# CLONING AND CHARACTERIZATION OF THE SISOMICIN-RESISTANCE GENE FROM *MICROMONOSPORA INYOENSIS*

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We cloned DNA fragments of sisomicin-producing *Micromonospora inyoensis*[1] into *Streptomyces* plasmid vectors and identified *Streptomyces lividans* TK24 transformants expressing the *M. inyoensis* sisomicin-resistance (*sisA*) gene. The *sisA* gene was compared to the previously reported *Micromonospora purpurea* Kan-Gen (kanamycin-gentamicin)-resistance gene[2]. While the restriction endonuclease digestion patterns of the two determinants appear to be divergent, the genes are nonetheless closely related, based on similar patterns and levels of aminoglycoside-resistance and their ability to cross-hybridize under stringent conditions. We have transformed recombinant plasmid pMD5-2, which carries the *sisA* gene, into our *M. purpurea* gentamicin-production strain and determined that gentamicin biosynthesis was not improved.

Sisomicin (Fig. 1) is a broad spectrum aminoglycoside antibiotic active against both Gram-positive and Gram-negative bacteria, as well as mycobacteria[3]. The structure of sisomicin, a pseudotrisaccharide composed of an aminocyclitol aglycone, 2-deoxystreptamine (DOS), bonded *via* glycoside linkages to 2-amino-2-deoxy-D-glucose and D-xylose, was reported by REIMANN *et al.*[4]. While the biosynthetic pathway of sisomicin has not been studied in detail, experiments using blocked mutants, and analysis of the products of mutasynthesis and accumulated pathway intermediates, indicate the existance of a complex biosynthetic mechanism that requires at least seven consecutive enzymatic steps[5,6]. The genetic characterization of this

Fig. 1. Structure of sisomicin and gentamicin.



Sisomicin

Gentamicin

C-1    $R_1 = R_2 = CH_3$
C-1a   $R_1 = R_2 = H$
C-2    $R_1 = CH_3$   $R_2 = H$
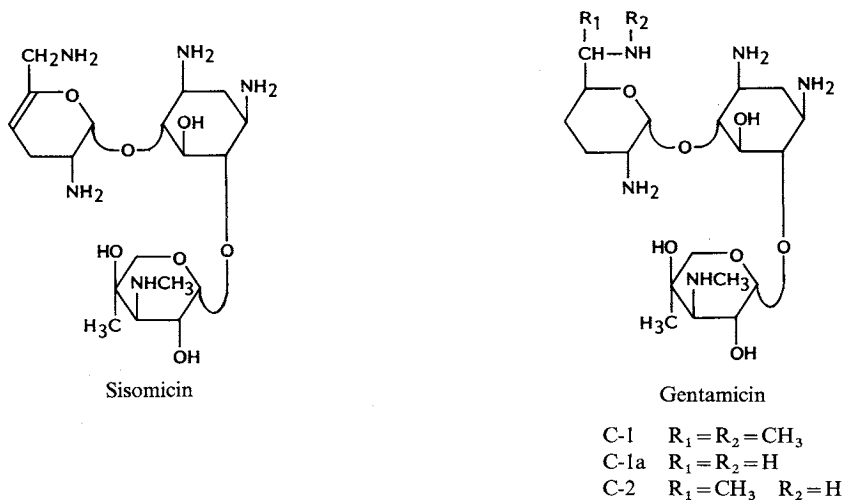
or any other *Micromonospora* antibiotic biosynthetic pathway has not been addressed.

Recently, the development of host-vector systems for *Streptomyces* has enabled investigators to clone several antibiotic-resistance and biosynthetic genes[7~9]. In addition, molecular cloning experiments have shown that structural genes coding for enzymes of a given antibiotic pathway are commonly clustered and closely linked to their respective resistance genes. The genetics of the synthesis of other aminoglycoside antibiotics, such as streptomycin and kanamycin, have been investigated in detail[10,11]. Until recently, plasmid vectors propagated in *Streptomyces* were not able to stably transform *Micromonospora*. In this report, we describe the cloning and expression, in *Streptomyces lividans*, of a chromosomal sisomicin-resistance (*sisA*) gene from *Micromonospora inyoensis*. We have also developed a procedure whereby our gentamicin-producing *M. purpurea* strain can be transformed with plasmid DNA. Using this transformation system, the *sisA* gene-containing recombinant plasmid pMD5-2 was introduced into *M. purpurea* and examined for its effect on gentamicin. Our data indicate that the high copy number of the plasmid vector has a detrimental effect on gentamicin biosynthesis.

## Materials and Methods

### Bacterial Strains and Plasmids

A derivative of the sisomicin-producing *M. inyoensis* 66-40 (NRRL 3292/22) was used as the source of the *sisA* and biosynthetic complex genes. A derivative of *M. purpurea* NRRL 2953 used for DNA isolation and transformation is a producer of gentamicin. *Streptomyces kanamyceticus* ATCC 12853 was purchased from the American Type Culture Collection. *S. lividans* TK24 and the plasmid vector pIJ703[12] were kindly provided by D. A. HOPWOOD. Plasmid pLST14[2,13] was supplied by E. CUNDLIFFE.

### Media and Buffers

Media and buffers for protoplasting and regeneration of *S. lividans* in transformation experiments were those described by HOPWOOD et al.[14]. *M. inyoensis* and *M. purpurea* were grown in MFM-11[15] and/or S-2 medium[16] for protoplast formation, and were maintained in P3 buffer[17]. *M. purpurea* protoplasts were regenerated on DSR medium, containing disodium succinate 48.8 g, soluble starch 20 g, $K_2SO_4$ 0.25 g, trace element solution ($ZnCl_2 \cdot 2H_2O$ 40 mg, $FeCl_3 \cdot 6H_2O$ 10 mg, $MnCl_2 \cdot 4H_2O$ 10 mg, $NaB_4O_7 \cdot 10H_2O$ 10 mg, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 10 mg per liter of deionized water) 2.0 ml, TES buffer 5.73 g, $KH_2PO_4$ 0.05 g, $MgCl_2 \cdot 6H_2O$ 1.01 g, $CaCl_2 \cdot 2H_2O$ 3.67 g, and agar 20 g, pH 7.5, per liter of deionized water. Soft agar medium contained 0.7% agar.

### Mutagenesis of *M. purpurea*

Fifty ml of MFM-11 medium was inoculated with 1 ml of previously frozen *M. purpurea* culture and incubated at 28°C, 260 rpm, for 18 hours. Cells were pelleted at $4,000 \times g$ for 10 minutes in a Sorvall SS-34 rotor and resuspended in 20 ml of MFM-11. The culture was sonicated at 50% power, setting 2.5, for 30 seconds using a microtip (Branson Sonic Power). After washing with 40 ml MFM-11, the pellet was resuspended in 20 ml of MFM-11.

*N*-Methyl-*N*-nitro-*N*-nitrosoguanidine (Sigma) was added to a final concentration of 10 μg/ml and the culture was shaken at 100 rpm for 2 hours at 22°C. The mutagenized cells were washed twice with 30 ml MFM-11, and resuspended in 50 ml MFM-11. The culture was incubated at 28°C, 18 hours, 260 rpm, centrifuged, resuspended in 20 ml S-2 medium, and grown at 28°C, 24 hours, 50 rpm. After centrifuging, the cells were subjected to protoplasting and transformation.

### Protoplast Preparation and Transformation

*Streptomyces* protoplasts were prepared and transformed according to HOPWOOD et al.[14]. Transformants were selected on R2YE medium containing 50 μg/ml thiostrepton (E. R. Squibb and Sons) and/or 50 μg/ml sisomicin. *M. purpurea* protoplasts were prepared as follows: 50 ml of MFM-11 medium

was inoculated with 1 ml of a frozen broth culture and shaken at 250 rpm, 32°C, for 48 hours. A 5 ml aliquot of this culture was transferred to 50 ml of S-2 medium, which was incubated for 24 hours at 250 rpm, 32°C. Cells were collected by centrifugation (10 minutes, $4,300 \times g$, Sorvall SS-34 rotor), then washed once with 40 ml 0.5 M filter-sterilized sucrose and once with P3 buffer. The pellet was resuspended in 25 ml of a filter-sterilized P3 buffer containing 1 mg/ml lysozyme (Sigma) and 0.63 mg/ml Achromopeptidase (Wako Pure Chemical Industries Ltd.). The cells were transferred to a 125-ml Erlenmeyer flask and incubated overnight 32°C on a rotary shaker at 100 rpm. Protoplasts were centrifuged for 10 minutes as described above, and washed once with P3 buffer. Cell debris was removed by filtering the solution through sterile non-absorbant cotton, recentrifuged, and resuspended in 0.5 ml P3 buffer.

The transformation of M. purpurea was performed using a modification of the Streptomyces protocol. Plasmid DNA (1 μg in 1 ~ 5 μl of sterile deionized water) and 200 μl of 25% polyethylene glycol 1000 (Koch-Light) in P buffer were added to approximately $1 \times 10^9$ protoplasts and mixed by gentle pipetting.

Transformation was terminated by the addition of 2 ml of P3 buffer. One ml of this suspension was removed and vortexed in sterile glass test tubes containing 4 ml of soft DSR agar medium at 40°C, and then poured onto DSR agar plates. Following incubation at 32°C for 36 ~ 48 hours, the regeneration plates were overlaid with 2 ml of soft nutrient broth containing thiostrepton to give a final concentration of 15 μg/ml. Thiostrepton-resistant (Tsr$^r$) transformants appeared after further incubation at 32°C for approximately 7 days.

### In Vitro Translation

Preparation of ribosomes from Streptomyces and in vitro translation of polyuridylic acid has been described by JONES[18]. Streptomyces cells were grown in YEME + MgCl$_2$ + glycine[14] containing required selective antibiotics. [$^{14}$C]Phenylalanine ($> 450$ mCi/mmol) was obtained from Amersham. Sisomicin (0.1 ~ 100 μg/ml) was included in some samples.

### DNA Preparation and Manipulation

For preparation of Micromonospora and Streptomyces chromosomal DNA, the procedure described by BRENNER et al.[19] was used, with the following modifications: Micromonospora cultures were grown in S-2 medium for 48 hours and incubated in a buffer containing 0.3 M sucrose, 25 mM Tris-HCl, 25 mM EDTA, pH 7.0 with 10 mg/ml lysozyme (Sigma) for 1 hour at 35°C before lysis, while Streptomyces cells were grown in YEME + MgCl$_2$ + glycine for 36 hours and resuspended in the same buffer for 30 minutes at 35°C prior to lysis. Plasmid DNA was prepared from cultures grown as described above plus 5 μg/ml of thiostrepton and/or 5 μg/ml of sisomicin according to HOPWOOD et al.[14]. Restriction analysis, ligations, and nick translations were performed as recommended by the manufacturer (International Biotechnologies, Inc.). Dephosphorylation using alkaline phosphatase (Boehringer Mannheim) and agarose gel electrophoresis in Tris-borate or Tris-acetate buffer were carried out accordingly to MANIATIS et al.[20]. For Southern blot analysis, 2 μg of total DNA was digested and electrophoresed for 640 V-hour before transfer to nitrocellulose (Schelcher and Schuell BA85, pore size 0.45 μm). DNA restriction fragments used as probes were purified after electrophoresis using Gene-Clean (Bio 101), and labeled with [$^{32}$P]dCTP (specific activity, $> 1 \times 10^9$ cpm/μg) using a Bio-Rad Random Primer Labeling Kit. Southern blot hybridizations were performed essentially as described by MOSELEY and FALKOW[21]. Prehybridization was carried out for 2 hours at 42°C in 5 × SSC, 50% formamide, 0.1% SDS, 1 × DENHARDT's solution, and hybridization was performed in the same solution with approx. $1 \times 10^7$ cpm of probe DNA at 42°C for 18 hours. Blots were washed twice with 150 ml of 2 × SSC, 0.1% SDS for 15 minutes each, followed by the same volume of 0.2 × SSC, 0.1% SDS at 60°C for 30 minutes (high stringency conditions). Filters were exposed to Kodak X-OMAT X-ray film at −70°C for 24 hours with two intensifying screens (Cronex Lightning Plus, DuPont).

## Results and Discussion

Cloning, Identification, and Characterization of the M. inyoensis sisA Gene

M. inyoensis chromosomal DNA was digested to completion with restriction endonuclease BamH I

Fig. 2.   A restriction map of pMD5-2.

(A) Restriction endonuclease cleavage map of pMD5-2. *mel*: melanin, *tsr*: thiostrepton-resistance determinant, *sisA*: fragment encoding sisomicin-resistance. (B) Detailed restriction map of *sisA* region. Numbers indicate relative locations of cleavage sites in base pairs. This fragment was not digested by *Aat* I, *Bam*H I, *Eco*R I, *Hin*d III, *Kpn* I, *Pvu* II, *Sph* I, *Xba* I, or *Xho* I.
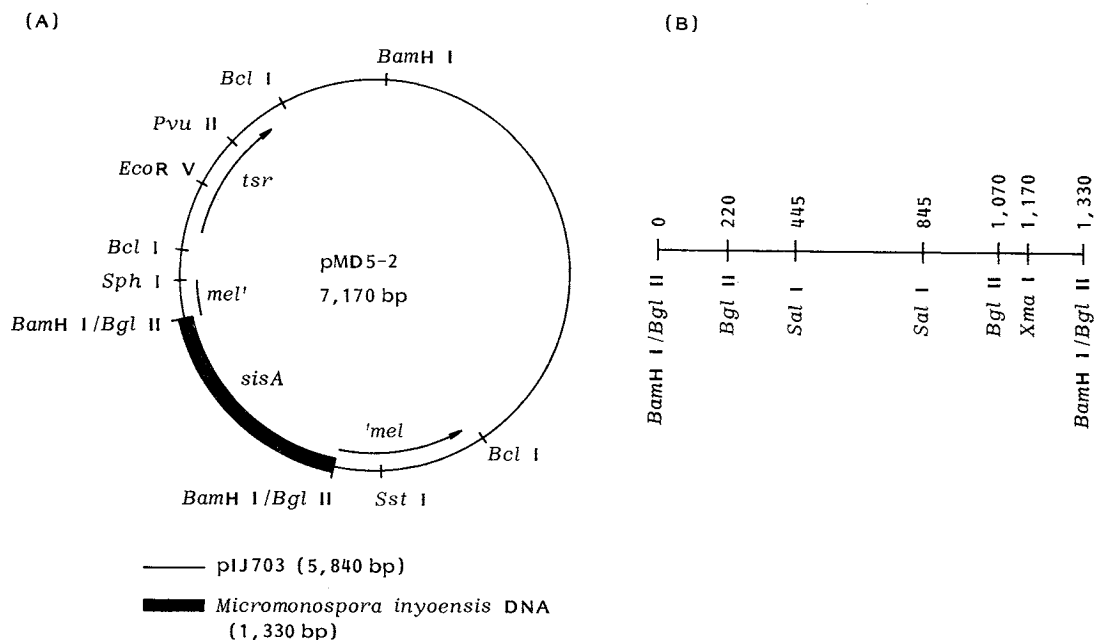


and ligated to *Bgl* II-digested pIJ703. The ligated DNA was added to *S. lividans* TK24 protoplasts and transformants were selected for thiostrepton-resistance. Following replica plating to medium containing sisomicin, two Sis[r] colonies appeared, and were confirmed to be simultaneously resistant to sisomicin and thiostrepton. Restriction analysis of the plasmids obtained from these isolates revealed the presence of a common 1,330 bp insert in pIJ703. When *S. lividans* was retransformed with one of the recombinant plasmids (pMD5-2), Sis[r]Tsr[r] colonies appeared at a high frequency, confirming that sisomicin-resistance (the gene for which was named *sisA*) was plasmid-linked. A restriction map of pMD5-2 is shown in Fig. 2.

It is known that many aminoglycoside-resistance factors modify target antibiotics and/or sensitive ribosomes so that they are insensitive to the effects of multiple antibiotics. *S. lividans* TK24 (pMD5-2) was tested for the spectrum and level of resistance to a number of aminoglycoside antibiotics. The presence of the *sisA* gene confers very high level resistance (up to 10 mg/ml) to sisomicin, gentamicin, and kanamycin, but not neomycin (Table 1). We then isolated ribosomes from *S. lividans* TK24 containing plasmids pIJ703, pMD5-2, and pLST14 (possessing the cloned Kan-Gen resistance (Kan-Gen[r]) gene from *M. purpurea*), and tested their ability to carry out *in vitro* translation in the presence of increasing concentrations of sisomicin. The results of this experiment (Fig. 3) reveal that ribosomes derived from cells containing pMD5-2 or pLST14 were able to efficiently synthesize polyphenylalanine at antibiotic concentrations up to 100 $\mu$g/ml, while those from *S. lividans* (pIJ703) were extremely sensitive to the antibiotic. Thus, the *sisA* gene apparently confers antibiotic-resistance by modifying sensitive ribosomes. Although it cannot be ruled out that the cloned DNA fragment also encodes an inactivating enzyme, the small size of the region makes it unlikely that a second functional gene is present. These data are in agreement with those

Table 1. Resistance of *Streptomyces lividans* TK24 and *S. lividans* TK24 (pMD5-2) to aminoglycoside antibiotics.

| | *S. lividans* TK24 (pMD5-2) | *S. lividans* TK24 |
|---|---|---|
| | μg/ml | |
| Sisomicin | 10,000 | <25 |
| Gentamicin | 10,000 | <25 |
| Kanamycin | 10,000 | <25 |
| Neomycin | <25 | <25 |

seen by THOMPSON *et al.* for pLST14 containing the Kan-Gen[r] gene[2].

Given the nearly identical function and spectrum of resistance conferred by the cloned *sisA* and Kan-Gen[r] genes, which were both isolated from *Micromonospora* species producing closely related antibiotics, it was possible that the two loci possessed a correspondingly close DNA sequence homology. However, a comparison of the restriction maps indicated no obvious similarity. To extend our analysis, an 850-bp *Bgl* II internal fragment of the *sisA* gene necessary for resistance was used as a probe in Southern blot hybridization experiments. As expected, the probe hybridized strongly to a 1,330-bp *Bam*H I fragment of *M. inyoensis* chromosomal DNA (Fig. 4). There was also significant homology between the probe and a 1,950-bp *M. purpurea Bam*H I fragment, suggesting that the chromosomal DNA region containing the resistance genes for *M. inyoensis* and *M. purpurea* are closely related. Supporting this conclusion was the fact that a 1,700-bp *Sph* I-*Bam*H I DNA fragment of pLST14 containing the Kan-Gen[r] gene also hybridized to the identical *Micromonospora* restriction fragments. The additional regions of weaker homology seen using this probe are indicative of distantly related sequences present elsewhere in these genomes. Dot blot hybridizations confirmed that significant sequence homology exists between the genes cloned on pMD5-2 and pLST14 (data not shown). Finally, despite the ability of the *sisA* and Kan-Gen[r] genes to confer resistance to high levels of kanamycin in *S. lividans*, neither probe hybridized to *S. kanamyceticus* DNA. This lack of homology with the chromosome of a kanamycin

Fig. 3. Effect of sisomicin on poly U-directed synthesis of polyphenylalanine.

Incorporation of [$^{14}$C]phenylalanine in *in vitro* translation.

● Ribosomes prepared from *Streptomyces lividans* TK24 (pIJ703), ▲ ribosomes prepared from *S. lividans* TK24 (pMD5-2), ■ ribosomes prepared from *S. lividans* TK24 (pLST14).
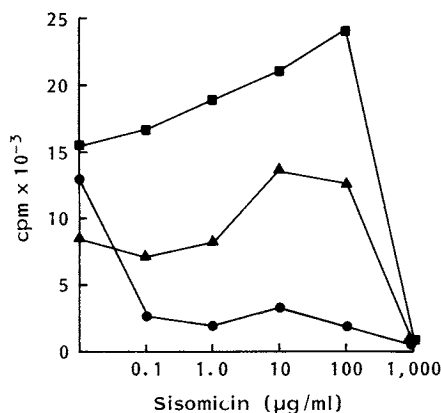


Fig. 4. Southern blot hybridization of DNA fragments containing the Kan-Gen[r] (panel A) and *sisA* (panel B) genes to *Streptomyces* and *Micromonospora* DNA.

Lane 1, *M. inyoensis* chromosomal DNA; lane 2, *M. purpurea* chromosomal DNA; lane 3, *S. kanamyceticus* chromosomal DNA; lane 4, *S. lividans* TK24 chromosomal DNA. All samples were digested with restriction endonuclease *Bam*H I. Numbers identify the size of the two major hybridizing fragments in base pairs.
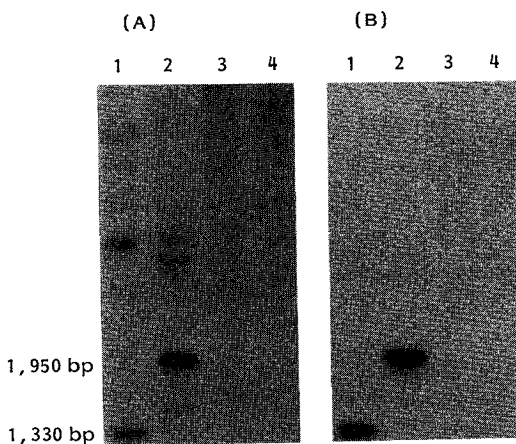
producer known to encode a ribosomal resistance factor suggests that the *Micromonospora* resistance genes have evolved independently or that they have greatly diverged from an ancestral locus common to *Streptomyces* and *Micromonospora*.

### Transformation of *M. purpurea* with the Cloned *sisA* Gene

Having cloned the *sisA* gene on a high copy number plasmid (pIJ703), we were interested in seeing if the presence of pMD5-2 in *Micromonospora* would lead to an increase of antibiotic production. CRAMEREI and DAVIES reported such an increase in kanamycin and neomycin upon the introduction of a cloned 6'-*N*-acetyltransferase gene present on pIJ702 back into their respective parental *Streptomyces* strains[22]. The host organism presumably becomes more resistant to the toxic effects of its own antibiotic and is thus capable of synthesizing higher levels of the compound. Because *M. inyoensis* protoplasts required extensive incubation times (>2 weeks at 32°C) before regenerating colonies were visible, *M. purpurea* was chosen as the host strain for transformation with pMD5-2. As demonstrated, the *sisA* gene also imparts high level resistance to gentamicin, and the resistance determinants of *M. inyoensis* and *M. purpurea* are closely related at DNA level. In addition, the biosynthetic pathways for sisomicin and gentamicin are very similar[5,6].

Wild-type *M. purpurea* protoplasts were completely refractory to transformation. It is quite likely that a restriction-modification system(s) is preventing the stable maintenance of DNA derived from another organism[23,24]. To overcome this barrier, mutagenesis has been successfully employed in *Streptomyces fradiae*[23,24] and recently, protocols for successfully transforming *Micromonospora* have been reported[25,26]. By exposing our *M. purpurea* production strain to nitrosoguanidine prior to transformation with pIJ703, we obtained 1~2 Tsr[r] colonies per µg of DNA in about half of the attempts to transform the culture. (The *mel* gene also present on pIJ703, however, was not visibly active; similar results have been observed in other systems[26,27].) Isolates cured of this plasmid were obtained by growing transformants on medium without thiostrepton and screening for colonies sensitive to this antibiotic. One such strain was transformed with pIJ703 or pMD5-2 DNA purified from *M. purpurea*, whereupon 50 Tsr[r] colonies/µg were isolated. Passage of the plasmids through *M. purpurea* evidently modified the DNA to a certain extent, since a non-mutagenized *M. purpurea* culture could now be transformed at a frequency of approximately 50 colonies/µg input DNA. However, the cured *M. purpurea* isolate may not be a restrictionless mutant, because it could only be transformed by pIJ703 derived from *S. lividans* at about 1~5 colonies/µg using our procedure (Table 2). It should be emphasized, however, that we have not yet optimized the transformation conditions for our strain. Restriction analysis of pIJ703 and pMD5-2 obtained from *M. purpurea* showed no obvious rearrangements or deletions and the plasmids retained their ability

Table 2. Transformation of mutagenized and non-mutagenized *Micromonospora purpurea* strains by pIJ703 derived from *Micromonospora* and *Streptomyces* hosts.

| Source of DNA | Transformation frequency of | | |
| --- | --- | --- | --- |
| | *M. purpurea* (wild type)[a] | *M. purpurea* (mutagenized)[a] | *M. purpurea* (cured strain)[a] |
| *S. lividans* TK24 | 0 | 0~2 | 1~5 |
| *M. purpurea* (mutagenized) | 50 | ND | 50 |

[a] Colonies/µg.

ND: Not determined.

to transform *S. lividans* to thiostrepton- and/or sisomicin-resistance. Based on preliminary observation of the amount of plasmid DNA obtained per ml of cell culture, the high copy number characteristic of the vector used was maintained in *Micromonospora*.

If gentamicin synthesis in *M. purpurea* is limited by the presence of unmodified ribosomes, then extra copies of the resistance gene may improve antibiotic titers. The antibiotic productivity of *M. purpurea* (pIJ703) and *M. purpurea* (pMD5-2) were compared with the mutant strain without plasmid. Both the

Table 3. Antibiotic productivity of *Micromonospora purpurea* transformed with pIJ703 or pMD5-2.

| Strain | Gentamicin (μg/ml) | %[a] |
|---|---|---|
| | Packed cell volume | |
| *M. purpurea* (wild-type) | 228 | 100 |
| *M. purpurea* (mutagenized) | 218 | 95 |
| *M. purpurea* (wild-type) (pIJ703) | 188 | 82 |
| *M. purpurea* (mutagenized) (pIJ703) | 112 | 49 |
| *M. purpurea* (mutagenized) (pMD5-2) | 152 | 67 |

[a] % of wild-type control.

parent and transformed strain were found to be resistant up to 3 mg/ml of gentamicin on MFM-11 agar medium. The presence of a high copy number plasmid apparently results in the depression of gentamicin titers, since both pIJ703 and pMD5-2 cause a 46.5 and 28.5% decrease, respectively, compared to the plasmidless control (Table 3). Similar reductions in antibiotic titer were observed when a cloned phenoxazone synthase (on pIJ702) or a high copy number plasmid were introduced into the parental *Streptomyces antibioticus* or *Streptomyces rimosus* hosts, respectively[28,29]. It is not known if the smaller loss of productivity seen in *M. purpurea* (pMD5-2) vs. *M. purpurea* (pIJ703) is significant, nor why the mutagenized *M. purpurea* isolate is more sensitive to the presence of pIJ703 than the wild-type strain. These and other questions raised by the results of transformations of *M. purpurea* will require more extensive analysis.

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