STRAIN- AND SPECIES-SPECIFIC DISTRIBUTION OF THE STREPTOMYCIN GENE CLUSTER AND *kan*-RELATED SEQUENCES IN *STREPTOMYCES GRISEUS*

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The streptomycin (SM) gene cluster was investigated for its distribution in streptomycetes by Southern hybridization using nick-translated DNA probes, which were isolated from the SM-6-phosphotransferase (SPH) and amidinotransferase (ADT) regions of the SM gene cluster of *Streptomyces griseus* SS-1198. *Bgl* II-digested genomic DNAs from SM-producing strains of *S. griseus* yielded the same size fragment (7.0 kb) which hybridized to both the SPH and ADT probes as expected from the restriction endonuclease cleavage map of the SM gene cluster. By contrast, no genomic DNA fragments from heterologous *Streptomyces* strains hybridized to the probes. Thus, only SM-producing strains of *S. griseus* possess the highly homologous SM gene cluster.

Similarly, distribution of DNA sequences homologous to the kanamycin (KM)-resistance determinant (*kan*) from a KM-resistant regenerant of *S. griseus* SS-1198 protoplasts was also examined. Using the *kan* gene fragment as the probe it was revealed that the *kan*-related sequences are present in all the strains of *S. griseus* tested, irrespective of the type of antibiotics they produce. However, no hybridization to the *kan* gene probe (KAN) was observed with DNA digests derived from other *Streptomyces* species.

It is well recognized that antibiotic production by actinomycetes is not species-specific but strain-specific. However, little is known concerning the biochemical and genetic basis for the strain specificity of antibiotic production in spite of numerous studies on antibiotic biosynthesis and its regulation. We have shown that *Streptomyces* strains which produce aminoglycoside (AG) antibiotics have individual AG-resistance patterns correlated with the type of antibiotics they produce[1,2]. In addition to this biochemical relationship, the genetic linkage between antibiotic biosynthetic genes and antibiotic-resistance genes has also been demonstrated in various antibiotic producers[3~8]. Consequently, it was of particular interest to determine whether the strain-specific production of antibiotics was associated with a localized distribution or expression of the relevant antibiotic gene cluster in antibiotic-producing strains.

Streptomyces griseus includes strains that produce various types of antibiotics such as streptomycin (SM), holomycin and grisein. Since high DNA homologies have been demonstrated among *S. griseus* strains[9], it was of interest to establish the genetic basis associated with the strain-specific production of antibiotics in *S. griseus*. In the SM-producing strains of this species, a gene cluster involving SM-biosynthetic genes and SM-resistance gene has been cloned and well characterized by different laboratories[3,10~15]. We have also cloned a gene segment (3.8 kb *Sph* I) directing SM-resistance from an SM-producing soil-isolate of *S. griseus*[16]. Interestingly, the cloned segment was found to be identical with the region covering SM-resistance and amidinotransferase determinants of the known SM gene cluster in terms of size and restriction site. This fact led us to a hypothesis that distribution of homologous SM gene clusters might be responsible for the strain-specific produc-

tion of SM in *S. griseus*. In order to establish this point, we performed Southern hybridization between gene segments located in the SM gene cluster and genomic DNA digests of SM producers as well as nonproducers.

Of further interest to us was the finding that kanamycin (KM)-hyper-resistant clones emerged following protoplast regeneration of a streptomycin-producing strain of *S. griseus*[16] in which no KM-resistance had been reported previously. Characterization of the cloned KM-resistance determinant (*kan*) revealed that a cryptic gene (*kan*^{0}) had mutated to *kan* directing the synthesis of an aminoglycoside acetyltransferase, AAC(3)[17]. This dramatic change in antibiotic-resistance seemed to be so unique that we were interested in the distribution of *kan*-related sequences in *Streptomyces*. Therefore, Southern hybridization experiments were carried out to establish whether *kan* exhibited any homology with genomic DNA from other *S. griseus* strains and other AAC(3)-producing streptomycetes.

In this report we reveal that the SM gene cluster is homologous and specifically distributed among SM-producing strains of *S. griseus* and that the distribution of homologous sequences to *kan* appears to be limited to *S. griseus* strains regardless of the types of antibiotics they produce.

Materials and Methods

Strains Used

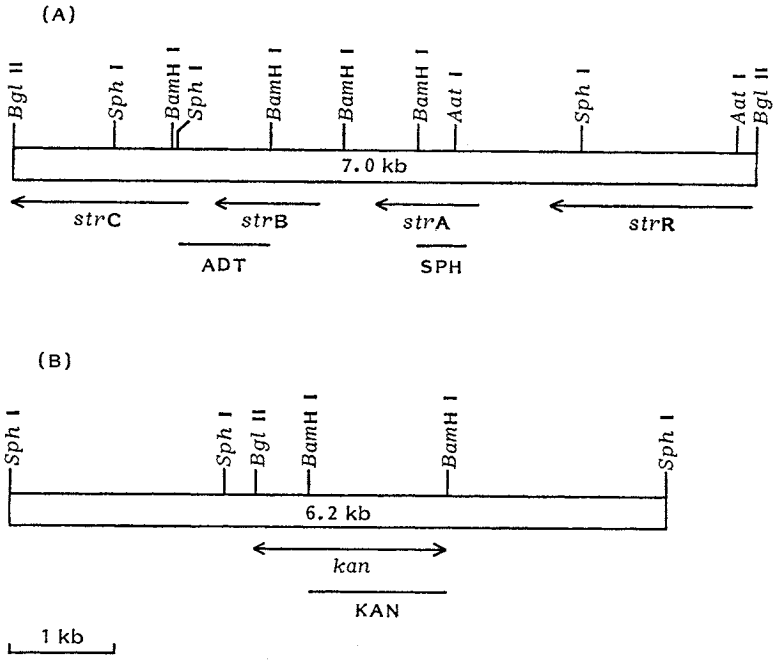
S. griseus strains which produce SM or other antibiotics, SM-producing strains of *Streptomyces bikiniensis* and *Streptomyces* sp., and other *Streptomyces* species which produce aminoglycoside antibiotics are listed in Table 1.

Table 1. Strains used.

Species	Strains	Antibiotics	Remarks	refs
Streptomyces griseus	SS-1198	Streptomycin	Sapporo, Japan	16
S. griseus	SS-1254[a]	Streptomycin	Tenjin Island, Japan	This work
S. griseus	ISP 5236	Streptomycin	Rutgers Univ.	9
S. griseus	HUT6037	Streptomycin	Hiroshima Univ.	28
S. griseus	N2-3-11	Streptomycin	Kaken, Japan	22
S. griseus	NIHJ 018	Grisein	NIH Japan	This work
S. griseus	NIHJ 060	Grisein	NIH Japan	This work
S. griseus	SS-1429[a]	Holomycin	SM-resistance	This work
S. griseus	MH541-f'-F3	Holomycin	IMC	This work
S. griseus	MH885-SF1	Chromomycin	IMC	This work
S. griseus	MH324-22-F9	Cycloheximide	IMC	This work
S. bikiniensis	ISP 5581	Streptomycin	SM-6-phosphotransferase	22, 29
Streptomyces sp.	SS-1696	Streptomycin	Gray color surface	This work
Streptomyces sp.	SS-1740	Streptomycin	Gray color surface	This work
S. fradiae	ISP 5063	Neomycin	APH(3'), AAC(3)	2, 30, 31
S. kasugaensis	MB273	Kasugamycin	AAC(3)-I (unpublished)	20
S. lavendulae	SS-1364	Neomycin	APH(3'), AAC?	2
S. lavendulae	SS-1365	Ribostamycin	APH(3'), AAC?	2
S. tenebrarius	ISP 5477	Tobramycin	SM-6-phosphotransferase	32
S. tenjimariensis	SS-939	Istamycin		33
S. lividans	TK21			34

[a] Taxonomic properties fell into those of *S. griseus* (data not shown).
IMC: Institute of Microbial Chemistry.

Fig. 1. Probes for detection of the streptomycin gene cluster and *kan*-related gene.



(A) Streptomycin gene cluster (7.0 kb *Bgl* II fragment) and ADT and SPH probes.
(B) Genomic DNA segment (6.2 kb *Sph* I fragment) containing *kan* region and KAN probe.

Preparation of DNA

Total DNA was isolated from *Streptomyces* strains as previously described[16]. DNA fragments used as probes for the SM gene cluster and the *kan* gene were isolated from the plasmids pANT1 and pANT3-1, respectively, by the method of GIRVITZ *et al.*[18] after digestion with appropriate restriction endonucleases and separation by agarose gel-electrophoresis. The 0.4-kb *Sal* I and 0.9 kb *Bam*H I-*Sph* I fragments corresponding to the SM-6-phosphotransferase (SPH) and amidinotransferase (ADT) determinants, respectively, isolated from pANT1 were used as the SPH and ADT probes. The 1.3-kb *Bam*H I fragment (pANT3-1) was used as the KAN probe (Fig. 1). Radiolabeled fragments ($1 \sim 2 \times 10^8$ cpm/μg) were obtained with [α-^{32}P]dCTP using a nick translation kit (Amersham).

Southern Blot Hybridization

Total DNA was digested completely with *Bam*H I or *Bgl* II and the fragments were separated by electrophoresis using 0.8% agarose in 1×TAE buffer. DNA fragments were denatured with 0.5 N NaOH - 1.5 M NaCl, neutralized with 0.5 M Tris-HCl (pH 8.0) and transferred to a nitrocellulose filter (BA85, 0.45 μm; SCHLEICHER and SCHUELL) with 10×SSC; the filter was then air-dried and baked at 80°C for 2 hours as described by SOUTHERN[19]. Prehybridization was performed overnight at 55~65°C in 6×SET (0.9 M NaCl, 12 mM EDTA and 180 mM Tris-HCl, pH 8.0), 10×DENHARDT's solution, 0.1% SDS, 100 μg/ml of sonicated and denatured calf thymus DNA; hybridization was carried out under the same conditions for 45 hours in fresh using 5~10 ng/ml of a probe. After hybridization the filter was washed 3 times with 2×SSC (200 ml) at increasing temperatures (60, 65 and 75°C) for 30 minutes each. Autoradiography was carried out by exposing X-ray film (Fuji RX) to the filter overnight at −80°C.

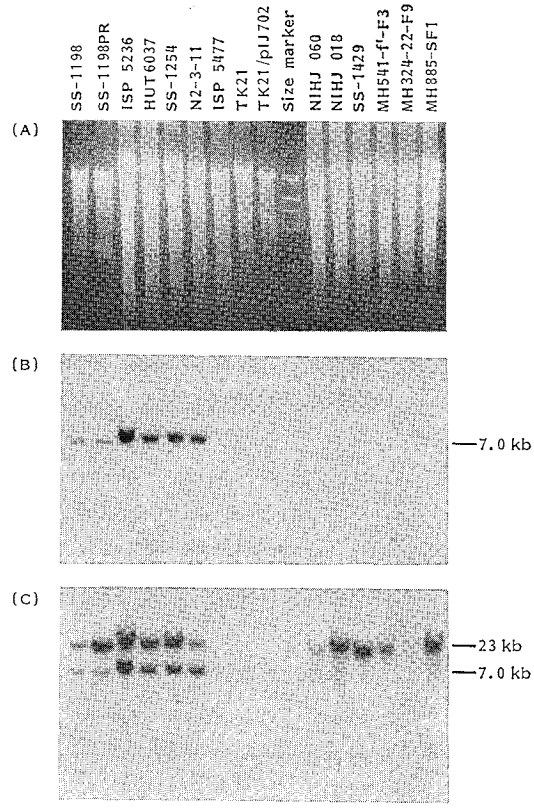
Results

Distribution of the SM Gene Cluster

In order to examine the distribution of the SM gene cluster in *Streptomyces* strains, we carried

out hybridization experiments using *Bgl* II-digested genomic DNA with the SPH (0.4 kb *Sal* I) and ADT (0.9 kb *Bam*H I - *Sph* I) gene probes. If the SM gene cluster was homologous in *Streptomyces* strains, it would be expected that *Bgl* II-digests of genomic DNA would possess a 7.0-kb hybridizing fragment (Fig. 1). Indeed, *Bgl* II-digests from all of the SM-producing strains (left 6 lanes) of *S. griseus* contained the same sized fragment (7.0 kb) which hybridized strongly to both the SPH and ADT probes (Fig. 2B). It is of interest that these strains included isolates collected from soils in the U.S.A. and Japan (Table 1). Hybridization was so intense that the radiolabeled probes could not be removed from the filter by washing with 2× SSC at 90°C (30 minutes); dissociation necessitated the treatment of the filter in boiling water for 15 minutes. These results clearly indicate that there is a high degree of homology between the probes and the target sequences. In contrast, DNA digests from *S. griseus* strains (right 6 lanes) that produce grisein, holomycin, cycloheximide or chromomycin, or from different *Streptomyces* species failed to hybridize to the probes. Interestingly, digests from other SM-producing organisms (*e.g. S. bikiniensis* ISP 5581 and *Streptomyces* sp. SS-1696 and SS-1740) also failed to hybridize to the SPH probe (data not shown). Furthermore, no homologous sequence (to SPH) was seen in the tobramycin-producing *Streptomyces tenebrarius* ISP 5477 or holomycin-producing *S. griseus* SS-1429 digests, although the former organism is known to produce an SM-6-phosphotransferase[20] and the latter is resistant to SM (100 μg/ml). Thus, it appears that the homologous SM gene cluster of *S. griseus* is specifically present in SM-producing strains of *S. griseus*.

Fig. 2. Hybridization between *Bgl* II-digested genomic DNA and probes for streptomycin gene cluster and *kan*-related genes.



(A) Agarose gel electrophoresis of *Bgl* II-digested genomic DNA. Size marker: Mixture of *Hind* III-digested λDNA and *Hae* III-digested φX174DNA was used.

(B) Hybridization with ADT and SPH probes. Hybridization between *Bgl* II-digested DNA fragments and SPH probe was erased by washing the filter in boiling water and then hybridization with ADT probe was performed. Exactly the same hybridization signals were obtained.

(C) Hybridization with KAN probe was carried out by using the filter used for hybridization with SPH and ADT probes (B).

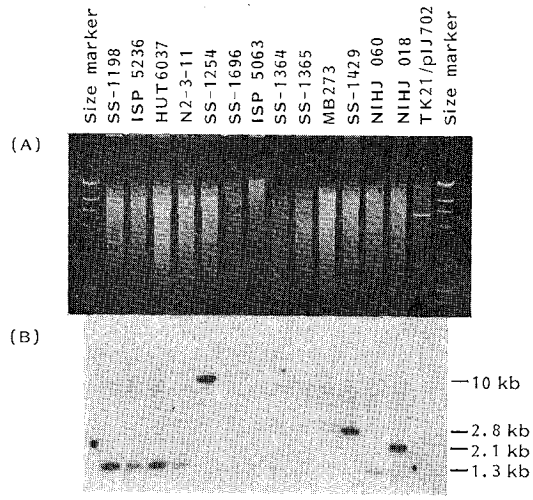
Distribution of Genes Homologous to the *kan* Gene

The filter previously used for the hybridization experiment with *Bgl* II-digested genomic DNA was subsequently subjected to Southern analysis with the KAN probe (Fig. 2C). Large fragments (20~23 kb) hybridizing to the probe were observed in all of the SM-producing strains of *S. griseus* (left 6 lanes). Unexpectedly, hybridization signals were also observed in the other *S. griseus* strains

(right 6 lanes) which produce different antibiotics. However, no hybridization signals were detected with the DNA digests from the other *Streptomyces* sp. tested.

Hybridization analysis using the *Bam*H I-digests of genomic DNAs and the KAN probe provided more conclusive results. Thus, all of the SM-producing *S. griseus* strains examined (left 4 lanes) except SS-1254 contained the same sized fragment (1.3 kb) which hybridized to the KAN probe. In comparison, the *Bam*H I-digest of genomic DNA from the latter strain possessed a larger hybridizable fragment, about 10 kb (5th lane from the left), although its DNA finger print and the SM gene cluster were indistinguishable from those of the other SM-producing strains of *S. griseus*. The digests from the other *S. griseus* strains (SS-1429, NIHJ 060 and NIHJ 018) also contained hybridizing fragments (2.8, 1.3 and

Fig. 3. Hybridization between *Bam*H I-digested genomic DNA and KAN probe.



(A) Agarose gel electrophoresis of *Bam*H I-digested genomic DNA. Size marker: Same as Fig. 2(A).
(B) Hybridization with KAN probe.

Table 2. Summary of hybridization between genomic DNA digests and SPH, ADT and KAN probes.

Species	Strains	Antibiotics	Hybridization		
			SPH and ADT	KAN	
			Bgl II-digest (kb)	*Bam*H I-digest (kb)	*Bgl* II-digest (kb)
S. griseus	SS-1198	Streptomycin	7.0	1.3	20~23
S. griseus	ISP 5236	Streptomycin	7.0	1.3	20~23
S. griseus	HUT6037	Streptomycin	7.0	1.3	20~23
S. griseus	N2-3-11	Streptomycin	7.0	1.3	20~23
S. griseus	SS-1254	Streptomycin	7.0	10	20~23
S. griseus	NIHJ 018	Grisein	—	2.1	20~23
S. griseus	NIHJ 060	Grisein	—	1.3	20~23
S. griseus	SS-1429	Holomycin	—	2.8	20~23
S. griseus	MH541-f'-F3	Holomycin	—	nt	20~23
S. griseus	MH885-SF1	Chromomycin	—	nt	20~23
S. griseus	MH324-22-F9	Cycloheximide	—	nt	20~23
S. bikiniensis	ISP 5581	Streptomycin	—	—	—
Streptomyces sp.	SS-1696	Streptomycin	—	—	—
Streptomyces sp.	SS-1740	Streptomycin	—	—	—
S. fradiae	ISP 5063	Neomycin	—	—	—
S. kasugaensis	MB273	Kasugamycin	—	—	—
S. lavendulae	SS-1364	Neomycin	—	—	—
S. lavendulae	SS-1365	Ribostamycin	—	—	—
S. tenebrarius	ISP 5477	Tobramycin	—	—	—
S. tenjimariensis	SS-939	Istamycin	—	—	—
S. lividans	TK21		—	—	—

nt: Not tested.

2.1 kb, respectively). However, no hybridization signals were detected in the other *Streptomyces* species irrespective of the type of aminoglycoside antibiotic and AAC produced. Thus, it appears reasonable to conclude that the *kan*-related genes are specifically associated with *S. griseus* species, as summarized in Table 2.

Discussion

As described, hybridization studies have revealed that a SM gene cluster, which is highly homologous, is specifically distributed in SM-producing strains of *S. griseus*. Further, DNA sequences homologous to this gene cluster in heterologous *Streptomyces* strains including SM-producing *S. bikiniensis* appear to be absent. In this context, DISTLER *et al.*[13] have shown that a genomic digest from a dihydro-SM-producing strain of *Streptomyces glaucescens* did not hybridize to the SPH gene of an SM-producing strain of *S. griseus*. It should also be noted that a gene segment, cloned from SM-producing *S. bikiniensis* IFO 13350 (derived from *S. bikiniensis* ISP 5235) and directing the biosynthesis of the *N*-methyl-L-glucosamine moiety of SM, possesses a region that overlaps part of the SM gene cluster of *S. griseus*[21]. Such homology may be attributable to the fact that this strain is accidentally derived from *S. griseus* ISP 5236 and had been improperly designated as an *S. bikiniensis* strain[22,23]. In support of this assignment, OKANISHI *et al.* have demonstrated that there is a 100% DNA-DNA homology between the strains ISP 5235 and ISP 5236[9]. Thus, it is obvious that the strain specific distribution of a SM gene cluster is associated with the strain specific production of SM in *S. griseus*. It would be of interest to establish whether the SM-biosynthetic genes of SM-producing strains of other *Streptomyces* species are clustered or not.

Genes (*afs*A and *afs*B) specifying A-factor have been reported to regulate positively SM production in *S. griseus*[24,25]. These regulatory genes may represent other genetic determinants associated with SM production. However, based on hybridization studies, distribution of genes homologous to these A-factor-related genes is not limited to SM-producing strains of *S. griseus*. Therefore, the SM gene cluster can be regarded as the marker genotype for SM production in *S. griseus*. In this regard, the antibiotic biosynthetic gene clusters described for several other antibiotic-producing strains of actinomycetes do not possess the same limited distribution. It has been shown that the "early" gene(s) (*act*I) of the actinorhodin gene cluster are widely distributed among heterologous polyketide-producing *Streptomyces* species[26]. Likewise, DNA fragments hybridizing to the undecylnorprodigiosin: *S*-Adenosylmethionine *O*-methyltransferase gene involved in the biosynthesis of undecylnorprodigiosin[27] were also reported to be distributed widely and thus are not confined to specific strains or species that produce the antibiotic. Based on these reports it appears that the SM gene-cluster of *S. griseus* is unique.

Unexpectedly, all of the *S. griseus* strains tested had sequences homologous to the *kan* gene directing an AAC(3). In contrast, no heterologous species examined possessed hybridizing DNA fragments regardless of their synthesis of AAC (3). It should be noted, in the case of *S. griseus* strains, that *kan*-related sequences represent the first case of species-specific DNA sequence in *Streptomyces*. This finding suggests some specific role(s) for the *kan*-related gene(s) in the life of *S. griseus*, although no information is available about their function at present.

The species-specific distribution of *kan*-related sequence may have taxonomic importance. For instance, strain SS-1429 had not been subjected to taxonomic identification until the sequence homologous to *kan* was discovered. A detailed study revealed that the strain SS-1429 belonged taxonomically to *S. griseus* (data not shown). Thus, *kan*-related sequences may be a useful genotypic marker for identification of *S. griseus* strains.

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