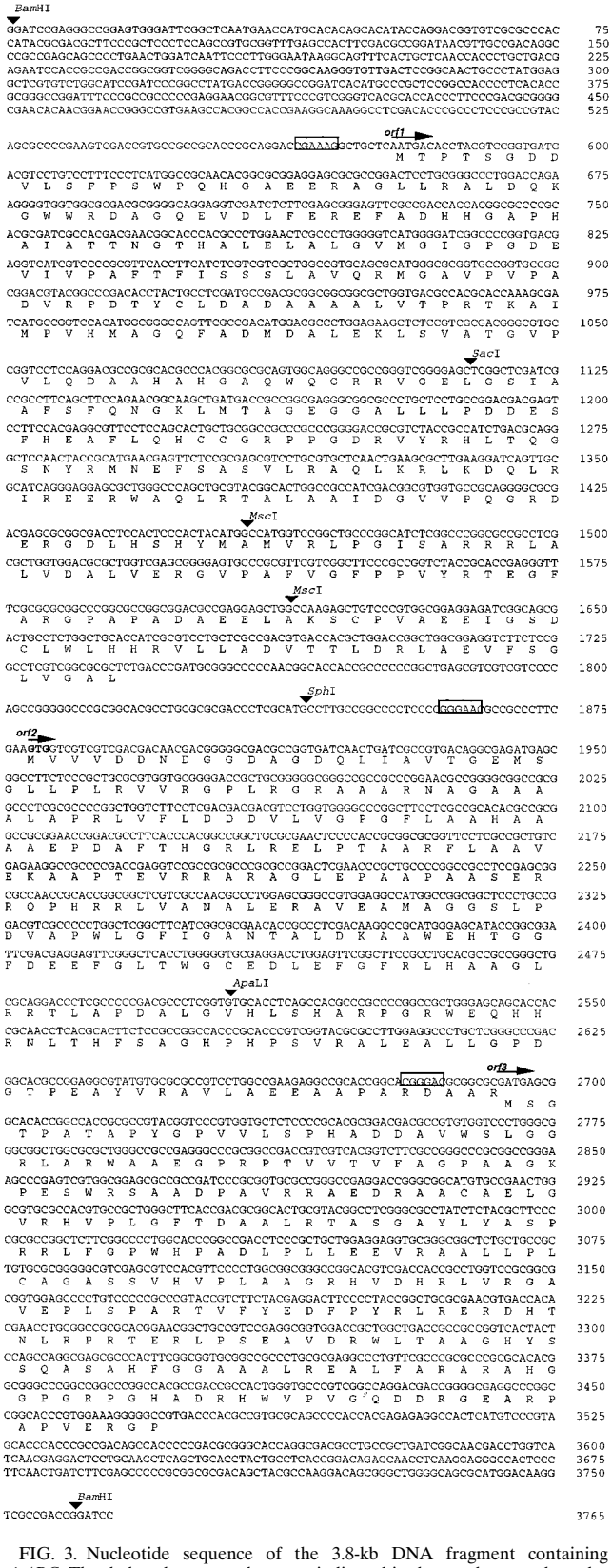

FIG. 3. Nucleotide sequence of the 3.8-kb DNA fragment containing *mitABC*. The deduced gene products are indicated in the one-letter code under the DNA sequence. Possible RBSs are boxed. The presumed translational start site and direction of transcription for each ORF are indicated by a labeled arrow. Restriction enzyme sites are marked by arrowheads.

Insertional disruption of the *mitA* and *mitB* genes in *S. lavendulae*. To test the dependence of functional *mitA* and *mitB* genes for MC biosynthesis, gene disruption constructs were generated for subsequent isolation of the corresponding *S. lavendulae* isogenic mutant strains.

The *mitA* disruption construct was made by replacing a 155-bp fragment between the two *Msc*I sites (located at the C terminus of the *mitA* gene in pDHS7601) with the 1.1-kb *Sma*I-*Bam*HI fragment containing a thiostrepton resistance gene from pDHS5000 (Fig. 5A). This replacement regenerated a *Bam*HI site at the junction, and the resulting construct was then subcloned into the *E. coli-Streptomyces* conjugative shuttle plasmid pKC1139, followed by conjugation into *S. lavendulae*. A double-crossover mutant strain (MV100) was selected based on the expected phenotype (thiostrepton resistant and apramycin sensitive) and further confirmed by Southern blot hybridization. Genomic DNAs from wild-type *S. lavendulae* and MV100 were digested with *Bam*HI and *Sph*I and hybridized with the 4.9-kb *Eco*RI-*Hin*dIII *tsr*-disrupted *mitA* fragment from pDHS2001. As expected, the 4.0-kb *Sph*I-hybridized band in the wild-type strain was shifted to 4.9 kb in MV100, whereas the 3.8-kb *Bam*HI hybridization band in the wild type was converted to two bands (2.2 and 2.5 kb) in the mutant (Fig. 5B).

The *mitB* gene was disrupted by inserting a neomycin resistance gene (*aphII*) into the *Bst*BI site (located at the 5′ end of *mitB*) (Fig. 6A). Transconjugants were selected on kanamycin-apramycin plates, and a double-crossover mutant strain (MM101) with a kanamycin-resistant, apramycin-sensitive phenotype was identified and subsequently confirmed by Southern blot hybridization. As expected, the 3.8-kb *Bam*HI hybridization band in wild-type *S. lavendulae* was shifted to 5.2 kb in MM101, whereas a 5.2-kb *Sac*I hybridization band was shifted to 6.6 kb (Fig. 6B).

***mitA*- and *mitB*-disrupted strains (MV100 and MM101) are blocked in MC biosynthesis.** The growth characteristics and morphologies of MV100 and MM101 in liquid medium and on agar plates were identical to those of wild-type *S. lavendulae*. HPLC was used to quantify production of MC in MV100 and MM101 (Fig. 7A), and culture extracts were used in a biological assay to test for the presence of the drug (Fig. 7B). Injection of 1 mg of wild-type *S. lavendulae* culture extract gave a peak in the HPLC that eluted with the same retention time as the MC standard. Upon injection of 1 mg of culture extract from the *mitA*- or *mitB*-disrupted strains (MV100 and MM101), no MC peak was observed. To corroborate the lack of production of MC, the HPLC eluant obtained from the MV100 culture extracts was collected over the retention time range determined for MC. This eluant completely lacked biological activity against *B. subtilis* (the MC target strain), while the fraction collected from the same retention time region of wild-type *S. lavendulae* and the vector control strain culture extracts showed substantial levels of biological activity (Fig. 7B).

It is important to note that the presence of the vector pKC1139 in *S. lavendulae* reduced the percentage of MC in the total crude extract while simultaneously increasing the total amount of material extractable by ethyl acetate. The combination of these two effects reduces the absolute amount of MC by approximately 25% in the vector control culture crude extract compared to that in the wild-type crude extract.

Exogenous AHBA can restore MC production in the MC-deficient MitA K191A mutant. Although complementation of MV100 (the *mitA* insertional disruptant) was attempted by providing exogenous AHBA in the culture medium, MC production was not restored as measured by HPLC or biological

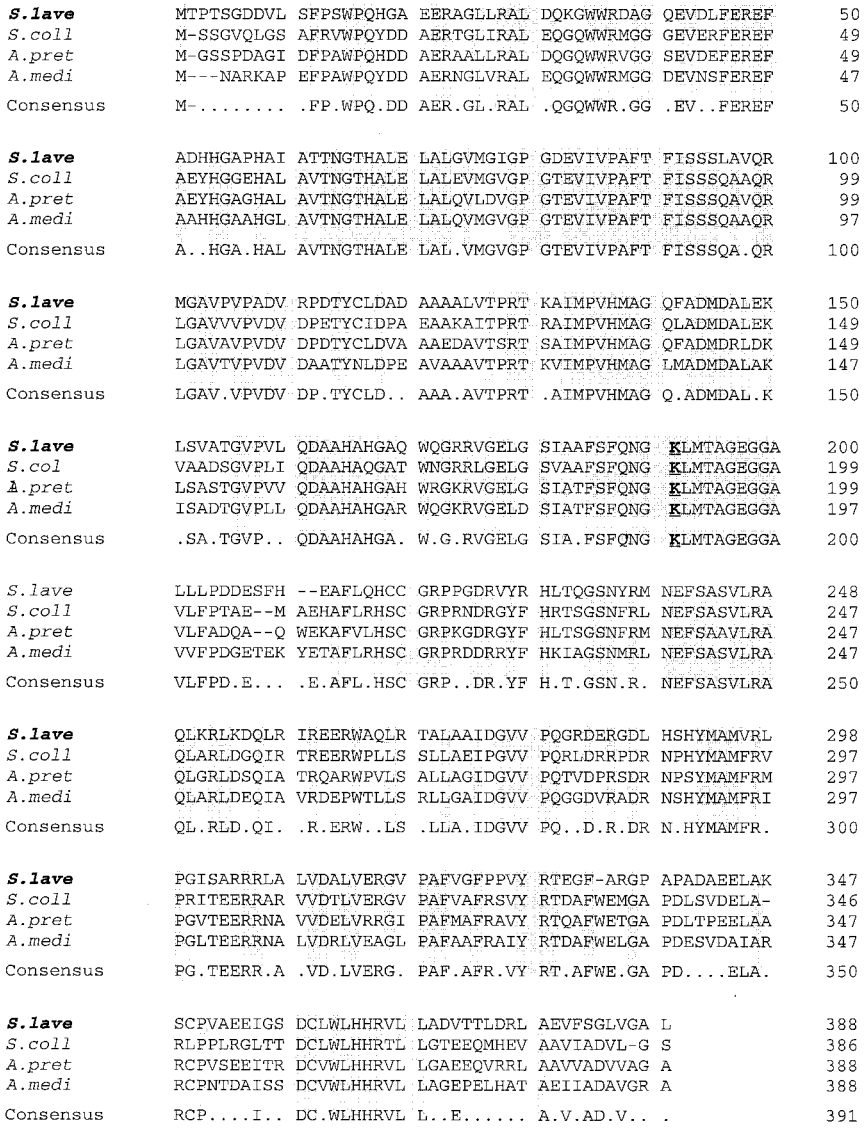

FIG. 4. Alignment of MitA with three other AHBA synthases. The deduced amino acid sequence comparison from AHBA synthase genes derived from *S. lavendulae* (*mitA*), *S. collinus* (Z54208), *A. pretiosum* (I39657), and *A. mediterranei* (I39657) is shown with the conserved lysine in the PLP-binding motif in boldface and underlined. Residues conserved in all sequences are shaded.

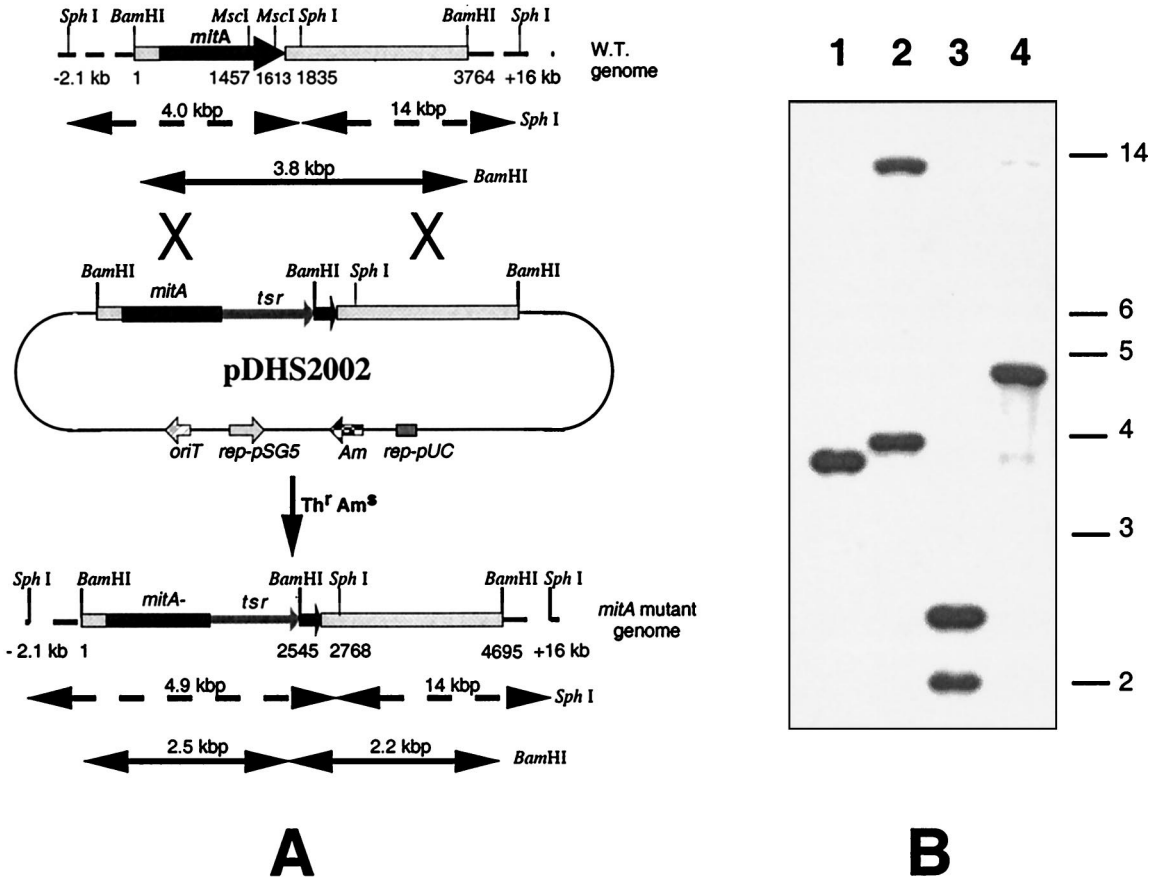

FIG. 5. Southern blot analysis of the *mitA* mutant strain MV100. (A) Construction of *mitA* disruption mutant and restriction map of the wild type and *mitA* disruption mutant showing expected band sizes in the Southern blot. The maps are not drawn to scale. (B) *S. lavendulae* genomic DNAs from the wild type (lanes 1 and 2) and the double-crossover mutant MV100 (lanes 3 and 4) were digested with *Bam*HI (lanes 1 and 3) and *Sph*I (lanes 2 and 4), respectively. The 4.9-kb *Eco*RI-*Hin*dIII fragment from pDHS2001 containing *tsr*-disrupted *mitA* was used as the probe.

assay. A polar effect on genes downstream of *tsr*-disrupted *mitA* in MV100 appeared likely, since supplying *mitA* in *trans* on a medium-copy-number plasmid (MV103) also failed to restore MC production. Therefore, site-directed mutagenesis was employed to generate a MitA K191A mutant, resulting in strain MV102. Kim et al. had demonstrated that the AHBA synthase from *A. mediterranei* is PLP dependent and catalyzes the aromatization of aminoDHS (36). Thus, the nitrogen of conserved lysine 191 is supposed to form a Schiff base with the PLP cofactor. Replacement of lysine 191 with alanine prevents binding of the cofactor and eliminates enzymatic activity. Replacement of the AGG encoding lysine 191 in wild-type *S. lavendulae* with a GCC codon in MV102 was confirmed by nucleotide sequence analysis. As expected, MV102 did not produce MC; however, when the culture medium was supplemented with exogenous AHBA, MC production was restored, as determined by MS ($[M+Na]^+ = 357$), HPLC, and TLC analysis (Table 2).

DISCUSSION

An effective strategy for the identification of natural product biosynthetic gene clusters in actinomycetes has included cloning of antibiotic resistance genes followed by investigation of adjacent DNA for the presence of structural and regulatory genes (13, 20, 40, 57). Although linkage of antibiotic resistance and biosynthetic genes appears to be a general feature in procaryotes, a growing number of examples involve the existence of multiple-resistance loci that may be linked or unlinked to the biosynthetic gene cluster (16, 49, 51). The identification and characterization of two genetically unlinked resistance loci (4, 50) for MC created a dilemma for mounting an effective search for the MC biosynthetic gene cluster. However, the use of the AHBA synthase gene from *A. mediterranei* provided an effective probe for identifying cosmid clones bearing a linked MC resistance gene. Thus, the isolation of several cosmid clones from an *S. lavendulae* genomic DNA library that hybridized to both the *A. mediterranei* AHBA synthase gene and the *S. lavendulae mrd* gene indicated that the MC biosynthetic gene cluster resided on DNA adjacent to *mrd*. DNA sequence analysis of the 3.8-kb *Bam*HI fragment revealed three ORFs whose deduced protein sequences corresponded to an AHBA synthase, a glycosyltransferase, and an *lmbE*-like product.

As determined by precursor feeding experiments, the mitosane core is formed through the condensation of AHBA and D-glucosamine (27). AHBA is thought to be derived from the ammoniated shikimate pathway from PEP and E4P, in which the last step from aminoDHS to AHBA is catalyzed by AHBA synthase (Fig. 1) (35, 36). Meanwhile, the reaction of attaching an activated sugar residue to a core compound is usually catalyzed by a group of enzymes called glycosyltransferases as specified by macrolide, glycopeptide antibiotic, and polysaccharide biosynthesis (31, 43, 52, 60). In principle, the condensation of AHBA with D-glucosamine can be initiated in two

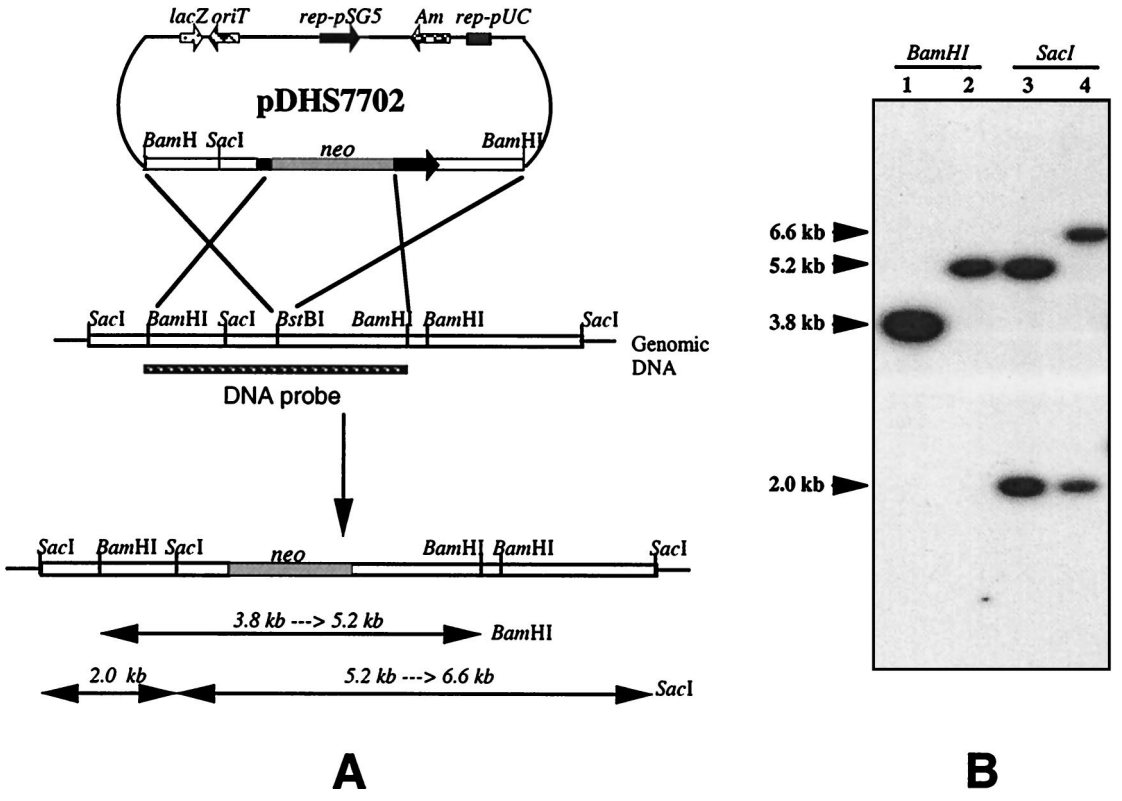

FIG. 6. (A) Construction of *mitB* disruption mutant and restriction map of the wild type and *mitB* disruption mutant showing expected band sizes in the Southern blot. (B) Southern blot analysis of *mitB* mutant MM101. *S. lavendulae* genomic DNAs from the wild type (lanes 1 and 3) and *mitB* mutant MM101 (lanes 2 and 4) were digested with *Bam*HI (lanes 1 and 2) and *Sac*I (lanes 3 and 4). The DNA probe was the 3.8-kb *Bam*HI fragment insert from pDHS7601. (B) Southern blot hybridization of *S. lavendulae* genomic DNA of the double-crossover disruption mutant showing the expected hybridization bands.

ways (Fig. 1). One would involve the formation of the C-8a–C-9 bond by an electrophilic aromatic alkylation or acylation. A second possibility would be formation of a Schiff base between the nitrogen of AHBA and the D-glucosamine C-1 aldehyde, followed by ring closure at C-8a–C-9. In either case, a *C*- or *N*-glycosyltransferase instead of an *O*-glycosyltransferase is expected. Although previously described glycosyltransferases display a high degree of sequence divergence (60), the mechanistic similarity with *O*-glycosyl transfer may suggest that *mitB* encodes a *N*-glycosyltransferase that initiates the formation of the mitosane system by linking glucosamine to AHBA. The *mitA* and *mitB* genes and their corresponding products are likely candidates to mediate formation of AHBA and the mitosane ring system, respectively. However, the possible function of the *lmbE*-like protein remains unclear, since its current role within the lincomycin biosynthetic pathway of *Streptomyces lincolnensis* is not known (44).

The involvement of AHBA synthase (encoded by *mitA*) and the putative glycosyltransferase (encoded by *mitB*) in MC biosynthesis was established by gene disruption to create mutants with MC biosynthesis blocked. This required development of a method to introduce DNA into *S. lavendulae* NRRL 2564, since the strain remains refractory to traditional *Streptomyces* protoplast- and electroporation-mediated transformation procedures. The modified protocol of Bierman et al. (12) was used to effect efficient conjugative transfer into *S. lavendulae* by using the *E. coli-Streptomyces* shuttle plasmid pKC1139. This result is significant because it has enabled the development of an effective system for analyzing in detail the genes involved in MC biosynthesis.

The function of *mitA* was probed by providing strains MV100 and MV102 with exogenous AHBA in the culture medium. Despite repeated attempts to complement MV100, MC production was not restored as measured by HPLC or biological assay. It is believed that insertion of the *tsr* gene into *mitA* resulted in disruption of biosynthetic genes immediately downstream, since supplying *mitA* in *trans* on a medium-copy-number plasmid also failed to restore MC production to MV100. This putative polar effect was eliminated by generating the MitA K191A mutant strain MV102. Providing exogenous AHBA to this mutant strain of *S. lavendulae* restored production of MC as shown by TLC, HPLC, and mass spectrometry. When MV102 was grown in the absence of AHBA there was no detectable production of MC. The ability of AHBA to complement the mutant MitA protein further supports the function of MitA as an AHBA synthase, as indicated by the database protein sequence alignment and previous studies of *rifK* (36).

TABLE 2. Complementation results with or without AHBA

S. lavendulae strain	MC production[a]	
	With AHBA	Without AHBA
Wild type	+	+
MV100	−	−
MV103	−	−
MV102	+	−

[a] +, production; −, no production.

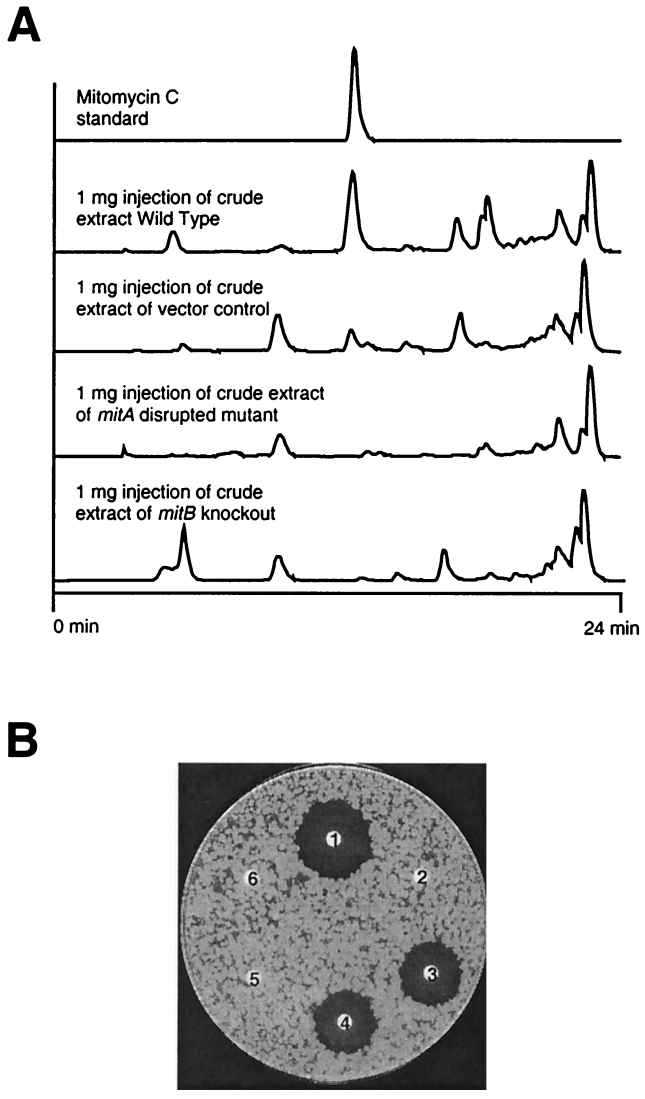

FIG. 7. Chemical analysis and biological activities of extracts from *S. lavendulae* wild-type and mutant strains. (A) HPLC analysis of authentic mitomycin C standard, mitomycin C production in the wild-type *S. lavendulae*, wild type with vector control, and *mitA* and *mitB* disruption mutants of *S. lavendulae*. For each analysis, 1 mg of crude extract was injected; 1 μg of MC was injected as a standard. (B) *B. subtilis* bioassay of MC production in *mitA* disruption mutant strain of *S. lavendulae*. Filter discs: 1, 100-μg injection of wild type (collected from 12.5 to 13.5 min); 2, 100-μg injection of *mitA* disruption mutant (collected from 12.5 to 13.5 min); 3, 100-μg injection of the wild type containing the vector (collected from 12.5 to 13.5 min); 4, 1 μg of MC collected from HPLC from 12.5 to 13.5 min; 5, Tris buffer negative control; 6) methanol solvent negative control.

Although our studies have focused on the identification of genes involved in the early steps of MC biosynthesis, further characterization of the genes and functions that mediate individual steps in the pathway will provide information required to understand more fully the details of gene regulation, molecular assembly, and cellular resistance for this important molecule.

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