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Cloning and Insertional Inactivation of *Streptomyces argillaceus* Genes Involved in the Earliest Steps of Biosynthesis of the Sugar Moieties of the Antitumor Polyketide Mithramycin

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Two genes (*mtmD* and *mtmE*) were cloned and sequenced from the mithramycin producer *Streptomyces argillaceus*. Comparison with proteins in databases and enzymatic assays after expression in *Escherichia coli* showed that they encode a glucose-1-phosphate:TTP thymidylyl transferase and a TDP-D-glucose 4,6-dehydratase, respectively. The *mtmD* gene was inactivated by gene replacement, generating a nonproducing mutant that accumulates a tetracyclic compound designated premithramycinone. The identification of premithramycinone reveals new aspects of the mithramycin biosynthetic pathway and suggests that at least some glycosylations occur before breakage of the fourth ring.

Mithramycin (also designated aureolic acid, plicamycin, mithracin, LA7017, and A2371) is an antitumor drug synthesized by different actinomycete species and has clinical application in the treatment of several tumors (14). It also possesses antibiotic activity against gram-positive but not gram-negative bacteria. Structurally, it is an aromatic polyketide containing a three-ring chromophoric aglycon derived from the condensation of 10 acetates (11). Genes for a type II polyketide synthase were cloned and sequenced from a mithramycin producer, *Streptomyces argillaceus*; their involvement in mithramycin biosynthesis has been demonstrated by insertional inactivation (9), and evidence in favor of the biosynthesis of the aglycon through a single polyketide backbone instead of two has been provided elsewhere (3). The mithramycin aglycon (mithramycinone) is glycosylated at positions 6 and 2 with disaccharide and trisaccharide moieties, respectively. These sugars belong to the large family of the 6-deoxyhexoses (6DOHs), which are synthesized through biosynthetic pathways that have early biosynthetic steps in common (8, 12). In 6DOH biosynthesis, first glucose-1-phosphate is converted into TDP-D-glucose by the action of a glucose-1-phosphate:TTP thymidylyl transferase. Then, a TDP-D-glucose 4,6-dehydratase is responsible for the conversion of TDP-D-glucose into TDP-D-4-keto-6-deoxyglucose. Further action of a 3,5-epimerase can direct TDP-D-4-keto-6-deoxyglucose into the L series of 6DOHs; in contrast, if there is no participation of an epimerase, the final sugars will be D-6DOHs. Further modifications in both L- and D-6DOHs via isomerization, methylation, reduction, dehydration, etc., can produce a great variety of 6DOHs, which are part of the structures of different actinomycete metabolites (12, 18).

Here we report the cloning, sequencing, and heterologous expression of two genes involved in the biosynthesis of the sugar moieties of the antitumor drug mithramycin. These genes (*mtmD* and *mtmE*) encode a glucose-1-phosphate:TTP thymidylyl transferase and a TDP-D-glucose 4,6-dehydratase, respectively. We also report the construction of a non-mithra-

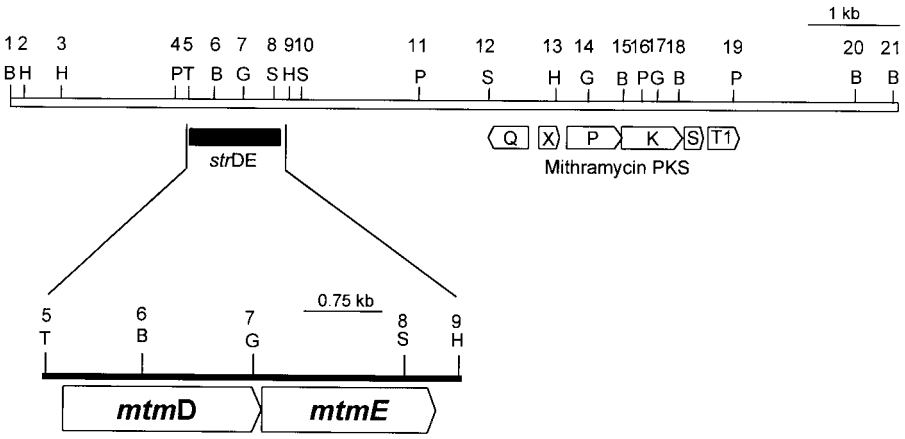

FIG. 1. Schematic representation of the region sequenced from cosAR7 and the different deduced ORFs and its location with respect to the mithramycin polyketide synthase (PKS). The bar indicates the region showing homology to the *strD* and *strE* genes of the streptomycin biosynthetic pathway in *S. griseus*. B, *Bam*HI; G, *Bgl*II; H, *Sph*I; P, *Pst*I; S, *Sac*I; T, *Stu*I.

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TABLE 1. Assay of TDP-D-glucose synthase and TDP-D-glucose-4,6-dehydratase activities after expression of *mtmD* and *mtmE* genes in *E. coli*

Gene construction	TDP-D-glucose synthase (μmol/min/mg of protein)		TDP-D-glucose-4,6-dehydratase (nmol/min/mg of protein)	
	−IPTG	+IPTG	−IPTG	+IPTG
pALTER-1 (vector)	18.21 ± 6.89	16.58 ± 4.96	11.97 ± 1.35	11.47 ± 3.11
pFLADE (plus *mtmDE*)	26.12 ± 4.60	63.99 ± 7.67	13.40 ± 2.36	32.15 ± 2.07

mycin-producing mutant through insertional inactivation of the *mtmD* gene; from the knowledge of the structure of the intermediate accumulated by this mutant, a hypothetical pathway for mithramycin biosynthesis is proposed.

Cloning and sequencing of *mtmD* and *mtmE* from *S. argillaceus*. From a cosmid library of chromosomal DNA from *S. argillaceus* ATCC 12956, we have isolated a cosmid clone (cosAR7) containing a region that encodes a type II polyketide synthase involved in the biosynthesis of the mithramycin aglycon (9). To isolate genes involved in the biosynthesis of the sugar moieties of mithramycin, we used the *strD* and *strE* genes of *Streptomyces griseus*, which encode two enzymes catalyzing early stages of the biosynthesis of the sugar moieties in streptomycin (5, 13). We found that the cosAR7 clone hybridized with these sugar probes under high-stringency conditions (two washes in 0.2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% sodium dodecyl sulfate at 68°C) in the same 10-kb *Bam*HI fragment that contains part of the mithramycin polyketide synthase (9). By subcloning and further hybridizations, the minimal hybridizing region was reduced to a 1.75-kb *Bam*HI-*Sac*I fragment (sites 6 to 8 in Fig. 1). A *Stu*I-*Sph*I fragment (sites 5 to 9 in Fig. 1) containing the region hybridizing with *strD* and *strE* was sequenced by the dideoxynucleotide chain termination method (16), and the nucleotide sequence, 2,403 bp, was analyzed for coding regions by the CODONPREFERENCE program (4) to reveal the presence of two open reading frames (ORFs) transcribed in the same direction. The first ORF (designated *mtmD*) would code for a polypeptide of 355 amino acids with an estimated M_r of 38,228, and the second ORF (designated *mtmE*) would code for a polypeptide of 331 amino acids with an estimated M_r of 36,352. The starting codon of *mtmE* overlaps with the stop codon of *mtmD* (ATGA). Both genes displayed the biased codon usage typical of *Streptomyces* genes, and neither of them was preceded by a sequence that could be clearly proposed as a ribosomal binding site.

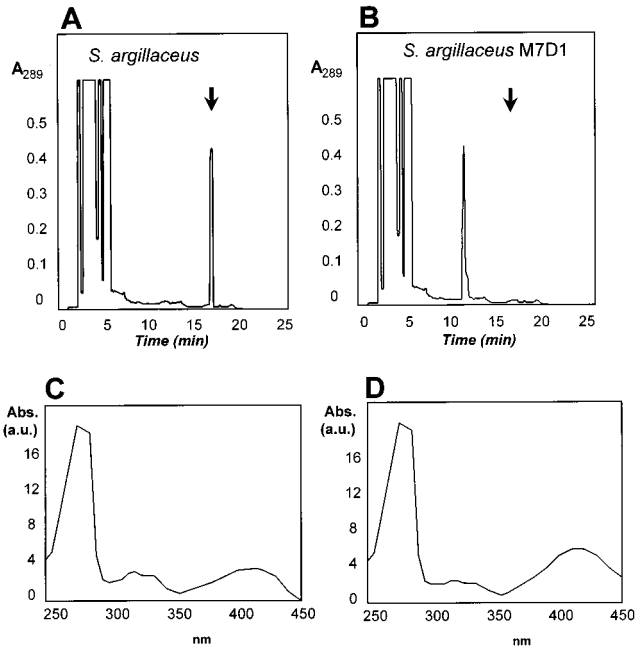

FIG. 2. High-pressure liquid chromatography analysis of ethyl acetate extracts of wild-type *S. argillaceus* (A) and M7D1 mutant (B) and absorption spectrum of mithramycin (C) and the major product (premithramycinone) accumulated by the M7D1 mutant (D). The arrow indicates the mobility of mithramycin. Abbreviations: a.u., arbitrary units; Abs., absorbance.

Identification of the functions of the *mtmD* and *mtmE* products. To gain insight about the possible function of the *mtmD* and *mtmE* gene products, a database search with the deduced amino acid sequence of the two genes was made. The putative MtmD protein showed similarity to putative glucose-1-phosphate:TTP thymidylyl transferases from streptomycetes involved in the biosynthesis of different antibiotics. The highest homologies were with StrD (68.7% identity and 80.6% similarity) from the streptomycin pathway (13), GraD (54.1% identity and 71.9% similarity) from the granaticin pathway (1), SnoD (52.0% identity and 72.2% similarity) from *Streptomyces nodosus* with unknown function (GenBank accession no. A25110), and DnrL (68.3% identity and 79.7% similarity) from the daunorubicin pathway (6). The MtmE protein strongly resembled TDP-D-glucose 4,6-dehydratases from different antibiotic pathways: StrE (69.2% identity and 81.4% similarity) from the streptomycin pathway (13); TylA2 (62.8% identity and 73.8% similarity) from the tylosin pathway (10); GraE (60% identity and 75% similarity) from the granaticin pathway (1); and Gdh (65.6% identity and 76.2% similarity), a dehydratase from the erythromycin producer *Saccharopolyspora erythraea*, which has been shown not to be involved in antibiotic biosynthesis (7). The MtmE protein shows an amino acid sequence (GGAGFIG) close to the amino terminus corresponding to the motif GXGXXG (X representing any amino acid), which has been described as a βαβ fold with an NAD binding motif (17). This nucleotide is a required cofactor for the TDP-D-glucose 4,6-dehydratase activity of *S. erythraea* (20) and those of equivalent dehydratases from anthracycline-producing actinomycetes (19).

Expression of the *mtmD* and *mtmE* genes. To verify that the cloned genes encoded the putatively assigned enzyme functions, the two genes were expressed together in *Escherichia coli* and the activities were separately assayed in cell extracts. A 5-kb *Sph*I fragment (sites 3 to 9 in Fig. 1) was first subcloned into the *Sph*I site of pUC18, generating pFL7SPB. Then, a 2.7-kb *Pst*I-*Sph*I fragment (sites 4 to 9 in Fig. 1) was rescued as a *Pst*I fragment (by using this site from the polylinker) and cloned into the *Pst*I site of pALTER-1 (Promega) downstream of the T7 promoter, generating pFLADE. Gene expression was induced by IPTG (isopropyl-β-D-thiogalactopyranoside) as described previously (15), and cell extracts of induced cells were used as a source of enzyme activities. Glucose-1-phos-

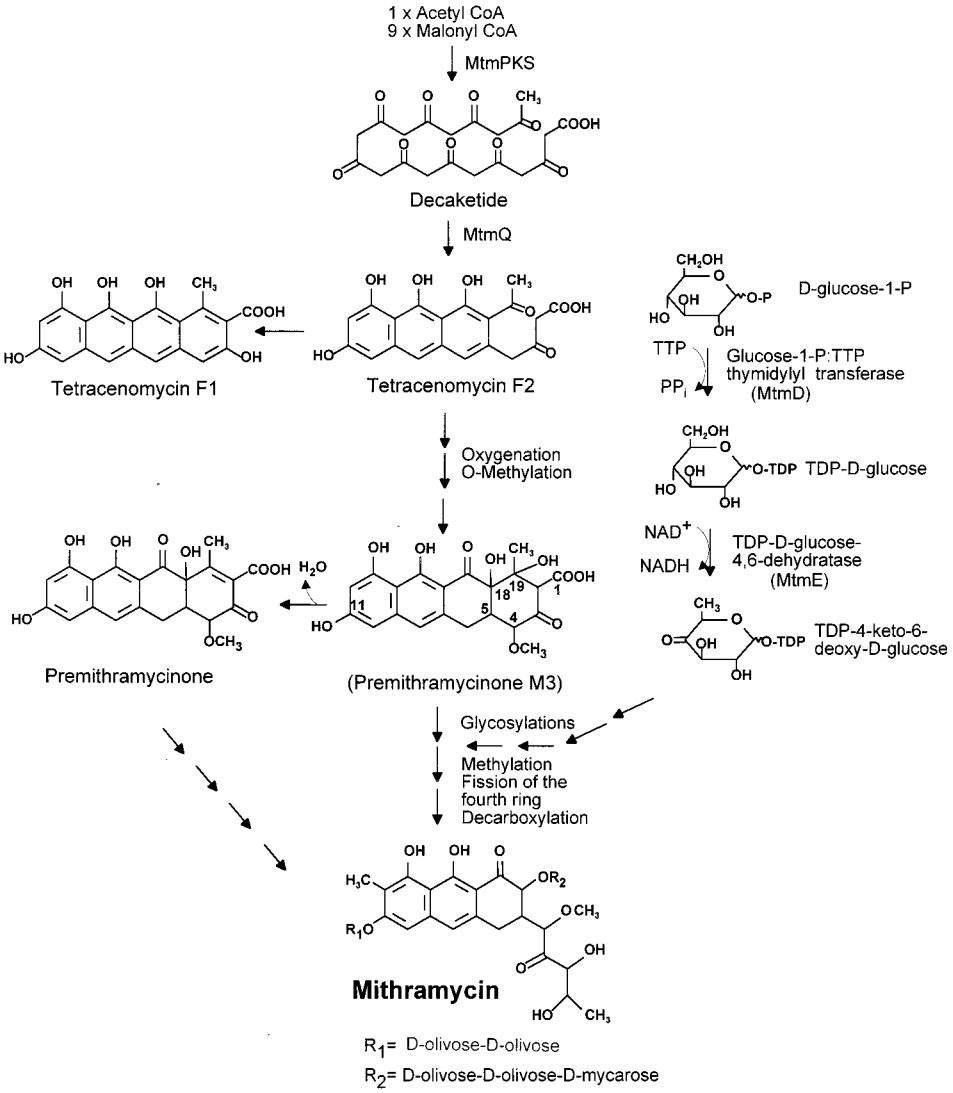

FIG. 3. Proposed pathway for the early steps in the biosynthesis of mithramycin aglycon and 6DOH. PKS, polyketide synthase.

phate:TTP thymidylyl transferase and TDP-D-glucose 4,6-dehydratase activity were determined as described elsewhere (10, 20). After IPTG induction, a three- to fourfold increase in both enzyme activities was observed compared with the controls (pALTER-1) (Table 1). The relatively high levels of both activities in the controls can be explained by the presence of equivalent enzymes in the *E. coli* host.

Generation of a non-mithramycin-producing mutant by insertional inactivation. A non-mithramycin-producing mutant was generated by replacing the *mtmD* wild-type gene by an in vitro-altered one. A 5-kb *Sph*I fragment (sites 3 to 9 in Fig. 1) was rescued from pFL7SPB as a *Xba*I-*Hin*dIII fragment and subcloned into pBT(+) [Bluescript SK(+) containing a thiostrepton resistance cassette (8a)]. Then, an apramycin resistance cassette was subcloned as a 1.5-kb *Bam*HI-*Bgl*II fragment from pUO9090 (9a) into the *Bam*HI site located inside the *mtmD* gene (Fig. 1). This construction (pM7D0) was used to transform *S. argillaceus* protoplasts and transformants selected on R5 plates containing 25 μg of apramycin per ml. Only one recombinant clone was obtained (M7D0), and it was found to be also resistant to thiostrepton (50 μg/ml). In this clone, the plasmid was integrated by a single crossover into the *mtmD* chromosomal homologous region. To force a second crossover essential to replace the wild-type region by the in vitro-altered one, the clone was inoculated into tripticasein soy broth liquid medium and incubated for 48 h at 30°C without antibiotics. Then, serial dilutions were plated onto agar plates containing 25 μg of apramycin per ml, and apramycin-resistant colonies were replica plated onto the same medium containing 50 μg of thiostrepton per ml. Colonies not growing on thiostrepton were considered to be the result of a second crossover. One colony (M7D1) was selected for the gene replacement to be verified by Southern hybridization (data not shown). Ethyl acetate extracts (under acidic conditions) of this mutant were analyzed by high-pressure liquid chromatography for mithramycin production. No mithramycin was found, but a major peak with shorter retention time than mithramycin was detected in the chromatogram (Fig. 2B). The UV absorption spectrum of the corresponding compound (Fig. 2D) was very similar to that of mithramycin (Fig. 2C): a first maximum at 289 nm and a second maximum slightly displaced with respect to that of mithramycin (412 nm for mithramycin [Fig. 2C] and 417 nm for this compound [Fig. 2D]). The major compound accumulated by the M7D1 mutant (designated premithramy-

cinone) was purified, and its structure was elucidated (a detailed description on the isolation and structure elucidation of the premithramycinone will be published elsewhere). It showed a tetracyclic ring structure (Fig. 3) derived from the action of the mithramycin polyketide synthase (*mtm* polyketide synthase genes [3, 9]) through the generation of a tetracenomycin F1-like intermediate and further oxygenation and methylation steps. The formation of premithramycinone cannot be the consequence of a polar effect on other mithramycin biosynthetic genes, since immediately downstream of *mtmDE* there is another gene (*mtmT2*) that is transcribed in the opposite direction (13a).

Proposed biosynthetic pathway for mithramycin biosynthesis. The carbon skeleton of mithramycin (mithramycin aglycon) consists of a tricyclic ring system with a side chain at C-5 (for a better understanding of the biosynthetic pathway, we are using not the International Union of Pure and Applied Chemistry numbering but a biosynthetic numbering system). It has been proposed that, for the biosynthesis of this aglycon, a type II polyketide synthase would synthesize a decaketide intermediate which would undergo successive aromatizations and cyclizations with the formation of a tetracenomycin F1-like compound (3). After decarboxylation at C-1, oxidative cleavage between C-18 and C-19 would generate a tricyclic structure on which further oxidation, reduction, and methylation reactions could take place, producing the mithramycinone aglycon. Such oxidative cleavage has been demonstrated to open ring B of a cyclized polyketide intermediate in the biosynthesis of the angucycline jadomycin B (21). According to this hypothesis, the incorporation of the 6DOH disaccharide and trisaccharide by glycosylation at C-11 and C-18 of the aglycon would occur after cleavage of the fourth ring. Conversion of D-glucose-1-phosphate into TDP-D-glucose by a glucose-1-phosphate:TTP thymidylyl transferase is the earliest step in 6DOH biosynthesis (8, 12), and therefore, the inactivation of this enzyme in the mithramycin producer should produce a mutant blocked in the biosynthesis of the three different 6DOHs attached to the mithramycin molecule. Consequently, such a mutant should accumulate the mithramycinone aglycon: a tricyclic ring structure with a lateral chain at C-5. To test this hypothesis, we determined the structure of the major product accumulated (premithramycinone) in the M7D1 mutant. Surprisingly, its structure (Fig. 3) was different from that expected and showed a tetracyclic ring. The existence of premithramycinone indicates that, after cyclization of the fourth ring in a tetracenomycin F2 intermediate (Fig. 3), two hydroxyl groups should be incorporated at C-4 and C-18. Then an O methylation should take place at the C-4 hydroxyl group. We suppose that the isolated compound premithramycinone is formed via the elimination of H_2O from a hypothetical intermediate, premithramycinone M3 (Fig. 3). It is not clear whether premithramycinone is a biosynthetic intermediate on the mithramycin biosynthetic pathway or a shunt product. There are plausible mechanisms for the formation of mithramycin via premithramycinone or premithramycinone M3. Feeding experiments with labelled $^{18}O_2$ gas and ^{18}O-labelled acetate will show whether the oxygen atom at C-19 is derived from acetate (premithramycinone is a shunt product) or from molecular oxygen (premithramycinone is an intermediate). These feeding experiments are under way now (14a). However, our results strongly support the idea that some (or all) glycosylations take place before fission of the fourth ring. In support of this hypothesis, a compound resembling glycosylated premithramycinone (designated chromocyclomycin) that contains a monosaccharide (D-mycarose) and a trisaccharide (D-olivose-D-oliose and D-mycarose) has been isolated from a mithramycin-producing streptomycete and possibly represents an aureolic acid intermediate (2).

Nucleotide sequence accession number. The 2,403-bp sequence has GenBank accession no. Y10907.

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