CLONING OF MIDECAMYCIN(MLS)-RESISTANCE GENES FROM *STREPTOMYCES MYCAROFACIENS*, *STREPTOMYCES LIVIDANS* AND *STREPTOMYCES COELICOLOR* A3(2)

OSAMU HARA[a,b] and C. RICHARD HUTCHINSON[b]

[a] Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd., Morooka-cho, Kohoku-ku, Yokohama 222, Japan
[b] School of Pharmacy and Department of Bacteriology, University of Wisconsin, Madison, WI 53706, U.S.A.

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DNA containing genes for midecamycin(Mdm)-resistance was cloned from *Streptomyces mycarofaciens* ATCC 21454 (*mdmA* gene), *Streptomyces lividans* 66 (*lrm* gene) and *Streptomyces coelicolor* A3(2). The phenotype imparted to *S. lividans* and *Streptomyces griseofuscus* transformants by the cloned DNA segments indicates that they encode an MLS-type of resistance activity. The *mdmA* and *lrm* genes could be distinguished by the phenotype they conferred in *S. lividans* and *S. griseofuscus*, whereas the *S. lividans lrm* and *S. coelicolor* MLS genes appear to be identical on the basis of their restriction maps and behavior in *S. lividans* and *S. griseofuscus*. The DNA sequence of a 1.4-kb *Bam*H I DNA fragment containing the *mdmA* gene indicates the presence of one complete *orf* whose deduced product exhibits a high similarity to the deduced product of the *Streptomyces thermotolerans carB* gene and several other bacterial MLS-resistance genes.

Studies of the genetics of antibiotic production have established that the genes for macrolide antibiotic biosynthesis and self-resistance are closely linked in *Streptomyces ambofaciens*[1)], *Saccharopolyspora erythraea*[2)], *Streptomyces fradiae*[3)], and *Streptomyces thermotolerans*[4)]. As a prelude to cloning genes governing the production of the 16-membered macrolide antibiotic midecamycin (Mdm), we isolated and sequenced a Mdm-resistance (*mdmA*) gene from *Streptomyces mycarofaciens*. The single Mdm-resistance (Mdm^R) gene found confers the macrolide-lincosamide-streptogramin B (MLS) type of resistance[5)] to *Streptomyces lividans* and *Streptomyces griseofuscus* transformants, and appears to encode an MLS determinant very similar to the products of other bacterial MLS genes. In contrast, two[4,6)] or three[3,7)] resistance genes have been cloned from the producing organism for the other 16-membered macrolides investigated to date. The *S. thermotolerans carA* and *carB* resistance genes, for instance, are linked to at least four carbomycin biosynthetic genes[4)]. The *carB* gene confers resistance to carbomycin, whose structure is very similar to Mdm[8)], and to some other macrolide antibiotics[6)].

During the course of our work, we also found that *S. lividans* exhibited Mdm-resistance. Mdm^R genes different from the *mdmA* gene were then isolated from *S. lividans* 66 and *Streptomyces coelicolor* A3(2). The latter two genes have identical restriction maps, but they could be distinguished from the *S. mycarofaciens mdmA* and *S. thermotolerans carB* genes by the pattern of MLS-resistance conferred to *S. lividans* and *S. griseofuscus* transformants.

Materials and Methods

Bacterial Strains and Plasmids

S. griseofuscus ATCC 23916 and *S. thermotolerans* ATCC 25500 were obtained from the American Type Culture Collection, and *S. mycarofaciens* HP 201 and SF 837 (ATCC 21454) from the Meiji Seika

Table 1. Plasmids constructed in this study.

Plasmid	Genotype	Vector	Source of cloned DNA
pOH1-4	*tsr, mdmA*	pIJ680	*Streptomyces mycarofaciens* ATCC 21454
pOH5	*tsr, mdmA*	pIJ702	*S. mycarofaciens* HP 201
pOH23	*tsr, mdmA*	pIJ680	*S. mycarofaciens* ATCC 21454
pOH51-4	*tsr, kan*	pIJ486/487	*S. mycarofaciens* ATCC 21454
pOH61	*tsr, mdmA*	pIJ680	*S. mycarofaciens* 1748
pOH241-43	*apm, mdmA*	pKC505	*S. mycarofaciens* ATCC 21454
pOH251-53	*apm, mdmA*	pKC505	*S. mycarofaciens* ATCC 21454
pOH301-04	*tsr, lrm*	pIJ680	*S. lividans* 66
pOH311	*tsr, carBE*	pIJ680	*S. thermotolerans* ATCC 25500
pOH331-32	*tsr, kan*	pIJ486/487	*S. lividans* 66
pOH333-34	*tsr. lrm, kan*	pIJ486/487	*S. lividans* 66
pOH342	*tsr, mdm*^{R}	pIJ680	*S. coelicolor* M110

tsr: thiostrepton-resistance, *kan*: kanamycin (neomycin)-resistance, *apm*: apramycin-resistance.

Kaisha Ltd. culture collection, and *S. fradiae* C373.1 from Eli Lilly and Co. *S. coelicolor* M110 (*hisA1 uraA1 strA1*) (derived from the A3(2) strain), *S. lividans* 66 (same as the 1326 strain) and the plasmid vectors pIJ486, pIJ487, pIJ680 and pIJ702 were obtained from DAVID HOPWOOD and MERVYN BIBB, John Innes Institute and AFRC Institute of Plant Science, Norwich, UK. pKC505 was obtained from NAGARAJA RAO, Eli Lilly and Co. The plasmids constructed in this work are listed in Table 1. Midecamycin A_1 was obtained from Meiji Seika Kaisha Ltd., apramycin from Eli Lilly and Co., and thiostrepton from Squibb Research Institute; all other antibiotics were obtained from Sigma Chemical Co., St. Louis, MO.

Growth Media and Conditions for Determining Antibiotic Resistance

The *Streptomyces* strains were grown in Tryptic Soy (TS) broth in 125 or 250-ml baffled Erlenmeyer shake flasks on a New Brunswick G25 gyratory shaker or in 13 ml test tubes on a New Brunswick G24 gyratory shaker at 28~30°C and 300 rpm unless specified otherwise. *S. lividans* and *S. griseofuscus* protoplasts were prepared and transformed as described by HOPWOOD *et al.*[9] using the R2YE[9] and modified R2[10] media, respectively. The resistance of *S. lividans* transformants to neomycin (50 μg/ml), Mdm and other antibiotics was determined by replica plating sporulated colonies to Difco nutrient agar supplemented with the antibiotic and scoring growth after 2 to 4 days incubation at 30°C; or by growing cells in liquid S1 medium[11] containing 5 μg/ml of thiostrepton (Thio) for 2 days at 28°C, then plating the cells on Difco nutrient agar containing various concentrations of another antibiotic. *S. griseofuscus* transformants were grown overnight in TS broth containing Thio (10 μg/ml) before plating on TS agar supplemented with another antibiotic.

Two methods were used to test for inducible MLS-resistance. (1) *S. lividans* and *S. griseofuscus* transformants were grown, respectively, in S1 or TS broth containing 5 μg/ml of Thio for 2 days at 28°C, 1 μg/ml of Mdm was added, and the culture was grown for 1 more hour. The resulting cells were plated on Difco nutrient agar plates containing various concentrations of an MLS-type antibiotic and the growth was scored after 2 to 4 days incubation at 30°C. (2) TS agar plates supplemented with erythromycin, Mdm, spiramycin, or tylosin (1 μg/ml) were overlaid with soft agar containing the culture broth of *S. lividans* or *S. griseofuscus*, grown as described in method 1 but without the addition of Mdm, and 8.0 mm paper discs spotted with 20 μg of erythromycin, Mdm, spiramycin or tylosin were placed on top of the plates. Zones of growth inhibition around the discs were measured after 3 days incubation at 30°C.

Cloning of Mdm^R Genes

Three different methods were employed. (1) Genomic DNA isolated from *S. mycarofaciens* 21454 and HP 201 was digested with *Bam*H I, *Bgl* II, *Kpn* I, *Pst* I, *Sph* I or *Sst* I and ligated to pIJ680 that had been linearized with *Bam*H I, *Kpn* I or *Pst* I, or to pIJ702 linearized with *Bgl* II, *Sph* I or *Sst* I. Similarly, DNA from *S. lividans* 66 was digested with *Bam*H I, *Bgl* II, *Sph* I or *Kpn* I; and DNA from *S. coelicolor* M110 was digested with *Bam*H I before ligation to the vector DNA. *S. lividans* 66 protoplasts were transformed with the ligation mixture and the resulting Thio-resistant (Thio^R) transformants were screened

for the Mdm^R phenotype by replica plating as described above. An identical experiment was performed with *S. mycarofaciens* 21454 DNA partially digested with *Sau*3 AI and ligated to *Bam*H I-digested pIJ680. *S. lividans* was retransformed with the plasmids isolated from experiment (1) to confirm that they conferred Mdm^R to this host. (2) A library of *S. mycarofaciens* 21454 DNA was constructed in *Escherichia coli* in the *E. coli-Streptomyces* shuttle cosmid pKC505 following the procedures of RICHARDSON *et al.*[7]. *S. griseofuscus* protoplasts were transformed with the plasmid DNA isolated from about 5,000 of these *E. coli* recombinants, and about 20,000 of the resulting apramycin-resistant ($apramycin^R$) transformants were pooled and incubated in 500 ml of TS broth containing 25 μg/ml of Mdm in a 2-liter flask at 30°C for 2 days. The culture was plated on TS agar containing 25 μg/ml of Mdm and Mdm^R colonies were isolated after 3 days growth at 30°C. (3) Colonies that hybridized to the *mdmA* gene cloned from *S. mycarofaciens* 21454 were isolated from the *S. mycarofaciens* library and tested for their ability to confer the Mdm^R phenotype in *S. lividans* transformants.

DNA Manipulation and Hybridization

Total DNA and plasmid DNA were isolated from *Streptomyces* strains as described by HOPWOOD *et al.*[9] or from *E. coli* as described by MANIATIS *et al.*[12]. Restriction enzyme digestions and DNA ligations were performed according to literature protocols[9,12]. Restriction maps of cloned DNA were determined from the results of single and double digestions according to literature protocols[9,12]. DNA was labeled with ^{32}P using the Multiprime kit from Amersham, Chicago, IL and [^{32}P]dCTP. Colony hybridization was performed as described by MANIATIS *et al.*[12] using 3×SSC/4×DENHARDT's/calf thymus DNA (100 μg/ml) at 62°C followed by washing for 30 minutes at 62°C with 2×SSC/0.1% SDS, then with 1×SSC/0.1% SDS. Southern blot-transfer and DNA hybridization were performed according to HOPWOOD *et al.*[9] or to the protocols of Amersham. The hybridization conditions were: (low stringency) hybridize at 42°C and wash twice for 30 minutes each with 2×SSC/0.1% SDS at 42°C; or (high stringency) hybridize at 70°C and wash once for 30 minutes with 2×SSC/0.1% SDS at 70°C, then twice for 30 minutes each with 0.2×SSC/0.1% SDS at 70°C.

DNA Sequencing and Analysis

Nested deletions of the 1.4-kb *Bam*H I fragment from pOH1 subcloned in pTZ18U (Toyobo) were generated using the Kilo-sequence Deletion Kit (Takara Shuzo). DNA sequencing was performed on single strand templates by the dideoxy-chain termination method using dye-labeled primers (Promega) for the Model 370A automated DNA sequencer (Applied Biosystems) and *Taq* polymerase (Promega) in the presence of 7-deaza-dGTP and following the manufacturer's directions. Both DNA strands were sequenced at least 3 times each.

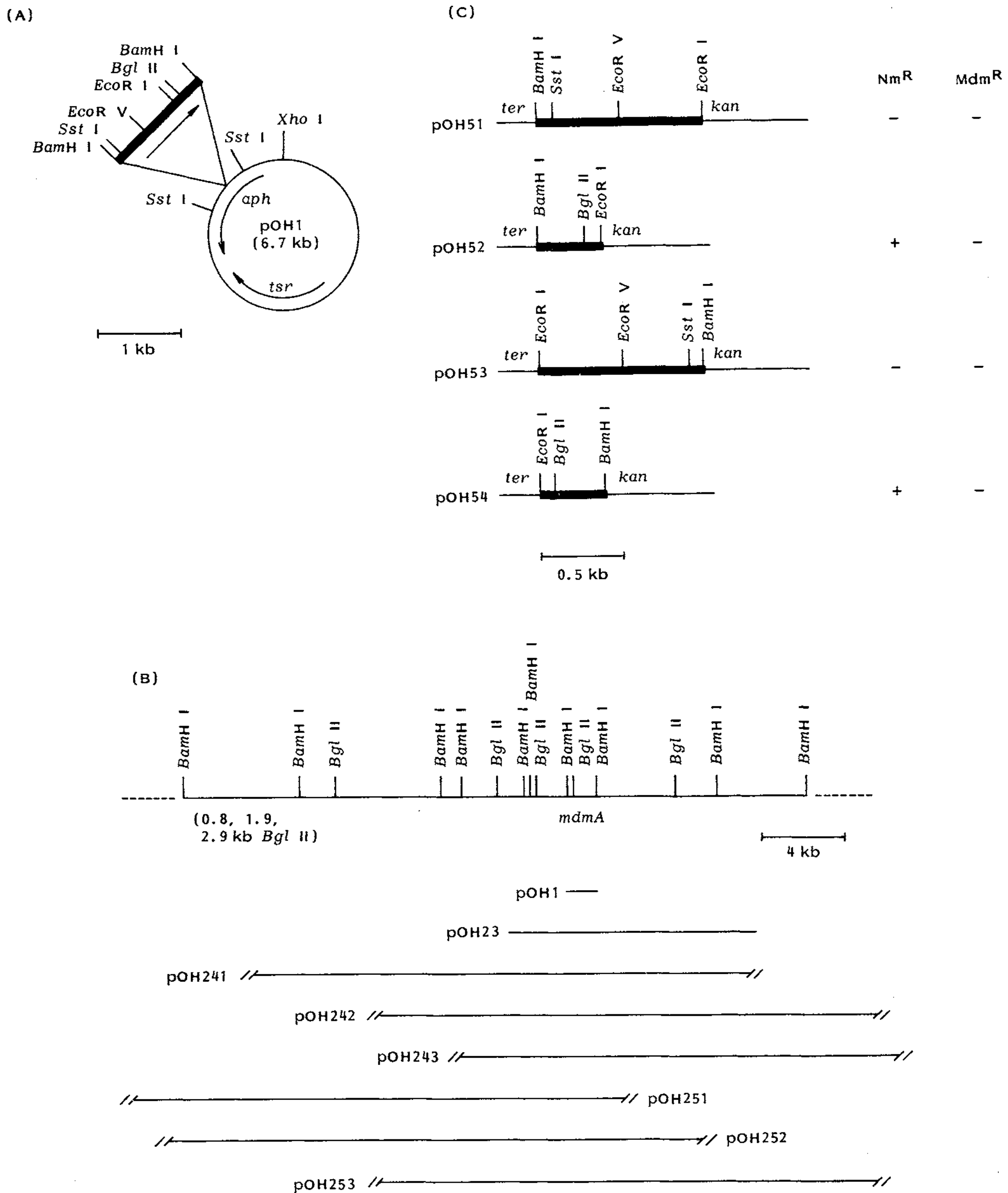
Results

A Single Mdm^R Gene Cloned from *S. mycarofaciens*, *S. lividans* and *S. coelicolor*

Four clones (pOH1-4) with identical 1.4-kb *Bam*H I fragments conferring Mdm^R in *S. lividans* transformants were shotgun cloned from the wild-type, 21454 strain of *S. mycarofaciens* using pIJ680[9] and *Bam*H I-digested genomic DNA. A 1.4-kb *Sst* I fragment was cloned from the *S. mycarofaciens* HP 201 strain (a Mdm-high producing strain derived from the wild-type SF 837 strain at Meiji Seika Kaisha, Ltd.) in pIJ702[9] as pOH5 using *Sst* I-digested DNA. No resistance clones were isolated from either strain by using DNA digested with *Bgl* II, *Kpn* I, *Pst* I or *Sph* I. Comparison of the restriction maps of these five clones indicated that a 1.3-kb *Bam*H I-*Sst* I fragment contains the *mdmA* gene as shown by pOH1 in Fig. 1(A). Six clones were isolated in pIJ680 using DNA from the 21454 strain that had been partially digested with *Sau*3 AI; one of them (pOH23) contained the same 1.4-kb *Bam*H I fragment as part of a 12.2-kb insert (Fig. 1(B)).

Ten clones containing overlapping DNA inserts were isolated from a library of *S. mycarofaciens* 21454 DNA made in the *E. coli-Streptomyces* shuttle cosmid vector pKC505[7] by selecting for inducible Mdm^R

Fig. 1. Restriction maps of Mdm^R clones from *Streptomyces mycarofaciens*.



(A) pOH1 contains the *mdmA* gene from *S. mycarofaciens* ATCC 21454. All the restriction sites mapped for the insert containing this gene (but not for the pIJ680 vector) are shown. The arrow indicates the direction of *mdmA* transcription. *Aph*, the gene conferring neomycin-resistance; *tsr*, the gene conferring thiostrepton-resistance[9]. (B) The relative locations of the DNA inserts in the primary clones (see Table 1) that contain the *mdmA* gene are shown below a partial restriction map of the region in the *S. mycarofaciens* ATCC 21454 genome surrounding this gene. // indicates uncertainty as to the exact end of these DNAs; --- indicates the unmapped region of the genomic DNA. (C) The antibiotic resistance phenotype of *S. lividans* after transformation with pOH51 ~ 54 is indicated to the right of partial restriction maps of pIJ486/7 clones (thin line) containing the *mdmA* gene (thick line). Only the insert DNA is drawn to scale. *Ter*, the fd transcription terminator[14]; *kan*, the gene conferring kanamycin(neomycin)-resistance[14]; Nm^R, neomycin-resistance; Mdm^R, midecamycin-resistance. '+', growth, and '−', no growth, on 50 μg/ml of kanamycin.

in *S. griseofuscus*. Restriction analysis of the plasmid DNAs isolated from the Mdm^R clones indicated three types of restriction patterns (represented by the plasmids pOH251~253 in Fig. 1(B)), but these plasmids all had the same 1.4 kb *Bam*H I fragment as pOH1.

Three clones (pOH241~243, Fig. 1(B)) with overlapping inserts were isolated from the same library by screening for colonies that hybridized to ^{32}P-labeled pOH1. Restriction analysis of the plasmid DNA isolated from these clones showed that the plasmids all contained the same 1.4 kb *Bam*H I fragment as in pOH1.

Genomic DNA isolated from *S. thermotolerans* ATCC 25500 (the carbomycin producer), *S. fradiae* C373.1 (the tylosin producer) and *Saccharopolyspora erythraea* WMH22 (the erythromycin producer) did not hybridize to ^{32}P-labeled pOH1 under low stringency conditions. In contrast, the *Bam*H I and *Bgl* II digests of the genomic DNA isolated from *S. mycarofaciens* 21454, HP 201 and the 1748 strain, a Mdm producer isolated in China in 1977 (Y. WANG, personal communication), showed an identical hybridization pattern when probed with [^{32}P]pOH1 (not shown). A 1.4-kb *Bam*H I fragment conferring Mdm^R to *S. lividans* was then cloned from the 1748 strain in pIJ680 as pOH61 (not shown) by the same method used for pOH1.

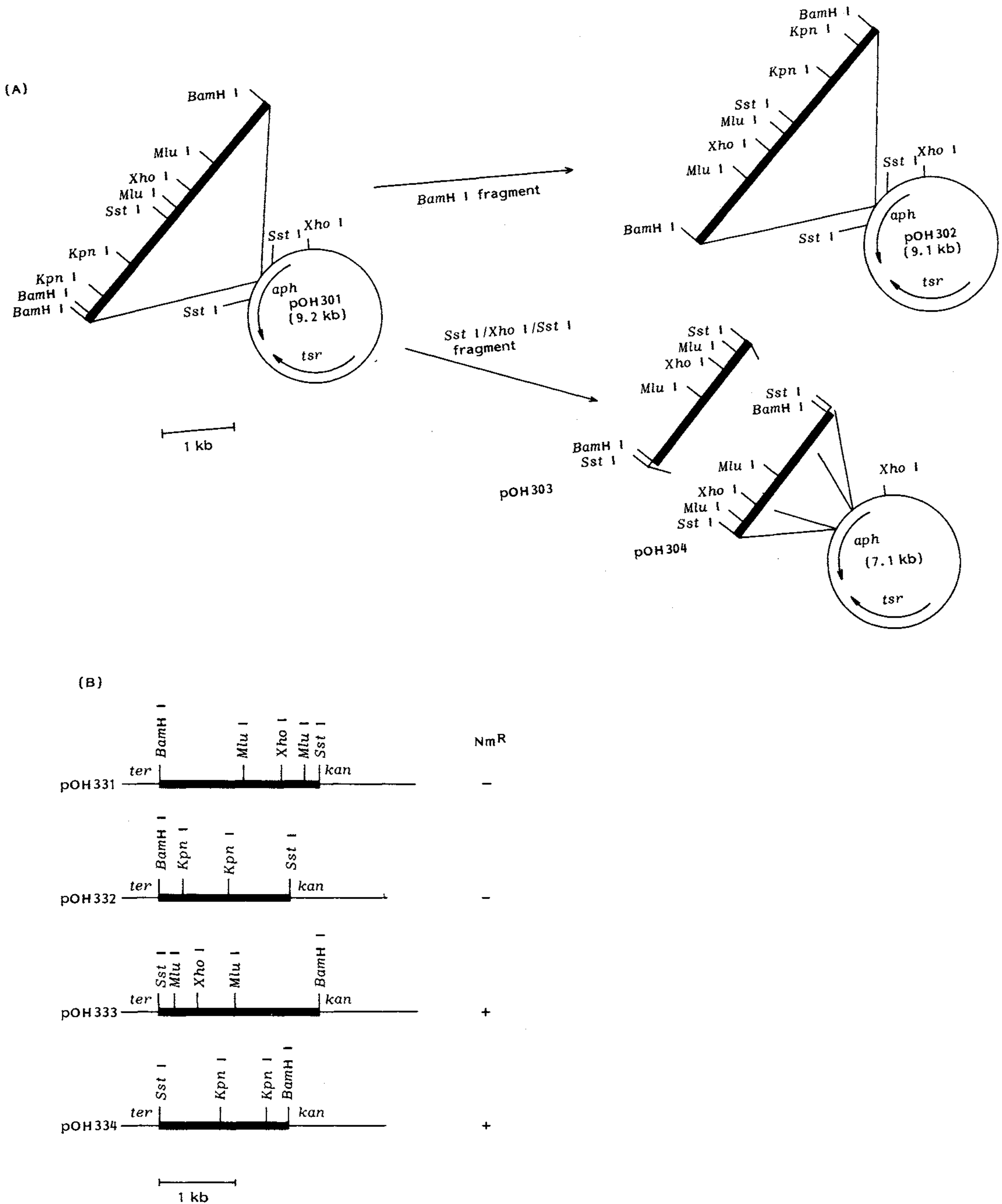
The collective results establish that *S. mycarofaciens* strains contain only one (clonable) *mdmA* gene.

During the above work, we noticed that *S. lividans* exhibited inducible antibiotic resistance when grown on nutrient agar containing Mdm. A 3.8-kb *Bam*H I fragment conferring resistance to 50~100 μg/ml of Mdm was shotgun cloned from *S. lividans* 66 in pIJ680 as pOH301 (Fig. 2(A)) using *Bam*H I-digested genomic DNA. By subcloning experiments, this Mdm^R determinant (the *lrm* gene: See Discussion) was shown by the loss of a small *Bam*H I fragment to reside in pOH302 and then in a 2.1-kb *Sst* I/*Xho* I/*Bam*H I fragment as represented by pOH304 (Fig. 2(A)). Subsequently, a plasmid conferring Mdm^R to *S. lividans* that had a 3.8-kb *Bam*H I insert and the same restriction pattern as pOH302 was cloned as pOH342 from *S. coelicolor* M110 by the same method (not shown).

The Cloned Mdm^R Genes Confer MLS resistance to *S. lividans* and *S. griseofuscus*

The Mdm^R exhibited by *S. lividans* and *S. griseofuscus* after transformation with different plasmids containing the Mdm^R genes was determined under non-inducing conditions and after growth for 1 hour in the presence of 1 μg/ml of Mdm to induce antibiotic resistance. Tables 2 and 3 give the results of these experiments and include the data from *S. lividans* and *S. griseofuscus* transformed with pOH311, which contains the *S. thermotolerans carBE* genes[4,6] cloned in pIJ680 as a 3.0-kb *Bam*H I fragment. [The *carB* gene is believed to encode an RNA methylase[6]; in *S. griseofuscus*, *carB* confers resistance to carbomycin, spiramycin, roseramicin, lincomycin and vernamycin B, and inducible resistance to tylosin[6].] *S. lividans* (pOH1) grew on Mdm concentrations as high as 800 μg/ml whereas *S. griseofuscus* (pOH1) essentially ceased growing when the concentration of Mdm was greater than 50 μg/ml (Table 2). No difference was noted between the level of resistance conferred by pOH1 and by pOH5, which contained DNA cloned from the Mdm-high producing HP 201 strain. *S. lividans* (pOH1) was resistant to Mdm, spiramycin and erythromycin, and weakly resistant to tylosin. This pattern of macrolide-resistance did not noticeably change after induction by Mdm (compare the data indicated by '+' and '*' in Table 2). *S. lividans* (pOH311) had a similar behavior. In contrast, *S. lividans* (pOH302) exhibited approximately the same level of resistance to Mdm and spiramycin, but was more sensitive to erythromycin and less sensitive to tylosin than *S. lividans* (pOH1). On induction with Mdm, its level of resistance to all four MLS antibiotics was increased

Fig. 2. Restriction maps of Mdm[R] clones from *Streptomyces lividans*.



(A) The derivation of pOH302 to pOH304 from pOH301, which contains the *lrm* gene from *S. lividans* 66. All the restriction sites mapped for this gene (but not for the pIJ680 vector) are shown. *Aph*, the gene conferring neomycin-resistance; *tsr*, the gene conferring thiostrepton-resistance[9]. (B) The antibiotic resistance phenotype of *S. lividans* after transformation with pOH331 ~ 334 is indicated to the right of partial restriction maps of pIJ486/7 clones (thin line) containing the *lrm* gene (thick line). Only the insert DNA is drawn to scale. *Ter*, the fd transcription terminator[14]; *kan*, the gene conferring kanamycin (neomycin)-resistance[14]; Nm[R], neomycin-resistance. '+', growth, and '−', no growth, on 50 μg/ml of kanamycin.

Table 2. MLS-resistance phenotype of *Streptomyces lividans* transformants[a].

Antibiotic (μg/ml)		Plasmid					
		pIJ680	pOH1	pOH311	pOH302	pOH304	pOH342
None		+++[b] ***[c]	+++ ***	+++ ***	+++ ***	+++ ***	+++ ***
Midecamycin	(50)	+/- *	+++ ***	+++ ***	+++ ***	+++ ***	+++ ***
	(100)	+/- */-	+++ ***	+++ ***	+++ ***	+++ ***	+++ ***
	(200)	-[d] */-	+++ ***	+++ ***	+++ ***	++ ***	++ ***
	(400)	- -	+++ ***	+++ ***	+ ***	+ **	+/- **
	(800)	- -	++ **	++ **	- */-	- */-	- -
Spiramycin	(50)	+ **	+++ ***	+++ ***	+++ ***	+++ ***	+++ ***
	(100)	+ *	+++ ***	+++ ***	+++ ***	+++ ***	+++ ***
	(200)	+/- */-	+++ ***	+++ ***	+++ ***	+++ ***	++ **
	(400)	- */-	+++ ***	+++ ***	+ **	++ **	+ *
	(800)	- -	+++ ***	+++ ***	+/- **	++ **	+/- *
Tylosin	(25)	++ **	+++ ***	+++ ***	+++ ***	+++ ***	+++ ***
	(50)	+/- */-	+++ ***	+++ ***	+++ ***	+ **	+++ ***
	(100)	- -	++ **	++ **	+++ ***	- -	+++ ***
	(200)	- -	- -	+/- */-	+++ ***	- -	+ **
	(400)	- -	- -	- -	+/- *	- -	- *
Erythromycin	(12.5)	+/- *	+++ ***	+++ ***	+++ ***	+++ ***	++ **
	(25)	- */-	+++ ***	+++ ***	+++ ***	+ **	+ *
	(50)	- -	+++ ***	+++ ***	+/- **	- */-	+/- *
	(100)	- -	++ **	++ **	- */-	- -	- -
	(200)	- -	+/- */-	+ *	- -	- -	- -
	(400)	- -	- -	+/- */-	- -	- -	- -

[a] The data shown are the average of three experiments.
[b] Relative growth on nutrient agar after 4 days without induction by midecamycin.
[c] Relative growth on nutrient agar after 4 days upon induction with midecamycin (1 μg/ml).
[d] Indicates no growth under the conditions tested.

somewhat. *S. lividans* (pOH304) behaved the same towards Mdm and spiramycin, but was significantly more sensitive to erythromycin and tylosin. The behavior of *S. lividans* (pOH342) paralleled that of *S. lividans* (pOH302). *S. lividans* (pIJ680), as the control, was comparatively sensitive to all four of the macrolides even under the inducing conditions.

Table 3. MLS-resistance phenotype of *Streptomyces griseofuscus* transformants[a].

Antibiotic (μg/ml)		Plasmid				
		pIJ680	pOH1	pOH311	pOH302	pOH342
None		+++[b] ***[c]	+++ ***	+++ ***	+++ ***	+++ ***
Midecamycin	(12.5)	—[d] —	+++ ***	+++ ***	+++ ***	+++ ***
	(25)	— —	+++ ***	+++ ***	+++ ***	+++ ***
	(50)	— —	+/— *	+/— *	+/— **	+/— **
	(100)	— —	— —	— */—	— *	— —
	(200)	— —	— —	— —	— —	— —
Spiramycin	(12.5)	— —	+++ ***	+++ ***	+++ ***	+++ ***
	(25)	— —	+++ ***	+++ ***	+++ ***	+++ ***
	(50)	— —	+++ ***	+++ ***	+++ ***	++ ***
	(100)	— —	+++ ***	+++ ***	++ ***	+ **
	(200)	— —	++ **	+ **	+ **	— */—
	(400)	— —	— */—	+/— *	— **	— —
Tylosin	(12.5)	— —	++ **	++ **	+++ ***	+++ ***
	(25)	— —	+ *	+ *	+++ ***	+++ ***
	(50)	— —	— */—	— */—	+++ ***	+++ ***
	(100)	— —	— —	— —	++ ***	++ ***
	(200)	— —	— —	— —	+/— **	+/— *
	(400)	— —	— —	— —	— —	— —
Erythromycin	(25)	+++ ***	+++ ***	+++ ***	+++ ***	+++ ***
	(50)	— —	+++ ***	+++ ***	+++ ***	+++ ***
	(100)	— —	+++ ***	+++ ***	+++ ***	+++ ***
	(200)	— —	+++ ***	+++ ***	++ **	+ *
	(400)	— —	+ **	+ **	— —	— —

[a] The data shown are the average of two experiments.
[b] Relative growth on nutrient agar after 4 days without induction by midecamycin.
[c] Relative growth on nutrient agar after 4 days upon induction with midecamycin (1 μg/ml).
[d] Indicates no growth under the conditions tested.

The data for the *S. griseofuscus* transformants (Table 3) reveal some interesting differences among the behavior of the *mdmA*, *carBE* and *lrm* genes. Compared with *S. lividans*, this host is more sensitive to Mdm, spiramycin and tylosin but more resistant to erythromycin. As such, it has served as a useful

host for the cloning and analysis of MLS genes from streptomycetes[1,3,4,6,7]. *S. griseofuscus* exhibited a similar pattern of resistance to Mdm and spiramycin when transformed with pOH1, pOH311 and pOH302 or pOH342 under non-induced and induced conditions (compare the data indicated by '+' and '*' in Table 3). Its resistance to tylosin was apparently greater when transformed with the *lrm* gene than with the *mdmA* or *carBE* genes, but its resistance to erythromycin showed an opposite trend: the *lrm* gene provided significantly less resistance than the *mdmA* and *carB* genes. Induction with Mdm did not significantly alter this behavior. The resistance properties of *S. griseofuscus* (pOH342) paralleled those of *S. griseofuscus* (pOH302) and for this reason are not shown.

In experiments in which *S. lividans* and *S. griseofuscus* were transformed with the plasmids listed in Tables 2 and 3, and the transformants tested for inducible MLS-resistance due to erythromycin, Mdm, spiramycin or tylosin in the growth medium by scoring the zones of inhibition due to the same four antibiotics by a paper-disc assay as described in the Materials and Methods, we observed that the cloned genes imparted considerable resistance to these MLS-type antibiotics but gave no evidence of inducible MLS-resistance.

The properties of the Mdm^R genes from *S. mycarofaciens* and *S. lividans* parallel the behavior of several other inducible MLS-resistance genes from *Streptomyces* that have been cloned and studied[5,13]. The *S. mycarofaciens mdmA* gene in particular seems to impart the same resistance phenotype to *S. lividans* and *S. griseofuscus* as the *S. thermotolerans carB* gene[6].

DNA Fragments Containing the Mdm^R Genes from *S. mycarofaciens* and *S. lividans* Exhibit Promoter Activity

We had found that the ability of pOH1 and pOH301 to confer Mdm-resistance in *S. lividans* transformants was independent of the orientation of the cloned fragment. pOH303 and pOH304 differ only in the orientation of their inserts although pOH304 conferred a higher level of Mdm^R than pOH303 (data not shown), which we attribute to a synergistic effect of the *aph*I promoter on expression of the insert in pOH304. These observations suggested that each DNA fragment contained at least the promoter for the resistance gene. To test this idea, the 1.4-kb *Bam*H I fragment in pOH1 containing the *S. mycarofaciens mdmA* gene was subcloned into the promoter-probe vectors pIJ486 and pIJ487[14] as 1.0 and 0.4 kb *Eco*R I-*Bam*H I fragments (Fig. 1(C)). Among the four plasmids obtained, only pOH52 and pOH54 conferred neomycin-resistance to *S. lividans* transformants; none of the four plasmids conferred Mdm^R. Since pOH52 and -54 differ only in the orientation of the 0.4-kb *Eco*R I-*Bam*H I fragment, the bidirectional promoter activity suggested that this fragment contains two promoters. However, the location of this fragment in relation to the *mdmA* gene, as determined from the DNA sequence data shown below, indicates that the position of either promoter is inconsistent with it being the promoter of the *mdmA* gene.

A similar experiment was carried out with pOH301, resulting in the subcloning of the two orientations of its 2.1 kb *Bam*H I/*Xho* I/*Sst* I fragment as pOH331 and -333, and its 1.7-kb *Bam*H I/*Sst* I fragment as pOH332 and -334 (Fig. 2(B)). The comparative neomycin-resistance of *S. lividans* transformed with these four plasmids indicated that only one orientation of each cloned fragment (pOH333 and pOH334) exhibited promoter activity. Since the results presented above indicate that the 2.1-kb DNA fragment contains the *S. lividans* Mdm^R gene, its promoter may lie in the fragment cloned in pOH333 (these data do not exclude the presence of another promoter in this fragment, however). The adjacent 1.7-kb *Bam*H I/*Sst* I fragmentin pOH301 appears to contain the promoter for another *S. lividans* gene.

Fig. 3. The nucleotide sequence of the 1382 nt *Bam*H I fragment that contains the *mdmA* gene.

```
      BamH I
      GGATCCGCGCACACGACAGACTGTCCGGGCGCGATGGCCCAACAGAGAACGCCGATACGG
    1 ---------+---------+---------+---------+---------+---------+ 60
      CCTAGGCGCGTGTGCTGTCTGACAGGCCCGCGCTACCGGGTTGTCTCTTGCGGCTATGCC
        I  R  A  H  D  R  L  S  G  R  D  G  P  T  E  N  A  D  T  A
                                                 1------->      Sst I
      CCGATGACGTCGGAACGGCGTATTTCAGAAGTGCACGGATGGATGCGGCGGAGCAGAGCT
   61 ---------+---------+---------+---------+---------+---------+ 120
      GGCTACTGCAGCCTTGCCGCATAAAGTCTTCACGTGCCTACCTACGCCGCCTCGTCTCGA
        D  D  V  G  T  A  Y  F  R  S  A  R  M  D  A  A  E  Q  S  S
         2------->                                     1------->
      CGACGCGGCGACACCCTCAACTATTACAGAGAACCCATAACATCCACTCTATGCGGCAAA
  121 ---------+---------+---------+---------+---------+---------+ 180
      GCTGCGCCGCTGTGGGAGTTGATAATGTCTCTTGGGTATTGTAGGTGAGATACGCCGTTT
        T  R  R  H  P  Q  L  L  Q  R  T  H  N  I  H  S  M  R  Q  M
                                            2------->  3------->
                                                          4------->
      TGACTGTCGTGGCAAGCGTGTCGACCATGAAGAGACGGCGACGAGTGCCGGGTCGCATCG
  181 ---------+---------+---------+---------+---------+---------+ 240
      ACTGACAGCACCGTTCGCACAGCTGGTACTTCTCTGCCGCTGCTCACGGCCCAGCGTAGC
        T  V  V  A  S  V  S  T  M  K  R  R  R  R  V  P  G  R  I  A
                          orf0       3------->         5------->
      CACCGGAGTGCAATCCAATGCCCGGGACCTCCCGGGTCATCCGGAGGATTCGCCGGAAGT
  241 ---------+---------+---------+---------+---------+---------+ 300
      GTGGCCTCACGTTAGGTTACGGGCCCTGGAGGGCCCAGTAGGCCTCCTAAGCGGCCTTCA
        P  E  C  N  P  M  P  G  T  S  R  V  I  R  R  I  R  R  K  C
                     4------->         5------->
      GCCTGTTCAGGCGGGGGTCGCCATCAAATCCCGAAGGGCTCGCCGGTCGTGATCGCGCCT
  301 ---------+---------+---------+---------+---------+---------+ 360
      CGGACAAGTCCGCCCCCAGCGGTAGTTTAGGGCTTCCCGAGCGGCCAGCACTAGCGCGGA
        L  F  R  R  G  S  P  S  N  P  E  G  L  A  G  R  D  R  A  F

      TCAGGTGGCTGAATTGCATCAGGGTTGTGGACCTTTGATGGACTTTCGGATCATGGATAG
  361 ---------+---------+---------+---------+---------+---------+ 420
      AGTCCACCGACTTAACGTAGTCCCAACACCTGGAAACTACCTGAAAGCCTAGTACCTATC
        R  W  L  N  C  I  R  V  V  D  L  *  W  T  F  G  S  W  I  G
         6-------->                     mdmA orf1   EcoR V
      GTAGGTTCGGCGGGGTGAAGTCAAGACCTCCGATGTCGCCGATATCTGCATCCGCTCCCG
  421 ---------+---------+---------+---------+---------+---------+ 480
      CATCCAAGCCGCCCCACTTCAGTTCTGGAGGCTACAGCGGCTATAGACGTAGGCGAGGGC
        R  F  G  G  V  K  S  R  P  P  M  S  P  I  S  A  S  A  P  A
                           6-------->
      CCGCGTCGCGCAGCACCGCTCGGCGGGAATTGGGGCAAAATTTCTTCCGATCGGCAGCCG
  481 ---------+---------+---------+---------+---------+---------+ 540
      GGCGCAGCGCGTCGTGGCGAGCCGCCCTTAACCCCGTTTTAAAGAAGGCTAGCCGTCGGC
        A  S  R  S  T  A  R  R  E  L  G  Q  N  F  F  R  S  A  A  A

      CTGCCTGCCGTTTTTCCGATCAACTCGATGCTTTTTGTGCCGACTTGCCCGGCTCCTTAG
  541 ---------+---------+---------+---------+---------+---------+ 600
      GACGGACGGCAAAAAGGCTAGTTGAGCTACGAAAAACACGGCTGAACGGGCCGAGGAATC
        A  C  R  F  S  D  Q  L  D  A  F  C  A  D  L  P  G  S  L  A

      CCGACGTTTTAACCGTTGAGATAGGCGCGGGCTCCGGCCGGGTGACCAAGGCACTGGCGT
  601 ---------+---------+---------+---------+---------+---------+ 660
      GGCTGCAAAATTGGCAACTCTATCCGCGCCCGAGGCCGGCCCACTGGTTCCTTGACCGCA
        D  V  L  T  V  E  I  G  A  G  S  G  R  V  T  K  A  L  A  S

      CGGCTGGACGCTCCTTACTCGCGGTGGAGATCGATGCCTATTGGGCACGTCGGCTGACCG
  661 ---------+---------+---------+---------+---------+---------+ 720
      GCCGACCTGCGAGGAATGAGCGCCACCTCTAGCTACGGATAACCCGTGCAGCCGACTGGC
        A  G  R  S  L  L  A  V  E  I  D  A  Y  W  A  R  R  L  T  A
```

Fig. 3. (Continued)

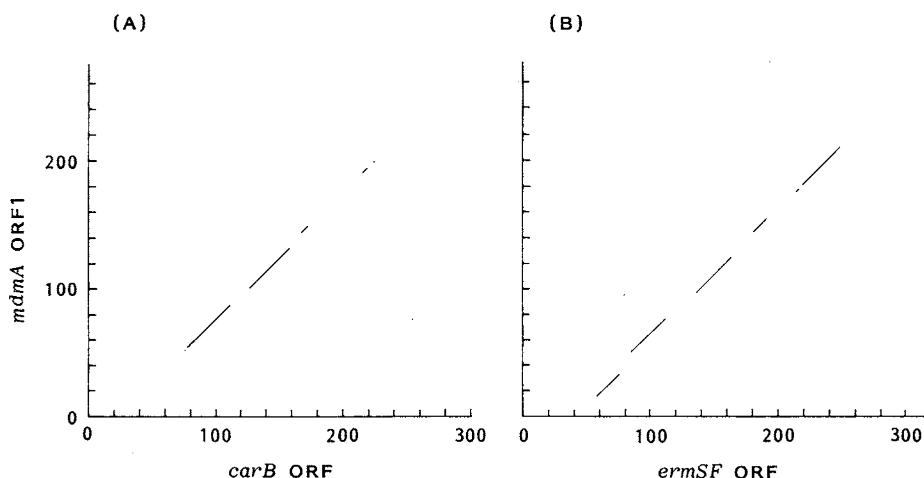
```
      CCGAATCACTTCCCGATGTCACGGTGGTGAACGAGGACTTTCTGAACTTGCAGCTGCCCA
  721 ---------+---------+---------+---------+---------+---------+ 780
      GGCTTAGTGAAGGGCTACAGTGCCACCACTTGCTCCTGAAAGACTTGAACGTCGACGGGT
        E  S  L  P  D  V  T  V  V  N  E  D  F  L  N  L  Q  L  P  R

      GGCAGCCAATCCGTCTGATTGGCAATCTTCCCTTTGTGTCCGGAACCAAGATACTGAGGC
  781 ---------+---------+---------+---------+---------+---------+ 840
      CCGTCGGTTAGGCAGACTAACCGTTAGAAGGGAAACACAGGCCTTGGTTCTATGACTCCG
        Q  P  I  R  L  I  G  N  L  P  F  V  S  G  T  K  I  L  R  R

      GCTGCCTGGAGCTGGGGCCGAATCGGATGTGCCAGGCGGTATTCCTGCTTCAGCGTGAGT
  841 ---------+---------+---------+---------+---------+---------+ 900
      CGACGGACCTCGACCCCGGCTTAGCCTACACGGTCCGCCATAAGGACGAAGTCGCACTCA
        C  L  E  L  G  P  N  R  M  C  Q  A  V  F  L  L  Q  R  E  Y

      ATGTGGGCAAGCGGACCGGTGCCTGGGGCGGCAATCTTTTCAACGCCCAGTGGGAGCCGT
  901 ---------+---------+---------+---------+---------+---------+ 960
      TACACCCGTTCGCCTGGCCACGGACCCCGCCGTTAGAAAAGTTGCGGGTCACCCTCGGCA
        V  G  K  R  T  G  A  W  G  G  N  L  F  N  A  Q  W  E  P  W
                                                EcoR I
      GGTATACGTTCGAAGGGGGGCTGGCTTTCTCCCGTAACGAATTCAGCCCTGTACCGCGCG
  961 ---------+---------+---------+---------+---------+---------+ 1,020
      CCATATGCAAGCTTCCCCCCGACCGAAAGAGGGCATTGCTTAAGTCGGGACATGGCGCGC
        Y  T  F  E  G  G  L  A  F  S  R  N  E  F  S  P  V  P  R  A

      CCGACACCCAGACGCTGGTGGTGATGCCGCGCCGTCGGCCGTCCGTGCCCTGGCGTGAGC
1,021 ---------+---------+---------+---------+---------+---------+ 1,080
      GGCTGTGGGTCTGCGACCACCACTACGGCGCGGCAGCCGGCAGGCACGGGACCGCACTCG
        D  T  Q  T  L  V  V  M  P  R  R  R  P  S  V  P  W  R  E  R
                                 Bgl II
      GCACCGACTATCAGCGGTTCACCCAACAGATCTTCGACACTGGTCAGATGACGATCGGTG
1,081 ---------+---------+---------+---------+---------+---------+ 1,140
      CGTGGCTGATAGTCGCCAAGTGGGTTGTCTAGAAGCTGTGACCAGTCTACTGCTAGCCAC
        T  D  Y  Q  R  F  T  Q  Q  I  F  D  T  G  Q  M  T  I  G  E

      AGGCCGCCCGGAAGGTGCTGCGCCGCGGCCATGCACAGTTCGTGCGCAGTGCCGGGGTGC
1,141 ---------+---------+---------+---------+---------+---------+ 1,200
      TCCGGCGGGCCTTCCACGACGCGGCGCCGGTACGTGTCAAGCACGCGTCACGGCCCCACG
        A  A  R  K  V  L  R  R  G  H  A  Q  F  V  R  S  A  G  V  R

      GGCCGGCCGATCGAGTCAAGGATCTCACGGTCCGGGACTGGGCCGCACTGTTCCGCGCGA
1,201 ---------+---------+---------+---------+---------+---------+ 1,260
      CCGGCCGGCTAGCTCAGTTCCTAGAGTGCCAGGCCCTGACCCGGCGTGACAAGGCGCGCT
        P. A  D  R  V  K  D  L  T  V  R  D  W  A  A  L  F  R  A  N

      ACCCTTAGCGGGCCGACTGATGGCGCCTCCCGGGCCCTGCCCGGGGGGGCGAACCGTCTG
1,261 ---------+---------+---------+---------+---------+---------+ 1,320
      TGGGAATCGCCCGGCTGACTACCGCGGAGGGCCCGGGACGGGCCCCCCCGCTTGGCAGAC
        P  *  R  A  D  *  W  R  L  P  G  P  A  R  G  G  E  P  S  V
                                                             BamH I
      TGTACGAAAGGCTGTATACAGGCAAGTTCTCTCAGGGAGGGTCCTCACGATGCGGGGGAT
1,321 ---------+---------+---------+---------+---------+---------+ 1,380
      ACATGCTTTCCGACATATGTCCGTTCAAGAGAGTCCCTCCCAGGAGTGCTACGCCCCCTA
        Y  E  R  L  Y  T  G  K  F  S  Q  G  G  S  S  R  C  G  G  S

      CC
1,381 -- 1,382
      GG
```

The translation of the third reading frame only is shown since this is the only one with *orf*s containing a third position GC bias consistent with the calculated 63.2% GC content of the DNA[16]. The restriction sites relevant to the discussion in the text are indicated in italics. The pairs of direct repeats containing ≥7 nts in the region from positions 1 to 510 are numbered consecutively and indicated by broken arrows above the DNA sequence. Translational start and stop sites are in bold face. The putative rbs for *mdmA orf0* is underlined.

Sequence Analysis of the *mdmA* Gene Suggests that it Encodes a Protein like the Products of Other Bacterial MLS-resistance Genes

The sequence of a 1,382 nucleotide (nt) *Bam*H I DNA fragment containing *mdmA* (Fig. 3) was analyzed by the Wisconsin Genetic Computer Group (GCG) CODONPREFERENCE program[15] to locate likely open reading frames (orf) by virtue of a high bias towards *G* or *C* in the third position of their codons[16]. The most probable translational start sites for the *mdmA orf1* (Fig. 3) are an ATG at position 453 or an GTG at position 642, and the stop site is an TAG at position 1,268. Since neither of these start sites is preceded by a suitable ribosomal binding site (rbs) with satisfactory complementarity to the 3′-end of *S. lividans* 16*S* rRNA (GAUCACCUCCAAACA)[17], a choice between them was made by comparing the deduced protein product of the two possible *orf*s with those of other bacterial MLS genes (specifically the *S. thermotolerans carB*[6], *Arthrobacter* sp. *ermA*[18], *S. erythraea ermE*[19], *S. fradiae ermSF*[20], and *Bacillus sphaericus ermG*[21] genes), using the GCG COMPARE and GAP programs[15]. ATG_{453} resulted in a basic protein (*p*I = 11.1) of 272 amino acids (MW 30,140) that closely resembles the putative products of *carB* and *ermSF* (Fig. 4). In particular, this ORF, but not the one beginning with GTG_{642}, contains

Fig. 4. Comparison of the deduced products of the *mdmA orf1*, *carB* and *ermSF* genes by the GCG COMPARE/DOTPLOT programs[15].



A window of 30 and stringency of 17 was used; all other values were the default settings. The relative position from the *N*- to *C*-terminus of each protein is indicated by numbers on the horizontal and vertical axes.

Table 4. Comparison of amino acid sequences around the probable nucleotide binding region[a] of the deduced products of representative bacterial MLS genes.

Gene	Amino acid sequence[b]	Location[c]
mdmA RNA MT	T V E I G A G S G R V T K A L A S	54～ 71
carB RNA MT[6]	V L E V G A G N G A I T R E L A R	78～107
ermA RNA MT[18]	V V E A G F G E G L L T R E L A R	61～ 77
ermC RNA MT[26]	I F E I G S G K G H F T L E L V K	34～ 50
ermE RNA MT[19]	V L E A G P G E G L L T R E L A D	65～ 81
ermG RNA MT[21]	I F E I G A G K G H F T A E L V Q	34～ 50
ermSF RNA MT[20]	L L E V G A G R G V L T E A L A P	89～106

[a] The consensus sequence for this region is K(R)xxxVxGxGxxGxxxxxxxxxxxxxxxxxD(E)[22,27,28].
[b] Strongly conserved residues are shown in bold face.
[c] The numbers indicate the position relative to the *N*-terminus of each protein.
RNA MT: RNA methyltransferase.

Fig. 5. Comparison of the deduced products of the *carB* and *mdmA* genes by the GCG GAP program[15] using the default values.

```
                .         .         .         .         .
  1 MAALLKRILRRRMAEKRSGRGRMAAARTTGAQSRKTAQRSGRSEADRRRR 50
                           . : . ...|.||.||          ||
  1 .....................MSPISASAPAASRSTA...........RR 18

                .         .         .         .         .
 51 VHGQNFLVDRETVQRF........VRFADPDPGEVVLEVGAGNGAITREL 92
      ||||: . ... ||        . :::. :: :.:|:|||.| :|:.|
 19 ELGQNFFRSAAAACRFSDQLDAFCADLPGSLADVLTVEIGAGSGRVTKAL 68

                .         .         .         .         .
 93 ARLCRRVVAYEIDRHFADRLREATAED.PRIEVVAGDFLKTSQPKVPFSV 141
    |. .|.::| ||| .:| ||     |||. | :.||.:|||.    |: |:.:
 69 ASAGRSLLAVEIDAYWARRL...TAESLPDVTVVNEDFLNLQLPRQPIRL 115

                .         .         .         .         .
142 VGNIPFGNTADIVDWCLN..ARRLRTTTLVTQLEYARKRTGGYRRWSRLT 189
    :||:||.....|: :||:  :.|:  ..:: | ||. ||||::    :.|
116 IGNLPFVSGTKILRRCLELGPNRMCQAVFLLQREYVGKRTGAWG..GNLF 163

                .         .         .         .         .
190 VATW.PEVEWRMGERISRRWFRPVPAVDSAVLRLERRPVPLIPPGLMHDF 238
     | | |   .:  |   :||. |.||| .|...| : .|. | :|   . |:
164 NAQWEPWYTFEGGLAFSRNEFSPVPRADTQTLVVMPRRRPSVPWRERTDY 213

                .         .         .         .         .
239 RDLVETGFTGKGGSLDASLRRRFPARRVAAGF.RRARLEQGVVVAYVTPG 287
    . :.:  |.. . .::.. |: :  ||. |.| |.| : .:  |. :|.
214 QRFTQQIFDTGQMTIGEAARKVL..RRGHAQFVRSAGVRPADRVKDLTVR 261

                .
288 QWITLFEELHGR* 300
    :| .|| . .
262 DWAALFRANP*.. 272
```

The vertical lines between the *carB* (top line) and *mdmA* (lower line) protein sequences indicate exact matches, and the single or two vertical dots indicate conservative amino acid replacements according to the GCG amino acid comparison table. The dots above each pair of protein sequence data indicate distances of 10 amino acids. The '*' indicates the translational stop point.

the region between residues 54 and 71 corresponding to a dinucleotide binding motif, which is highly conserved in proteins that bind nucleotides such as NADH, ATP or *S*-adenosylmethionine[22]. Table 4 illustrates this fact for the putative products of *mdmA orf1* and several other bacterial MLS genes. By GAP analysis (Fig. 5), *mdmA* ORF1 showed an overall 51% similarity and 32.8% identity to the product of the *carB* gene[6]. The latter gene[6] and *mdmA orf1* contain two and three leucine TTA codons, respectively, which occur only rarely in *Streptomyces orf*s and are possibly important in the post-transcriptional regulation of gene expression[23]. The *mdmA orf1* has a GC content of 63.2%. No evidence was found by the GCG STEMLOOP analysis[15] for inverted repeats downstream of TAG_{1268} that might serve as a *rho*-independent transcriptional termination site, unlike the *carB* gene which has an 18-bp perfect inverted repeat immediately after the 3'-end of its *orf*[6]. Direct repeats 7 nt or longer upstream of *mdmA orf1* are indicated in Fig. 3; no perfect inverted repeats longer than 5 nt were present in this region.

There are six possible translational start sites for *orf*s in the region upstream of ATG_{453}, all of which end at TAG_{398}. The *orf* beginning at ATG_{258} has the expected third position GC bias and is the only one preceded by a satisfactory rbs (GGAG). Analysis of the reverse direction of the sequence shown in Fig. 3 by CODONPREFERENCE did not result in unambiguous evidence for the presence of additional *orf*s.

Discussion

The genes for MLS-resistance determinants cloned from *Streptomyces* species are known in several

cases to encode RNA methylases that act on specific nucleotides in 23*S* ribosomal RNA[5,13,19~21,24,25]. The close relationship between the deduced product of the *mdmA* gene and several other *S*-adenosylmethionine dependent, RNA methyltransferases (Table 4) supports the assumption that *mdmA* encodes such a methyltransferase. Induction of the bacterial MLS-resistance genes by macrolide antibiotics has been reported in homologous and heterologous genetic backgrounds,[5,13] and is thought to be one mechanism by which microorganisms acquire resistance to the macrolide antibiotic they produce[13]. Thus, a role in the production of Mdm is a likely function of the *S. mycarofaciens mdmA* gene, which is consistent with the discovery that it is linked to at least one other gene for midecamycin biosynthesis (O. HARA and C. R. HUTCHINSON, unpublished work). A parallel situation exists in *S. thermotolerans* where the *carE* gene, which encodes an isovalerylCoA transferase apparently required for the acylation of the 4″ position of mycarose during carbomycin biosynthesis, is adjacent to the *carB* gene[4].

The presence of genes in *S. lividans* and *S. coelicolor* conferring inducible MLS-resistance has been independently discovered by G. JENKINS and E. CUNDLIFFE (personal communication). They have established that *S. lividans* possesses inducible ribosomal RNA methylase activity that confers high level resistance to lincomycin and lower levels of resistance to certain macrolide antibiotics (G. JENKINS *et al.*, J. Gen. Microbiol., in press). The methylase gene (*lrm*) is inducible by erythromycin and to a lesser extent by some other macrolides. A 3.5-kb DNA fragment cloned by JENKINS and CUNDLIFFE from an *S. lividans* mutant that is constitutively resistant to lincomycin contains the *lrm* gene and a second MLS-resistance gene immediately adjacent. Since a major portion of this DNA fragment and the one we cloned as pOH301 have identical restriction maps for the portions that overlap, and confer similar resistance phenotypes, the two genes are likely to be the same and should be referred to as the *S. lividans lrm* gene (JENKINS and CUNDLIFFE have found that the resistance phenotypes conferred by *lrm per se* and *carB* are indistinguishable in *S. lividans* and *S. griseofuscus* under carefully determined conditions).

The presence of at least two MLS genes in *S. lividans* and the lack of detailed knowledge about what regulates the expression of these or heterologous MLS genes in this and other streptomycete hosts prohibits a meaningful discussion of the behavior of the *mdmA* gene in *S. lividans* and *S. griseofuscus*, including the question of its inducibility. Clear differences exist, apart from the mere fact that *S. griseofuscus* is inherently more sensitive to macrolide antibiotics, but comparative studies of gene transcription and mRNA translation, plus the activity of the RNA methyltransferases, will have to be done before the phenotypic behavior reported here for the two different strains transformed with the *mdmA* gene can be explained.

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