Identification of Two Genes from *Streptomyces argillaceus* Encoding Glycosyltransferases Involved in Transfer of a Disaccharide during Biosynthesis of the Antitumor Drug Mithramycin

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Mithramycin is an antitumor polyketide drug produced by *Streptomyces argillaceus* that contains two deoxy-sugar chains, a disaccharide consisting of two D-olivoses and a trisaccharide consisting of a D-olivose, a D-oliose, and a D-mycarose. From a cosmid clone (cosAR3) which confers resistance to mithramycin in streptomycetes, a 3-kb *Pst*I-*Xho*I fragment was sequenced, and two divergent genes (*mtmGI* and *mtmGII*) were identified. Comparison of the deduced products of both genes with proteins in databases showed similarities with glycosyltransferases and glucuronosyltransferases from different sources, including several glycosyltransferases involved in sugar transfer during antibiotic biosynthesis. Both genes were independently inactivated by gene replacement, and the mutants generated (M3G1 and M3G2) did not produce mithramycin. High-performance liquid chromatography analysis of ethyl acetate extracts of culture supernatants of both mutants showed the presence of several peaks with the characteristic spectra of mithramycin biosynthetic intermediates. Four compounds were isolated from both mutants by preparative high-performance liquid chromatography, and their structures were elucidated by physicochemical methods. The structures of these compounds were identical in both mutants, and the compounds are suggested to be glycosylated intermediates of mithramycin biosynthesis with different numbers of sugar moieties attached to C-12a-O of a tetracyclic mithramycin precursor and to C-2-O of mithramycinone: three tetracyclic intermediates containing one sugar (premithramycin A1), two sugars (premithramycin A2), or three sugars (premithramycin A3) and one tricyclic intermediate containing a trisaccharide chain (premithramycin A4). It is proposed that the glycosyltransferases encoded by *mtmGI* and *mtmGII* are responsible for forming and transferring the disaccharide during mithramycin biosynthesis. From the structures of the new metabolites, a new biosynthetic sequence regarding late steps of mithramycin biosynthesis can be suggested, a sequence which includes glycosyl transfer steps prior to the final shaping of the aglycone moiety of mithramycin.

Many bioactive drugs contain sugars attached to their aglycones which are usually important or, in some cases, essential for bioactivity. Most of these sugars belong to the family of the 6-deoxyhexoses (6-DOH) (18, 20, 27) and are transferred to the different aglycones as late steps in biosynthesis. Genes involved in the biosynthesis of different 6-DOH have been reported elsewhere and participate in the biosynthesis of erythromycin (9, 12, 31, 38, 39), daunorubicin (13, 26, 36), mithramycin (22), granaticin (2), streptomycin (10, 28), and tylosin (14, 23). However, information about the glycosyltransferases (GTFs) responsible for the transfer of the sugars to the respective aglycones is quite scarce. So far, only two GTFs from antibiotic producers have been biochemically characterized in detail, and they are involved in macrolide inactivation: Mgt, from *Streptomyces lividans*, a nonmacrolide producer (7, 17);

and OleD, from the oleandomycin producer *Streptomyces antibioticus* (15, 29), which inactivates oleandomycin by addition of glucose to the 2'-OH group of the desosamine attached to the macrolactone ring (40). In the last several years, a few genes have been proposed to encode GTFs involved in the transfer of sugars to various aglycones during biosynthesis: *dnrS* and *dnrH*, from *Streptomyces peucetius*, involved in daunorubicin (26) and baumycin (36) biosynthesis, respectively; *graorf5*, involved in granaticin biosynthesis (2); *eryCIII* and *eryBV*, involved in the transfer of desosamine and mycarose, respectively, in erythromycin biosynthesis (12, 32, 38); and *tylM2*, from *Streptomyces fradiae*, involved in sugar transfer during tylosin biosynthesis (14).

Mithramycin (Fig. 1) is an aromatic polyketide which shows antibacterial activity against gram-positive bacteria and also antitumor activity (30, 37). Together with the chromomycins and the olivomycins, mithramycin constitutes the so-called aureolic acid group of antitumor drugs. The polyketide moiety of mithramycin is derived from the condensation of 10 acetate building blocks in a series of reactions catalyzed by a type II polyketide synthase (5, 21). The mithramycin aglycone is glycosylated at positions 6 and 2 with disaccharide (D-olivose-D-olivose) and trisaccharide (D-olivose-D-oliose-D-mycarose) moieties, respectively. All of these sugars belong to the 6-DOH

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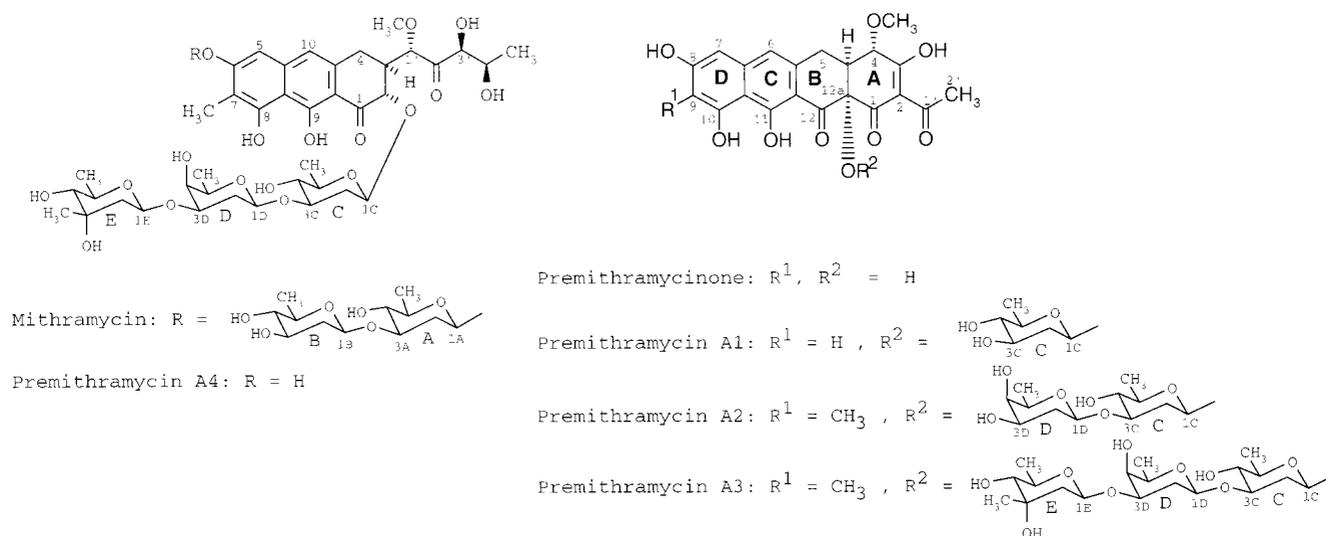
FIG. 1. Structures of mithramycin, premithramycinone, and the new premithramycins.

family. In the mithramycin pathway, two genes (*mtmD* and *mtmE*) encoding two enzymes (glucose-1-phosphate:TTP thymidylyl transferase and dTDP-4,6-dehydratase, respectively) involved in the biosynthesis of the mithramycin 6-DOH have been cloned, and their participation in mithramycin biosynthesis has been demonstrated by insertional inactivation (22). Here we report the characterization of two *Streptomyces argillaceus* genes (*mtmGI* and *mtmGII*) that encode two putative GTFs responsible for the formation and transfer of the disaccharide chain. Inactivation of these genes by gene replacement showed identical accumulated compounds and allowed the isolation of four glycosylated compounds which are likely to be intermediates in mithramycin biosynthesis.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and vectors. *S. argillaceus* ATCC 12956, a mithramycin producer, was used as a source of chromosomal DNA. For sporulation, it was grown for 7 days at 30°C on plates containing medium A, consisting of MOPS (morpholinepropanesulfonic acid) (Sigma), 21 g/liter; glucose, 5 g/liter; yeast extract (Difco), 0.5 g/liter; meat extract (Lab-Lemco Powder; Oxoid), 0.5 g/liter; Casamino Acids (Difco), 1 g/liter; final pH, 7.0, adjusted with KOH. For protoplast regeneration, the organism was grown on R5 solid medium plates (16). Liquid medium for production and isolation of mithramycin intermediates was modified R5 medium containing the following: sucrose, 100 g/liter; K_2SO_4, 0.25 g/liter; $MgCl_2 \cdot 6H_2O$, 10.12 g/liter; glucose, 10 g/liter; Casamino Acids, 0.1 g/liter; yeast extract (Difco), 5 g/liter; and MOPS, 21 g/liter. Two milliliters of R5 trace elements solution was added per liter, the pH was adjusted to 6.85, and the medium was sterilized by autoclaving. *Escherichia coli* XL1-Blue (6) was used as the host for subcloning and was grown at 37°C in Trypticase soy broth medium (Oxoid). M13mp18 and M13mp19 phage vectors were used for DNA sequencing. pUC18, pBSKT (a pBSK derivative containing a thiostrepton resistance cassette), and pIAGO (a pWHM3 derivative [39] containing the promoter of the erythromycin resistance gene [*ermE*] from *Saccharopolyspora erythraea* [4]) were used for subcloning.

DNA manipulation and sequencing. Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, ligations, and other DNA manipulations were performed or made according to standard procedures for *E. coli* (33) and for *Streptomyces* (16). Southern hybridization was performed according to standard procedures (16). Sequencing was performed on single-stranded templates derived from different clones in M13 phage by the dideoxynucleotide chain-termination method (34) with [α-^{35}S]dCTP (1,200 Ci/mmol; Amersham) and modified T7 DNA polymerase (Sequenase version 2.0; U.S. Biochemicals). To overcome band compression artifacts, 7-deaza-dGTP was routinely used instead of dGTP (24). Single-stranded DNA was prepared by the polyethylene glycol precipitation method as described elsewhere (33). Both DNA strands were sequenced with primers supplied in the Sequenase kit or with internal primers (17-mer). Computer-aided database searching and sequence analyses were carried out with the University of Wisconsin Genetics Computer Group program package (8) and the BLAST program (1).

Insertional inactivation. For inactivation of the *mtmGI* gene, a 1.6-kb *Sma*I-*Xho*I fragment (sites 4 to 12 in Fig. 2A) was subcloned between the *Sma*I-*Sal*I restriction sites of pUC18. The resulting construct was opened by the unique internal *Sal*I site present in the insert (site 6 in Fig. 2A) and made blunt ended with the large fragment of *E. coli* DNA polymerase and an apramycin resistance cassette inserted as a 1.5-kb *Sma*I-*Eco*RV fragment. A thiostrepton resistance cassette was further added to this latter construct as a 1.7-kb *Sma*I fragment in the *Sca*I site located within the ampicillin resistance gene of pUC18, thus causing its inactivation. This final construct was designated pEFG1K (Fig. 3A). For inactivation of the *mtmGII* gene, a 2.1-kb *Pst*I-*Not*I fragment (sites 1 to 7 in Fig. 2A) was subcloned into the same restriction sites of pBSKT containing the thiostrepton resistance cassette, and the apramycin resistance cassette was then subcloned in the unique *Sma*I site (site 4 in Fig. 2A) of the insert as a 1.5-kb *Sma*I-*Eco*RV fragment, generating pEFG2K (Fig. 3A). The orientation of the apramycin resistance gene in both constructs was in the same direction as that of the inactivated gene. Both constructs were then used to transform *S. argillaceus* protoplasts, integrants were selected for apramycin resistance (25 µg/ml) on R5 agar plates, and their susceptibility to thiostrepton (50 µg/ml) was tested. Am^r Thio^r integrants obtained in this primary screening were the consequence of a single-crossover event, and the screening for the second crossover essential to replace the wild-type region by the in vitro-disrupted one was carried out as described elsewhere (22).

Production and isolation of mithramycin intermediates. A seed culture was prepared in Trypticase soy broth medium inoculated with spores at an optical density at 600 nm of 0.3. After incubation for 24 h at 30°C and 200 rpm, this culture was used to inoculate (at 2.5% [vol/vol]) eight 2-liter Erlenmeyer flasks, each containing 400 ml of modified R5 medium. Production of mithramycin-related compounds was monitored at intervals as follows. Fifteen milliliters of culture was centrifuged, and the supernatant fluid was adjusted to pH 3.5 with formic acid and extracted with 5 ml of ethyl acetate. The ethyl acetate extract was then run on high-performance liquid chromatography (HPLC) with a µBondapak C_{18} column (Waters) and eluted with a linear gradient from 10 to 100% acetonitrile in 0.1% trifluoroacetic acid (TFA) in water for 30 min, at 1.5 ml/min. Detection and spectral characterization of peaks were performed with a photodiode array detector (Waters). After 72 h of incubation, the whole culture was centrifuged and the supernatant was filtered (Supor membrane; 0.2-µm pore size; Gelman) and applied to a solid-phase extraction cartridge (Supelclean LC-18; 10 g; Supelco). The cartridge was washed with 0.1% TFA in water and eluted with a mixture of acetonitrile and 0.1% TFA in water. A linear gradient from 0 to 100% acetonitrile in 60 min, at 10 ml/min, was used. Fractions were taken every 5 min and subsequently analyzed by HPLC (see above). Products with spectral characteristics resembling those of mithramycin were found in fractions between 15 and 35 min. The products were purified by preparative HPLC in a µBondapak C_{18} radial compression cartridge (PrepPak Cartridge; 25 by 100 mm; Waters). Short gradients with acetonitrile and 0.1% TFA in water, at a flow rate of 10 ml/min, were optimized for resolution of individual peaks. The material collected in each case was concentrated in vacuo and finally lyophilized.

Physicochemical characterization and structure elucidation of the new premithramycins. R_f values of putative mithramycin intermediates were determined on silica with a $CHCl_3/CH_3COOH/CH_3OH/H_2O$ ratio of 58:34:7:1 as the solvent. Relative retention times (R_{rel}) in HPLC were determined on a Kontrosorp 10 C_{18} semipreparative column with a flow rate of 5 ml/min with an H_2O (1%
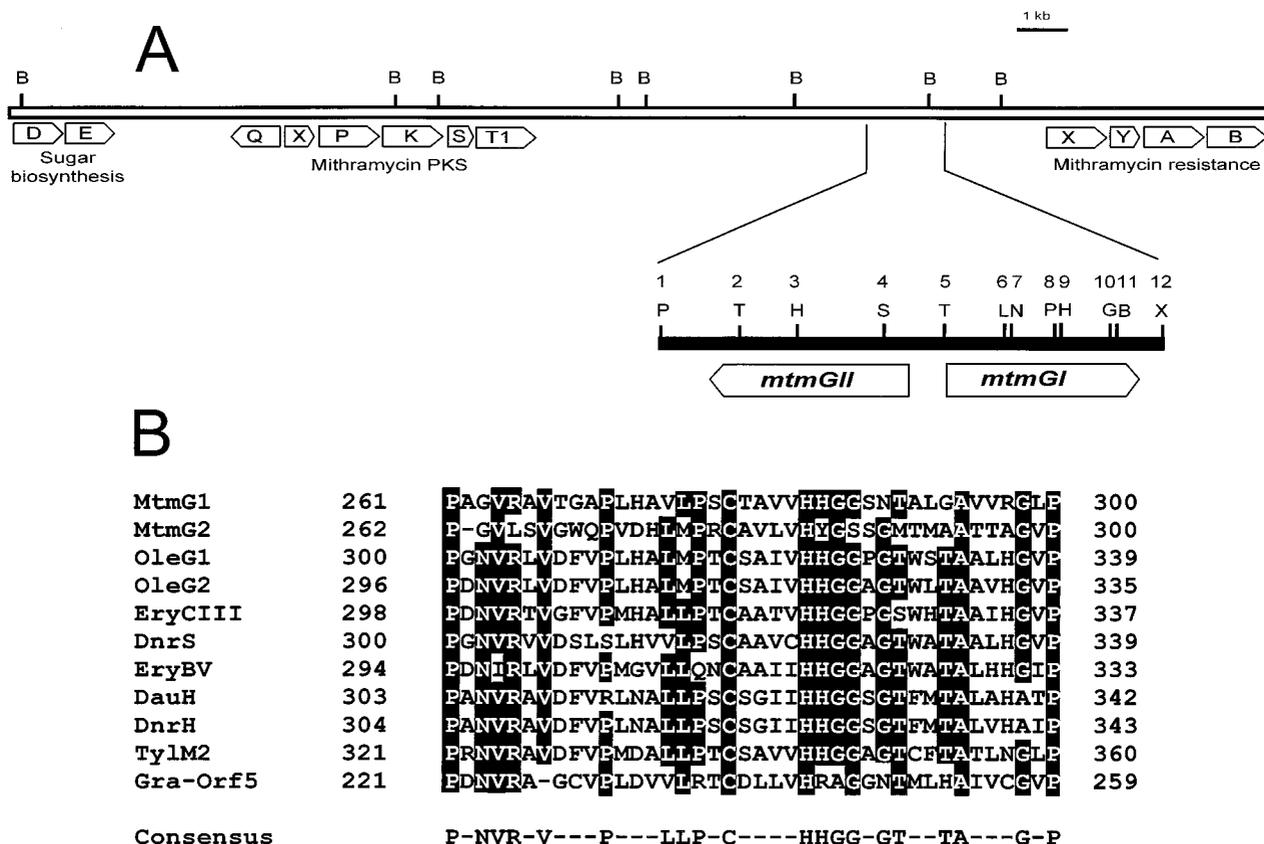
FIG. 2. (A) Schematic representation of the region sequenced from cosAR3 and location of the *mtmGI* and *mtmGII* genes with respect to previously reported genes from the mithramycin gene cluster. Mithramycin sugar biosynthetic genes (22) and mithramycin polyketide synthase genes (21) were designated *mtm* genes, while mithramycin resistance genes (11) were designated *mtr* genes. B, *Bam*HI; G, *Bgl*II; H, *Sph*I; L, *Sal*I; N, *Not*I; P, *Pst*I; S, *Sma*I; T, *Stu*I; X, *Xho*I. (B) Alignment of the deduced amino acid sequences of different GTFs involved in antibiotic biosynthesis. MtmGI and MtmGII, mithramycin GTFs from *S. argillaceus* (this work); OleG1 and OleG2, oleandomycin GTFs from *S. antibioticus* (25); EryCIII and EryBV, desosaminyl and mycarosyl GTFs from *Saccharopolyspora erythraea* (12, 38); DnrH, baumycin GTF from *S. peucetius* (36); DnrS, daunorubicin GTF from *S. peucetius* (26); DauH, daunomycin GTF from *Streptomyces* sp. strain C5 (GenBank accession no. U43704); Gra-Orf5, granaticin GTF from *S. violaceoruber* (2); TylM2, tylosin GTF from *S. fradiae* (14).

HCOOH)/CH$_3$CN ratio of 63:37 as the eluent. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Inova 500 instrument in d$_6$-acetone with instruments at field strengths of 7.05 and 11.94 T. Fast atom bombardment (FAB) mass spectra were recorded with nitrobenzylic alcohol as the matrix. UV and circular dichroism (CD) spectra were recorded in methanol. To obtain an acidic and alkaline UV spectrum, 1 drop of concentrated HCl and NaOH, respectively, was added to the methanol solution. Infrared (IR) spectra were measured as KBr pellets. The new structures were predominantly elucidated through FAB mass spectroscopy (MS) and NMR spectroscopy. Various standard NMR methods including ^{1}H NMR, broad-band H-decoupled ^{13}C NMR, attached proton test (APT), homonuclear correlation spectroscopy, and heteronuclear correlation spectroscopy (hetero-multiple quantum correlation and hetero-multiple bond correlation [HMBC]) have been used to detect all $^1J_{C-H}$ and $^nJ_{C-H}$ long-range couplings ($n = 2$ to 4), which allowed unambiguous assignments of all protons and carbons (if not stated otherwise in Table 1 or 2).

Values determined for individual intermediates were as follows. Premithramycin A1: $R_f = 0.26$; HPLC: $R_{rel} = 9$ min; IR (KBr): $\nu = 3,426, 2,924, 1,634, 1,460, 1,351, 1,163, 1,056,$ and 535 cm^{-1}; FAB MS (negative ions): m/z 543 (100% [M-H]$^-$); UV (CH$_3$OH) λ_{max} nanometers (ε): 426 (7,400), 281 (26,900), 230 (16,700), and 202 (16,900); (CH$_3$OH/NaOH) λ_{max} nanometers (ε): 422 (11,100), 280 (27,000), and 205 (18,200); (CH$_3$OH/HCl) λ_{max} nanometers (ε): 423 (7,400), 342 (4,000), 328 (4,400), 282 (24,000), 230 (22,200), 202 (19,200); CD (c = 2.85 · 10^{-5} mol/liter, CH$_3$OH): $\lambda_{extr.}$ nanometers ([Θ]^{24}) = 424.4 (−16,300), 345.8 (10,300), 323.0 (5,100), 311.8 (7,400), 305.2 (8,800), 281.2 (54,500), 261.0 (−7,100), 247.6 (8,600), and 229.6 (−13,200). NMR data: see Tables 1 and 2. Premithramycin A2: $R_f = 0.36$; HPLC: $R_{rel} = 23$ min; IR (KBr): $\nu = 3,422, 2,926, 2,361, 1,628, 1,420, 1,348, 1,158, 1,067,$ and 668 cm^{-1}; FAB MS (negative ions): m/z 687 (100%, [M-H]$^-$); UV (CH$_3$OH) λ_{max} nanometers (ε): 428 (7,700), 343 (6,800), 327 (6,700), 280 (31,100), 232 (21,900), and 202 (15,100); (CH$_3$OH/NaOH) λ_{max} nanometers (ε): 426 (11,400), 281 (31,300), and 205 (25,600); (CH$_3$OH/HCl) λ_{max} nanometers (ε): 432 (7,600), 346 (6,700), 284 (26,900), 232 (26,400), and 202 (17,900); CD (c = 3.81 · 10^{-5} mol/liter, CH$_3$OH): $\lambda_{extr.}$ nanometers ([Θ]^{24}) = 424.4 (−8,700), 347.4 (15,900), 322.6 (9,100), 289.8

(67,700), 265.6 (−8,400), 248.6 (6,300), and 227.2 (−17,800). NMR data: see Tables 1 and 2. Premithramycin A3: $R_f = 0.40$; HPLC: $R_{rel} = 29$ min; IR (KBr): $\nu = 3,425, 2,928, 1,629, 1,419, 1,369, 1,344, 1,158, 1,067,$ and 606 cm^{-1}; FAB MS (negative ions): m/z 831 (100% [M-H]$^-$); UV (CH$_3$OH) λ_{max} nanometers (ε): 430 (8,400), 344 (6,900), 282 (33,300), 232 (23,200), and 201 (14,800); (CH$_3$OH/NaOH) λ_{max} nanometers (ε): 427 (12,300), 282 (33,900), and 205 (18,900); (CH$_3$OH/HCl) λ_{max} nanometers (ε): 433 (8,400), 346 (7,200), 285 (29,200), 232 (28,900), and 201 (18,100); CD (c = 2.67 · 10^{-5} mol/liter, CH$_3$OH): $\lambda_{extr.}$ nanometers ([Θ]^{24}) = 427.8 (−11,700), 346.8 (17,900), 323.0 (10,500), 289.6 (81,100), 265.0 (−8,600), 247.8 (9,700), and 226.2 (−24,100). NMR data: see Tables 1 and 2. Premithramycin A4: $R_f = 0.24$; HPLC: $R_{rel} = 14$ min; IR (KBr): $\nu = 3,425, 2,919, 2,353, 1,628, 1,411, 1,375, 1,255, 1,068, 803,$ and 610 cm^{-1}; FAB MS (negative ions): m/z 823 (100% [M-H]$^-$); UV (CH$_3$OH) λ_{max} nanometers (ε): 418 (5,000), 323 (5,200), 282 (21,000), 231 (12,300), and 202 (14,100); (CH$_3$OH/NaOH) λ_{max} nanometers (ε): 412 (7,100), 279 (21,000), 236 (10,600), and 205 (24,300); (CH$_3$OH/HCl) λ_{max} nanometers (ε): 413 (4,400), 324 (5,600), 279 (18,900), 231 (13,600), and 205 (24,800); CD (c = 2.67 · 10^{-5} mol/liter, CH$_3$OH): $\lambda_{extr.}$ nanometers ([Θ]^{24}) = 441.0 (9,700), 398.2 (−2,800), 357.0 (−500), 335.4 (−4,900), 313.8 (−1,400), 295.0 (−6,300), 283.6 (3,200), 268.8 (−4,000), 248.8 (−1,600), and 218.8 (1,000). NMR data: see Tables 1 and 2.

Nucleotide sequence accession number. The GenBank accession number for the fragment is AF077869.

RESULTS

Sequencing of the *mtmGI* and *mtmGII* genes. We have previously reported the cloning and characterization of a region of the chromosome of *S. argillaceus* ATCC 12956 (mithramycin producer) from clone cosAR3 that confers a high level of resistance to mithramycin in a heterologous host (11). We have now cloned (from clone cosAR3) and sequenced a 3-kb *Pst*I-
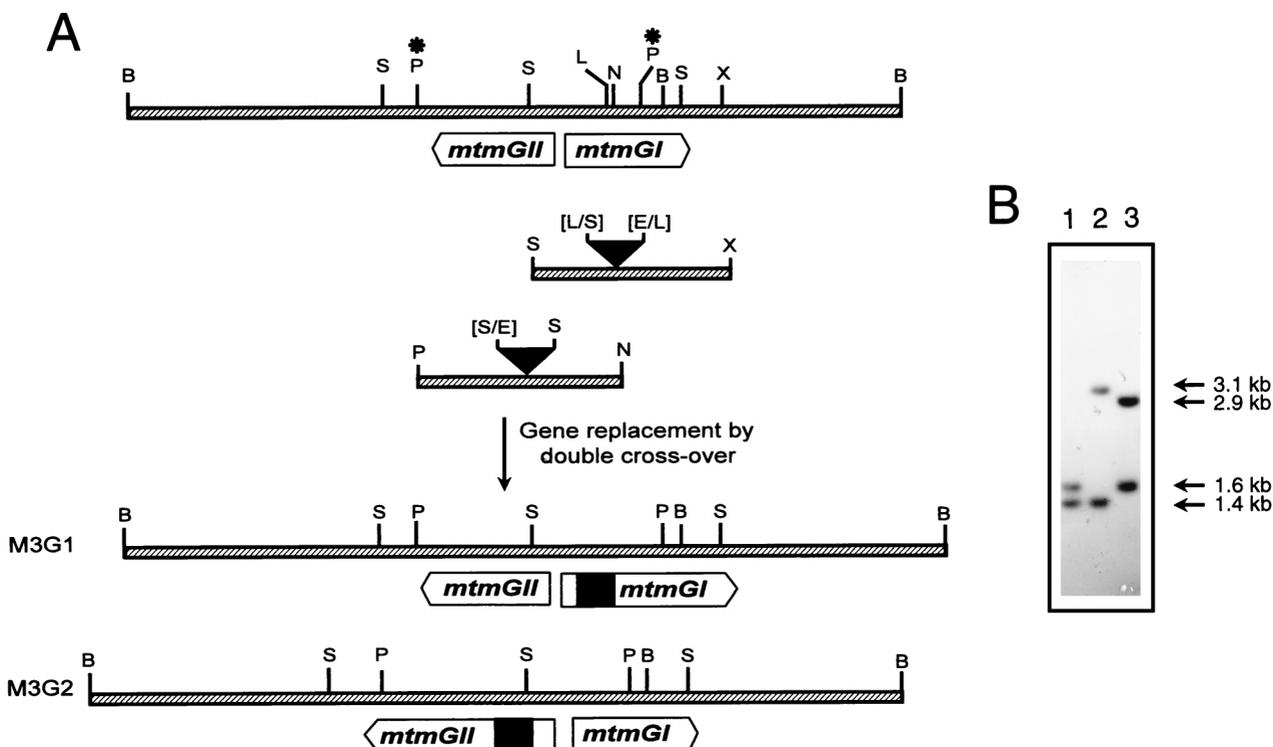
FIG. 3. Analysis of gene replacement in the generation of mutants M3G1 and M3G2. (A) Scheme representing the replacement in the chromosome of the wild-type *mtmGI* and *mtmGII* genes by the in vitro-mutated ones. In the case of the *mtmGI* gene, an apramycin resistance cassette (black inverted triangle) was subcloned into the blunt-ended *Sal*I site of *mtmGI* (generating mutant M3G1). In the case of *mtmGII*, the apramycin resistance cassette was subcloned into the unique *Sma*I site of *mtmGII* (generating mutant M3G2). B, *Bam*HI; L, *Sal*I; N, *Not*I; P, *Pst*I; S, *Sma*I; X, *Xho*I. The asterisks above the two *Pst*I sites indicate the boundaries of the probe used. (B) Southern hybridization with the 2.3-kb *Pst*I fragment as the ^{32}P-labeled probe. Chromosomal DNA from the wild-type strain and that from mutants M3G1 and M3G2 were digested with *Sma*I and analyzed by Southern hybridization. Lane 1, *Sma*I-digested chromosomal DNA from the wild-type strain. Lane 2, *Sma*I-digested chromosomal DNA from mutant M3G1. Lane 3, *Sma*I-digested chromosomal DNA from mutant M3G2.

*Xho*I fragment located approximately 1.7 kb upstream of the *mtrX* gene and 13.5 kb downstream of the mithramycin polyketide synthase genes, *mtmQXPKST1* (21) (Fig. 2A). The nucleotide sequence was analyzed for coding regions with the CODONPREFERENCE program (8), and two divergent open reading frames (ORFs) were observed. The first ORF (designated *mtmGII*) comprises 1,140 nucleotides and would code for a polypeptide with an estimated M_r of 40,308. The starting codon (ATG) of the second ORF (designated *mtmGI*) is 220 bp from *mtmGII* and is divergently transcribed from *mtmGII*. It comprises 1,182 nucleotides and would code for a polypeptide with an estimated M_r of 41,563. Both genes have a high GC content and the bias in the third codon position characteristic of *Streptomyces* genes.

Deduced functions of the MtmGI and MtmGII proteins. Computer analysis of *mtmGI* and *mtmGII* gene products with the BLAST program (1) identified similarities between the MtmGI and MtmGII proteins and GTFs involved in sugar transfer in biosynthesis of a number of antibiotics and antitumor drugs: DnrS from the daunorubicin pathway in *S. peucetius* (26), DnrH from the baumycin pathway in *S. peucetius* (36), DauH from the daunomycin pathway in *Streptomyces* sp. strain C5 (GenBank accession no. U43704), EryBV and EryCIII from the erythromycin pathway in *Saccharopolyspora erythraea* (12, 38), Gra-orf5 from the granaticin in *Streptomyces violaceoruber* (2), TylM2 from the tylosin pathway in *S. fradiae* (14), and OleG1 and OleG2 from the oleandomycin pathway in *S. antibioticus* (25). Both MtmGI and MtmGII proteins showed similar percentages of identity and similarity of amino acids with all of these antibiotic GTFs, ranging from 30 to 37% identity and 53 to 60% similarity of amino acids. MtmGI and MtmGII also resemble, although to a lesser extent, two GTFs that have been shown to be involved in macrolide inactivation in *S. lividans* (Mgt [17]) and in *S. antibioticus* (OleD [15, 29]). All of these GTFs and also MtmGI and MtmGII contain a characteristic motif present in UDP-glycosyltransferases and UDP-glucuronosyltransferases which is close to the C termini of the proteins (Fig. 2B).

Inactivation of the *mtmGI* and *mtmGII* genes. To assay the role of the *mtmGI* and *mtmGII* genes in sugar transfer during mithramycin biosynthesis, each gene was insertionally inactivated by insertion of an antibiotic resistance cassette. After the gene replacement experiments, one colony was selected from each experiment (designated M3G1 and M3G2 for inactivation of *mtmGI* and *mtmGII*, respectively). To verify that the DNA replacement was actually taking place, DNA from the wild-type strain and from the mutants M3G1 and M3G2 was prepared, *Sma*I digested, and analyzed by Southern hybridization. With a 2.3-kb *Pst*I fragment (*Pst*I sites are indicated by asterisks in Fig. 3A) as a probe, two *Sma*I hybridizing bands (1.4 and 1.6 kb) were observed in the wild-type strain, while in the mutants the *Sma*I bands changed as a consequence of the gene replacement to 1.4 and 3.1 kb (for mutant M3G1) and 1.6 and 2.9 kb (for mutant M3G2) (Fig. 3B). According to the restriction map of the region and that of the inserted apramycin resistance cassette, this Southern analysis indicated that the *mtmGI* and *mtmGII* genes had been replaced by the in vitro-mutated copies. To verify that the gene replacement was affect-

TABLE 1. ^{1}H NMR data of premithramycins A1 to A4 in d_6-acetone[a]

Position	A1[b,c]	A2[c,d]	A3[e,f]	A4[f,g]
2-H				4.75 d (11.5)
3-H				2.77 tdd (11.5, 3.5, 2)
4-H	4.10 d (11)	4.08 s br[h]	4.04 s br[h]	a: 2.95[i]
				e: 2.62 dd (16.5, 3.5)
4-OMe	3.53 s	3.52 s	3.52 s	
4a-H	3.11 ddd (11, 4, 3)	3.13 ddd (11, 4, 3)	3.12 ddd (11, 4, 3)	
5-H	a: 3.88 ddd (16.5, 4, 1.5)	a: 3.85 ddd (16.5, 4, 1)	a: 3.79 ddd (16.5, 4, 1)	6.64 s
	e: 3.04 dd (16.5, 3)	e: 3.06 dd (16.5, 3)	e: 3.05 dd (16.5, 3)	
6-H	6.98 d (1.5)	6.93 d (1)	6.93 d (1)	
7-H	6.65 d (2)	6.70 s	6.71 s	
7-Me				2.14 s
9-H	6.42 d (2)			
9-CH$_3$		2.15 s	2.16 s	
10-H				6.72 s
1′-H				4.84 d (2)
1′-OMe				3.42 s
2′-H$_3$	2.60 s	2.60 s	2.59 s	
3′-H				4.26 d (2.5)
4′-H				4.26 m[i]
5′-H$_3$				1.25 d (6)
1A-H	4.91 dd (9.5, 2)	4.89 dd (9.5, 2)	4.88 dd (9.5, 2)	5.10 dd (10, 2)
2A-H$_a$	1.52 ddd (12, 12, 9.5)	1.56 ddd (12, 12, 9.5)	1.55 ddd (12, 12, 9.5)	1.60 ddd (12, 12, 10)
2A-H$_e$	2.49 ddd (12, 5, 2)	2.54 ddd (12, 5, 2)	2.55 m (broad)	2.50 ddd (12, 5, 2)
3A-H	3.51 ddd (12, 9, 5)[i]	3.55 ddd (12, 9, 5)[i]	3.55 ddd (12, 8.5, 5)[i]	3.68 m[i]
4A-H	2.90 dd (9, 9)	2.90 dd (9, 9)	2.90 dd (9, 8.5)[j]	2.98 dd (9, 9)
5A-H	3.30 dq (9, 6)	3.29 dq (9, 6)	3.29 dq (9, 6)	3.29 dq (9, 6)
6A-H$_3$	1.24 d (6)	1.25 d (6)	1.24 d (6)	1.29 d (6)
1B-H		4.60 dd (10, 2)	4.62 dd (10, 2)	4.69 dd (10, 2)
2B-H$_a$		1.70 ddd (12, 12, 10)	1.74 ddd (12, 12, 10)	1.78 ddd (12, 12, 10)
2B-H$_e$		1.88 ddd (12, 5, 2)	1.90 ddd (12, 4.5, 2)	1.94 m[k]
3B-H		3.77 ddd (12, 5, 3)	3.86 ddd (12, 4.5, 3)	3.89 ddd (12, 4.5, 3)
4B-H		3.49 s	3.69 s	3.72 s (broad)
5B-H		3.64 q (6.5)	3.67 q (6.5)	3.71 q[i]
6B-H$_3$		1.26 d (6.5)	1.26 d (6.5)	1.28 d (6.5)
1C-H			4.94 dd (9.5, 2)	4.96 dd (9.5, 2)
2C-H$_a$			1.52 dd (13.5, 9.5)	1.54 dd (13.5, 9.5)
2C-H$_e$			1.86 dd (13.5, 2)	1.88 dd (13.5, 2)
3C-Me			1.20 s	1.21 s
4C-H			2.91 d (9.5)[j]	2.93 d (9.5)
5C-H			3.62 dq (9.5, 6)	3.64 dq (9.5, 6)
6C-H$_3$			1.2 d (6)	1.21 d (6)

[a] δ in parts per million relative to trimethylsilyl (*J* in hertzes).
[b] OH signals not observed except one at δ 9.56.
[c] At 300 MHz.
[d] OH signals: δ 4.59, 9.50, 9.90, and 14.9 (all broad).
[e] OH signals: δ 3.20, 3.39, 4.58, 9.44, and 9.91 (all broad).
[f] At 500 MHz.
[g] OH signals: δ 9.91 and 16.18 (broad).
[h] After exchange with D_2O: d (10).
[i] Partially obscured.
[j] Partially obscured by water; fully visible only after D_2O exchange.
[k] Broad signal; no couplings visible.

ing only the *mtmGI* or *mtmGII* gene, both genes were independently subcloned into the shuttle vector pIAGO under the control of the erythromycin resistance promoter (see Materials and Methods) and introduced by transformation into both mutants. Each gene complemented the corresponding mutant, restoring mithramycin biosynthesis. However, cross-complementation was not observed: the *mtmGI* gene did not complement the *mtmGII* mutant and vice versa.

Characterization of the products accumulated by the M3G1 and M3G2 mutants. Both mutants (M3G1 and M3G2) were incubated in modified R5 liquid medium for 72 h at 30°C, the culture supernatants were extracted with ethyl acetate, and extracts were analyzed by HPLC (Fig. 4). Interestingly, the HPLC patterns from the extracts of both mutants were nearly

identical. In both cases, no mithramycin was detected, but six major peaks showing the characteristic absorption spectra of mithramycin biosynthetic intermediates were observed. The material contained in four of these peaks (A, B, C, and D) from both mutants was purified by preparative HPLC (as described in Materials and Methods), and the structures of the compounds were elucidated and found to be identical in both mutants. Three compounds consist of nearly identical tetracyclic ring frame aglycones and a glycan chain attached at C-12a-O which varies in the amount of its deoxysugar units: premithramycin A1 (peak A) with an aglycone lacking a 9-CH$_3$ group and one D-olivose moiety and premithramycins A2 (peak C) and A3 (peak D) with aglycones containing a 9-CH$_3$ group and a disaccharide (D-olivose-D-oliose) and trisaccharide (D-olivose-

TABLE 2. ^{13}C NMR data of premithramycins A1 to
A4 at 125.7 MHz in d$_6$-acetone^a

Position	A1	A2	A3	A4
C-1	NO^b	195.5 s^c	196.2 s^c	202.4 s
C-2	113.1 s	113.3 s	113.2 s	75.9 d^c
C-3	NO^b	189.5 s^c	189.0 s^c	41.9 d
C-4	78.5 d	78.3 d	78.0 d	26.4 t
C-4a	43.0 s	43.1 s	43.0 s	135.3 s
4-OCH$_3$	61.1 q	61.1 q	61.4 q	
C-5	26.6 t	26.6 t	26.6 t	101.0 d
C-5a	136.2 s	134.8 s	134.6 s	
C-6	117.9 d	117.9 d	117.8 d	160.0 s
C-6a	142.8 s	140.3 s	140.1 s	
C-7	103.2 d	102.8 d	102.8 d	109.0 s
7-CH$_3$				6.8 q
C-8	160.8 s^c	162.2 s	162.1 s	155.8 s
C-8a				105.9 s
C-9	102.2 d	110.8 s	110.7 s	NO^b
C-9a				106.9 s
9-CH$_3$		8.2 q	8.2 q	
C-10	163.3 s^c	157.7 s	157.5 s	115.2 d
C-10a	107.8 s	107.3 s	107.1 s	138.5 s
C-11	168.0 s	168.1 s	168.0 s	
C-11a	108.3 s	108.3 s	108.0 s	
C-12	NO^b	193.1 s^c	193.0 s^c	
C-12a	85.5 s	85.5 s	85.5 s	
C-1'	204.1 s	204.3 s	204.3 s	81.0 d^d
1'-OCH$_3$				57.7 q
C-2'	28.2 q	28.4 q	28.4 q	210.3 s
C-3'				78.1 d^e
C-4'				67.4 d^e
C-5'				18.7 q
C-1A	98.2 d	98.3 d	98.3 d^d	100.0 d
C-2A	40.5 t	38.6 t	38.5 t	37.0 t
C-3A	71.9 d	82.0 d	81.9 d	80.9 d^d
C-4A	77.9 d	75.7 d	75.6 d	74.4 d
C-5A	72.9 d	72.9 d	72.8 d	71.6 d
C-6A	18.2 q	18.4 q	18.5 q	17.1 q
C-1B		100.7 d	100.5 d	99.4 d
C-2B		35.6 t	32.8 t	31.6 t
C-3B		69.3 d	77.1 d	75.9 d
C-4B		70.6 d	69.2 d	67.9 d
C-5B		71.7 d	71.2 d	70.0 d^f
C-6B		16.9 q	16.9 q	15.6 q
C-1C			98.2 d^d	97.0 d
C-2C			44.6 t	43.4 t
C-3C			71.1 s	69.7 s
3C-CH$_3$			27.7 q	26.1 q
C-4C			77.1 d	75.8 d^c
C-5C			71.3 d	70.1 d^f
C-6C			18.7 q	17.4 q

^{a} δ in parts per million relative to internal trimethylsilyl. Multiplicities and assignments are from the APT, hetero-multiple quantum correlation, and HMBC experiments.

^{b} NO, not observed due to tautomerization.

$^{c \text{ to } f}$ Assignments are interchangeable pairwise.

D-oliose-D-mycarose) chain, respectively. The fourth identified compound, premithramycin A4 (peak B), consists of the tricyclic mithramycinone as the aglycone moiety and contains the same trisaccharide chain as that of premithramycin A3, which is attached at C-2-O (due to the different numbering in this molecular frame [Fig. 1 and below]). Although the four compounds show very similar UV spectra, they exhibit slight differences in their UV maxima: premithramycins A2 and A3 show identical absorption spectra, and premithramycins A1 and A4 show similar patterns but with maxima displaced at higher wavelengths (data not shown). The biosynthesis of these four compounds was monitored during growth, and we found

that the patterns of biosynthesis of the different compounds were similar in both mutants (data not shown).

Elucidation of structure of the new premithramycins. The negative ion FAB MS spectra of the four new premithramycins, A1 to A4 (A1, m/z 543; A2, m/z 687; A3, m/z 831; A4, m/z 823), are in agreement with molecular formulae of $C_{27}H_{28}O_{12}$ (A1), $C_{34}H_{40}O_{15}$ (A2), $C_{41}H_{52}O_{18}$ (A3), and $C_{40}H_{56}O_{18}$ (A4).

The NMR data (Tables 1 and 2) indicate premithramycinone (31) to be the aglycone moiety of premithramycin A1. Typical for this aglycone is the acetyl group (C-1', C-2'), the OCH$_3$ group at C-4, three aromatic protons of rings C and D, and, in addition to the carbonyl of the acetyl side chain, six sp^2 carbons attached to an oxygen atom. As a result of this unusual arrangement, an intensive tautomerization in rings A through C can be observed. As a consequence, some of the carbons (C-1, C-3, C-11, and C-12) show broad signals or variation of their chemical shifts and cannot be assigned unambiguously due to the lack of observable long-range C-H couplings. One olivose can be recognized as an additional structural element due to its typical ^{1}H and ^{13}C NMR signals and its ^{1}H NMR signal patterns, which also indicate its β-glycosidic linkage (1-H shows a 10-Hz transaxial coupling to 2-H$_a$). The position of the sugar moiety is directly deducible from $^3J_{C-H}$ coupling observed between 1A-H and C-12a in the HMBC spectrum.

In the ^{1}H NMR spectrum of the aglycone moiety of premithramycin A2, the 9-H signal is lacking, and an aromatic methyl group at δ 2.15 can be observed instead. In the ^{13}C NMR spectrum, C-9 appears as a quaternary carbon, as opposed to the corresponding ^{13}C NMR spectrum of premithramycin A1 in which this carbon bears a proton (deducible from the APT spectrum). All other aglycone NMR signals are identical with those of premithramycin A1. Thus, C-9 is methylated in premithramycin A2. In addition, two sugar moieties (12 additional carbon signals in the ^{13}C NMR spectrum and 21 additional proton signals in the ^{1}H NMR spectra) can be identified from the NMR data. The analysis of the chemical shifts and especially of the coupling patterns indicates the presence of a disaccharide fragment consisting of one olivose and one oliose moiety, both β-glycosidically linked. The interglycosidic linkage (between 3A-O and C-1B) as well as the position in which this disaccharide is attached to the aglycone (12a-O) could be directly deduced from the HMBC spectrum, which shows a $^3J_{C-H}$ long-range coupling between 1B-H and C-3A and between 1A-H and C-12a, respectively.

The structure of premithramycin A3 could be similarly deduced by comparison of its NMR data with those of premithramycin A2 (Tables 1 and 2). This revealed identical aglycones, as well as two identical sugar moieties, and one additional sugar unit. The ^{1}H NMR coupling analysis of the signals of this additional sugar proved that this moiety is mycarose, as was expected from the mithramycin structure (19). The couplings observable in the HMBC spectrum (between 1C-H and C-3B, 1B-H and C-3A, and 1A-H and C-12a) led to the given structure (Fig. 1) and confirm the interglycosidic bondage positions as well as the position of linkage of the trisaccharide to the aglycone. All sugar units are β-glycosidically linked, because they all show a large transaxial coupling of 10 Hz.

In contrast to the premithramycins A1 to A3, premithramycin A4 has an aglycone identical to that of mithramycin. This is obvious from the comparison of the NMR data in which, e.g., both keto carbonyls (at δ 202.4 and 210.3 for C-1 and C-2', respectively), the terminal methyl group of the side chain (C-5' at δ 18.7, 5'-H$_3$ [d, J = 6 Hz] at δ 1.25), and the typical 2-H signal (δ 4.75 [d, J = 11.5 Hz]) are observable. The trisaccharide chain is identical to that of premithramycin A3 (Tables 1 and 2) and is linked at C-2-O, as can be directly observed in the
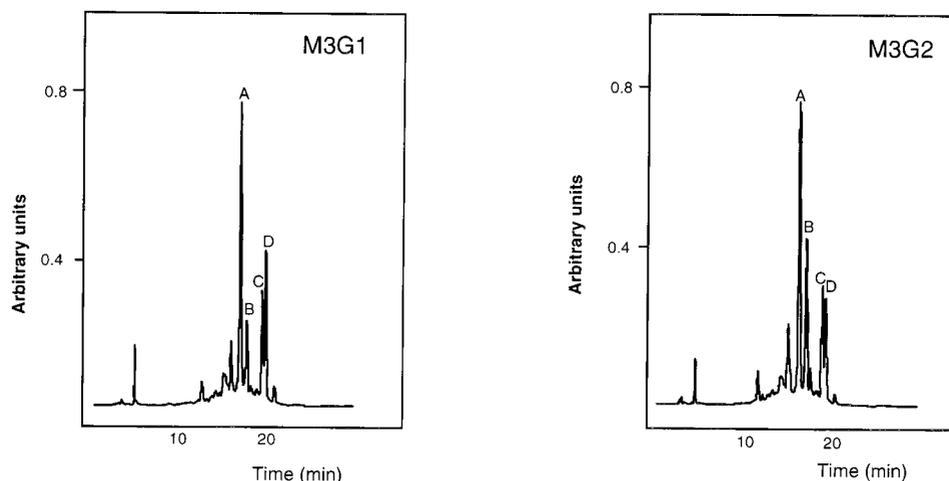
FIG. 4. HPLC analysis of the products accumulated by mutants M3G1 and M3G2. The mobility of mithramycin under the chromatographic conditions used was 17.8 min, a retention time between peaks B and C.

HMBC spectrum, which shows $^3J_{C-H}$ couplings between 1C-H and C-3B, 1B-H and C-3A, 2-H and C-1A, and 1A-H and C-2. These couplings confirm the interglycosidic linkage positions as well as the connection of the trisaccharide chain with the aglycone, as shown in Fig. 1.

That all of the sugar moieties in the premithramycins A1 to A4 are D-sugars is indicated from their β-glycosidic linkage (in context with Klyne's rule) and is also in agreement with the mithramycin structure (19).

DISCUSSION

Many bioactive compounds contain 6-DOH in their structures which, in many cases, are very important features for their biological activities. In mithramycin, DNA binding and antitumor activity are dependent on the presence of the sugars attached to the aglycone (35). The transfer of D-olivose (three molecules), D-oliose (one molecule), and D-mycarose (one molecule) in their activated forms (dTDP-sugars) to the mithramycin aglycone requires the participation of GTFs. We report here the identification of two genes from the mithramycin producer *S. argillaceus* encoding two GTFs presumably responsible for the transfer of the D-olivose disaccharide to the aglycone. These two genes (*mtmGI* and *mtmGII*) were identified in a cosmid clone, cosAR3, in which a mithramycin resistance determinant was previously found (11) and were located approximately 13.5 kb downstream of the mithramycin polyketide synthase (21). Independent insertional inactivation of both genes generated two nonproducing mutants which accumulated the same compounds as determined by HPLC analysis of the culture supernatants of both mutants followed by elucidation of the structures of the four major compounds. All four compounds lack the D-olivose disaccharide normally connected at C-6-O. Thus, the MtmGI and MtmGII GTFs should be involved in the transfer of this disaccharide. There are two alternative ways for the transfer of the disaccharide: (i) sequential and successive addition of both D-olivose molecules by independent GTFs or (ii) formation of the diolivosyl and transfer of the disaccharide to the aglycone. According to the former hypothesis, inactivation of one of the GTFs (that responsible for adding the second sugar) would result in a monosaccharide at C-6-O, while inactivation of the other GTF (responsible for transferring the first sugar unit) would produce compounds with no sugars in this position. In contrast, pro-

duction of compounds not glycosylated at C-6-O would be obtained as a consequence of the inactivation of either GTF gene on the basis of the latter hypothesis. Identical compounds were isolated from culture supernatants of mutants M3G1 and M3G2 (premithramycins A1, A2, A3, and A4), thus supporting the disaccharide transfer hypothesis.

It has been proposed that the biosynthesis of mithramycin proceeds in its early stage through several tetracyclic intermediates, two of which (premithramycinone and 4-demethylpremithramycinone) have been isolated elsewhere (5, 22, 31). As a late step in biosynthesis, an enzyme (possibly an oxygenase) would be responsible for a C—C bond scission, leading to the typical tricyclic aureolic acid chromophore (31). Experimental evidence in support of this view comes from the isolation of premithramycins A3 and A4, glycosylated tetracyclic and tricyclic compounds differing only in the breakage of the fourth ring. However, it is still not yet clear when the corresponding oxygenase causes this C—C bond scission in the fourth ring, i.e., before, during, or after sugar addition. The isolation of premithramycin A4 (a glycosylated compound containing the trisaccharide at C-2-O but lacking the disaccharide at C-6-O) suggests that fourth ring breakage takes place at least after addition of the trisaccharide chain, but it is still unclear whether this happens before or after the disaccharide has been attached. Chromocyclomycin, a tetracyclic glycosylated compound containing a monosaccharide (D-mycarose) and a trisaccharide (D-olivose-D-oliose and D-mycarose), has been isolated from a mithramycin-producing streptomycete (3). This compound possibly represents a mithramycin-related compound in which the fission of the fourth ring has not yet occurred but which has been monoglycosylated at C-8-O (here with D-mycarose instead of with D-olivose) and suggests that breakage of the fourth ring might be a late step in biosynthesis, probably occurring after all sugars have been transferred into the aglycone (Fig. 5). The isolation of premithramycin A4 from the cultures of mutants M3G1 and M3G2 possibly indicates the existence of some kind of relaxed substrate specificity of the responsible oxygenase acting also on those tetracyclic intermediates in which some sugar moieties are lacking. Four oxygenase genes (*mtmOI*, *mtmOII*, *mtmOIII*, and *mtmOIV*) have been identified in the mithramycin gene cluster (28a), and recent experimental evidence strongly suggests that the *mtmOIV* product could be a candidate for breakage of the fourth ring (10a).
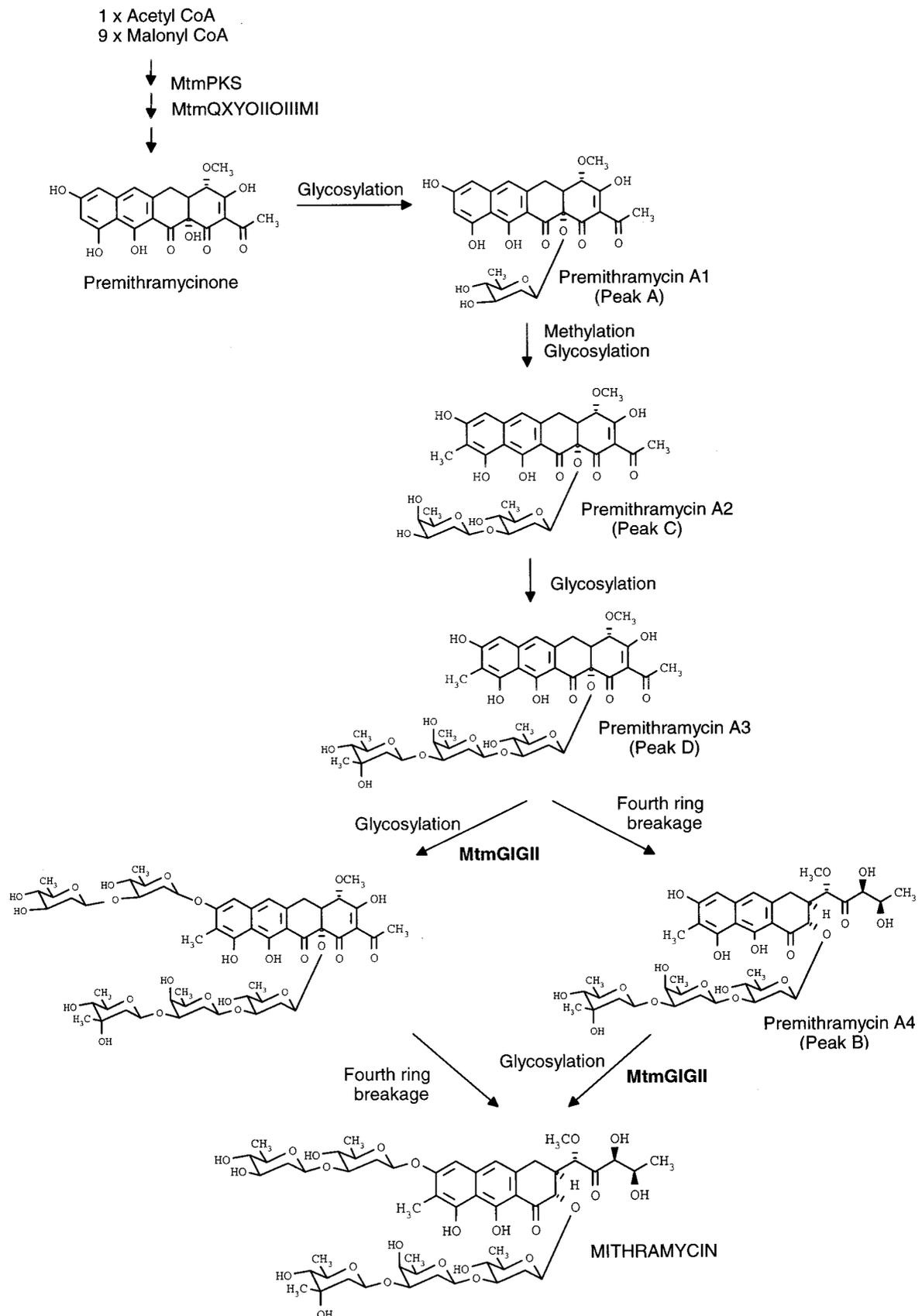
FIG. 5. Proposed pathways for mithramycin biosynthesis. CoA, coenzyme A.

Two methyl groups in the mithramycin aglycone must be introduced by methyltransferases: the O-methyl group at carbon 4 and the C-methyl group at carbon 9. Premithramycinone (31) is accumulated by the M7D1 mutant of *S. argillaceus* in which the *mtmD* gene encoding a glucose-1-phosphate:TTP thymidylyl transferase has been inactivated (22). Premithramycinone contains the O-methyl group but lacks the C-methyl group, thus indicating that O-methylation occurs before sugar transfer. Recent experimental evidence demonstrates that the *mtmMI* gene product encodes this *O*-methyltransferase (11a). Interestingly, premithramycins A2 and A3 contain the C-methyl group at carbon 9 while premithramycin A1 does not. This suggests that C methylation (possibly catalyzed by the MtmMII protein) must take place before the second D-olivose is transferred to the trisaccharide chain to convert premithramycin A1 into premithramycin A2.

ACKNOWLEDGMENTS

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Bechthold, A., J. K. Sohng, T. M. Smith, X. Chu, and H. G. Floss.** 1995. Identification of *Streptomyces violaceoruber* Tü22 genes involved in the biosynthesis of granaticin. Mol. Gen. Genet. **248:**610–620.
3. **Berlin, Y. A., M. N. Kolosov, I. V. Vasina, and I. V. Yartseva.** 1968. The structure of chromocyclomycin. J. Chem. Soc. Chem. Commun. **1968:**762–763.
4. **Bibb, M. J., G. R. Janssen, and J. M. Ward.** 1985. Cloning and analysis of the promoter region of the erythromycin resistance gene (*ermE*) of *Streptomyces erythraeus*. Gene **38:**215–226.
5. **Blanco, G., H. Fu, C. Méndez, C. Khosla, and J. A. Salas.** 1996. Deciphering the biosynthetic origin of the aglycone of the aureolic acid group of antitumor agents. Chem. Biol. **3:**193–196.
6. **Bullock, W. O., J. M. Fernández, and J. N. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5:**376–379.
7. **Cundliffe, E.** 1992. Glycosylation of macrolide antibiotics in extracts of *Streptomyces lividans*. Antimicrob. Agents Chemother. **36:**348–352.
8. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
9. **Dhillon, N., R. S. Hale, J. Cortes, and P. F. Leadlay.** 1989. Molecular characterization of a gene from *Saccharopolyspora erythraea* (*Streptomyces erythraeus*) which is involved in erythromycin biosynthesis. Mol. Microbiol. **3:**1405–1414.
10. **Distler, J., A. Ebert, K. Mansouri, K. Pissowotzki, M. Stockmann, and W. Piepersberg.** 1987. Gene cluster for streptomycin biosynthesis in *Streptomyces griseus*: nucleotide sequence of three genes and analysis of transcriptional activity. Nucleic Acids Res. **15:**8041–8056.
10a.**Fernández, E.** Unpublished results.
11. **Fernández, E., F. Lombó, C. Méndez, and J. A. Salas.** 1996. An ABC transporter is essential for resistance to the antitumor agent mithramycin in the producer *Streptomyces argillaceus*. Mol. Gen. Genet. **251:**692–698.
11a.**Fernández, M. J., C. Méndez, and J. A. Salas.** Unpublished results.
12. **Gaisser, S., G. Böhm, J. Cortés, and P. F. Leadlay.** 1997. Analysis of seven genes from the eryAI-eryK region of the erythromycin biosynthetic gene cluster in Saccharopolyspora erythraea. Mol. Gen. Genet. **256:**239–251.
13. **Gallo, M. A., J. Ward, and C. R. Hutchinson.** 1996. The *dnrLM* gene in *Streptomyces peucetius* contains a naturally occurring frameshift mutation that is suppressed by another locus outside of the daunorubicin-production gene cluster. Microbiology **142:**269–275.
14. **Gandecha, A. R., S. L. Large, and E. Cundliffe.** 1997. Analysis of four tylosin biosynthetic genes from the *tylLM* region of the *Streptomyces fradiae* genome. Gene **184:**197–203.
15. **Hernández, C., C. Olano, C. Méndez, and J. A. Salas.** 1993. Characterization of a *Streptomyces antibioticus* gene cluster encoding a glycosyltransferase involved in oleandomycin inactivation. Gene **134:**139–140.
16. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of *Streptomyces*. A laboratory manual. The John Innes Foundation, Norwich, England.

17. **Jenkins, G., and E. Cundliffe.** 1991. Cloning and characterization of two genes from *Streptomyces lividans* that confer inducible resistance to lincomycin and macrolide antibiotics. Gene **108:**55–62.
18. **Kirschning, A., A. Bechthold, and J. Rohr.** 1997. Chemical and biochemical aspects of deoxysugars and deoxysugar oligosaccharides. Top. Curr. Chem. **188:**1–84.
19. **Künzel, E., S. E. Wohlert, R. Machinek, C. Méndez, J. A. Salas, and J. Rohr.** Reinvestigation of the structure of mithramycin. Submitted for publication.
20. **Liu, H., and S. J. Thorson.** 1994. Pathways and mechanisms in the biogenesis of novel deoxysugars by bacteria. Annu. Rev. Microbiol. **48:**223–256.
21. **Lombó, F., G. Blanco, E. Fernández, C. Méndez, and J. A. Salas.** 1996. Characterization of *Streptomyces argillaceus* genes encoding a polyketide synthase involved in the biosynthesis of the antitumor mithramycin. Gene **172:**87–91.
22. **Lombó, F., K. Siems, A. F. Braña, C. Méndez, K. Bindseil, and J. A. Salas.** 1997. Cloning and insertional inactivation of *Streptomyces argillaceus* genes involved in earliest steps of sugar biosynthesis of the antitumor polyketide mithramycin. J. Bacteriol. **179:**3354–3357.
23. **Merson-Davies, L. A., and E. Cundliffe.** 1994. Analysis of five tylosin biosynthetic genes from the *tylBA* region of the *Streptomyces fradiae* genome. Mol. Microbiol. **13:**349–355.
24. **Mizusawa, S., S. Nishimura, and F. Seila.** 1986. Improvement of the dideoxy chain termination method of DNA sequencing by the dideoxy-7-deazaguanosine triphosphate in place of dGTP. Nucleic Acids Res. **14:**1319–1324.
25. **Olano, C., A. M. Rodriguez, J.-M. Michel, C. Méndez, M.-C. Raynal, and J. A. Salas.** Analysis of a *Streptomyces antibioticus* chromosomal region involved in oleandomycin biosynthesis that contains two glycosyltransferases responsible for glycosylation of the macrolactone ring. Mol. Gen. Genet., in press.
26. **Otten, S. L., X. Liu, J. Ferguson, and C. R. Hutchinson.** 1995. Cloning and characterization of the *Streptomyces peucetius dnrQS* genes encoding a daunosamine biosynthesis enzyme and a glycosyltransferase involved in daunorubicin biosynthesis. J. Bacteriol. **177:**6688–6692.
27. **Piepersberg, W.** 1994. Pathway engineering in secondary metabolite-producing actinomycetes. Crit. Rev. Biotechnol. **14:**251–285.
28. **Pissowotzki, K., K. Mansouri, and W. Piepersberg.** 1991. Genetics of streptomycin production in *Streptomyces griseus*: molecular structure and putative functions of *strELMB2N*. Mol. Gen. Genet. **231:**113–123.
28a.**Prado, L., F. Lombó, C. Méndez, and J. A. Salas.** Unpublished results.
29. **Quirós, L. M., I. Aguirrezabalaga, C. Olano, C. Méndez, and J. A. Salas.** 1998. Two glycosyltransferases and a glycosidase are involved in oleandomycin modification during its biosynthesis by *Streptomyces antibioticus*. Mol. Microbiol. **28:**1177–1186.
30. **Remers, W. A.** 1979. The chemistry of antitumor antibiotics, vol. 1, p. 133–175. Wiley-Interscience, New York, N.Y.
31. **Rohr, J., U. Weißbach, C. Beninga, E. Künzel, K. Siems, K. Bindseil, L. Prado, F. Lombó, A. F. Braña, C. Méndez, and J. A. Salas.** 1998. The structure of premithramycinone and demethylpremithramycinone, early intermediates of the aureolic acid group antibiotic mithramycin. Chem. Commun. **1998:**437–438.
32. **Salah-Bey, K., M. Doumith, J. M. Michel, S. Hadock, J. Cortés, P. F. Leadlay, and M. C. Raynal.** 1998. Targetted gene inactivation for the elucidation of deoxysugar biosynthesis in the erythromycin producer *Saccharopolyspora erythraea*. Mol. Gen. Genet. **257:**542–553.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
35. **Sastry, M., and D. J. Patel.** 1993. Solution structure of the mithramycin dimer-DNA complex. Biochemistry **32:**6588–6604.
36. **Scotti, C., and C. R. Hutchinson.** 1996. Enhanced antibiotic production by manipulation of the *Streptomyces peucetius dnrH* and *dnmT* genes involved in doxorubicin (adriamycin) biosynthesis. J. Bacteriol. **178:**7316–7321.
37. **Skarbek, J. D., and M. K. Speedie.** 1981. Antitumor antibiotics of the aureolic acid group: chromomycin A3, mithramycin A, and olivomycin A, p. 191–235. *In* A. Aszalos (ed.), Antitumor compounds of natural origin: chemistry and biochemistry, vol. I. CRC Press, Inc., Boca Raton, Fla.
38. **Summers, R. G., S. Donadio, M. J. Staver, E. Wendt-Pienkowski, C. R. Hutchinson, and L. Katz.** 1997. Sequencing and mutagenesis of genes from the erythromycin biosynthetic gene cluster of Saccharopolyspora erythraea that are involved in L-mycarose and D-desosamine production. Microbiology **143:**3251–3262.
39. **Vara, J., M. Lewandowska-Skarbek, Y.-G. Wang, S. Donadio, and C. R. Hutchinson.** 1989. Cloning of genes governing the deoxysugar portion of the erythromycin biosynthesis pathway in *Saccharopolyspora erythraea* (*Streptomyces erythreus*). J. Bacteriol. **171:**5872–5881.
40. **Vilches, C., C. Hernández, C. Méndez, and J. A. Salas.** 1992. Role of glycosylation and deglycosylation in biosynthesis of and resistance to oleandomycin in the producer organism, *Streptomyces antibioticus*. J. Bacteriol. **174:**161–165.