CLONING AND ANALYSIS OF A GENE (*SMS13*) ENCODING SANNAMYCIN B-GLYCYLTRANSFERASE FROM *Streptomyces sannanensis* AND ITS DISTRIBUTION AMONG ACTINOMYCETES

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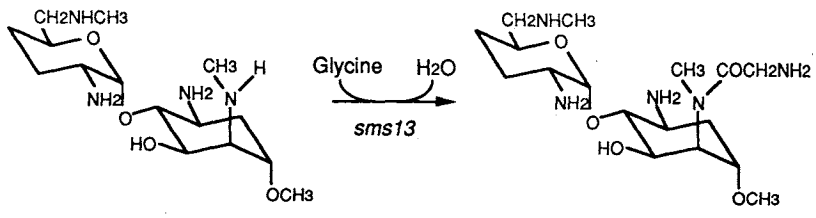
A gene encoding sannamycin B-glycyltransferase (*sms13*) of *Streptomyces sannanensis* IFO 14239 was identified by cloning and complementation of *S. sannanensis* mutant SN13 which is blocked at the interconversion of sannamycins B and A. The cloned DNA fragment also permitted the conversion of fortimicin B to A both in *S. sannanensis* SN13 and *Streptomyces lividans* TK23. DNA sequences similar to *sms13* were detected in all five producers of the fortimicin-group antibiotics, *Micromonospora olivasterospora* ATCC 21819 (fortimicin-producer), *Micromonospora* sp. strain SF-2098 ATCC 31580 (SF-2052), *Dactylosporangium matsuzakiense* ATCC 31570 (dactimicin), *Streptomyces tenjimariensis* ATCC 31603 (istamycin), and *Saccharopolyspora hirsuta* ATCC 20501 (sporaricin). This suggests that these genes of similar function from different genera were derived from a common ancestral gene.

Antibiotics with similar structures are often produced by taxonomically distant microorganisms, and the sequences of such antibiotic biosynthetic genes are well conserved, *e.g.* in polyketide antibiotics[1] and in β-lactam antibiotics[2~5]; this is of considerable interest from the point of view of the evolution and distribution of the secondary metabolic pathways. Fortimicin A (astromicin)[6] produced by *Micromonospora olivasterospora* ATCC 21819, has an unique pseudodisaccharide structure. A number of other fortimicin-group antibiotics of similar structures are known, which are produced by the following five members of four different genera; *Streptomyces sannanensis* IFO 14239 (sannamycin)[7], *Streptomyces tenjimariensis* ATCC 31603 (istamycin)[8], *Saccharopolyspora hirsuta* ATCC 20501 (sporaricin)[9], *Micromonospora* sp. SF-2098 ATCC 31580 (SF-2052 compounds)[10] and *Dactylosporangium matsuzakiense* ATCC 31570 (dactimicins)[11].

The biosynthetic pathway of fortimicin A in *M. olivasterospora* is believed to consist of more than 14 steps[12]. All members of the fortimicin-group antibiotics are thought to be synthesized by related biosynthetic pathways[13,14]. It is unlikely that such complex biosynthetic routes evolved by chance independently in different genera, and dissemination of these antibiotic productivities by some form of

Fig. 1. Proposed reaction of sannamycin B to sannamycin A in *Streptomyces sannanensis* IFO 14239.



sannamycin B sannamycin A

the horizontal transfer could be considered. We have previously reported a self-cloning system in *S. sannanensis*[15] and also obtained nonproducing mutants by mutagenesis[16]. In this study, we cloned the sannamycin B-glycyltransferase gene by complementation in the nonproducing mutant *S. sannanensis* SN13, which was blocked at the conversion of sannamycin B to A (Fig. 1). A study of the distribution of sequences similar to *sms13* in other actinomycetes is presented.

Materials and Methods

Bacterial Strains and Plasmids

S. sannanensis IFO 14239 DNA was used for the construction of a gene library in the shotgun-cloning of *sms13*. *S. sannanensis* SN13, blocked at sannamycin B-glycyltransferase step[16], was used as a host. *S. lividans* TK23 was employed to assay the fortimicin B to A converting activity by *sms13*. Either *Bacillus subtilis* ATCC 6633 or *Escherichia coli* ATCC 26 were used as indicators for antibacterial activity. Plasmid pEN101[17] was used as a plasmid vector for *S. sannanensis* cloning.

Cultivation Conditions

S. lividans strains were grown in SK No. 2 medium at 30°C for 3 days. SK No. 2 medium consisted of Stabilose K (Matsutani Kagaku) 2.0% (w/v), glucose 0.5%, yeast extract (Nippon Seiyaku) 0.5%, peptone (Nippon Seiyaku) 0.5%, meat extract (Kyokuto Seiyaku) 0.3%, KH_2PO_4 0.02%, $MgSO_4 \cdot 7H_2O$ 0.06%, pH 7.6. *S. sannanensis* strains were grown in SA medium[16] at 30°C for 3 days. For strains harboring pEN101 or its derivatives, 2 μg/ml of thiopeptin (Fujisawa) was added.

Protoplasting and Transformation

Protoplasting and transformation were carried out according to the method described for *S. sannanensis* IFO 14239[15]. Protoplasting and transformation of *S. lividans* were according to CHATER *et al.*[18].

Preparation and Manipulation of DNA

Genomic DNA was isolated as described by CHATER *et al.*[18]. Plasmid DNA was isolated according to KIESER[19]. Restriction endonucleases, calf intestine alkaline phosphatase and T4 DNA ligase (Boehringer or Takara) were used under the conditions specified by the suppliers.

Shotgun-cloning and Subcloning of *sms13*

Total DNA (300 μg) prepared from *S. sannanensis* IFO 14239 was partially digested with *Sau*3A I and size-fractionated by 10~40% sucrose density gradient ultracentrifugation to obtain 4~6 kb DNA fragments. Plasmid pEN101 prepared from *S. sannanensis* (10 μg) was completely digested with *Bgl* II and dephosphorylated with calf intestine alkaline phosphatase. The digested genomic DNA (22 μg) and vector DNA (10 μg) were ligated with T4 DNA ligase at 16°C for 15 hours and transformed into *S. sannanensis* SN13 as described above. Thiopeptin-resistant transformants were incubated at 30°C for 6 days in SA medium containing 2 μg/ml of thiopeptin, and the colonies were overlaid with E medium[16] containing 10^5 cells/ml of *E. coli* ATCC 26 and incubated at 37°C for 16 hours. Transformants which formed inhibitory zones were transferred to SY2 medium[16] containing 50 μg/ml of fortimicin A and incubated at 30°C for 3 days to eliminate *E. coli*.

Plasmid pSN13-1, obtained in the shotgun-cloning, was completely digested with *Eco*R I, and dephosphorylated with alkaline phosphatase. Subsequently, it was partially digested with *Sau*3A I and ligated with pEN101 cut by *Bgl* II. *S. sannanensis* SN13 was transformed with this DNA and the transformants restoring sannamycin A production were obtained. Plasmid containing insertion fragments smaller than that of pSN13-1 were selected.

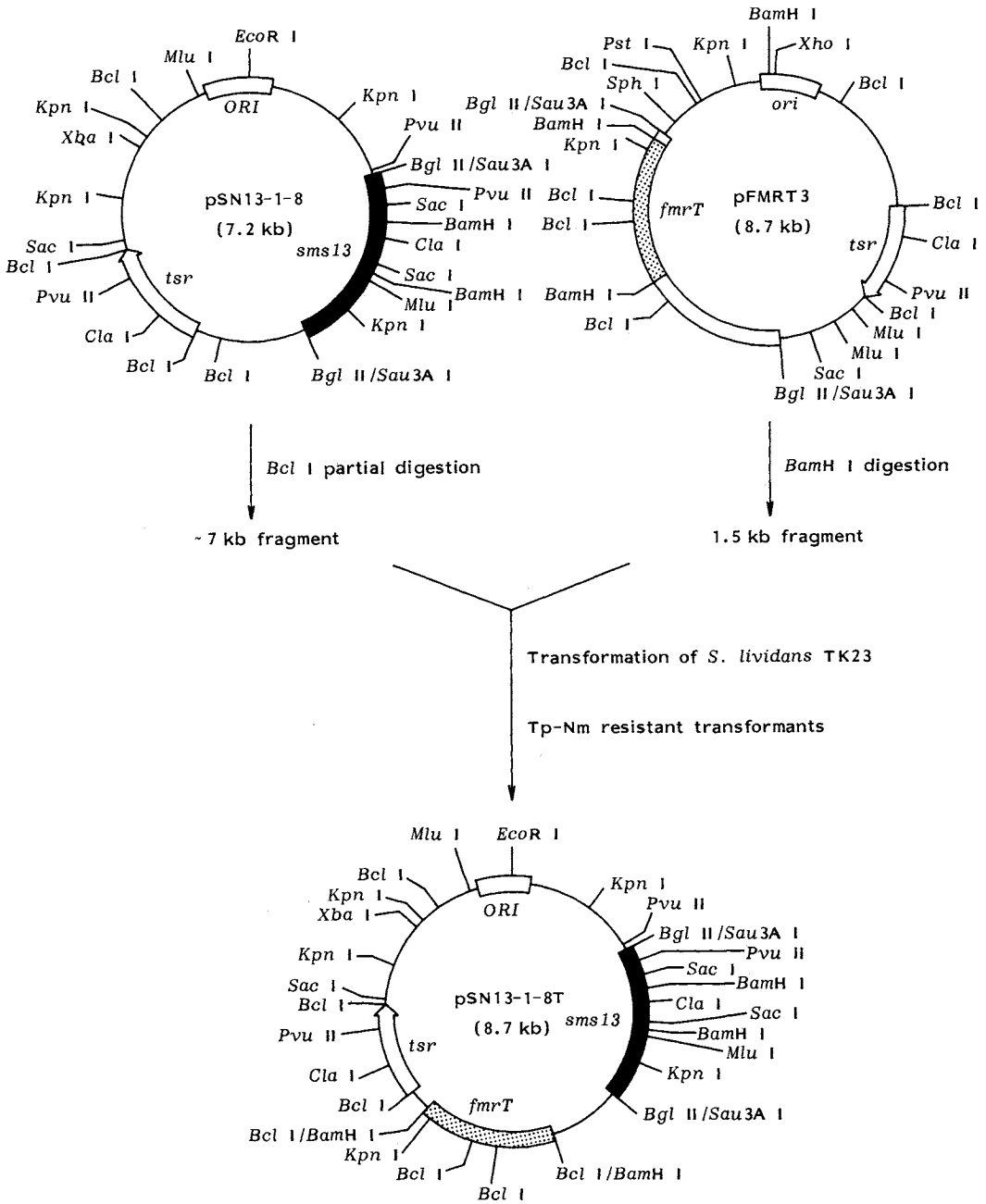
Construction of pSN13-1-8T Carrying *sms13* and a Fortimicin-resistance Gene

A scheme for the formation of pSN13-1-8T carrying both *sms13* and a fortimicin-resistance gene was

shown in Fig. 2. Plasmid pSN13-1-8 which was obtained by subcloning was partially digested with *Bcl* I and ligated to the 1.5 kb *Bam*H I fragment of pFMRT3[20] containing the fortimicin A-resistance gene (*fmrT*) cloned from *S. tenjimariensis* ATCC 31603. *S. lividans* TK23 was transformed with this DNA preparation. Thiopeptin-resistant transformants were transferred onto ATCC No. 5 medium containing neomycin B at 50 μg/ml to select the transformants harboring *fmrT*. *S. lividans* TK23 (pSN13-1-8T) carrying both *sms13* and *fmrT* was selected from these transformants by determination of the physical

Fig. 2. Construction scheme of pSN13-1-8T.

The DNA fragments containing *sms13*, *fmrT* and its flanking sequence are represented by filled, dotted and open boxes, respectively.



maps of the harbored plasmids.

Detection of Fortimicin B to Fortimicin A Converting Activity

Activity of sannamycin B-glycyltransferase in the washed mycelia was examined using fortimicin B as substrate according to DAIRI and HASEGAWA[13]. The products were detected by HPLC[13].

Alkaline Treatment of the Bioconversion Products of Fortimicin B

The fortimicin B-bioconverted products by the washed mycelia of *S. lividans* TK23 (pSN13-1-8T) were treated in 0.16 M NH_4OH or 0.16 N NaOH at room temperature for an hour and analyzed by using HPLC after neutrization with H_2SO_4.

Southern-blot Hybridization

Total DNAs isolated from various actinomycetes were digested with *Bam*H I and separated in 0.7% agarose gel electrophoresis. They were blotted to Genescreen plus (NEN) by the alkaline transfer method[21]. The 0.8 kb *Sal*I fragment of pSN13-1 was labeled with α-^{32}P-dCTP (Amersham) by nick-translation. Hybridization was performed at 65°C for 16 hours in a hybridization solution consisting of 6 × SSC[22], 5 × DENHARDT's solution[22], 100 μg/ml sonicated and denatured calf thymus DNA, with the radioactive DNA probe. Filters were washed twice at room temperature in 150 ml of 0.5 × SSC - 0.2% SDS for 15 minutes, and four times at 65°C in 250 ml of 0.3 × SSC - 0.2% SDS for 30 minutes. After drying, the filters were exposed to X-ray film (Fuji) at −80°C for 1 ~ 2 days.

Results

Cloning of *sms13*

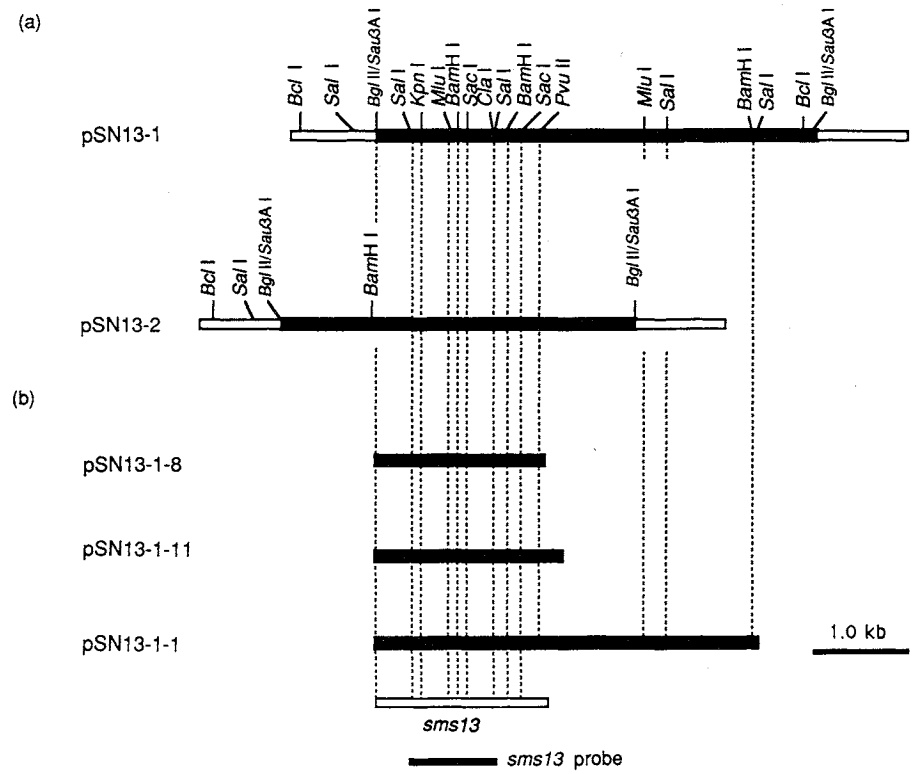
Glycylation of fortimicin B and sannamycin B (Fig. 1) are required to convert these antibiotics to biologically active molecules[6]. This amino acid transfer is an interesting characteristic of the biosynthesis of the fortimicin-group antibiotics. We have obtained the gene encoding the enzyme for this step by using *S. sannanensis* SN13, a mutant deficient in sannamycin B-glycyltransferase, as the host for cloning. Two transformants which restored antibiotic production were obtained from 6,126 thiopeptin-resistant transformants. These plasmids were designated pSN13-1 and pSN13-2, respectively (Fig. 3 a). A 2.7 kb overlapping region was observed in the inserts of the two plasmids. On subsequent subcloning, we obtained three types of plasmids from pSN13-1. Physical maps of their DNA inserts are shown in Fig. 3 b. Plasmid pSN13-1-8 contained a minimum 1.3 kb fragment which was common to the inserts of pSN13-1 and pSN13-2; *S. sannanensis* SN13 (pSN13-1-8) restored sannamycin A production. Thus, *sms13* could be located within this 1.3 kb. Fortimicin B to A converting activity was restored in the bioconversion using the washed mycelia of *S. sannanensis* SN13 (pSN13-1) (Fig. 4, a ~ c). Activity was also detected in the washed mycelia of *S. lividans* TK23 (pSN13-1-8T) harboring *sms13* together with the fortimicin-resistance gene (*fmrT*)[20] of *S. tenjimariensis* ATCC 31603 (Fig. 4, d ~ e). We also observed an unknown product at a retention time very close to that of *N*-formimidoylfortimicin A during HPLC analysis of the bioconverted products by *S. lividans* TK23 (pSN13-1-8T). However, it was not *N*-formimidoylfortimicin A because the peak was stable to alkaline treatment under conditions in which *N*-formimidoylfortimicin A was degradated to fortimicin A (Fig. 4 f). Thus, we concluded that the *sms13* gene product converted fortimicin B directly to fotimicin A.

Distribution of *sms13*-like Sequences in Actinomycetes

Southern-blot analysis was performed using the 0.8 kb *Sal*I fragment of pSN18-1-8 (Fig. 3 b) as a probe. Two signals, 0.8 and 0.55 kb, were detected in *Bam*H I-digested *S. sannanensis* DNA in agreement

Fig. 3. The physical maps of the cloned fragments containing *sms13*.

Inserted DNA fragments of pSN13-1, pSN13-2 (a) and the subcloned plasmids (b) are shown. Open box represents the proposal region of *sms13*. Thick bar represents the 0.8 kb *Sal* I fragment which was used as a probe in Southern blot hybridization analysis.



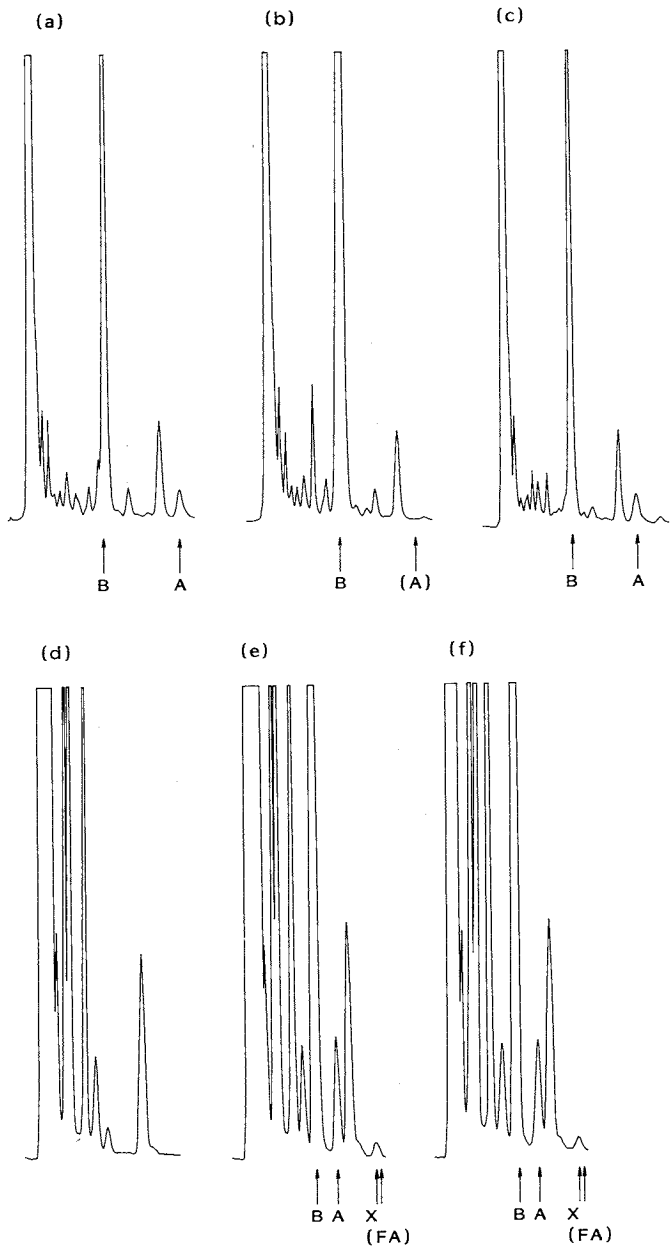
with the structure of pSN13-1 and pSN13-2 (Fig. 5). Positive signals of different size (0.6 to 6.0 kb) were detected in all the producers of fortimicin-group antibiotics. DNA homologous to *sms13* detected in *M. olivasterospora* is present in the gene cluster for fortimicin biosynthesis (DAIRI *et al.*; in preparation). The size of the DNA fragment detected in *M. olivasterospora* (6.0 kb *Bam*H I fragment) agreed with the organization of the gene cluster, which might encode fortimicin B-glycyltransferase (*fms13*). Analyses in which the genomic DNAs were digested with several restriction enzymes revealed significant conservation between *M. olivasterospora* and *Micromonospora* sp. SF-2098, independently isolated (data not shown). Under the hybridization-washing condition employed in this study, signals were detected in some strains of *Micromonospora* and *Dactylosporangium*, *i.e. M. purpurea* ATCC 15835 and *M. echinospora* subsp. *echinospora* ATCC 15837 both of which produced gentamicins, *M. zionensis* IFO 14116 which produced sisomicin, and *D. aurantiacum* ATCC 23491.

Discussion

We have cloned a DNA fragment encoding sannamycin B-glycyltransferase (*sms13*) using the self-cloning system of *S. sannanensis*. In both *S. sannanensis* and *S. lividans* carrying *sms13*, fortimicin B was efficiently converted to fortimicin A in spite of difference in structures between sannamycin B and fortimicin B. This agrees with the result of bioconversion studies using the washed mycelia of

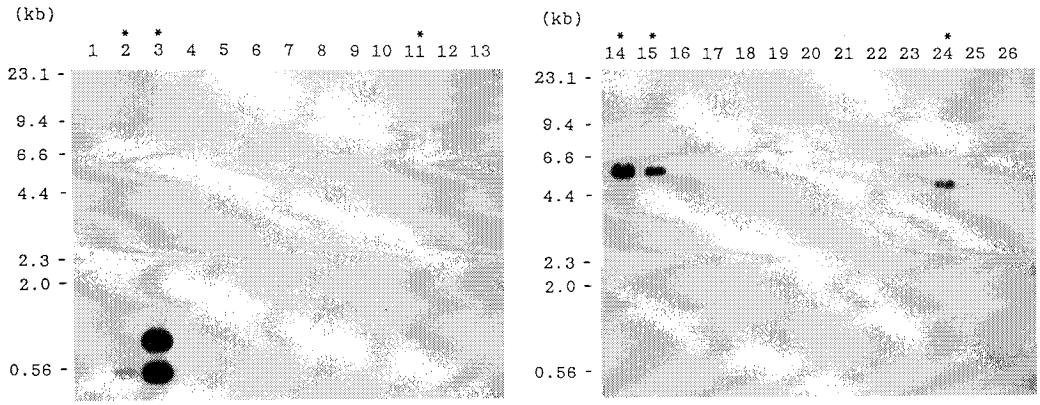
Fig. 4. Fortimicin B to fortimicin A converting activity by the *sms13* gene product.

(a)~(c); HPLC chromatograph of the bioconversion products of fortimicin B by the washed mycelia of *S. sannanensis* wild type (a), *S. sannanensis* SN13 (b), *S. sannanensis* SN13 (pSN13-1) (c). (d)~(f); HPLC chromatograph of the bioconversion products of fortimicin B by the washed mycelia of *S. lividans* TK23 (pSN13-1-8T), control experiment without the substrate (d), with the substrate (e) and the converted products after alkaline treatment in 0.16 N NH_4OH (f).



(a)~(c) and (d)~(f) were separated with 5.5% methanol and with 7.5% methanol, respectively. Positions of retention times of peaks, fortimicin B, fortimicin A, *N*-formimidoylfortimicin A and the unknown products are indicated as vertical arrows with B, A, (FA) and X, respectively.

Fig. 5. Distribution of DNAs homologous to *sms13*.



The 0.8 kb *Sal* I fragment within *sms13* was used as probe (see Fig. 3). Total DNAs were digested with *Bam*H I. Lanes are *Streptomyces lividans* TK23 (lane 1); *Streptomyces tenjimariensis* ATCC 31603 (istamycin producer) (lane 2); *Streptomyces sannanensis* IFO 14239 (sannamycin) (lane 3); *Streptomyces fradiae* ATCC 10745 (neomycin) (lane 4); *Streptomyces griseus* subsp. *griseus* (Waksman) (streptomycin) (lane 5); *Streptomyces kasugaensis* ATCC 15715 (kasugamycin) (lane 6); *Streptomyces hygroscopicus* subsp. *hygroscopicus* ATCC 27438 (hygromycin) (lane 7); *Streptomyces kanamyceticus* ATCC 12853 (kanamycin) (lane 8); *Streptomyces ribosidificus* ATCC 21294 (ribostamycin) (lane 9); *Streptomyces tenebrarius* IFO 13396 (nebramycin) (lane 10); *Saccharopolyspora hirsuta* ATCC 20501 (sporaricin) (lane 11); *Saccharopolyspora hirsuta* MK-220 (XK-220; a macrolide antibiotics) (lane 12); *Saccharopolyspora hirsuta* ATCC 27875 (lane 13); *Micromonospora olivasterospora* ATCC 21819 (fortimicin) (lane 14); *Micromonospora* sp. SF-2098 ATCC 31580 (SF-2052) (lane 15); *Micromonospora purpurea* ATCC 15385 (gentamicins) (lane 16); *Micromonospora sagamiensis* subsp. *nonreducans* ATCC 21803 (sagamicin and gentamicin A1) (lane 17); *Micromonospora sagamiensis* ATCC 21826 (sagamicin and gentamicin A1) (lane 18); *Micromonospora echinospora* subsp. *echinospora* ATCC 15837 (gentamicins) (lane 19); *Micromonospora echinospora* subsp. *pallida* ATCC 15838 (gentamicins) (lane 20); *Micromonospora inyonensis* ATCC 27600 (sisomicin) (lane 21); *Micromonospora zionensis* IFO 14116 (sisomicin) (lane 22); *Micromonospora echinospora* subsp. *ferruginea* ATCC 15836 (gentamicins) (lane 23); *Dactylosporangium matsuzakiense* ATCC 31570 (dactimicin) (lane 24); *Dactylosporangium aurantiacum* ATCC 23491 (lane 25); *Dactylosporangium variesporam* (capreomycin) (lane 26). Asterisks indicate the producers of the fortimicin-group antibiotics.

S. sannanensis[13]. Bioconversion of fortimicin B using *S. lividans* TK23 (pSN13-1-8T) confirmed that the cloned DNA fragments contained the structural gene of sannamycin B-glycyltransferase and not a regulatory gene.

Two different pathways have been postulated for the final stage of the biosynthesis of fotimicin A. In one, the final product, *N*-formimidoylfortimicin A, is directly synthesized from fortimicin B by addition of *N*-formimidoylglycine, and that fortimicin A is produced by a non-enzymatical decomposition of *N*-formimidoylfortimicin A[14]. The other is that fortimicin A is synthesized from fortimicin B by addition of one molecule of glycine, and consequently *N*-formimidoylfortimicin A is synthesized by addition of a formimidoyl group to the glycine moiety of fortimicin A[23]. Our results show the presence of a glycyltransferase directly converting fortimicin B to A in *S. sannanensis*; no activity converting fortimicin B to *N*-formimidoylfortimicin A was detected in *S. lividans* TK23 (pSN13-1-8T). The DNA fragment hybridizing with the *sms13* probe identified within the gene cluster for fortimicin A biosynthesis in *M. olivasterospora* (DAIRI *et al.*; in preparation) must encode fortimicin B-glycyltransferase. These results support the notion that the final steps of fortimicin A biosynthesis are fortimicin B → fortimicin A → *N*-formimidoylfortimicin A.

Sequences hybridizing to a DNA probe derived from the *sms13* gene were found in all six producers of the fortimicin-group antibiotics. They are likely to be genes similar to that for sannamycin B-glycyltransferase responsible for glycine transfer to their corresponding precursors. This suggests that

the sequences of the glycyltransferases are conserved among the producers of the fortimicin-group antibiotics. With respect to the common biosynthetic steps between *M. olivasterospora* and *S. sannanensis* and the sequence conservation of the biosynthetic genes among the producers of the fortimicin-group antibiotics[13], the biosynthetic genes might have evolved from a common set of ancestral genes and propagated beyond the genera.

In DNA hybridization studies using *sms13*, faint signals were detected in strains which produced the other aminoglycoside antibiotics, such as gentamicins and sisomicin. These signals could be due to similarity with *sms13* or its flanking sequences. These strains are not known to produce any of the fortimicin-group antibiotics. Considering the structures of gentamicins and sisomicin, glycyltransferase activity would not be involved in their biosynthesis. This suggests that they have silent glycyltransferase genes which are not expressed under laboratory conditions, or that there may be some sequence relationships within the biosynthetic genes for aminoglycoside antibiotics. Further work is needed to determine the sequences of these related genes and the functions of the gene products.

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