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A Macrolide 3-*O*-Acyltransferase Gene from the Midecamycin-Producing Species *Streptomyces mycarofaciens*

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The *Streptomyces mycarofaciens mdmB* gene encodes a 3-*O*-acyltransferase that catalyzes the addition of acetyl and propionyl groups to position 3 of the lactone ring in 16-member macrolide antibiotics like midecamycin and spiramycin. A putative *O*-methyltransferase gene (*mdmC*) is immediately downstream of *mdmB*, and both of these genes are closely linked to the *mdmA* midecamycin resistance gene.

Macrolide antibiotics are antiinfective, immunosuppressive, insecticidal, and parasiticidal agents produced by species of *Streptomyces* and a few other genera of the order *Actinomycetales* (15, 17). The antiinfective types typically consist of a 12- to 16-member lactone to which one or more deoxysugars are attached. In the 16-member class, such as leucomycin, midecamyin, spiramycin, and carbomycin (Fig. 1), some of the hydroxyls in both the lactone and sugar moieties are acylated with short-chain fatty acids (15). These appendages are important determinants of antibiotic potency; for this reason, variations in the acylation pattern have been made chemically as part of drug development programs (18).

With the advent of gene cloning technology for antibiotic-producing organisms (8, 10) has come the hope that hybrid actinomycetes that will produce derivatives of known antibiotics, as well as fundamentally new metabolites, can be constructed (11, 13). The surest way to accomplish this is to make derivatives known to have improved activity. Pioneering work for macrolides has been reported by Epp et al. (4), who cloned the *Streptomyces thermotolerans carE* gene that encodes a deoxyhexose *O*-acyltransferase. This gene causes conversion of the 4″-hydroxyl in the mycarose residue of spiramycin (Fig. 1) to its isovaleryl ester both in the spiramycin-producing species *S. ambofaciens* and in *S. lividans* through bioconversion of added spiramycin following introduction of the *carE* gene by transformation (4).

In a search for the midecamycin biosynthesis (*mdm*) genes of *S. mycarofaciens*, we examined the bioconversion properties of clones from an approximately 20-kb region near the *mdmA* midecamycin resistance gene (5) that carried *carE*-homologous DNA. In experiments with spiramycin I (Fig. 1), instead of the expected functional *carE* homolog, we found a 2.4-kb DNA segment that caused 3-*O*-acylation of spiramycin I to spiramycin II or III (Fig. 1).

Cloning and functional analysis of the *S. mycarofaciens* 3-*O*-acyltransferase gene. Clones of *S. mycarofaciens* ATCC 21454 DNA containing the *mdmA* midecamycin resistance gene (5) were screened for hybridization to the *S. thermotolerans carE* gene on the presumption that midecamycin biosynthesis requires a step like the one governed by *carE*, since midecamycin and carbomycin have nearly identical structures (Fig. 1). pOH23 (Fig. 2) was found to contain a 2-kb *Bgl*II-*Bam*HI DNA segment that hybridized weakly to *carE*. *S. lividans* TK24 (9) was transformed with pOH23 or pOH311 (this plasmid has a 3.0-kb *Bam*HI DNA segment, containing the *carBE* genes [4], that was cloned in the *Bam*HI site of pIJ680 [8], data not shown), and the transformants were cultivated in R2YE medium (8) for 1 to 7 days at 28°C in the presence of 100 μg of spiramycin I per ml. Analysis of ethyl acetate extracts of the cultures by silica gel thin-layer chromatography (chloroform-methanol ratio, 5:1 [vol/vol]) and bioautography with *Micrococcus luteus* showed that new antibiotic substances had been formed by the two types of transformants.

As expected (4), 7-day agar cultures of the pOH311 transformant caused spiramycin I to be metabolized to 4″-isovalerylspiramycin I. This was verified by chromatographic comparison of the new product to an authentic sample plus spectral analysis by field desorption mass spectrometry and 400-MHz nuclear magnetic resonance spectroscopy (data not shown). In contrast, *S. lividans* transformed with pOH77, which was made in pIJ680 from pOH23 (Fig. 2) and contains the 3.1-kb *Sst*I-*Bam*HI *mbmB* segment and a 1.4-kb *Bam*HI *mdmA* segment (to provide adequate midecamycin and spiramycin resistance [5]), caused spiramycin I [δ 3.80 ppm, br doublet, J = 10.8 Hz (C-3 -C<u>H</u>OH)] to be metabolized mainly to spiramycin II [M + 1 ion @ m/z 885; δ 5.15 ppm, br doublet, J = 11.0 Hz (C-3 -C<u>H</u>OCOCH$_3$); δ 2.29 ppm, s (C-3 -CHOCOC<u>H</u>$_3$)] plus smaller amounts of spiramycin III [M + 1 @ m/z 899; δ 5.16 ppm, br quartet (C-3 -C<u>H</u>OCOCH$_2$CH$_3$); δ 1.23 ppm, m (C-3 -OCOCH$_2$C<u>H</u>$_3$); δ 2.61 ppm, m (C-3 -OCOC<u>H</u>$_2$CH$_3$)] and a compound that is probably 3-*O*-butyrylspiramycin (M + 1 @ m/z 913). Figure 3 illustrates a typical time course for this biotransformation. When the same transformant was cultured in liquid R2YE medium for 3 days, spiramycin III was the major product, accompanied by a lesser amount of spiramycin II.

Leucomycins A_5 and A_7 (Fig. 1) appeared to be converted to their 3-*O*-acyl derivatives, leucomycin A_4 (3-acetyl) and/or midecamycin A_2 (3-propionyl) and midecamycin A_1 (3-propionyl) and/or leucomycin A_6 (3-acetyl), respectively, by *S. lividans*(pOH77) transformants. These structure assignments were not confirmed by spectral analysis. Leucomycin A_5 was not noticeably metabolized by cultures of the *S. lividans*(pOH311) transformant, and midecamycin M_1 was not metabolized by cultures of the *S. lividans*(pOH77) transformant. These results establish the unique characteristics of *carE* (C-4″ *O*-acylation) versus *mdmB* (C-3 *O*-acylation). The essential role of the *S. mycarofaciens mdmB* acyltransferase

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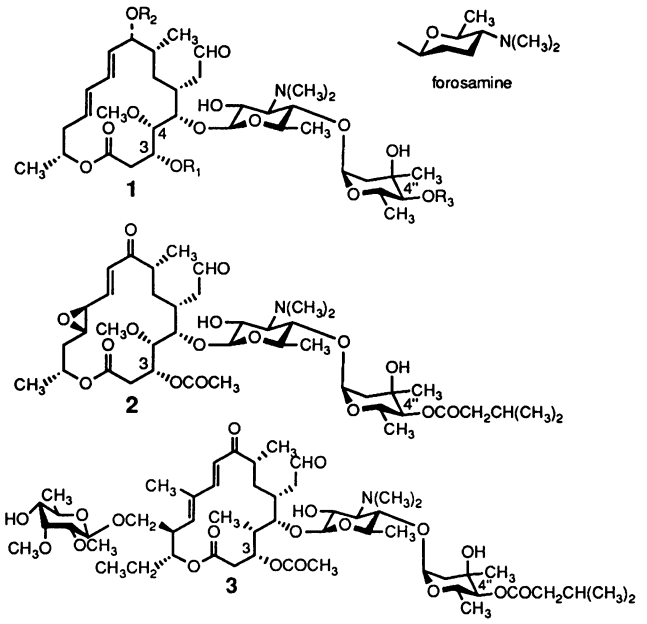

FIG. 1. Structures of representative 16-member macrolide antibiotics leucomycin A_4 (1: R_1 = $COCH_3$, R_2 = H, R_3 = $COCH_2CH_2CH_3$), leucomycin A_5 (1: R_1 = R_2 = H, R_3 = $COCH_2CH_2CH_3$), leucomycin A_6 (1: R_1 = $COCH_3$, R_2 = H, R_3 = $COCH_2CH_3$), leucomycin A_7 (1: R_1 = R_2 = H, R_3 = $COCH_2CH_3$), midecamycin A_1 (1: R_1 = $COCH_2CH_3$, R_2 = H, R_3 = $COCH_2CH_3$), midecamycin A_2 (1: R_1 = $COCH_2CH_3$, R_2 = H, R_3 = $COCH_2CH_2CH_3$), midecamycin M_1 (1: R_1 = $COCH_2CH_3$, R_2 = R_3 = H), spiramycin I (I: R_1 = R_3 = H, R_2 = forosaminyl), spiramycin II (1: R_1 = $COCH_3$, R_2 = forosaminyl, R_3 = H), spiramycin III (1: R_1 = $COCH_2CH_3$, R_2 = forosaminyl, R_3 = H), carbomycin A (2), and tylosin (3).

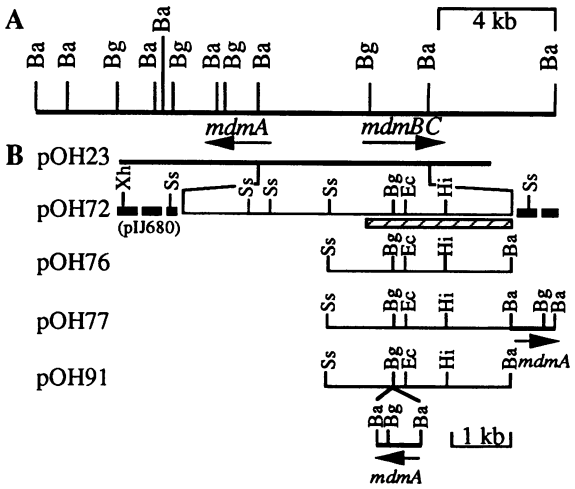

FIG. 2. (A) Restriction map of *Bam*HI (Ba) and *Bgl*II (Bg) sites in an approximately 18-kb region of *S. mycarofaciens* DNA cloned as described by Hara and Hutchinson (5). (B) Restriction map of clones discussed in the text. pOH23 is a pIJ680 clone (5); the region that hybridized to *carE* is defined by the *Bgl*II-*Bam*HI sites in panel A above the *mdmBC* arrow. pOH72 contains the 5.6-kb *Bam*HI DNA segment cloned from pOH23 into the *Bam*HI site of pIJ680. The thick dashed line represents pIJ680 vector DNA. The cross-hatched box indicates the region of DNA sequenced that contains the *mdmBC* genes. Additional restriction site abbreviations: Xh, *Xho*I; Ss, *Sst*I; Ec, *Eco*RI; Hi, *Hin*dIII. The ≈3-kb *Sst*I DNA segment obtained from pOH72 was ligated with *Sst*I-digested pIJ680 to obtain pOH76. pOH76 was digested with *Bam*HI and ligated with the 1.4-kb *Bam*HI *mdmA* fragment of pOH1 (5) to obtain pOH77 or digested with *Bgl*II and ligated with the *mdmA* fragment to obtain pOH91. The arrows indicate the relative locations and directions of transcription of the *mdmA*, *mdmB*, and *mdmC* genes.

gene was proven by a bioconversion experiment with an *S. lividans*(pOH91) transformant; in pOH91, *mdmB* is inactivated by insertion of the *mdmA* gene into a *Bgl*II site internal to the *mdmB* coding region (Fig. 2). Cultures of this transformant failed to metabolize spiramycin I to spiramycin II, indicating that *mdmB* encodes 3-*O*-acyltransferase activity.

The availability of two types of *O*-acyltransferase genes for 16-member macrolides will facilitate manufacture of hybrid antibiotics of this class. In fact, 3-*O*-acetyl-4″-*O*-isovaleryltylosin (Fig. 1) has been made in this way recently, by using the 3- and 4″-*O*-acyltransferase genes from the carbomycin-producing species *S. thermotolerans* (1).

Sequence analysis of the *mdmB* and *mdmC* genes. The sequence of both strands of a 2,381-nucleotide (nt) portion of the DNA cloned in pOH77 (Fig. 2) was performed on single-strand templates by the dideoxy-chain termination method using dye-labeled primers (Promega) for a 370A automated DNA sequencer (Applied Biosystems) and *Taq* polymerase (Promega) in the presence of 7-deaza-dGTP. Analysis of the sequence data with CODON PREFERENCE (3) revealed only two likely open reading frames (data not shown). The first was designated *mdmB*, and the second was designated *mdmC*. Both open reading frames have unusually low N-terminal G+C contents: 61.4% for the first 376 nts of *mdmB* and 68.9% for the first 74 nts of *mdmC*. *mdmB* begins at nt 126 and is preceded by a satisfactory ribosome-binding site (GGAG, 11 nts upstream) for *Streptomyces* genes (2); it ends at nt 1289 (TGA) and thus should encode a protein with a molecular weight of 42,171 and a pI of 10.43, as determined by PEPTIDESORT (3) analysis. The *Bgl*II site into which

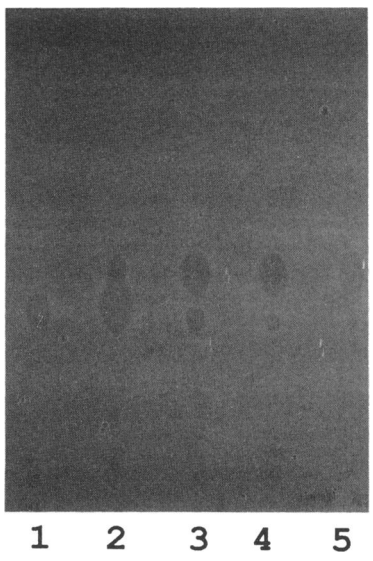

FIG. 3. Bioautogram of the extract of *S. lividans*(pOH77) grown on R2YE agar medium containing 100 μg of spiramycin I per ml. The zones of clearing resulted from lack of growth of *M. luteus*. Lanes: 1, spiramycin I reference standard; 2 to 5, extracts from 1-, 3-, 5-, and 7-day-old cultures. The upper spots in lanes 2 to 5 are a mixture of spiramycin II (major product) and spiramycin III (minor product).

```
A      1                                                                100
CarE}  .mPlpkhLPa LgGMRFIsAL LVFtSHIstQ pFFknteIns ALqFpLnrLG pltVSfFFML SGFVLtWagl pdkSkVnFWR RRtvRaYsLH LP....vLLV
MdmB}  mpPrvvrLPs LtGLRWfAAL aVFacHIaqQ qFFAdqqVgt AL.LhittLG sIAVSVFFLL SGFVLaWSAr dkDSvttFWR RRfakIYPLH Lv....TFLI
 Sf6}  ....mhksnc FdtaRLVAAM MVLvSH.... .hYAlsgqpe pylFgFesaG gIAViIFFsi SGYliskSAi rsDSfIdFma kRarRIFPal vPcsilTYFl
Consen --P----LP- L-G-R--AAL -VF-SHI--Q -FFA------ AL-F----LG -IAVS-FF-L SGFVL-WSA- --DS---FWR RR--RIYPLH LP----T-L-

       101                                                              200
CarE}  tllIVLaLnE PnM.GrSVWD GLltnLL..L IQAWFPDHhe ygsmNpvaWS LSCELFFYam FPFlFaFfTK VrtdRLWRwA AaVsvAaVsI PLVaLlLPAS
MdmB}  aGvIIFsLaE PtLpGgSVWD GLvpdLL..L VQsWLPEptI iAGfNtpsWS LSCEFaFYlt FPLwYrLVrK IpVrRLWWcA AGIAaAVIcV PFVtsqFPAS
 Sf6}  fGwIlndFsa eyFshdiVRk tissiFMsqa pdAditsHlI hAGiNgslWt LplEFLcY.. ......iITg VaVahLk... nGkAfiVIll vFVsLsLigS
Consen -G-I---L-E P---G-SVWD GL---LL--L -QAW-P-H-I -AG-N---WS LSCEF-FY-- FP------TK V-V-RLW--A AG-A-AVI-- PFV-L-LPAS

       201                                                              300
CarE}  pplpWDPdMP qlrWWFiYmF PPVRLLEFVL GmlMAqIvir GrWRGPrpla cVaLFsAvFA .VTFaVPnhY dpgAltVpvi ALLlasvAVG DVrGVRsWLg
MdmB}  aeta..PgMP lneLWFaCWL PPVRMLEFVL GivMALIlrt GvWRGPgVvs salLLaAaYg .VTqvVPpmF TiaACsIVpa ALLitalAna DVqGlRtgLr
 Sf6}  vsenRD.... .vmFsiplWL yPlRgLaFfF GatMAMyeks ..WnvsnVki tVvsLlAmYA yasYgkgidY TmtcYilVsf stiaictsVG Dpl.VkgRF.
Consen -----DP-MP ----WF--WL PPVR-LEFVL G--MA-I--- G-WRGP-V-- -V-LL-A-YA -VT--VP--Y T--A---V-- ALL----AVG DV-GVR--L-

       301                                                          398
CarE}  trtMVlLGEl tFAFYLVHYL IIQYGHRFaG GkqGYYRQWd TpaAvgLtLl AFTlALgLSa FLHfFVEKPv MRtLGRpRRs pdagstPrsE pAPsgTp*
MdmB}  savLVrLGEW SFAFYLVHFM VIrYGHRLmG GeLGYaRQWs TasAgaLaLa mLaVAivagg LLHtvVEnPc MRLLGR.RRp vatapdPatD eAPklTra
 Sf6}  ........DY SYgvYiyaFp VqQvv..int lhMGFYp... .....sMlLs AvTV.LfLSh LswnLVEK.. .RFLtRsspk lsld...... ........
Consen ----V-LGE- SFAFYLVHF- VIQYGHR--G G--GYYRQW- T--A--L-L- A-TVAL-LS- LLH--VEKP- MR-LGR-RR- ------P--- -AP--T--

B      1                                                                100
Ccoamt}masngeskhs eVghkslLqS dALyqYIlet SVypREpeaM kELrEvTAkh Pwn.lMttsa DEGQFLnMLl kLInAkntME IGvYTGYSlL atAlALPddG
  MdmC}.......... .VadqttL.S pALLdY..ar SValREdglL rELhDmTAQl PggRAMqImp EEaQFLgLLI RLVgArrVLE IGtFTGYStL cMARALPaGG
Ratcom}.......... ....mgdtke qriLrYVqqn akpgdpqsvL eaidtyctQ. .keWAMnVgd akGQiMdavI ReyspslVLE lGaYcGYSav rMARlLqpGa
Consen ---------- -V-----L-S -ALL-Y---- SV--RE---L -EL---TAQ- P---AM---- -EGQFL--LI RL--A--VLE IG-YTGYS-L -MARALP-GG

       101                                                              200
Ccoamt}kILaMDINrE nyeIGlPiie kAGVghKIDF ReGpAlpvLd hMlEdgkyhG TFDFVFVDAD KDnYinYhkr lidLVkiGGL IgyDNTLWnG sVAqP.ADaP
  MdmC}RIvTcDIsdk WpgIGaPfwq rAGVdglIDL RiGdAartLa eLrErdg.DG aFDLVFVDAD KagYLhYyeq alaLVrpGGL VaiDNTLFfG rVAdPaADDP
Ratcom}RlLTMEmNpD YaaItqqmln fAGlqdKVti lnGasqdlip qLkkkydvD. TLDMVFlDhw KDrYL....p dtlLlekcGL lrkgtvLLad nVivPgtpD.
Consen RILTMDIN-- ---IG-P--- -AGV--KID- R-G-A---L- -L-E----DG TFD-VFVDAD KD-YL-Y--- ---LV--GGL ---DNTL--G -VA-P-ADDP

       201                                                    250
Ccoamt}mrkyVRyYrd FVieLNkaLa aDpRIEIcML pVgDGVTLcR Ris.......
  MdmC}dtvaVR.... ...tLNdLLr dDeRVDIaLL tVaDGITLAR Rre*......
Ratcom}.......Fla YVrgsssFec thyssyleyM kVvDGlekAi yqgpsspdks
Consen ----VR---- -V--LN--L- -D-R--I--L -V-DG-TLAR R---------
```

FIG. 4. Comparisons of three *O*-acyltransferases (A) and three *O*-methyltransferases (B) by the PILEUP method (3), which uses the Needleman-Wunsch algorithm (16). The Consen data, in which a capital letter indicates the presence of at least two identical residues in a vertical column, were generated with PRETTY (3).

the *mdmA* gene was inserted (Fig. 2) occurs at nt 376, near the N terminus of *mdmB*. *mdmC* most likely begins at nt 1327 with a similarly placed but less satisfactory ribosome-binding site (GGAAAG) and ends at nt 1992 (TAA); it should encode a protein with a molecular weight of 24,020 and a pI of 4.54.

Comparisons of the deduced products of *mdmB* and *mdmC*, using FASTA and TFASTA (3) analyses, identified strong similarities to known proteins in the GenBank and SwissProt data bases (as of 11 March 1992). When viewed by the COMPARE and DOTPLOT methods (3), MdmB most strongly resembles CarE and the product of the *Shigella* bacteriophage SF6 *O*-acetyltransferase gene, an enzyme that *O*-acetylates a lipopolysaccharide in the *Shigella flexneri* O antigen (21) (data not shown). All three proteins appear to be quite hydrophobic when analyzed by the method of Kyte and Doolittle (14) using PEPPLOT (3) (data not shown), and several conserved regions can be seen by PILEUP (3) analysis (Fig. 4A). However, none of these proteins exhibit significant similarity to acetyltransferases with a verified R(C=O)X-binding site. MdmC, in contrast, strongly resembles the caffeoylcoenzyme A 3-*O*-methyltransferase (Ccoamt) of parsley (20) and a catechol *O*-methyltransferase (Ratcomt) from rats (19) (Fig. 4B). MdmC is much less similar to several other aromatic *O*-methyltransferases examined (data not shown) and, like caffeoylcoenzyme A 3-*O*-methyltransferase, lacks the consensus binding region characteristic of *S*-adenosylmethionine-dependent methyltransferases (6, 12) (Fig. 4B). Moreover, its only distant similarity by COMPARE (3) analysis to EryG, the 3″-*O*-mycarosylmethyltransferase of erythromycin biosynthesis (6), suggests that MdmC is the 4-*O*-methyltransferase for the lactone ring of midecamycin and related 16-member macrolides, instead of 3″-*O*-mycarosylmethyltransferase.

Nucleotide sequence accession number. The nucleotide sequence of the *mdmBC* genes has been assigned GenBank accession number M93958.

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