

Cloning and Characterization of Genes Encoded in dTDP-D-mycaminose Biosynthetic Pathway from a Midecamycin-producing Strain, *Streptomyces mycarofaciens*

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Abstract Two subclusters from *Streptomyces mycarofaciens*, a midecamycin producer, were cloned and partially sequenced. One region was located at the 5' end of the mid polyketide synthase (PKS) genes and contained the genes *midA*, *midB* and *midC*. The other region was at the 3' end of the PKS genes and contained *midK*, *midI* and *midH*. Analysis of the nucleotide sequence revealed that these genes encode dTDP-glucose synthase (*midA*), dTDP-glucose dehydratase (*midB*), aminotransferase (*midC*), methyltransferase (*midK*), glycosyltransferase (*midI*) and an assistant gene (*midH*). All of these genes are involved in the biosynthesis of dTDP-D-mycaminose, the first deoxysugar of midecamycin, and in transferring the mycaminose to the midecamycin aglycone in *S. mycarofaciens*. Similar to gene pairs *desVIII/desVII* in *S. venezuelae* and *tylMIII/tylMII* in *S. fradiae*, the product of *midH* probably functions as an auxiliary protein required by the MidI protein for efficient glycosylation in midecamycin biosynthesis.

Key words deoxysugar; dTDP-D-mycaminose; midecamycin; *Streptomyces mycarofaciens*

Macrolides constitute a class of antibiotics that contain a macrocyclic lactone ring composed of a polyketide-derived backbone to which one, two or three sugars are commonly attached. They are produced as secondary metabolites by mycelium-forming soil bacteria from the order *Actinomycetales*; the majority are from members of the genera *Streptomyces*, *Micromonospora* and *Saccharopolyspora*. Macrolide antibiotics are widely used as anti-infective, immunosuppressive, insecticidal, and parasiticidal agents in the clinic or for agricultural purposes. The known mechanism of the biological function of the main group of classical macrolides (erythromycin, tylosin and so on) is to bind to the peptidyltransferase center of the 50S subunit of the bacterial ribosome, thereby inhibit-

ing bacterial protein synthesis [3].

Streptomyces mycarofaciens produces midecamycin, a 16-membered macrolide (Fig. 1). During the biosynthesis of midecamycin, two deoxyhexose sugars, mycaminose and mycarose, are added to the polyketide lactone ring in sequential order [4]. The genetic organization of macrolide antibiotics has shown a common feature: the genes of polyketide synthase (PKS) involved in the lactone ring formation are clustered in the center of the chromosome with flanking genes of sugar biosynthesis on both sides [5–8]. To date, the PKS gene clusters have been cloned and characterized in the biosynthesis of several macrolide antibiotics [5–10]. However, the genetic analysis of sugar moieties that affect the bioactivity of macrolides has been studied at a much lower level.

Here we report the cloning and characterization of genes encoded in the dTDP-D-mycaminose biosynthetic pathway from a midecamycin-producing strain, *S. mycarofaciens*.

Received: November 11, 2006 Accepted: December 25, 2006
This study was supported by a grant from the Fifth Framework Program (FP5) of the European Community for Research, Technological Development and Demonstration Activities

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DOI: 10.1111/j.1745-7270.2007.00265.x

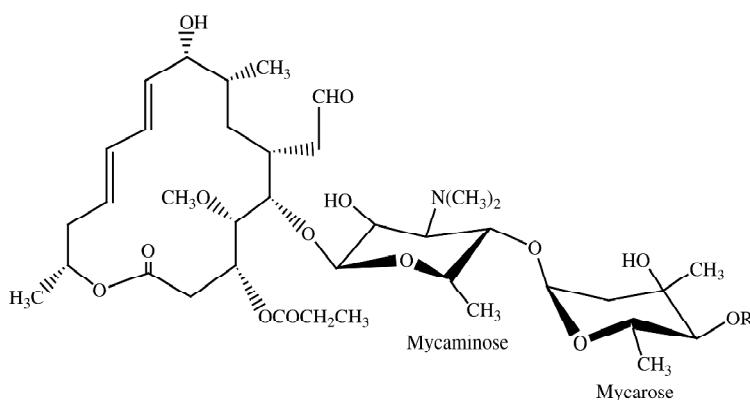


Fig. 1 Structure of midecamycin in *Streptomyces mycarofaciens*

Materials and Methods

Bacterial strains and culture conditions

Escherichia coli DH5 α was used throughout the study as a cloning host. *E. coli* JM108 was the host for a cosmid library derived from *S. mycarofaciens* genomic DNA. Luria Bertani medium was used in *E. coli* propagation [11]. *S. mycarofaciens* ATCC 21454 was obtained from the American Type Culture Collection. The wild-type strain was grown on ISP2 plates and sporulated at 28 °C for 5 d. The ISP2 contained Difco yeast extract 4 g, Difco malt extract 10 g, Difco dextrose 4 g and agar 20 g in 1 liter of tap water. To produce a seed culture, 10 ml of EP1 medium [12] was inoculated with spore suspension and cultivated at 28 °C for 3 d in a rotary shaker at 230 rpm. The EP1 consisted of corn steep liquor 5 g, defatted soya flour 10 g, CaCO₃ 2 g, and NaCl 5 g in one liter of tap water, pH 6.8. After autoclaving, 20 g of glucose per liter was added. Then 4.2 ml of the seed culture was inoculated into 60 ml of EP2 medium [12] and grown for 5–7 d by shaking (230 rpm) at 28 °C. The EP2 consisted of defatted soya flour 10 g, CaCO₃ 2 g, and CoCl₂·6H₂O 1 mg in one liter of tap water, pH 6.8–7.0. After autoclaving, 20 g of glucose per liter was added.

Vector, DNA manipulation and cosmid library construction

The *Streptomyces-E. coli* shuttle vector pKU206 was used for genomic DNA library construction [13]. pUC18 was the routine cloning vector. Total DNA isolation, plasmid DNA preparation, restriction endonuclease digestion, ligation and other DNA manipulations were carried out according to the standard protocols for *E. coli* and *Strep-*

tomyces [11,14]. DNA fragments were labeled with ³²P using the Rediprime DNA labeling system (Amersham, Piscataway, USA). For the construction of the cosmid genomic library, *S. mycarofaciens* chromosomal DNA was partially digested with *Sau*3AI, fragments in the range of 25–40 kb were cloned into pKU206 digested with *Bam*HI and packaged according to the protocol of the DNA packaging kit (Roche, Mannheim, Germany).

DNA probe preparation

The 303 bp DNA fragment (*midB'*) was obtained by polymerase chain reaction (PCR) amplification using primers AS2 and AS5 from the genomic DNA of *S. mycarofaciens*. AS2 (5'-GCCGCCGCGTCCCATGTCGAC-3') and AS5 (5'-CCCGTAGTTGGAGCAGCGGGT-3') were derived from the *strE* gene, encoding dTDP-glucose 4,6-dehydratase in the gene cluster of streptomycin biosynthesis from *S. griseus* [15]. The 420 bp DNA fragment (*midI'*) was first amplified by PCR using primers EryCIII/H and EryCIII/I from the genomic DNA of *S. mycarofaciens*, then reamplified using internal primers TylMII/H and EryCIII/I. Primers EryCIII/H (5'-CACGCG-CGGCTGCTGTGGGGACCCGAC-3') and EryCIII/I (5'-CGCCGCGCAG GTCGGCAGCAGCGCGTG CAT-3') were derived from the highly conserved sequence region of *eryCIII* in the gene cluster of erythromycin biosynthesis, whereas TylMII/H (5'-GAGGAGCCCCGGGAGGAC-CCGGTCGCCGA-3') was designed from *tylMII* in the gene cluster of tylosin biosynthesis. Both *eryCIII* and *tylMII* encoded macrolide glycosyltransferase [12,16].

Screening of the cosmid genomic library

The probes *midB'* and *midI'* were used to screen the established *S. mycarofaciens* genomic library by colony hybridization based on the protocol described by Weis and

Quertermous [17].

DNA sequencing and analysis

DNA sequencing was carried out with cosmid by standard shotgun cloning to obtain at least 4-fold coverage. Primer walking was used to close the gaps. The sequence was assembled using the Sequencher software package (Gene Codes, Ann Arbor, USA) and analyzed with MacVector (Accelrys, San Diego, USA) and the BLAST server of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST>).

Nucleotide sequence accession number

The nucleotide sequence reported here has been submitted to GenBank with accession numbers DQ672716, DQ672717, DQ672718, DQ672719, and DQ672720.

Results

Identification of pathway-specific gene probes

To identify the specific genes in midecamycin biosynthesis, the DNA fragments *midB'* (303 bp) and *midI'* (420 bp) were amplified by PCR from the genomic DNA of *S. mycarofaciens*. Sequencing analysis indicated that MidB' encodes dTDP-glucose 4,6-dehydratase and MidI' encodes a glycosyltransferase. The evidence is that the product of *midB'* in a 101 amino acid overlap is 76% identical to TylAII in *S. fradiae* and 70% identical to StrE in *S. griseus*. Both proteins TylAII and StrE are the putative dTDP-glucose 4,6-dehydratase [15,18]. The comparison of the *midI'* product to other known glycosyltransferases revealed that MidI' in a 142 amino acid overlap is 56% identical to TylMII and 53% identical to EryCIII in *Saccharopolyspora erythraea* [12,16]. Analysis of several previously identified macrolide deoxysugar clusters suggested that these two genes are separately located on both sides of the PKS gene cluster [9,15–18]. Therefore, the obtained gene fragments of *midB'* and *midI'* were used as pathway-specific probes to screen the genomic DNA library of *S. mycarofaciens*.

Cloning and sequencing of the partial *mid* biosynthetic gene cluster

Initial colony hybridization analysis with the *midB'* probe against the genomic DNA library of *S. mycarofaciens* revealed two positive cosmids, cosM1 (30.2 kb) and cosM2 (22.6 kb) [Fig. 2(A)]. Both cosmids are overlapped 12.4 kb, and sequencing showed that the extending region of cosM2 contains the 5' end sequence of the *mid* PKS genes. By chromosomal walking using a probe of a 2.3 kb DNA fragment close to the end of cosM1 to screen the library, a positive cosmid cosM4 (29.3 kb) was obtained that extended away from cosM1 in a region of approximately 18 kb. Using the *midI'* probe to screen the genomic library, a positive cosmid cosM3 (26.3 kb) was obtained. One end of cosM3 contains the 3' end sequence

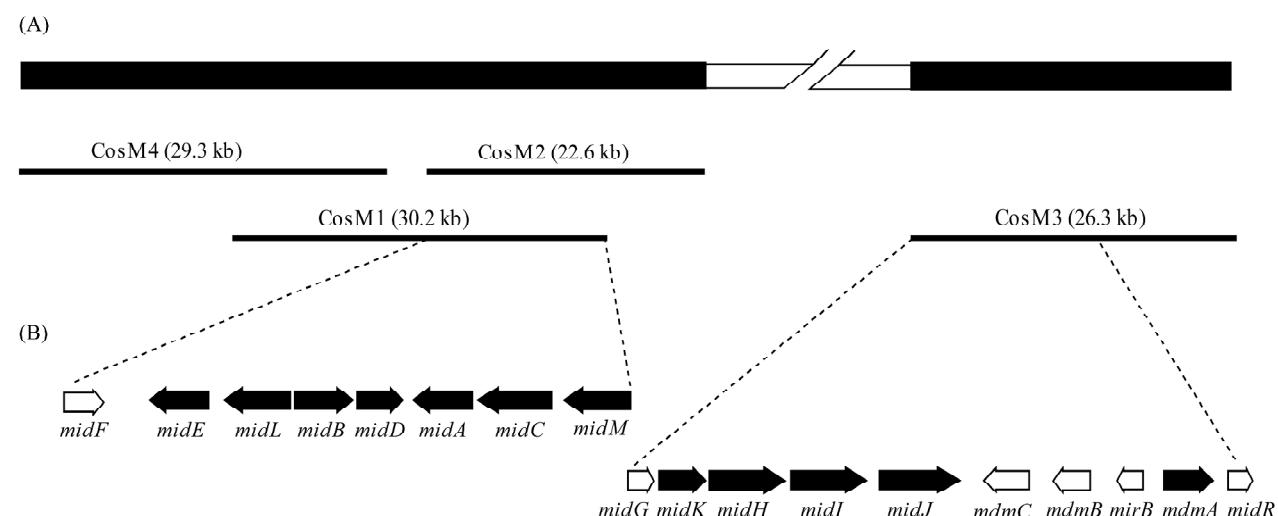


Fig. 2 Organization of the partial gene cluster in midecamycin biosynthesis

(A) Schematic representation of cosmid clone location. The black bars represent the region of *Streptomyces mycarofaciens* chromosomal DNA found in existing cosmids. The white bar indicates the unresearched region of the chromosome with a break shown as a double slant. (B) Genes identified within the analyzed sequence (cosM1 and cosM3). The arrowheads indicate transcription direction. Black arrow bars represent open reading frame genes and white arrow bars indicate incomplete genes.

of the *mid* PKS genes. Although finding the *mid* PKS genes was not the target of this study, it could facilitate the identification of the overall organization and orientation of the gene cluster and the genes encoding 6-deoxysugar biosynthetic enzymes. Therefore, cosmids cosM1, cosM2 and cosM4 should be upstream of the *mid* PKS genes, whereas cosmid cosM3 should be located downstream [Fig. 2(A)].

Extensive sequencing was done from cosmids cosM1 and cosM3. Computer-assisted analysis of the sequenced regions led to identification of the genes shown in Fig. 2(B) and listed in Table 1. They include genes for mycaminose biosynthesis and attachment as well as genes for PKS, resistance, regulation and chain modification.

Analysis of genes involved in mycaminose biosynthesis and attachment

The *midA*, *midB* and *midC* genes were found in a subcluster upstream of the *mid* PKS genes. The deduced product of *midA* showed significant sequence identity to putative dTDP-glucose syntheses from several streptomycetes [9,19,20]. The complete *midB* gene includes the DNA fragment of the *midB'* probe. The deduced product

of *midB* is highly similar in sequence to dTDP-glucose-4,6-dehydratases from several deduced gene products in macrolide antibiotics [18,19,21]. The deduced product of *midC* shows significant sequence similarity with putative aminotransferases from various deduced gene products in different antibiotic biosynthetic pathways [18,20,22]. The BLASTP analysis of the MidA, MidB and MidC proteins is shown in Table 2.

The *midK*, *midI* and *midH* genes were found in a subcluster downstream of the *mid* PKS genes. The deduced product of *midK* shows significant sequence similarity to a family of enzymes proposed to function as S-adenosylmethionine-dependent methyltransferases [16,20,23]. The complete *midI* gene includes the DNA fragment of the *midI'* probe. The deduced product of *midI* shows convincing end-to-end sequence similarity to the known glycosyltransferases of several macrolide antibiotics [9,16,20]. In database searches, the deduced product of *midH* shows sequence similarity to *TylMIII* in *S. fradiae*, *DnrQ*

Table 1 Deduced functions of the putative genes identified in the midecamycin biosynthetic gene cluster

Gene designation	aa (n)	Proposed function
<i>midA</i>	303	dTDP-glucose synthase
<i>midB</i>	326	dTDP-glucose-4,6-dehydratase
<i>midC</i>	398	Aminotransferase
<i>midK</i>	249	Methyltransferase
<i>midH</i>	414	Auxiliary protein
<i>midI</i>	421	Glycosyltransferase
<i>midM</i>	372	Cytochrome P-450
<i>midD</i>	264	Thioesterase
<i>midL</i>	372	dTDP-4-keto-6-deoxyglucose 2,3-dehydratase
<i>midJ</i>	448	Crotonyl CoA reductase
<i>midG</i> (incomplete)	133	Midecamycin PKS
<i>midF</i> (incomplete)	228	Regulation
<i>midR</i> (incomplete)	140	Regulation
<i>mdmA</i>	271	Resistance
<i>mirB</i> (incomplete)	135	Resistance
<i>midE</i>	338	4"-O-propionyl transferase
<i>mdmB</i> (incomplete)	213	3-O-acyltransferase
<i>mdmC</i> (incomplete)	252	O-methyltransferase

aa, amino acid.

Table 2 BLASTP analysis of gene products involved in mycaminose biosynthesis and attachment from *Streptomyces mycarofaciens*

Gene product	Best BLASTP matches (%identity/protein/organism)	Accession No.
MidA	70/ChmAII/S. <i>bikiniensis</i> 66/AveBIII/S. <i>avermitilis</i> 60/DesIII/S. <i>venezuelae</i>	AY509120 NC_003155 AF079762
MidB	72/AprE/S. <i>tenebrarius</i> 64/TylAII/S. <i>fradiae</i> 64/AveBII/S. <i>avermitilis</i>	AAG18457 U08223 NC_003155
MidC	58/OleN2/S. <i>antibioticus</i> 56/TylB/S. <i>fradiae</i> 54/DesV/S. <i>venezuelae</i>	AF055579 U08223 AF079762
MidK	57/TylMI/S. <i>fradiae</i> 54/OleM1/S. <i>antibioticus</i> 53/DesVI/S. <i>venezuelae</i>	X81885 AJ002638 AF079762
MidI	59/ChmCIII/S. <i>bikiniensis</i> 57/TylMII/S. <i>fradiae</i> 54/DesVII/S. <i>venezuelae</i>	AY509120 X81885 AF079762
MidH	35/TylMIII/S. <i>fradiae</i> 32/DnrQ/S. <i>peucetius</i> 30/DesVIII/S. <i>venezuelae</i>	X81885 L47164 AF079762

MidA, dTDP-glucose synthase; MidB, dTDP-glucose-4,6-dehydratase; MidC, aminotransferase; MidK, methyltransferase; MidI, glycosyltransferase; MidH, auxiliary protein.

in *S. peucetius*, and *DesVIII* in *S. venezuelae* [16,20,24]. **Table 2** shows the results of BLASTP analysis of the MidK, MidI and MidH proteins.

Discussion

Several studies have shown that various 6-deoxyhexoses, present in a range of antibiotic molecules, are made from D-glucose-1-phosphate by way of dTDP-glucose and dTDP-4-keto-6-deoxy-glucose before the pathway diverges [25,26]. Based on sequence analysis and comparison, the gene *midA* as dTDP-glucose synthase and *midB* as dTDP-glucose-4,6-dehydratase are supposed to be responsible for the early steps in the midecamycin biosynthetic gene cluster. In particular, a novel gene order of *midA* and *midB* was found in this study whereby both genes are convergent and separated by *midD*. In most other antibiotics *midA* and *midB* are usually co-directional neighbors [18,20].

The MidC protein deduced as an aminotransferase might catalyze the conversion of dTDP-3-keto-6-deoxyglucose to dTDP-3-amino-6-deoxyglucose during dTDP-D-mycaminose biosynthesis. The aminotransferase enzyme is thought to be dependent on pyridoxal phosphate as a co-factor [27]. The sequence similarities between MidC and other macrolide aminotransferases are most apparent in a region of the protein that contains the conserved lysine

residue, which is supposed to be the attachment site for pyridoxal phosphate (data not shown). The MidK protein displayed as a methyltransferase might act on the amino group of dTDP-3-amino-6-deoxyglucose during mycaminose biosynthesis, perhaps by introducing two methyl groups at that site. The sequence alignment of the MidK protein with other macrolide methyltransferases shows that they possess three of the consensus sequence motifs typical of methyltransferases that use S-adenosylmethionine as a co-substrate [28] (data not shown).

Based on sequence analysis and comparison, the MidI protein as a glycosyltransferase is believed to be responsible for attachment of mycaminose to midecamycin lactone. A sequence alignment of MidI with other known glycosyltransferases shows that all these proteins retain a characteristic motif, P-NVR-VDFVPL-ALLP-C---VHHGG-GT--TA--HG-P, present in UDP-glycosyltransferases [29] (data not shown). As the gene order of *midH* directly upstream from *midI* is the same as the gene pairs *tylMIII/tylMII* in *S. fradiae* and *desVIII/desVII* in *S. venezuelae* [1,2], and the sequence of MidH showed similarity with the TylMIII and DesVIII, the product of *midH* probably functions as an auxiliary protein required by the MidI protein for efficient glycosylation in midecamycin biosynthesis.

As shown in **Fig. 3**, a biosynthetic route from glucose-1-phosphate to dTDP-D-mycaminose, and the sugar at-

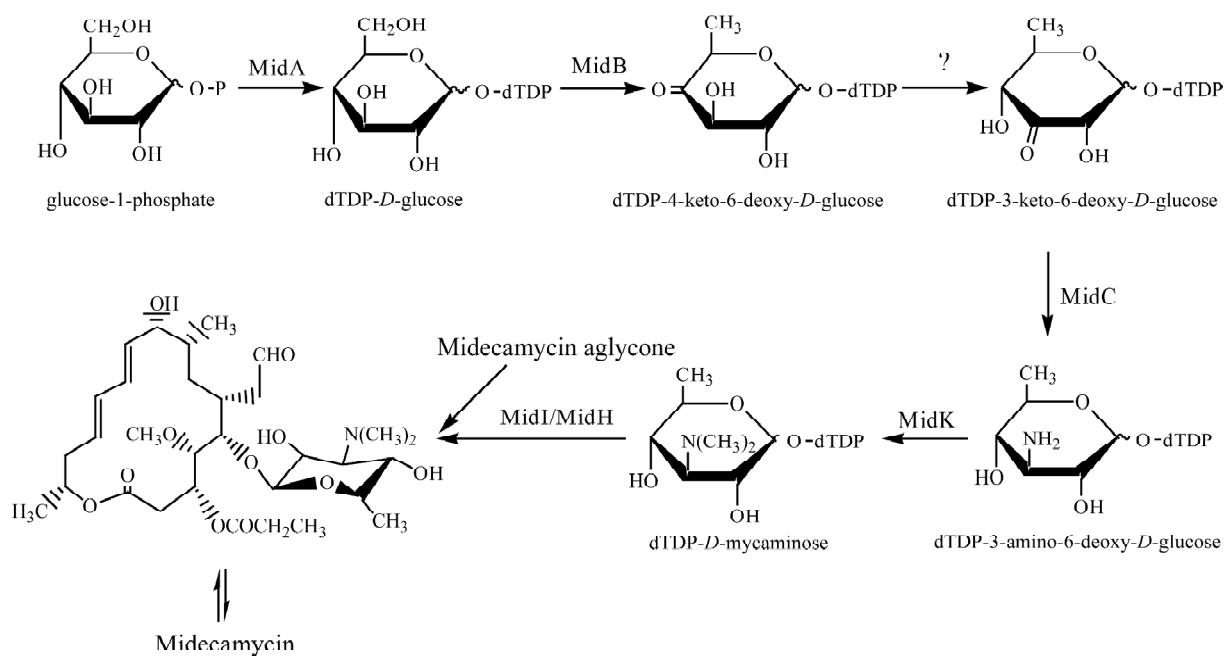


Fig. 3 Proposed biosynthetic route to dTDP-D-mycaminose in *Streptomyces mycarofaciens* and related *mid*-encoding proteins

tachment to midecamycin lactone in *S. mycarofaciens* is proposed. In this pathway, a gene encoding 3,4-isomerase, which is responsible for converting dTDP-4-keto-6-deoxy-D-glucose to dTDP-3-keto-6-deoxy-D-glucose, is still missing. We have since extended another 20 kb of sequencing region on both sides of CosM1 and CosM3 to identify the missing gene and genes involved in mycarose biosynthesis, the second deoxysugar of midecamycin. Based on the present study, it is concluded that the proposed biosynthetic pathway is very similar to the parallel genes identified in the tylisin biosynthetic gene cluster of *S. fradiae*.

Acknowledgement

We thank Prof. John SMITH for help in preparing the manuscript.

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