Characterization of Sparsomycin Resistance in *Streptomyces sparsogenes*

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The antitumor antibiotic sparsomycin, produced by *Streptomyces sparsogenes*, is a universal translation inhibitor that blocks the peptide bond formation in ribosomes from all species. Sparsomycin-resistant strains were selected by transforming the sensitive *Streptomyces lividans* with an *S. sparsogenes* library. Resistance was linked to the presence of a plasmid containing an *S. sparsogenes* 5.9-kbp DNA insert. A restriction analysis of the insert traced down the resistance to a 3.6-kbp DNA fragment, which was sequenced. The analysis of the fragment nucleotide sequence together with the previous restriction data associate the resistance to *srd*, an open reading frame of 1,800 nucleotides. Ribosomes from *S. sparsogenes* and the *S. lividans*-resistant strains are equally sensitive to the inhibitor and bind the drug with similar affinity. Moreover, the drug was not modified by the resistant strains. However, resistant cells accumulated less antibiotic than the sensitive ones. In addition, membrane fractions from the resistant strains showed a higher capacity for binding the drug. The results indicate that resistance in the producer strain is not connected to either ribosome modification or drug inactivation, but it might be related to an alteration in the sparsomycin permeability barrier.

The antitumor antibiotic sparsomycin is a universal translation inhibitor that blocks protein synthesis in all species (for a review, see reference 20). The broad spectrum of sparsomycin action indicated that the drug was targeted to a highly conserved component of the translation machinery. The relevance of the target was also supported by the fact that mutations inducing high resistance to sparsomycin have not been reported, and only a moderately resistant strain has been found in *Halobacterium salinarium* (14).

In fact, it was soon shown that sparsomycin blocks the peptide bond formation (10). The drug binds and causes important conformational changes in the peptidyl transferase active center. Thus, it was found that sparsomycin can block the binding of substrates at the A-site (24) but enhances binding to the P-site (11). Actually, sparsomycin has been a very powerful tool in the study of the structure and function of the ribosome (6). Recently, the antibiotic was found to interact with nucleotide A2602 in the peptidyl transferase center of the bacterial ribosome (23).

The drug was initially developed as a potential antitumor agent, although toxicity soon limited its clinical application (17). Nevertheless, the synthesis of a series of sparsomycin derivatives with higher inhibitory activities (30) has led to a reappraisal of its potential as an anticancer drug (5, 7).

Sparsomycin is produced by *Streptomyces sparsogenes*, which is obviously resistant to the drug. Organisms producing toxic compounds, including antibiotics, use different approaches to defend themselves from their own action (3). Not the least frequent approach is to modify the target, making it insensitive to the drug. Thus, methylation of specific residues within the 16S rRNA makes the ribosomes of many aminoglycoside antibiotic producers resistant to their respective products (27, 29). The same strategy is used by the producers of macrolides (13), lincosamides (28), pactamycin (1), and thiostrepton (4).

The study of these resistance mechanisms has provided relevant information on the antibiotic mode of action and on the ribosomal binding site at the molecular level. It would be important, therefore, to see whether *S. sparsogenes*, like other antibiotic-producing streptomycetes, has managed to modify the highly conserved sparsomycin target site to make the ribosomes resistant to the drug. Alternatively, as in the case of other producers, a different resistance mechanism, such as drug inactivation or permeability barrier alterations, might have evolved (for a review, see reference 3), perhaps because modification of the target is not possible without seriously affecting its activity.

We have approached the study of sparsomycin resistance by directly analyzing the producer and by trying to characterize genetic determinants from *S. sparsogenes* that provide resistance in *Streptomyces lividans*, an organism susceptible to the drug.

**MATERIALS AND METHODS**

**Bacterial strains and culture media.** Streptomyces strains were *S. sparsogenes* ISP5356 (ATCC 25498), *S. lividans* 1326, and *S. lividans* 3131, which corresponds to *S. lividans* 1326 transformed with plasmid pIJ702 (12). *Streptomyces* spp. were grown in yeast extract-malt extract (YEME) liquid medium or on R5 agar plates (9). R2YE plates were used for the regeneration of protoplasts after transformation (9). *Escherichia coli* DH5α was used for manipulating plasmids. Growth conditions for *E. coli* were as described elsewhere (25).

**Sensitivity of *Streptomyces* spp. to antibiotics.** The sensitivities of the different strains of *Streptomyces* spp. to antibiotics were assayed either in liquid medium (YEME) or on R5 plates containing the amount of antibiotic indicated below. Inhibition in liquid medium was estimated by monitoring the A_{550} of the cultures. Inhibition on agar plates was determined either by colony counting or by the size of the halo formed in the plate around 3-mm-diameter paper disks containing the antibiotics at the indicated concentrations. Unless otherwise indicated, thiostrepton was not included in the media, to avoid possible effects on antibiotic resistance (8).

**DNA manipulation.** Restriction enzyme digestions, ligations, agarose gel electrophoresis, etc., were performed according to well-established techniques (25).
Restriction endonucleases were purchased from Boehringer Mannheim, MBI Fermentas, New England Biolabs, and Amersham and were used as recommended by the suppliers. T4 DNA ligase, calf intestinal alkaline phosphatase, and the DNA polymerase I Klenow fragment were from Boehringer Mannheim.

Standard procedures were used for propagation and subcloning of plasmids in E. coli. Nucleotide sequences of DNA inserts were determined on both strands using a Dye-Terminator cycle sequencing ready reaction kit (Applied Biosystems) with custom-made oligonucleotides as primers. Sequencing reactions were run on an automated DNA sequencer (model 377; Applied Biosystems).

Preparation and screening of a genomic library of S. sparsogenes. Genomic DNA from S. sparsogenes (40 μg), prepared according to the methods described in reference 9, was partially digested with Sau3A (0.03 U of enzyme per μg of DNA; 15 min at 37°C). Six- to 8-kbp fragments were purified by agarose gel electrophoresis, and 10 μg of the sample was ligated to 2 μg of pIJ702 obtained from S. lividans 3131 and purified by cesium chloride-ethidium bromide gradient centrifugation. The plasmid was previously digested with BglII and treated with alkaline phosphatase to prevent recircularization. The ligation mixture was used to transform S. lividans 1326 protoplasts according to standard techniques (9).

The screening of the library was done in two steps. First, the transfectants were tested for a plasmid marker (resistance to the antibiotic tetracycline). The thioestrepton-resistant colonies, numbering approximately 5,000, were allowed to sporulate and the spores were tested for resistance to sparsomycin on plates containing 100 μg of the antibiotic/ml but in the absence of thioestrepton, which has been reported to affect the sensitivity of S. lividans to different translation inhibitors, including sparsomycin (8).

Preparation of cell extracts and ribosomes. Total cell extracts (S30 and S100 fractions) and ribosomes from the different Streptomyces strains were prepared as previously described (21).

Activity tests. (i) Binding of 125I-labeled phenol-sparomycin to ribosomes. Ribosomes (1.0 μM) were incubated for 30 min at 30°C with increasing amounts of 125I-labeled phenol-sparomycin (0.1 μM; 104 cpm/pmol) (15) in 50 μl of binding buffer (10 mM Tris-HCl, 10 mM MgCl2, 50 mM NH4Cl, 6 mM β-mercaptoethanol). After incubation the samples were diluted with 5 ml of binding buffer and filtered through nitrocellulose filters. After two washes with the same buffer, filters were counted in a gamma counter to estimate the amount of drug bound to ribosomes.

(ii) Polyphenylalanine synthesis. The polymerizing activity of the Streptomyces extracts was estimated by a poly(U)-dependent polyphenylalanine synthesis assay carried out as described elsewhere (21).

Analysis of in vivo sparsomycin modification. S. sparsogenes and S. lividans cells were grown to mid-exponential phase. Cells were collected by centrifugation (10 min at 10,000 rpm in an SS-34 rotor) and resuspended in binding buffer to an A260 of 5. A 200-μl aliquot of the suspension of cells was incubated with 100,000 cpm of 0.1 μM 125I-labeled phenol-alanine-sparomycin (104 cpm/pmol) (13) for 1 h at 30°C. Cells were recovered by centrifugation, resuspended again in 200 μl of binding buffer, and broken. The antibiotic was extracted from both pellet and supernatant fractions by mixing them with an equal volume of ethyl acetate, and the organic and the aqueous phases were separated by centrifugation. The radioactive antibiotic was analyzed by thin-layer chromatography on precoated silica gel plates, using chloroform-methanol (80:20 or 80:10) as a solvent.

Accumulation of sparsomycin by the cells. Streptomyces cells were grown until the A260 reached 0.2. Cells from 100 ml of medium were collected by centrifugation and resuspended in 4 ml of binding buffer, and 100 μl of the suspension was incubated with 100,000 cpm of 125I-labeled phenol-alanine-sparomycin (104 cpm/pmol) for different periods. After the incubation, the cells were diluted with 5 ml of binding buffer and filtered through glass fiber filters. The filters were washed two times with 5 ml of the same buffer and dried, and the radioactivity was estimated by scintillation counting.

Binding of radioactive sparsomycin to subcellular fractions. The different Streptomyces strains were grown in 50 ml of medium to an A260 of 0.2, collected by centrifugation, washed with binding buffer, and resuspended in the same buffer to an A260 of 5.0. Cells were broken in a French press, and the extracts were centrifuged at 15,000 × g for 30 min to obtain a membrane fraction. Ribosomes were prepared by centrifuging the supernatant at 100,000 × g for 3 h. Aliquots containing equivalent amounts of the different fractions were incubated with 100,000 cpm of 125I-labeled phenol-alanine-sparomycin (104 cpm/pmol) for 30 min at 30°C. The bound drug was estimated as described previously.

Nucleotide sequence accession number. The sequence of the S. sparsogenes DNA fragment reported here was submitted to the EMBL nucleotide sequence database and assigned the accession number AJ276161.

RESULTS

Isolation of S. lividans sparsomycin-resistant strains. S. lividans was transformed with an S. sparsogenes genomic DNA library in plasmid pIJ702 and prepared as described in Materials and Methods. Seven transformants were initially selected which were able to grow on agar plates containing 100 μg of sparsomycin/ml. The parental S. lividans was totally inhibited with 20 μg of drug/ml under similar growing conditions. When spores were prepared from resistant clones and allowed to grow again in the presence of sparsomycin, only one of them, SLT4, showed the same resistant phenotype, as well as conserving the thioestrepton resistance marker of the pIJ702 vector. S. lividans transformed with the empty pIJ702 did not show any resistance to the drug in the absence of thioestrepton.

The resistance of SLT4 to sparsomycin is associated with the presence of the transforming construct containing S. sparsogenes DNA, since curing the plasmid, which resulted in a loss of thioestrepton resistance, caused a concomitant loss of the sparsomycin resistance phenotype (Fig. 1).

Unexpectedly, the sparsomycin-resistant strain showed alterations in sensitivity to other protein synthesis inhibitors. Thus, S. lividans SLT4 shows a notable increase in its sensitivity to chloramphenicol and tetracycline while having a higher resistance to lincomycin and puromycin (Fig. 2).

Characterization of the resistance determinant. The plasmid present in S. lividans SLT4 was isolated and characterized. A DNA insert of 5.9-kbp was found, which was fragmented using appropriate restriction sites; the fragments were subcloned in the same plasmid, pIJ702. The new plasmids were used to transform S. lividans 1326, and the transformants were tested for resistance to sparsomycin. In this way, a minimal fragment of 3.6 kbp (Fig. 3, insert 3) was found to be able to induce resistance to the drug. None of the S. sparsogenes DNA fragments subcloned in a pBluescript plasmid were able to provide sparsomycin resistance in E. coli.

The fragment was sequenced, and the analysis of the sequence (Fig. 4) indicated the presence of two ORFs (srd and moxR) and a fragment of a third ORF encoding a transmembrane polypeptide (imp) at one of the ends. Restriction analysis and subcloning of the original fragment (Fig. 3, insert 1) indicated that insert 4, comprising the middle ORF—which
showed homology to \textit{moxR}—and the incomplete \textit{tmp} ORF did not induce resistance. In addition, disruption of \textit{srd} by cutting insert 3 to obtain inserts 4 and 5 (Fig. 3) resulted in loss of resistance. It seems, therefore, that the sparsomycin resistance determinant, \textit{srd}, must be associated with the first ORF in the sequenced fragment.

The \textit{srd} ORF carries GTG as a putative initiator codon and seven nucleotides at the 5' end of the fragment. It is probably expressed from a promoter in the vector and encodes a 601-amino-acid-long polypeptide (Fig. 5). No significant homology of this \textit{S. sparsogenes} DNA to any sequence in the data bank could be found. Similarly, no relevant standard functional or structural domain was detected in the polypeptide sequence, except for one weak transmembrane domain.

Biochemical characterization of the resistance mechanism. Three main resistance mechanisms are usually responsible for the resistance of cells to drugs: (i) reduction of the target affinity for the drug; (ii) inactivation of the drug; and (iii) alteration of the permeability barrier affecting the transport. In the first case, the reduction of the affinity can be due to either a direct alteration of the target or to the indirect action of an allosteric effector which increases the dissociation of the drug.

(i) Reduction of the target affinity for the drug. To explore this possibility, the capacity of sparsomycin to inhibit a poly(U)-dependent polyphenylalanine synthesis cell-free system derived from \textit{S. sparsogenes}, \textit{S. lividans} SLT4, and the parental \textit{S. lividans} 3131 was estimated. As shown in Fig. 6, all the extracts were equally sensitive to sparsomycin. Moreover, ribosomes from \textit{S. sparsogenes} and from \textit{S. lividans}, grown either in the absence (sparsomycin sensitive) or in the presence of thiostrepton (sparsomycin resistant) had a similar capacity to bind the drug (Fig. 7). These results indicate that the resistance shown either by the producer or by the resistant \textit{S. lividans} is not due to either a direct or an indirect effect on the affinity of the target, namely the ribosome, for sparsomycin.

(ii) Modification of the drug by the resistant cells. To test whether the resistant cells are able to inactivate the antibiotic by modifying its structure in some way, \textit{S. sparsogenes} and \textit{S. lividans} SLT4 cells were incubated with radioactive sparsomycin, $^{125}$I-labeled phenol-alanine-sparsomycin (16), for 1 h. The cells were broken afterwards and the extracts were centrifuged, yielding a supernatant and a pellet. Both fractions were treated with ethyl acetate to extract the drug, which was then analyzed by thin-layer chromatography using different solvents. No alteration of the drug mobility could be observed in any of the cases using untreated $^{125}$I-labeled phenol-alanine-sparsomycin as a control (data not shown).

(iii) Accumulation of sparsomycin by the cells. A possible alteration in sparsomycin transport in the resistant strains was checked by testing the capacity of the cells to accumulate the same radioactive derivative of sparsomycin. As shown in Fig. 8, the radioactivity found in \textit{S. sparsogenes} and \textit{S. lividans} SLT4 was considerably lower than that found in the sensitive \textit{S. lividans} 3131 strain.

In order to characterize in more detail the different abilities to accumulate sparsomycin of the resistant and sensitive strains, cells were fractionated and the capacities of different fractions to bind radioactive drug were estimated. As summarized in Table 1, the membrane fractions derived from \textit{S. sparsogenes} and SLT4 bound more radioactive drug than the equivalent fractions from sensitive cells.

**DISCUSSION**

The results from the sparsomycin producer \textit{S. sparsogenes}, as well as from the resistant \textit{S. lividans} SLT4 expressing a resistant genetic determinant from the producer, clearly show that the ribosomes from both types of strains bind the drug to a...
similar extent. These data indicate that the resistance mechanism working in both strains does not involve modification of the drug target, the ribosomal peptidyl transferase center. Similarly, since the drug inhibits the in vitro protein-synthesizing activity of extracts derived from the resistant and sensitive strains equally, the action of an allosteric effector inducing a rapid dissociation of sparsomycin, like the Tet(M) class of proteins in tetracycline resistance (2), can also be excluded.

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**FIG. 4.** Analysis of the nucleotide sequence of the 3.9-kbp fragment. Analysis of G+C content identified three putative ORFs in the sequence, marked srd, moxR, and tmp. The region included in the different inserts in Fig. 3 is indicated at the bottom. A vertical line marks the starting point of the *S. sparsogenes* DNA.

**FIG. 5.** Nucleotide and deduced amino acid sequences of the srd ORF.

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![Diagram of the nucleotide sequence of the 3.9-kbp fragment](image)

**TABLE 1**

<table>
<thead>
<tr>
<th>Nucleotide Sequence</th>
<th>Protein Sequence</th>
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<tbody>
<tr>
<td>3GACGCTGTCAGAG ,...</td>
<td>MRTTPIGAPVDLGACPGLGA</td>
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Sparsomycin is, indeed, a very efficient inhibitor of the peptide bond formation in the Bacteria as well as in Archaea and in Eucarya. Affinity labeling data have shown that the antibiotic seems to bind to nucleotide A2602 in the 23S rRNA peptidyl transferase domain (23), which is very close to the active center, as revealed by the 2.4 Å three-dimensional structure of the 50S ribosomal subunit recently reported (19). Sparsomycin is, therefore, interacting at a very critical site of the ribosome which, apparently, evolution has not been able to modify in order to produce a sparsomycin-resistant ribosome. In fact, the putative drug binding site, A2602, is a universally conserved nucleotide, which underlines its functional importance and accounts for the broad range of organisms sensitive to the drug. 

S. sparsogenes does not seem to resist exogenous sparsomycin by modifying the drug either, since radioactive drug incubated with the resistant cells was chromatographically indistinguishable from the untreated controls. Since the target is sensitive to sparsomycin and the drug is not inactivated by modification, the resistance mechanism in the resistant strains must lay at the level of the permeability barrier. Thus, it has been found that S. sparsogenes accumulates considerably less drug than the sensitive S. lividans cells when incubated in the presence of radioactive sparsomycin, suggesting that resistance probably results from differences in the cell permeability.

It was possible to transform sparsomycin resistance in sensitive S. lividans cells by using an S. sparsogenes DNA library. The biochemical characteristics of the resistance phenotype in the transformant S. lividans SLT4 cells are similar to those found in the producer, namely, sparsomycin susceptibility of the translation machinery, inability to modify the drug, and reduced cellular accumulation of antibiotic. These results sug-

TABLE 1. Binding of 125I-phenol-alanine-sparsomycin by different cellular fractions

<table>
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<tr>
<th>Fraction</th>
<th>Radioactivity (cpm) bound to the indicated fraction</th>
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<tbody>
<tr>
<td></td>
<td>S. lividans</td>
</tr>
<tr>
<td>Membrane</td>
<td>3,800</td>
</tr>
<tr>
<td>Ribosomes</td>
<td>37,480</td>
</tr>
</tbody>
</table>
gest, therefore, that resistance to sparsomycin in the producer might be associated with an alteration in the transport process. The fact that the sparsomycin resistance determinant also affects the sensitivity of the cells to several other inhibitors supports an effect at the level of permeability, although this hypothesis must be experimentally confirmed.

An increasing number of antibiotic-producing organisms have been shown to contain active transport systems that efficiently export the antibiotic molecules to the exterior. The mechanisms can be divided into two classes. One of them is connected to proton-driven membrane electrochemical gradients and involves mdr-type proteins (22). The other one is based on the ABC transporter system, which couples antibiotic transport to ATP hydrolysis (18).

The S. sparsogenes genetic determinant responsible for sparsomycin resistance in S. lividans SLT4 can be associated with srd, an ORF that encodes a 600-amino-acid polypeptide. How-

S. sparsogenes

real transport mechanism involved in sparsomycin resistance. Studies using the recombinant protein will be required to lo-

in the producer strain (26). Perhaps srd is only one part of a transport system present in S. sparsogenes.

Interestingly, a membrane fraction of the resistant strain, S. lividans SLT4, binds more radioactive sparsomycin than the equivalent fraction from sensitive S. lividans, suggesting that srd might encode a sparsomycin-binding protein, as mdr does in the case of mitomycin (26). Srd, in spite of not being a typical membrane protein, might be an accessory component of the transport mechanism required for optimal efficiency. In any case, these results must be considered as preliminary, and further studies using the recombinant protein will be required to locate the protein in the cell and to obtain further insight into the real transport mechanism involved in sparsomycin resistance.

ACKNOWLEDGMENTS

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antigens, and sparsomycin–sparsomycin in combination with antitu-

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