Cloning of Aminoglycoside-resistance Determinants from *Streptomyces tenebrarius* and Comparison with Related Genes from Other Actinomycetes

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At least two aminoglycoside-resistance determinants from *Streptomyces tenebrarius* have been cloned separately in *Streptomyces lividans*. In each case, resistance (to kanamycin plus apramycin or to kanamycin plus gentamicin) was expressed at the level of the ribosome and involved specific methylation of 16S ribosomal RNA. Hybridization and restriction analysis revealed that related genes were present in other aminoglycoside-producing actinomycetes.

INTRODUCTION

The manner in which aminoglycoside-producing actinomycetes are able to tolerate their products has received considerable attention. For example, it has been well documented that some such organisms possess aminoglycoside-modifying enzymes, most of which inactivate their substrates *in vitro* (for a review, see Benveniste & Davies, 1973). Moreover, unequivocal evidence that some of these enzymes can confer resistance to aminoglycosides (at varying levels) has been obtained by cloning the relevant genes in a sensitive host, such as *Streptomyces lividans* (Thompson *et al.*, 1982a; Crameri & Davies, 1986). On the other hand, several aminoglycoside producers have been shown to possess antibiotic-resistant ribosomes, regardless of whether they also possess drug-modifying enzymes. One such strain is *Streptomyces tenebrarius*, producer of the nebramycin complex which includes tobramycin and other derivatives of kanamycin B plus the novel aminoglycoside, apramycin. Ribosomes from this organism were found to be highly resistant *in vitro* to kanamycins A, B and C, to the gentamicin C complex and also to neamine and ribostamycin (which comprise, respectively, two or three rings of the four-ring neomycin molecule), although they were somewhat more sensitive to neomycin (Yamamoto *et al.*, 1982). Subsequently, these findings were confirmed and extended (Piendl *et al.*, 1984). Ribosomes from *S. tenebrarius* were shown to be resistant also to apramycin and the 30S sub-particle was implicated in the expression of these various effects. Furthermore, when hybrid 30S ribosomal subunits (containing combinations of RNA and proteins from *S. tenebrarius* and *S. lividans*) were reconstituted *in vitro* and assayed in the presence of gentamicin, it was shown that some property of 16S rRNA from *S. tenebrarius* was responsible for ribosomal resistance to that drug (Piendl *et al.*, 1984).

The aim of the present work was to define the mechanism(s) responsible for all the ribosomal resistance characters of *S. tenebrarius*. Given the complexity of the resistance-profile, it was decided at the outset to clone individual aminoglycoside-resistance genes and to study the effects of their products in a sensitive background, i.e. in *S. lividans*. This work was aided by the results of other studies, then in progress, concerning ribosome-associated resistance to aminoglycosides in *Micromonospora purpurea* (the gentamicin producer) and in *Streptomyces tenjimariensis* – producer of istamycin. Fragments of DNA from these two organisms had been cloned in *S. lividans*, thereby generating two populations of strains with characteristic and dissimilar resistance profiles. Both, however, possessed aminoglycoside-resistant ribosomes and, in either case, resistance was due to methylation of 16S rRNA. Clones containing DNA from *M.*

purpurea were highly resistant to kanamycin plus gentamicin (Thompson *et al.*, 1985), whereas those possessing DNA from *S. tenjimariensis* were highly resistant to kanamycin and apramycin but were sensitive to modest levels of gentamicin (Skeggs *et al.*, 1985). The availability of those strains for comparative purposes greatly facilitated the present analysis.

METHODS

Bacterial strains and media. The following strains were used: *Streptomyces tenebrarius* NCIB 11028 (nebramycin producer) and *Escherichia coli* MRE 600, plus several strains of *Streptomyces lividans*: TK 21 (a plasmid-free derivative of *S. lividans* 66 obtained from Professor D. A. Hopwood, John Innes Institute, Norwich, UK); TSK 1 control strain (strain TK 21 containing the high-copy-number cloning vector, pIJ702, see Katz *et al.*, 1983); JR 14 resistant to kanamycin plus gentamicin (strain TK 21 containing pLST14; i.e. pIJ702 with 3.6 kb of *M. purpurea* DNA inserted into the *SphI* site, see Thompson *et al.*, 1985); TSK 41 resistant to kanamycin and apramycin (strain TK 21 containing pLST41; i.e. pIJ702 with 3.7 kb of *S. tenjimariensis* DNA inserted into the *BglII* site, see Skeggs *et al.*, 1985); TSK 412 resistant to kanamycin and apramycin (strain TK 21 containing pLST412, i.e. pIJ702 with about 0.8 kb of *S. tenjimariensis* DNA inserted into the *SphI* site, see Skeggs *et al.*, 1985). All *Streptomyces* strains were grown at 30 °C in tryptic soy broth (Difco) or on NE plates. The latter contained 1% (w/v) glucose, 0.2% (w/v) yeast extract, 0.2% (w/v) Casamino acids, 0.1% (w/v) beef extract, 2% (w/v) agar, and the pH was adjusted to 7.0 with KOH prior to autoclaving. Soft NE agar contained 0.7% (w/v) agar.

Generation and selection of aminoglycoside-resistant clones of *S. lividans*. Total DNA from *S. tenebrarius* (prepared according to a standard procedure – Chater *et al.*, 1982) was partially digested with *Sau3A* and the products were size-fractionated to yield 5–10 kb fragments as described previously (Skeggs *et al.*, 1985). Of these, 5 µg was mixed with 1 µg of vector plasmid pIJ702 (previously linearized with *BglII*) and ligation was carried out using T4 DNA ligase for 1 h at 22 °C, followed by overnight incubation at 4 °C, at a final DNA concentration of 40 µg ml⁻¹. The ligation mixture was used to transform 4 × 10⁹ protoplasts from *S. lividans* TK 21, which were allowed to regenerate on ten R2YE agar plates (Thompson *et al.*, 1982b). After 20 h at 30 °C, the plates were overlaid with soft NE agar containing thiostrepton (20 µg ml⁻¹ final concentration per plate) to select for transformants containing the vector plasmid. These were then replica-plated onto NE agar plates containing thiostrepton (20 µg ml⁻¹, final concentration) plus an aminoglycoside (kanamycin, gentamicin, apramycin or neomycin at 50 µg ml⁻¹).

Preparation of probes for Southern analysis. Digests of pLST14 (with *EcoRI* plus *BamHI*) and pLST41 (with *KpnI* plus *BglII*) were prepared as recommended by the suppliers of the restriction endonucleases and the products were separated by electrophoresis in low-melting-temperature agarose. In either case, the desired restriction fragment was the fastest-moving species in the gel, from which it was excised, denatured and annealed to mixed sequence hexadeoxynucleotides (Pharmacia). These were extended using DNA polymerase Klenow fragment (BRL) as described by Feinberg & Vogelstein (1984) in the presence of [γ -³²P]dCTP [Amersham; 3000–4000 Ci mmol⁻¹ (111–148 TBq mmol⁻¹)]. Then, unincorporated nucleotides were removed using a column (3 × 0.5 cm) of Sephadex G50 equilibrated with 3 mM-Tris/HCl (pH 7.0 at 20 °C) containing 0.2 mM-Na₂EDTA. The radiolabelled probe was eluted from the column in about 450 µl of this buffer, mixed with 50 µl of an aqueous solution of denatured salmon sperm DNA (2 mg ml⁻¹, final concentration), heated for 10 min at 90–100 °C and then used directly in Southern analysis, as described below.

Southern analysis. Digests of pIJ702 and its derivatives were prepared using various restriction endonucleases as follows: for pIJ702, *KpnI*; for pLST14 and pLST31, *EcoRI* plus *BamHI*; for pLST41 and pLST51, *KpnI* plus *BglII*. The DNA fragments were separated on a 0.7% (w/v) agarose gel (approx. 100 ng per lane) using as running buffer 40 mM-Tris/acetate (pH 7.8 at 20 °C) containing 2 mM-Na₂EDTA. Then, DNA in the gel was denatured, neutralized and transferred onto Genescreen Plus (New England Nuclear) by the capillary blot procedure described by the manufacturers. The DNA on the filter was then reprocessed to ensure complete denaturation before being neutralized and dried, again as recommended by the manufacturer.

Prehybridization of the filter was carried out inside a sealed bag at 42 °C for 16 h with gentle agitation in 10 ml of solution containing 50% (v/v) deionized formamide, 1% (w/v) SDS, 1 M-NaCl and 10% (w/v) dextran sulphate. Then, 0.5 ml of a solution (prepared as above) containing radio-labelled probe and denatured salmon sperm DNA was added to the bag and hybridization was carried out at 42 °C for 18 h with gentle agitation. The filter was then washed with 2 × 100 ml of 2 × SSC (SSC is 150 mM-NaCl plus 15 M-sodium citrate) at 20 °C for 5 min followed by two further washes using 2 × SSC plus 1% (w/v) SDS at 55 °C for 30 min and two final washes with 0.1 × SSC at 20 °C for 30 min. The filter was then dried at 20 °C and subjected to autoradiography at 20 °C using Fuji RX film.

Preparation of ribosomes and assays of cell-free protein synthesis. Ribosomes, ribosomal subunits and 100000 g post-ribosomal supernatant (S100*; so designated because it contained materials removed from ribosomes by 'high-salt' buffer containing 30 mM-MgCl₂ plus 1 M-NH₄Cl) were prepared and the synthesis of polyphenylalanine directed by polyuridylic acid was followed as described previously (Skeggs *et al.*, 1985). Regardless of the source of ribosomes or their subunits, these were always used together with S100* from *S. lividans* TSK 1 (control strain).

Methylation of 30S ribosomal subunits. Ribosomal 30S subunits from *E. coli* (20 pmol) were incubated at 35 °C for 1 h together with 5 µl of S100* from *S. lividans* TSK 31 or TSK 51 (as sources of methylase activity) plus *S*-adenosylmethionine as cofactor under the conditions described previously (Skeggs *et al.*, 1985). The cofactor was omitted from control incubations. After precipitation with ethanol, 30S particles were resuspended in HRS buffer (10 mM-HEPES/KOH, pH 7.5 at 20 °C; 10 mM-MgCl₂; 50 mM-NH₄Cl; 3 mM-2-mercaptoethanol), supplemented with *E. coli* 50S ribosomal subunits plus S100* from *S. lividans* TSK 1 and assayed in cell free protein synthesis as above.

Antibiotics. These were obtained as follows: thiostrepton (Squibb Institute, Princeton, NJ, USA); sisomicin sulphate, (J. Davies, Pasteur Institute, Paris, France); neamine and neomycin sulphate (Upjohn Co., Kalamazoo, Mich., USA); butirosin sulphate (Warner-Lambert Co., Ann Arbor, Mich.); ribostamycin sulphate (Meiji Seika Kaisha, Ltd, Yokohama, Japan); gentamicin sulphate (Sigma).

RESULTS AND DISCUSSION

Cloning and genetic analysis of resistance determinants

Fragments of DNA from *S. tenebrarius* were cloned in *S. lividans* TK 21, using as vector the multicopy plasmid, pIJ702, and thiostrepton-resistant transformants were replica plated on kanamycin, gentamicin or apramycin (each at 50 µg ml⁻¹). Several colonies were obtained on gentamicin and on apramycin although, as it happened, none grew on the plates containing kanamycin. However, all the clones selected on the former two aminoglycosides subsequently proved to be resistant to kanamycin. Two such strains, designated *S. lividans* TSK 31 and TSK 51 (originally selected on gentamicin and apramycin, respectively), were chosen for further investigation. Each contained a recombinant plasmid (pLST31, about 9.2 kb, and pLST51, about 9.9 kb, respectively), that conferred the aminoglycoside resistance characteristic of strain TSK 31 or TSK 51 when retransformed into *S. lividans* TK 21. Those patterns of collateral resistance are given in Table 1 as MIC values, together with comparable data relating to *S. tenebrarius* and the control strain, *S. lividans* TSK 1 (the latter being *S. lividans* TK 21 containing the vector pIJ702). Evidently, strains TSK 31 and TSK 51 were both highly resistant to kanamycin, whereas gentamicin and apramycin discriminated between them. These resistance phenotypes seemed familiar and this prompted us to compare strains TSK 31 and TSK 51 with two clones that had been generated in earlier studies. They were *S. lividans* strains JR 14 and TSK 412 which, respectively, contain DNA from the gentamicin producer *M. purpurea* and from *S. tenjimariensis*, the producer of istamycin (Thompson *et al.*, 1985; Skeggs *et al.*, 1985). At this stage of the investigation, it was also confirmed by Southern analysis that probes derived from the inserts in pLST31 and pLST51 each hybridized with DNA separately prepared from *S. tenebrarius* (data not given). The data in Table 1 extended the range of aminoglycosides with which strains JR 14 and TSK 412 had previously been challenged and revealed close similarities between *S. lividans* JR 14 and TSK 31 on the one hand and strains TSK 412 and TSK 51 on the other. Given that *S. tenebrarius* was highly resistant to each of the drugs listed in Table 1, it was

Table 1. *Minimal inhibitory concentrations (µg ml⁻¹) of aminoglycoside antibiotics for S. lividans strains and for S. tenebrarius*

Spores were streaked on NE agar plates (see Methods) containing antibiotics and growth was monitored after 3–5 d incubation at 30 °C.

Antibiotic	<i>S. lividans</i> strain					<i>S. tenebrarius</i>
	TSK 1 (control)	JR 14	TSK 31	TSK 51	TSK 412	
Kanamycin	10–30	>1000	>1000	>1000	>1000	>1000
Tobramycin	3–10	300–1000	300–1000	100–300	100–300	>1000
Apramycin	<1	<1	1–3	300–1000	300–1000	>1000
Gentamicin	3–10	>1000	>1000	10–30	10–30	>1000
Sisomicin	3–10	>1000	>1000	100–300	100–300	>1000
Neamine	10–30	10–30	10–30	>1000	>1000	>1000
Ribostamycin	3–10	3–10	3–10	>1000	>1000	>1000
Butirosin	<1	<1	<1	>1000	>1000	>1000
Neomycin	1–3	3–10	3–10	30–100	30–100	>1000

Fig. 1. Restriction maps of DNA fragments carrying aminoglycoside-resistance genes. Aminoglycoside-resistance genes were cloned using pIJ702 as vector. Boxes represent inserted DNA from the following organisms: in pLST31 and pLST51, *S. tenebrarius*; in pLST14, *M. purpurea*; in pLST41 and pLST412, *S. tenjimariensis*. In pLST 14, ★ indicates loss of the *SphI* cloning site. The whole of the kanamycin-apramycin resistance gene from *S. tenjimariensis* is contained within pLST412 (insert size 0.8 kb) and has been subcloned from pLST41 (Skeggs *et al.*, 1985). The kanamycin-gentamicin resistance gene from *M. purpurea* has been located within pLST14 by subcloning (D. J. Holmes, unpublished data). It is contained within about 1.1 kb of the inserted DNA that begins between the *BamHI* and *BglII* sites and extends leftwards to a point between the *EcoRI* site and the proximal *SphI* site.

interesting that fairly high levels of resistance to most of those compounds were also displayed by *S. lividans* TSK 31 or by strain TSK 51 or by both. On the other hand, strain TSK 31 was only marginally resistant to neomycin and, although the resistance level of strain TSK 51 was significantly higher, it did not approach that seen with *S. tenebrarius*. Accordingly, repeated attempts were made, using *S. tenebrarius* DNA, to generate clones that would grow on higher concentrations of neomycin (e.g. $50 \mu\text{g ml}^{-1}$), but to no avail.

When the DNA fragments from *S. tenebrarius* cloned in pLST31 and pLST51 had been subjected to restriction analysis (Fig. 1), they were compared with previously unpublished maps of the *M. purpurea* DNA fragment present in pLST14 (isolated from *S. lividans* JR 14) and the *S. tenjimariensis* DNA contained in pLST41. The latter DNA fragment (about 3.7 kb) contained the kanamycin-apramycin resistance gene, which was subsequently isolated on pLST412 as a smaller insert (0.8 kb in length). The similarities between the inserts in pLST31 compared with pLST14 and in pLST51 compared with pLST41 were obvious, suggesting that the similar patterns of aminoglycoside resistance linking *S. lividans* strain JR 14 with TSK 31 and strain TSK 412 with TSK 51 resulted from their possession of related (and possibly homologous) resistance genes. Further evidence in support of this idea was obtained when a fragment of *M.*

Fig. 2. Southern analysis of aminoglycoside-resistance genes from various actinomycetes. Radio-labelled probes were prepared using (a) the *Bgl*II–*Kpn*I fragment from the insert in pLST41 and (b) the *Eco*RI – *Bam*HI fragment from the insert in pLST14 (see Fig. 1). These probes contained (a) at least 75% of the kanamycin-apramycin resistance gene from *S. tenjimariensis*, plus adjacent DNA, and (b) at least 55% of the kanamycin-gentamicin resistance gene from *M. purpurea*, again plus adjacent DNA from that organism. Hybridization was carried out with filters containing linearized pIJ702 plus the recombinant plasmids pLST14, pLST31, pLST41 and pLST51, each of which had been cleaved into three restriction fragments as follows: 5.0 kb, 2.5 kb and 1.6 kb from pLST14; 5.1 kb, 2.5 kb and 1.6 kb from pLST31; 5.0 kb, 3.0 kb and 1.4 kb from pLST41; 5.5 kb, 3.0 kb and 1.4 kb from pLST51 (for further details see Methods and Fig. 1).

purpurea DNA, isolated from pLST14 and known to contain most of the kanamycin-gentamicin resistance determinant (see legend to Fig. 1), was used to probe Southern blots containing restriction fragments from pIJ702, pLST41, pLST31, pLST51 and pLST14. Hybridization at high stringency was observed with the piece of *S. tenebrarius* DNA present in pLST31 but not with any part of pLST41, pLST51 or pIJ702 (Fig. 2). Conversely, when a similar Southern blot was probed with a radiolabelled restriction fragment derived from pLST41 and containing most of the kanamycin-apramycin resistance determinant (see legend to Fig. 1), hybridization at high stringency was observed with the *S. tenebrarius* DNA insert in pLST51 but not with any part of pLST31. These obvious similarities between the resistance genes from *S. tenebrarius* and those isolated from *M. purpurea* and *S. tenjimariensis* influenced our approach to the biochemical characterization of the mechanisms of aminoglycoside resistance in strains TSK 31 and TSK 51, as described below. Before that, however, another aspect of the homologies evident in Fig. 1 deserves further comment. The pieces of DNA being compared, between pLST14 and pLST31 on the one hand and between pLST41 and pLST51 on the other, appeared similar over much more than gene-sized contours, at least at this crude level of analysis. In the absence of additional data, the significance of these 'extra' homologies cannot be assessed and they may well be trivial. Nevertheless, it might be instructive to know whether resistance determinants in these various aminoglycoside-producing organisms are physically adjacent to other common genes involved in aminoglycoside metabolism (more of this, later).

Fig. 3. Effects of aminoglycosides on cell-free protein synthesis. Assays contained S100* from *S. lividans* TSK 1 (control strain) together with 5 pmol 70S ribosomes from *S. lividans* TSK 31 (□) (100% activity = 25 Phe per ribosome h^{-1}); *S. lividans* TSK 51 (●) (100% activity = 20 Phe per ribosome h^{-1}); *S. tenebrarius* (○) (100% activity = 15 Phe per ribosome h^{-1}); *S. lividans* TSK 1 (■) (100% activity = 15 Phe per ribosome h^{-1}). Activities are given as pmol phenylalanine incorporated per pmol ribosomes per hour (Phe per ribosome h^{-1}). Antibiotic concentrations have been corrected to account for sulphate and refer to free base.

Biochemical analysis of resistance mechanisms

Prior to the present work, it had already been shown that ribosomes from *S. tenebrarius* were resistant *in vitro* to a range of aminoglycosides, including kanamycin, gentamicin, apramycin and, to a lesser extent, neomycin (Yamamoto *et al.*, 1982; Piendl *et al.*, 1984). Those results were amply confirmed here (and extended) when ribosomes from *S. lividans* strains TSK 31 and TSK 51 were compared with those from *S. lividans* TSK 1 and *S. tenebrarius* (negative and positive controls, respectively) in their response to aminoglycosides *in vitro*. In these experiments, ribosomes from the various organisms were supplemented with S100* from strain TSK 1 and assayed in protein-synthesizing systems directed by polyuridylic acid. Some of the results are shown in Fig. 3. Ribosomes from *S. lividans* TSK 31 were resistant to kanamycin, tobramycin and gentamicin, whereas those from strain TSK 51 were highly resistant to kanamycin, apramycin and ribostamycin but showed only low-level resistance to gentamicin and neomycin. Also, in other studies (data not presented), ribosomes from *S. lividans* TSK 51 and from *S. tenebrarius* proved to be highly resistant *in vitro* to neamine and to butirosin. Two general conclusions were drawn from these data. First, for each *S. lividans* strain, the profile of ribosomal resistance observed *in vitro* reflected the phenotype of the intact organism. Secondly, the levels of resistance to individual aminoglycosides displayed by *S. tenebrarius* ribosomes were matched by the responses of ribosomes from *S. lividans* TSK 31 and/or strain TSK 51. Thus, it was concluded that the fragments of *S. tenebrarius* DNA that had been cloned in strains TSK 31 and TSK 51 probably contained all the aminoglycoside-resistance determinants that affected the ribosome. It was also evident that the patterns and levels of resistance to aminoglycosides

Fig. 4. Correlation between methylation of 30S ribosomal subunits and resistance to aminoglycosides. Ribosomal 30S subunits (5 pmol) from *E. coli* were preincubated with S100* (as a source of methylase activity) either from *S. lividans* TSK 31 or *S. lividans* TSK 51 in the presence or absence of *S*-adenosylmethionine (SAM). After ethanol precipitation, the particles were supplemented with *E. coli* 50S ribosomal subunits (5 pmol) and S100* from *S. lividans* TSK 1 and assayed in cell-free protein synthesis in the absence of drugs (○) or in the presence of tobramycin (■), apramycin (□), ribostamycin (●) or gentamicin (▲), each at 10 µg ml⁻¹ final concentration of free base.

observed with ribosomes from strains TSK 51 and TSK 31 were indistinguishable from those seen previously with ribosomes from *S. lividans* TSK 412 and JR 14 respectively (Skeggs *et al.*, 1985; Thompson *et al.*, 1985). Again, these results were compatible with the idea that *S. tenebrarius* contains resistance genes that are homologous with those from *S. tenjimariensis* and *M. purpurea* that had been cloned previously. That being so, the likely resistance mechanisms operating in *S. lividans* TSK 31 and TSK 51 were evident, since it had already been shown that ribosomal resistance to aminoglycosides in *S. lividans* JR 14 and in strain TSK 412 was due to methylation of 16S RNA. Accordingly, this point was addressed directly.

Using S100* preparations from *S. lividans* TSK 31 and TSK 51, as sources of putative methylase activity, and *S*-adenosyl[methyl-³H]methionine as cofactor, the incorporation of radiolabelled methyl groups into *E. coli* 30S ribosomal subunits was followed. In controls, S100* from *S. lividans* TSK 1 was used to indicate the 'background' level of incorporation due to other rRNA methylases, present in *S. lividans* but not in *E. coli*. The results were unequivocal. Substantially higher levels of incorporation (amounting to about 0.6 methyl groups per 30S particle) were observed with S100* extracts from the clones compared with the control strain and in each case it was 16S rRNA that was methylated (data not shown). Then, to confirm that methylation caused ribosomal resistance, the experiment was repeated using unlabelled *S*-adenosylmethionine. After incubation with S100*, plus or minus *S*-adenosylmethionine, the 30S ribosomal subunits were assayed for their response to aminoglycosides in a protein-synthesizing system. To do this, they were supplemented with 50S ribosomal subunits from *E. coli* plus S100* (source of protein synthesis factors, etc.) from *S. lividans* TSK 1. The results are shown in Fig. 4.

Resistance to specific aminoglycosides was observed only when 30S particles had been incubated with *S*-adenosylmethionine in addition to S100* from either of the clones. Moreover, the resistance profiles resembled those of native ribosomes from strains TSK 31 and TSK 51. These results proved that specific methylation of 16S RNA by the products of *S. tenebrarius* genes could render *E. coli* ribosomes resistant to aminoglycosides. Presumably, similar mechanisms also operate in *S. tenebrarius*; hence we conclude that ribosomes from that organism owe their complex pattern of aminoglycoside resistance to methylation at (at least two) specific sites within 16S RNA.

Conclusions

Although all the resistance characteristics of *S. tenebrarius* ribosomes can be adequately accounted for in terms of 16S RNA methylation, this is still not sufficient to account for the phenotype of that strain – even relating to antibiotics against which ribosomal resistance is expressed. Thus, *in vitro*, ribosomes from *S. tenebrarius* were indistinguishable from those of *S. lividans* TSK 31 and TSK 51 in their response to tobramycin, yet the MIC values were different for the three strains. Indeed, the data in Table 1 suggest that some other tobramycin-resistance determinant from *S. tenebrarius* might have been cloned in *S. lividans* TSK 31. Again, *S. tenebrarius* ribosomes were similar to those from *S. lividans* TSK 51 in their response to apramycin or neomycin but the intact organisms displayed different levels of resistance. We have no data to account for these observations but we note that, in common with other aminoglycoside producers, *S. tenebrarius* possesses aminoglycoside-modifying activity (Davies, 1980; Yamamoto *et al.*, 1982). Conceivably, this could contribute to the phenotype as could the permeability properties of the mycelial surface of *S. tenebrarius*. Data bearing upon these points are currently unavailable.

Finally, on a teleological note, it is intriguing to enquire why *S. tenebrarius* carries the seemingly redundant rRNA methylase gene(s) that we have cloned in *S. lividans* TSK 31. Possibly, *S. tenebrarius* requires that DNA for protection against some other antibiotic product as yet uncharacterized. Alternatively, it could be that the presence of the 'extra' resistance determinant(s) reflects a recurrent pattern in the genetic organization of antibiotic-producing organisms. Thus, in *S. tenebrarius*, genes involved in the production of apramycin and tobramycin might form separate clusters, each associated with a methylase gene. Such physical linkage between antibiotic biosynthetic genes and resistance determinants has already been observed in several contexts (for a review, see Hopwood *et al.*, 1986). Whether it occurs in *S. tenebrarius* and, if so, whether on the chromosome or on a plasmid (or on both) remains to be established.

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