Characterization of two different types of resistance genes among producers of fortimicin-group antibiotics

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Fortimicin-A (FTM-A; astromicin)-resistance genes (*fmr* genes) isolated from six producers of the FTM-group of antibiotics were analysed. These genes could be classified into two types by the resistance profiles to aminoglycoside antibiotics and by their DNA homologies. Three genes, *fmrT* from the istamycin producer *Streptomyces tenjimariensis* ATCC 31603, *fmrS* from the sannamycin producer *Streptomyces sannanensis* IFO 14239 and *fmrH* from the sporaricin producer *Saccharopolyspora hirsuta* ATCC 20501, conferred resistance to FTM-A, kanamycin (Km) and neomycin B (Nm-B), but not to gentamicin (Gm). The other three genes, *fmrO* from the FTM-A producer *Micromonospora olivasterospora* ATCC 21819, *fmrM* from the antibiotic SF-2052 producer *Micromonospora* sp. SF-2098 (ATCC 31580) and *fmrD* from the dactimicin producer *Dactylosporangium matsuzakiense* ATCC 31570, conferred resistance to FTM-A, Km and Gm, but not to Nm-B. No DNA homology was detected between the two types of the resistance genes in Southern-blot analysis. The present results revealed that, in spite of the similarity of their biosynthesis genes, there are at least two different types of resistance genes among the FTM-group antibiotic producers.

Introduction

It is interesting, from the point of view of the evolution and distribution of antibiotic biosynthetic genes, that antibiotics with similar structures are often produced by taxonomically distant micro-organisms (Malpartida *et al.*, 1987; Leskiw *et al.*, 1988; Schiffman *et al.*, 1988; Weigel *et al.*, 1988; Burnham *et al.*, 1989). Fortimicin (FTM)-group antibiotics with unique pseudodisaccharide structures are produced by six strains of four genera, namely *Micromonospora olivasterospora* ATCC 21819 (FTM-A) (Nara *et al.*, 1977), *Micromonospora* sp. SF-2098 ATCC 31580 (SF-2052 compounds) (Ohba *et al.*, 1981), *Dactylosporangium matsuzakiense* ATCC 31570 (dactimicins) (Inoue *et al.*, 1979), *Streptomyces tenjimariensis* ATCC 31603 (istamycins) (Okami *et al.*, 1979), *Streptomyces sannanensis* IFO 14239 (sannamycins) (Watanabe *et al.*, 1979) and *Saccharopolyspora hirsuta* ATCC 20501 (sporaricins) (Deushi *et al.*, 1979).

The biosynthetic pathway of FTM-A in *M. olivasterospora* is believed to consist of more than 14 steps (Odakura *et al.*, 1984), and all members of the FTM-group antibiotics are thought to be synthesized via similar biosynthetic pathways (Dairi & Hasegawa, 1989; Hotta *et al.*, 1989). Sequence conservation of the glycytransferase, one of the key enzymes for the biosynthesis of the FTM-group antibiotics, among the FTM-group antibiotic producers suggested that these biosynthesis genes were derived from a common set of ancestral genes (Ohta *et al.*, 1992).

All the FTM-group antibiotic producers exhibit high-level resistance to FTM-A. We previously isolated the FTM-A resistance gene (*fmrT*) from the istamycin producer *S. tenjimariensis*, and showed that sequences homologous to *fmrT* were present in the sannamycin producer *S. sannanensis* and the sporaricin producer *Sac. hirsuta* (Ohta & Hasegawa, 1989). These findings seemed to indicate that the resistance genes were also conserved among these antibiotic producers. However, very interestingly, recent preliminary hybridization experiments for an expanded range of strains unexpectedly indicated that this was not the case, suggesting the existence of two or more types of resistance genes. In the present study, we cloned the FTM-A-resistance genes (*fmr* genes) from the other five FTM-group antibiotics producers in order

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Abbreviations: FTM-A, fortimicin A; Km, kanamycin; Gm, gentamicin; Nm-B, neomycin B; Sm, streptomycin; CIAP, calf intestine alkaline phosphatase.

to compare them in the same genetic background and screen for sequences similar to *fmr* genes in other actinomycetes.

Methods

Bacterial strains and plasmids. *S. sannanensis* IFO 14239, *Sac. hirsuta* ATCC 20501, *M. olivasterospora* ATCC 21819, *Micromonospora* sp. ATCC 31580 and *D. matsuzakiense* ATCC 31570 were used as sources of total DNA for the shot gun cloning experiments of *fmr* genes. *S. lividans* TK23 was used as a cloning host. Plasmids pIJ702 (Katz *et al.*, 1983), pEN101 (Nagano *et al.*, 1988) and pEN101B in which a *Bgl*II site of pEN101 was converted to a *Bam*HI site using a synthetic DNA linker (unpublished data) were used as cloning vectors.

Culture conditions and media. All actinomycetes were grown at 30 °C in SK no. 2 medium (Ohta *et al.*, 1992), or maintained on ATCC no. 5 agar medium. Strains harbouring plasmids carrying *fmr* genes were grown in medium containing 50 µg FTM-A hydrochloride ml⁻¹ (Kyowa Hakko Kogyo).

Determination of the antibiotic resistance patterns of the strains. About 10⁹ spores were spread on ATCC no. 5 medium containing 0, 25, 50, 125, 250 and 500 µg ml⁻¹ of the antibiotics to be tested: FTM-A hydrochloride, Km sulphate (Sigma), Gm sulphate (Sigma), Nm-B sulphate (Sigma) and streptomycin (Sm) sulphate (Sigma). Growth was monitored after incubation at 30 °C for 3–5 d.

Preparation and manipulation of DNA. Total DNA and plasmid DNA were isolated by the methods described by Chater *et al.* (1982) and Kieser (1984), respectively. Restriction endonucleases, calf intestine alkaline phosphatase (CIAP) and T4 DNA ligase were purchased from Boehringer Mannheim or Takara Shuzo. They were used under the conditions specified by the suppliers.

Isolation of *fmr* genes. Total DNA of *S. sannanensis* IFO 14239 was partially digested with *Sau*3AI and ligated to pIJ702 cut by *Bgl*II. Total

DNA of *Sac. hirsuta* ATCC 20501 was digested with *Bam*HI and ligated to pEN101B cut by *Bam*HI. Total DNA of *M. olivasterospora* ATCC 21819 was partially digested with *Sau*3AI and ligated to pEN101 cut by *Bgl*II. Total DNA of *Micromonospora* sp. SF-2098 ATCC 31580 was partially digested with *Sau*3AI and ligated to pEN101 cut by *Bgl*II. Total DNA of *D. matsuzakiense* ATCC 31570 was digested with *Sac*I and ligated to pEN101 cut by *Sac*I. All of these digested plasmid vectors were used after CIAP treatment. Protoplasts of *S. lividans* TK23 were transformed with these ligated DNA preparations and regenerated on R5 medium (Hintermann *et al.*, 1984). Thiopeptin-resistant transformants were obtained by overlaying with soft-agar medium consisting of Nutrient broth (Difco) 0.5% (w/v), Bacto agar (Difco) 0.5% (w/v) and 20 µg thiopeptin ml⁻¹ (Fujisawa Pharmaceutical). These transformants were replica-plated onto ATCC no. 5 medium containing FTM-A (50 µg ml⁻¹). After incubation at 30 °C for 3–5 d, FTM-A-resistant transformants were selected.

Construction of a plasmid carrying both *fmrT* and *fmrO*. The 1.5 kb *Bam*HI fragment containing *fmrT* in pFMRT3 (Ohta & Hasegawa, 1989) was ligated to an *fmrO*-containing recombinant plasmid, pFMRO2, partially digested with *Bcl*II, and *S. lividans* TK23 was transformed with this DNA preparation. After regeneration on R5 medium without selection by antibiotics, the colonies were transferred to ATCC no. 5 medium containing 50 µg Nm-B ml⁻¹. They were then transferred to ATCC no. 5 containing 250 µg Gm ml⁻¹, and the transformants resistant to Nm and Gm were selected.

Southern-blot hybridization. For the analysis of the cloned *fmr* genes, each plasmid was digested as follows: pFMRTB1 with *Bam*HI, pFMRS1 with *Bcl*II, pFMRH1 with *Bam*HI and *Eco*RI, pFMRO1 with *Bcl*II, pFMRM1 with *Bgl*II, and pFMRD1 with *Sac*I. In the experiment in which the total DNA from various actinomycetes was probed, all total DNA samples were digested with *Bam*HI. To determine the location of *fmrO* within the gene cluster for FTM-A biosynthesis, a cosmid clone pGLM559 (Dairi *et al.*, 1993) was digested with *Mlu*I or *Xho*I. These preparations were separated by 0.7% agarose gel electrophoresis in TAE buffer (40 mM-Tris/acetate and 2 mM-EDTA, pH 8.0), and blotted onto Genescreen plus (New England Nuclear

Table 1. Sensitivities to aminoglycoside antibiotics of test strains and *S. lividans* transformants carrying various *fmr* genes

Bacterial growth was monitored after 3–5 d cultivation on ATCC no. 5 medium containing 0, 25, 50, 125, 250 and 500 µg ml⁻¹ of the antibiotics. Numbers represent the minimum concentrations (µg ml⁻¹) of the antibiotics which inhibit the growth of the bacteria. s, Sensitive to 25 µg of the antibiotic ml⁻¹.

	FTM-A	Km	Gms	Nm-B	Sm
<i>S. tenjimariensis</i>	> 500	> 500	s	250	s
<i>S. sannanensis</i>	> 500	> 500	s	250	s
<i>Sac. hirsuta</i>	> 500	> 500	s	250	s
<i>M. olivasterospora</i>	> 500	> 500	> 500	s	s
<i>Micromonospora</i> sp. SF-2098	> 500	> 500	> 500	s	s
<i>D. matsuzakiense</i>	> 500	> 500	> 500	s	s
<i>S. lividans</i> TK23	s	s	s	s	s
<i>S. lividans</i> (pFMRTB1)	> 500	> 500	s	250	s
<i>S. lividans</i> (pFMRS2)	> 500	> 500	s	250	s
<i>S. lividans</i> (pFMRH1)	> 500	> 500	s	250	s
<i>S. lividans</i> (pFMRO2)	> 500	> 500	> 500	s	s
<i>S. lividans</i> (pFMRM1)	> 500	> 500	> 500	s	s
<i>S. lividans</i> (pFMRD1)	> 500	> 500	> 500	s	s
<i>S. lividans</i> (pFMRT01)*	> 500	> 500	> 500	250	s

* pFMRT01 is a pFMRO2-derived plasmid carrying both *fmrT* and *fmrO*, but it does not carry *tsr*.

Research Products) according to the method of Reed & Mann (1985). The 0.8 kb *SalI* fragment within pFMRTB1 (Fig. 1a) and the 3.2 kb *PvuII* fragment within pFMRO2 (Fig. 1d) were used as the '*fmrT*' probe and the '*fmrO*' probe', respectively. These DNA fragments were labelled with [α - 32 P]dCTP (Amersham) by nick-translation. Hybridization was carried out at 65 °C for 16 h in $6 \times$ SSC ($1 \times$ SSC is 0.15 M-NaCl plus 0.015 M-sodium citrate, pH 7.2), $5 \times$ Denhardt's solution (Maniatis *et al.*, 1982), 100 μ g sonicated/denatured calf thymus DNA ml $^{-1}$, with the radioactive DNA probe. The filters were washed twice for 15 min at room temperature in 150 ml $0.5 \times$ SSC, 0.2% (w/v) SDS, and four times for 30 min at 65 °C in 250 ml $0.3 \times$ SSC, 0.2% (w/v) SDS. Autoradiograms were made by exposing the filters to Fuji X-ray films (type AIF RX) at -80 °C for 1-2 d.

Results

Resistance profiles of strains to aminoglycoside antibiotics

Table 1 shows sensitivities of the FTM-group antibiotic producers to various aminoglycoside antibiotics. All strains were highly resistant to FTM-A and Km, and were sensitive to Sm. *S. tenjimariensis*, *S. sannanensis* and *Sac. hirsuta* were resistant to Nm-B, but sensitive to Gm. In contrast, *M. olivasterospora*, *Micromonospora* sp. SF-2098 and *D. matsuzakiense* were resistant to Gm, but sensitive to Nm-B. The *fmrT* gene isolated from *S. tenjimariensis* was considered to be a resistance gene for self-defence because of the structural similarity of FTM-A and istamycin A, its own product (Ohta & Hasegawa, 1989). The gene conferred resistance to Nm-B; however, *M. olivasterospora*, *Micromonospora* sp. SF-2098 and *D. matsuzakiense* were sensitive to this antibiotic. These results indicated the presence of two or more different types of resistance genes in these producers. As *S. lividans* TK23 was sensitive to these antibiotics (Table 1), it was used as the host to clone the resistance genes.

Isolation of *fmr* genes

Isolation of *fmrT* from the istamycin producer *S. tenjimariensis* was previously described (Ohta & Hasegawa, 1989). The physical map of the cloned DNA in pFMRTB1 is shown in Fig. 1(a). Although Skeggs *et al.* (1987) cloned an aminoglycoside-resistance gene (*kamA*; kanamycin-apramycin resistance methyltransferase) from the same strain of *S. tenjimariensis*, its physical map was quite different from our clone (Skeggs *et al.*, 1985, 1987). In the present study, *fmr* genes were cloned into *S. lividans* TK23 from the other five strains. Plasmids pIJ702, pEN101 and pEN101B were used as vectors. Plasmid pEN101 is a multi-copy plasmid which is similar in length, copy number and stability to those of pIJ702 (unpublished data); pEN101B is a pEN101 derivative.

From the sannamycin producer *S. sannanensis* IFO 14239, four FTM-A-resistant transformants were ob-

tained in the shotgun cloning experiment. These plasmids, pFMRS1, pFMRS1, pFMRS2, pFMRS3 and pFMRS4, contained 1.5, 2.3, 1.6 and 1.4 kb inserts, respectively. The resistance determinant, *fmrS*, should be located within the common 1.3 kb region in these cloned DNA fragments. Fig. 1(b) shows the physical map of the insert in pFMRS2 and the presumed location of *fmrS*. From the sporaricin producer *Sac. hirsuta* ATCC 20501, one resistant transformant, harbouring pFMRH1, was obtained. pFMRH1 contained a 5.9 kb *Bam*HI fragment (Fig. 1c). During the preparation of this report, the isolation of *kamC* (see above for designation of *kam*) of

Fig. 1. Physical maps of the cloned DNA fragments containing *fmr* genes. The restriction maps of the cloned fragments of (a) pFMRTB1 from *S. tenjimariensis* (Ohta & Hasegawa, 1989), (b) pFMRS2 from *S. sannanensis*, (c) pFMRH1 from *Sac. hirsuta* (ATCC 20501), (d) pFMRO2 from *M. olivasterospora*, (e) pFMRM1 from *Micromonospora* sp. SF-2098, and (f) pFMRD1 from *D. matsuzakiense* are shown. The black regions represent the cloned fragments. The location of *fmrT*, *fmrS* and *fmrO* are indicated as open bars under the physical maps. Dotted lines represent the 0.8 kb *SalI* fragment of the *fmrT* probe (a) and the 3.2 kb *PvuII* fragment of the *fmrO* probe (d) used in Southern-blot hybridization analysis.

another sporaricin producer, *Sac. hirsuta* CL102, was reported by Holmes *et al.* (1991). However, the physical map of our clone was quite different from their *kamC*-containing DNA fragment; the taxonomic relationship of the two strains is unclear. As the structures of these two resistance genes were different and as the resistance mechanism of our gene has not been clarified, our resistance gene obtained from *Sac. hirsuta* ATCC 20501 was designated *fmrH*. From the FTM-A producer *M. olivasterospora*, three resistant transformants were obtained. The plasmids, pFMRO1, pFMRO2 and pFMRO3, carried 2.6, 3.6 and 3.0 kb inserts, respectively. The resistance determinant, *fmrO*, should be located within the common 1.9 kb region in the cloned DNA fragments of these three plasmids. Fig. 1(d) shows the physical map of the insert in pFMRO2 and the presumed location of *fmrO*. From the antibiotic SF-2052 producer *Micromonospora* sp. strain SF-2098, one resistant transformant, harbouring, pFMRM1, was obtained. pFMRM1 contained a 3.4 kb insert (Fig. 1e). The resistance determinant was designated *fmrM*. From the dactimicin producer *D. matsuzakiense*, one resistant transformant, harbouring pFMRD1 was obtained. Although the shotgun cloning was performed by *SacI* digestion, pFMRD1 contained two *sacI* fragments, of 3.1 and 0.7 kb (Fig. 1f).

Similarity was observed between the physical maps of *fmrO* and *fmrM*, cloned from two *Micromonospora*

strains; however, no structural similarities were observed among the other genes.

Resistance profiles of *fmr* genes

Resistance profiles of *S. lividans* TK23 transformants harbouring each *fmr* gene were examined. Exact comparison of the resistance levels conferred by resistance genes present in taxonomically different micro-organisms is difficult because of the difference in their optimum growth conditions and the difficulty of controlling their growth rates. We therefore used the homogeneous background of the host *S. lividans* carrying the *fmr* genes to compare the characteristics of these resistance genes. The results summarized in Table 1 clearly indicate that the resistance patterns of these transformants are similar to those of the corresponding producer strains. These results also show that *fmr* genes could undoubtedly be classified into two groups with respect to their resistance profiles, namely the Nm-resistant group (designated '*fmrT*-type', consisting of *fmrT*, *fmrS* and *fmrH*) and the Gm-resistant group (designated '*fmrO*-type', consisting of *fmrO*, *fmrM* and *fmrD*). The resistance pattern of the *fmrT*-type was similar to the *kam* genes isolated from *S. tenjimariensis* (*kamA*) (Skeggs *et al.*, 1985), a nebramycin producer *S. tenebrarius* NCIB 11028 (*kamB*) (Skeggs *et al.*, 1987) and a sporaricin producer *Sac. hirsuta* CL102 (*kamC*) (Holmes *et al.*, 1991), in spite of the discrepancy

Fig. 2. DNA homologies among the cloned *fmr* genes. Ethidium bromide-stained gel (a) and the results of Southern-blot hybridization analysis using the *fmrT* probe (b) and the *fmrO* probe (c) are shown. The 0.8 kb *SacI* fragment in pFMRTB1 (Fig. 1a) and the 3.2 kb *PvuII* fragment in pFMRO2 (Fig. 1d) as the *fmrT* and *fmrO* probes, respectively. Lanes: 1, size markers (λ phage DNA digested with *HindIII*); 2, pFMRTB1 digested with *BamHI* (*fmrT* is contained in the 2.8 kb fragment); 3, pFMRS1 digested with *BclI* (*fmrS* is contained in the 3.0 kb fragment); 4, pFMRH1 digested with *BamHI* and *EcoRI* (*fmrH* is contained in the 5.9 kb fragment); 5, pFMRO1 digested with *BclI* (*fmrO* is contained in the 5.3 kb fragment); 6, pFMRM1 digested with *BglII* (*fmrM* is contained in the 3.4 kb fragment); 7, pFMRD1 digested with *SacI* (*fmrD* is contained in the 3.2 kb fragment).

of the physical maps between *kamA* and our *fmrT*, and between *kamC* and our *fmrH*. On the other hand, the resistance pattern of the *fmrO*-type was similar to Km-Gm, resistance genes isolated from the Km producer *Streptomyces kanamyceticus* ISP 5500 (Nakano *et al.*, 1984), *S. tenebrarius* NCIB 11028 (*kgmB*) (Skeggs *et al.*, 1987), and the Gm producer *M. purpurea* (*grm*) (Kelemen *et al.*, 1991).

An 8.7 kb plasmid, pFMRT01, which carried both *fmrT* and *fmrO*, was constructed by introducing *fmrT* into pFMRO2, although the plasmid lost a portion of pFMRO2 including *tsr* during the construction. An *S. lividans* TK23 transformant harbouring pFMRT01 was resistant to both Nm-B and Gm (Table 1), indicating that these resistance mechanisms worked independently and simultaneously in *S. lividans*.

DNA homologies of the cloned *fmr* genes

Southern-blot hybridization analysis was performed to examine the DNA homologies among the *fmr* genes. The 0.8 kb *Sa*I fragment in pFMRTB1 (*fmrT*; Fig. 1a) and the 3.2 kb *Pvu*II fragment in pFMRO2 (*fmrO*; Fig. 1d) were used as the hybridization probes. The plasmids carrying the *fmr* genes described above were digested with appropriate restriction enzymes in order to distinguish the cloned DNA fragments from the vector sequences. When the *fmrT* probe was employed, significant homologies were found in *fmrS* and *fmrH*, but not in *fmrO*, *fmrM* and *fmrD* (Fig. 2b). The opposite result was obtained when the *fmrO* probe was employed (Fig. 2c).

Distribution of DNA fragments homologous to *fmrT* and *fmrO*

Southern hybridization analyses of the total DNA from various actinomycetes were performed to examine the distribution of DNA fragments homologous to *fmrT* and *fmrO*. When the *fmrT* probe was employed, a positive signal of 2.8 kb was detected in *S. tenjimariensis*, which agreed with the cloned fragment by *Bam*HI digestion (Fig. 3, lane 2; Fig. 1a). Positive signals were clearly detected in *S. sannanensis* and *Sac. hirsuta* ATCC 20501 as expected, with sizes of 7.0 and 5.9 kb, respectively (Fig. 3, lanes 3 and 11). The size of the signal detected in the sporaricin producer *Sac. hirsuta* ATCC 20501 agreed with the structure of the cloned fragment by *Bam*HI digestion (Fig. 1c); however, it differed in size from the 2.6 kb *Bam*HI fragment containing *kamC* in another sporaricin producer, *Sac. hirsuta* CL102 (Holmes *et al.*, 1991). *Sac. hirsuta* ATCC 27875 also gave a signal of 2.4 kb (Fig. 3, lane 13). This strain has not been reported

Fig. 3. Distribution of DNA homologous to *fmrT*. The result of Southern-blot hybridization analysis using the 0.8 kb *Sa*I fragment in pFMRTB1 (Fig. 1a) as a probe is shown. Total DNA preparations were digested with *Bam*HI and separated by 0.7% agarose gel electrophoresis in TAE buffer. Lanes: 1, *Streptomyces lividans* TK23; 2, *Streptomyces tenjimariensis* ATCC 31603 (istamycin producer); 3, *Streptomyces sannanensis* IFO 14239 (sannamycin); 4, *Streptomyces fradiae* ATCC 10745 (Nm-B); 5, *Streptomyces griseus* subsp. *griseus* (Sm); 6, *Streptomyces kasugaensis* ATCC 15715 (kasugamycin); 7, *Streptomyces hygroscopicus* subsp. *hygroscopicus* ATCC 27438 (hygromycin); 8, *Streptomyces kanamyceticus* ATCC 12853 (Km); 9, *Streptomyces ribosidificus* ATCC 21294 (ribostamycin); 10, *Streptomyces tenebrarius* ATCC 17920 (nebramycin); 11, *Saccharopolyspora hirsuta* ATCC 20501 (sporaricin); 12, *Saccharopolyspora hirsuta* (MK-220) (XK-220, a macrolide antibiotic, unpublished data); 13, *Saccharopolyspora hirsuta* ATCC 27875. DNA preparations from the strains in Fig. 4 (lanes 1–13) were also probed, but gave no hybridization signals with *fmrT* (not shown). Positions of the fragments of λ phage DNA digested with *Hind*III are indicated on the left. Asterisks above the lane numbers indicate the producers of the FTM-group antibiotics.

to produce FTM-group antibiotics; however, it showed a similar resistance profile to those of *fmrT*-type resistance genes – resistant to FTM-A, Km and Nm-B, but sensitive to Gm and Sm. These results suggest that *Sac. hirsuta* ATCC 27875 has an *fmrT*-type resistance gene. On the other hand, no signal was detected in *M. olivasterospora*, *Micromonospora* sp. SF-2098, *D. matsuzakiense* and other actinomycetes including producers of the other aminoglycoside antibiotics (see legend to Fig. 3).

When the *fmrO* probe was employed, positive signals of approximately 15, 15 and 25 kb were detected in *M. olivasterospora*, *Micromonospora* sp. SF2098 and *D. matsuzakiense*, respectively (Fig. 4, lanes 1, 2 and 11). No positive signal was detected in *S. tenjimariensis*, *S. sannanensis* and sporaricin-producing *Sac. hirsuta* (not shown). Hybridization signals were also commonly detected in *Micromonospora* strains which produced the other aminoglycoside antibiotics: 6.1 kb signals in *M. purpurea* ATCC 15835 and three *M. echinospora* strains,

Fig. 4. Distribution of DNA homologous to *fmrO*. The result of Southern-blot hybridization analysis using the 3.2 kb *PvuII* fragment in pFMRO2 (Fig. 1d) as a probe is shown. Lanes: 1, *Micromonospora olivasterospora* ATCC 21819 (FTM-A); 2, *Micromonospora* sp. SF-2098 ATCC 31580 (SF-2052); 3, *Micromonospora purpurea* ATCC 15835 (identical to strain DSM 43036; Gms); 4, *Micromonospora sagamiensis* subsp. *nonreducans* ATCC 21803 (sagamicins and Gm-A1); 5, *Micromonospora sagamiensis* ATCC 21826 (sagamicins and Gm-A1); 6, *Micromonospora echinospora* subsp. *echinospora* ATCC 15837 (Gms); 7, *Micromonospora echinospora* subsp. *pallida* ATCC 15838 (Gms); 8, *Micromonospora inyonensis* ATCC 27600 (sisomicins); 9, *Micromonospora zionensis* IFO 14116 (sisomicins); 10, *Micromonospora echinospora* subsp. *ferruginea* ATCC 15836 (Gms); 11, *Dactylosporangium matsuzakiense* ATCC 31570 (dactimicin); 12, *Dactylosporangium aurantiacum* ATCC 23491; 13, *Dactylosporangium variesporum* (capreomycin). DNA preparations from the strains in Fig. 3 (lanes 1–13) were also probed, but gave no hybridization signals with *fmrO* (not shown). Other details as for Fig. 3.

ATCC 15837, ATCC 15838 and ATCC 15836 (Gm producers; lanes 3, 6, 7 and 10), 4.1 kb in two *M. sagamiensis* strains, ATCC 21803 and ATCC 21826 (sagamicins and Gm-A1; lanes 4 and 5), an approximately 13 kb signal and a weak 4.7 kb signal in *M. inyonensis* ATCC 27600 (sisomicins; lane 8), and a 7.3 kb signal and a weak 13 kb signal in *M. zionensis* IFO 14116 (sisomicins; lane 9). All these strains were highly resistant to FTM-A (more than 500 µg ml⁻¹); however, they are not known to produce any FTM-group antibiotics.

Location of *fmrO* in the gene cluster of FTM-A biosynthesis

The genes for FTM-A biosynthesis are clustered within an approximately 25 kb region (Dairi *et al.*, 1993). The *fmrO* probe hybridized to the 6.4 kb *MluI* and the 6.0 kb *XhoI* fragments of pGLM559 carrying the FTM-A biosynthesis gene cluster (Dairi *et al.*, 1993). This

indicated that *fmrO* is adjacent to one end of the gene cluster for FTM-A biosynthesis in the *M. olivasterospora* genome. The size of the signal detected in the Southern analysis of the total DNA (Fig. 4, lane 1) is also consistent with the structure of the gene cluster.

Discussion

Self-defence against their own products is essential for the growth of antibiotic producers (Hotta *et al.*, 1983). Antibiotic biosynthesis genes are generally clustered in limited regions in the genome, and the genes conferring resistance to the organism's own products are usually located in or near the gene clusters (Rhodes *et al.*, 1984; Ohnuki *et al.*, 1985; Chater & Bruton, 1985; Malpartida & Hopwood, 1986; Murakami *et al.*, 1986; Stanzak *et al.*, 1986; Fishman *et al.*, 1987). These facts seem to suggest a tight genetic linkage between antibiotic biosynthesis genes and the resistance genes during the evolution and the horizontal distribution of antibiotic biosynthesis systems.

The FTM-group antibiotics, which are produced by six actinomycetes of four genera, are believed to be synthesized via similar biosynthetic pathways (Dairi & Hasegawa, 1989; Hotta *et al.*, 1989), and at least parts of these antibiotic biosynthetic genes are conserved among these producers (Ohta *et al.*, 1992). We had previously postulated that the resistance genes for self-defence would also be conserved, like the biosynthesis genes (Ohta & Hasegawa, 1989). However, the present study showed that the resistance genes of these strains could be clearly classified into two different families with respect to their resistance profiles and DNA homologies. The results suggest that the differences in the resistance patterns are caused by differences in the nucleotide sequences and hence differences in the enzyme structures. Thus there may exist at least two functionally different families of resistance genes among the FTM-group antibiotic producers.

Importantly, *fmrO* was found to be located adjacent to the gene cluster for FTM-A biosynthesis. It is very curious that the *fmrO*-type resistance genes could not be found in *S. tenjimariensis*, *S. sannanensis* and sporaricin-producing *Sac. hirsuta*, which carry the homologous glycytransferase gene that is located in this gene cluster. This fact suggests that the biosynthetic genes and the resistance genes have evolved separately in these producers. It can be considered that the genes for antibiotic biosynthesis and resistance are horizontally distributed by genetic transfer, such as conjugation or transduction via phages, in addition to the vertical succession accompanied by bacterial evolution. It is possible that part of the gene cluster for the biosynthesis of FTM-group antibiotics has been transferred inde-

pendently of its corresponding resistance gene to one of the FTM-group antibiotic producers which had another class of resistance genes. Our results suggest a looser genetic linkage between the antibiotic biosynthesis genes and the self-defence genes during their presumed dissemination among the FTM-group antibiotic producers.

The resistance pattern conferred by the *fmrO*-type resistance genes was similar to those conferred by the Km-Gm resistance gene conferring the ribosome modification isolated from *S. kanamyceticus* ISP 5500 (Nakano *et al.*, 1984) and by the kanamycin-gentamicin resistance methyltransferase gene (*kgmB*) of *S. tenebrarius* NCIB 11028 (Skeggs *et al.*, 1987). However, no hybridization signals were detected in these strains using the *fmrO* probe under the conditions employed in this study. This suggests that the resistance determinant found in *S. kanamyceticus* and *S. tenebrarius* is different from the *fmrO*-type resistance genes, or that the similarity in nucleotide sequences is too small to be detected by this hybridization experiment. The *fmrO*-type resistance genes of *Micromonospora* and *Dactylosporangium* are more similar to each other than to those of the *Streptomyces* strains. This seems to be consistent with the taxonomic distances between the genera *Micromonospora*, *Dactylosporangium* and *Streptomyces*. Recent comparisons of 16S rRNA sequences showed that the former two genera are very closely related, and are distant from *Streptomyces* and *Saccharopolyspora* (Stackebrandt & Charfreitag, 1990).

Positive signals hybridizing to the *fmrO* probe were commonly detected in *Micromonospora* strains which produced other aminoglycoside antibiotics, i.e. gentamicins, sagamicins or sisomicins. The signals detected in *Micromonospora* strains should be resistance genes similar to *fmrO* considering the high resistance of these strains to FTM-A. The resistance genes with resistance patterns similar to *fmrO* isolated from *S. kanamyceticus* (Nakano *et al.*, 1984), *S. tenebrarius* (*kgmB*) (Skeggs *et al.*, 1987) and *M. purpurea* (*grm*) (Kelemen *et al.*, 1991) confer ribosome modification. Ribosomal resistance is a widespread mechanism among aminoglycoside-producing *Micromonospora* strains (Matkovic *et al.*, 1984). No FTM-A-inactivation activity was detected in *M. olivasterospora* (data not shown). Thus, one can suppose that the *fmrO*-type resistance genes confer such a ribosome modification. It was reported that *grm* of *M. purpurea* encoded a specific methyltransferase of 16S ribosomal RNA (Kelemen *et al.*, 1991). However, the size of the band that hybridized to the *fmrO* probe in *M. purpurea* ATCC 15835 (identical to strain DSM 43036 used by Piendl *et al.*, 1984) (6.1 kb *Bam*HI fragment; Fig. 4, lane 3) was not the same as that which hybridized to the *grm* probe in their strain (1.9 kb *Bam*HI fragment).

It has been shown that the self-defence mechanism of

S. tenjimariensis is ribosomal resistance (Yamamoto *et al.*, 1981, 1986; Piendl *et al.*, 1984; Skeggs *et al.*, 1985), and that its *kamA* encodes a specific methyltransferase of 16S ribosomal RNA (Beauclerk & Cundliffe, 1987). The *kamA* of *S. tenjimariensis*, *kamB* of *S. tenebrarius* NCIB 11028 and *kamC* of another sporaricin-producing *Sac. hirsuta* strain, CL102, have been reported to have DNA similarity (Skeggs *et al.*, 1987; Cundliffe, 1989; Holmes *et al.*, 1991). The *fmrT*-type resistance genes obtained in this study gave a similar resistance profile to that of the *kam* type resistance genes. However, the physical map of *fmrT* disagreed with that of *kamA* cloned from the same strain of *S. tenjimariensis* (Skeggs *et al.*, 1987), and no signal hybridizing to the *fmrT* probe was detected in *S. tenebrarius* ATCC 17920 under the conditions used in this study. *fmrT* seems to be the only gene which confers resistance to FTM-A, Km and Nm-B in our strain. At this stage we cannot explain why *fmrT* does not appear to be related to *kam*. Further comparative studies, including sequence analysis, between the *fmrT* and *kam* genes are needed.

Although the two *Micromonospora* strains which produce the FTM-group antibiotics were independently isolated by different research groups (Nara *et al.*, 1977; Ohba *et al.*, 1981), the patterns of hybridization signals obtained with the *fmrO* probe agreed well. Such agreement was also observed using the sannamycin B-glycyltransferase gene as a probe (Ohta *et al.*, 1992). These observations suggest that the biosynthetic gene clusters for FTM-A and SF-2052 compounds are well conserved. Similarities in the hybridization signals to the *fmrO* probe were observed among the producers of the Gm-group antibiotics, *M. purpurea* ATCC 15835, *M. echinospora* subsp. *echinospora* ATCC 15837, *M. echinospora* subsp. *pallida* ATCC 15838 and *M. echinospora* subsp. *ferruginea* ATCC 15836, and between *M. sagamiensis* subsp. *nonreducans* ATCC 21803 and *M. sagamiensis* ATCC 21826. The difference between the former and the latter groups is only in the level and composition of their Gm-group antibiotics. The properties of these antibiotics seem to be correlated with the degree of conservation of the gene organizations around the resistance genes, probably including the biosynthetic genes for these antibiotics.

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