Ribosomal Resistance in the Gentamicin Producer Organism

*Micromonospora purpurea*

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The mechanism of resistance of the gentamicin-producing organism *Micromonospora purpurea* was analyzed. Determination of minimal inhibitory concentrations revealed high resistance to the 4,6-substituted deoxystreptamine aminoglycosides amikacin, gentamicin, kanamycin, netilmicin, sisomicin, and tobramycin and also to lividomycin A and hygromycin B, but susceptibility to streptomycin, dihydrostreptomycin, paromomycin, and neomycin during all phases of the growth cycle. The nonproducing, closely related *Micromonospora melanosporea* was susceptible to these compounds. In agreement with results from previous studies (R. Benveniste and J. Davies, Proc. Natl. Acad. Sci. U.S.A. 70:2276–2280, 1973), extracts from *M. purpurea* showed no activity of enzymes specifically modifying gentamicin. 70S ribosomes from *M. purpurea* but not from *M. melanosporea* were resistant to inhibition by gentamicin, kanamycin, tobramycin, and lividomycin in a polyuridylic acid-dependent polyphenylalanine synthesis system and susceptible to those compounds which were inhibitory in vivo. The former antibiotics were also unable to induce misreading. Subunit exchange experiments between *M. purpurea* and *M. melanosporea* showed that the main site for inhibition and induction of misreading is the 30S subunit (up to gentamicin concentrations of 10 μg/ml).

There are two principal ways by which antibiotic-producing organisms protect themselves against their own product: (i) one group of organisms possesses sensitive target sites, and they prevent the action of the antibiotic by its enzymatic modification to a biologically inactive compound (10, 12, 20); (ii) the second group possesses altered target sites which are no longer recognized by the drug (8, 15).

For aminoglycoside-producing organisms, it has been shown that enzymatic detoxification is the main resistance mechanism. Most of the producers contain enzymes which acetylate or phosphorylate the antibiotic and which are very similar in their properties to the plasmid-coded modifying enzymes present in resistant clinical isolates (3, 9, 11, 17, 21, 23). They possess sensitive ribosomes and produce the modifying enzyme usually only under the same conditions necessary for antibiotic production; in the nonproducing growth phase they are therefore phenotypically susceptible.

Ribosomal resistance to aminoglycosides has until recently mainly been found in laboratory mutants, first in streptomycin, in which single-step mutations provide high-level resistance (24), and later in 2-deoxystreptamine compounds, in which no single-step high-level resistant mutants are known. Rather, resistance to this class of aminoglycosides is brought about by multiple mutations either in the antibiotic uptake system or in ribosomal proteins of the 30S or 50S subunit (1, 4, 5). These multiple mutational resistance sites at the ribosome are in accordance with biochemical data which indicate that 2-deoxystreptamine aminoglycosides show multiple interaction with the ribosome (18, 27) and bind to both the 30S and the 50S subunits (6, 7, 19).

Recently, it was shown that ribosomal resistance also exists in an aminoglycoside-producing bacterium, namely, *Streptomyces tenjimarianus*, the istamycin producer (26). This organism is highly resistant to several aminoglycosides but does not contain any modifying enzymes. We report here on a similar finding for *Micromonospora purpurea*. *M. purpurea* synthesizes gentamicin, does not contain any specific gentamicin-modifying enzymes, and possesses ribosomes resistant to this drug. Ribosomal subunit exchange between *M. purpurea* and a closely related, gentamicin-susceptible organism, *Micromonospora melanosporea*, allowed the subunit localization of the main sites of gentamicin action on translation and misreading.

**MATERIALS AND METHODS**

**Organisms and media.** *M. purpurea* DSM43036 and *M. melanosporea* DSM43141 were obtained from the German Collection of Microorganisms, Göttingen.
The gentamicin-producing capacity of *M. purpurea* was verified by thin-layer chromatography (see below) of samples of medium from a stationary-phase culture. The thin-layer plates, after drying, were put on equal-sized agar plates with enriched TGYES medium (1% tryptone, 0.5% yeast extract, 0.2% glucose, 0.5% NaCl) for 30 min. The medium had been seeded with 10° *Bacillus subtilis* cells per ml as indicator organisms. After incubation at 37°C, two zones of inhibition developed which corresponded in their positions to the migration of the two main components of the gentamicin C complex. *M. melanospore* did not produce any detectable inhibitory compound. The medium used for cultivation of *Micromonospora* species contained 0.3% meat extract, 0.5% tryptone, 0.1% glucose, 2.4% starch, 0.5% yeast extract, 0.6% CaCO₃, and 0.4% KCl (3). The growth temperature was 30°C. *Escherichia coli* strain K802 with an adenylating enzyme was from the Schering Inc. strain collection. *E. coli* and *K. pneumoniae* were grown in TGYES medium at 37°C.

**Determination of minimal inhibitory concentrations.** A loopful of a culture from the exponential and stationary (i.e., nonproducing and producing) growth phases was streaked on complete medium plates containing different concentrations of the antibiotics. Growth was monitored after 5 to 9 days of incubation.

**Measurement of the effect of aminoglycosides on incorporation of L-[3H]leucine.** Portions (10 ml each) of an exponentially growing culture were transferred to a 100-ml Erlenmeyer flask with 4 ml of fresh medium containing the antibiotic to be tested and L-[3H]leucine at 1 μCi/ml. Samples (1 ml) were taken at the times indicated and mixed with 1 ml of 20% trichloroacetic acid. The precipitate was collected on glass fiber filters and washed three times with 5% trichloroacetic acid and once with 70% ethanol. After drying, radioactivity was determined in a liquid scintillation spectrometer.

**Preparation of ribosomes, ribosomal subunits, and 100,000 x g supernatant.** Cells of *Micromonospora* or *E. coli* were harvested by centrifugation and washed two times with TMNSH buffer (10 mM Tris-chloride [pH 7.4], 10 mM magnesium acetate, 30 mM ammonium chloride, 6 mM 2-mercaptoethanol). They were suspended in the same buffer (10 g [wt weight] per 15 ml of buffer) containing 5 μg of DNase I per ml and broken in a French press cell at 10,000 lb/in². Supernatants (30,000 x g) were prepared by two steps of centrifugation: first, 10 min at 12,000 x g, and then 30 min at 30,000 x g. Portions (1 ml) of the 30,000 x g extracts were centrifuged through 10 to 30% sucrose gradients (45 min at 41,000 rpm in a Beckman VTI 50 rotor), and the 70S material was pooled, collected by ultracentrifugation, resuspended in TMNSH buffer, and frozen at -70°C.

Ribosomal subunits were prepared from cells which had been incubated for 15 min at 10°C before harvesting. The 30,000 x g supernatant was prepared as described above except that a buffer of the following composition was used: 20 mM Tris-chloride (pH 7.4), 1 mM magnesium acetate, 150 mM potassium chloride, and 6 mM 2-mercaptoethanol. 30S and 50S subunits were purified by two consecutive gradient centrifugations (10 to 30% sucrose; 80 min at 44,000 rpm in a VTI 50 rotor). The subunits were collected by centrifugation (6 h at 150,000 x g) and stored frozen at -70°C. The 100,000 x g supernatants were obtained by centrifugation of 30,000 x g extracts (in TMNSH) for 150 min at 120,000 x g. Electrophoretic separation of ribosomal proteins was carried out according to Geyl et al. (14).

**Polyuridylic acid-dependent incorporation of phenylalanine.** The reaction mixture contained, in a volume of 250 μl: 70 μl of Mix II (22), 30 μl of L-[14C]phenylalanine (10 μCi/μl; 1 mM), 20 μl of polyuridylic acid (4 mg/ml), 10 μl of 100,000 x g supernatant (about 150 μg of protein), 50 μl of antibiotic dissolved in water, and 6 units of absorbance at 260 nm (A₂₆₀ units) of 70S ribosomes or 2 A₂₆₀ units of 30S plus 3 A₂₆₀ units of 50S subunits. Two magnesium concentrations were used: 8 and 12 mM. Mixtures were first incubated for 10 min at 37°C for activation of the ribosomes, and the reaction was started by the addition of polyuridylic acid. Incubation was for 30 min at 30°C, and incorporation was stopped by the addition of 1 ml of 10% trichloroacetic acid. After heating to 95°C for 20 min, the radioactivity of the precipitate was determined as described above.

Polyuridylic acid-dependent misreading was determined in assay mixtures in which radioactive phenylalanine was replaced by a mixture of 1 mM unlabeled phenylalanine plus 14C-labeled L-serine, L-isoleucine, and L-tyrosine (each 1 mM and 10 μCi/μmol).

**Analysis of aminoglycoside-modifying enzymes.** Aminoglycoside-acetylating enzymes were analyzed as described by Haas and Dowding (16). The assays were carried out at pH 5.8 and 7.2. The 100,000 x g extracts were dialyzed against TMNSH buffer before analysis. Aminoglycoside-phosphorylating and -adenylating enzymes were tested by the method described by Davies (9). Reaction products of the acetylation assays were separated by thin-layer chromatography according to Aszalos and Frost (2) with n-propanol-pyridine-acetic acid–water (15:10:3:12) as a solvent. The plates were dried and exposed to Kodak Regulix films for 7 days.

**Antibiotics.** The antibiotics used were from the following suppliers: gentamicin sulfate (C complex) and amikacin, Sigma Chemical Co., St. Louis, Mo.; neomycin sulfate, streptomycin sulfate, and dihydrostreptomycin sulfate, Serva, Heidelberg, West Germany; kanamycin sulfate, Boehringer, Mannheim, West Germany; lvidomycin sulfate A, Laboratoire R. Belon, Neuilly-sur-Seine, France; spectinomycin sulfate, Mycofarm, Delft, The Netherlands; paromomycin sulfate, Roussel Uclaf, Paris, France; tobramycin, Eli Lilly & Co., Indianapolis, Ind.; sisomicin sulfate, Bayer, Elberfeld, West Germany; netilmicin sulfate, Schering Corp., Bloomfield, N.J.; hygromycin B, Bio- gal Corp., Debrecen, Hungary.

**RESULTS**

**Minimal inhibitory concentrations.** The minimal inhibitory concentrations of aminoglycosides for the gentamicin-producing organism *M. purpurea* and the nonproducing species *M. melanospore* are given in Table 1. In contrast to *M. melanospore*, *M. purpurea* showed very high resistance to all 4,6-disubstituted deoxystrepta-
TABLE 1. Minimal inhibitory concentrations for *M. purpurea* and *M. melanosporea* of aminoglycosides

| Antibiotic                | MIC* (µg/ml) |
|---------------------------|-------------|
|                           | *M. purpurea* | *M. melanosporea* |
| 4,6-Substituted deoxystreptamines |             |                |
| Amikacin                  | >640        | 0.6            |
| Gentamicin                | >640        | 2.5            |
| Kanamycin                 | >640        | 5              |
| Netilmicin                | >640        | 40             |
| Sisomicin                 | >640        | 5              |
| Tobramycin                | >640        | 5              |
| 4,5-Substituted deoxystreptamines |             |                |
| Lividomycin A             | 160         | 2.5            |
| Neomycin                  | 1.25        | 1.25           |
| Paromomycin               | 5           | 0.6            |
| Others                    |             |                |
| Dihydrastreptomycin       | 0           | 5              |
| Hygromycin B              | >640        | 5              |
| Spectinomycin             | 160         | 160            |
| Streptomycin              | 10          | 5              |

* MIC, Minimal inhibitory concentration.

mine aminoglycosides (amikacin, gentamicin, kanamycin, netilmicin, sisomicin, and tobramycin) and a varying response to 4,5-disubstituted deoxystreptamine aminoglycosides (lividomycin A, neomycin, and paromomycin) and other compounds like hygromycin B, spectinomycin, and streptomycin.

The intrinsic resistance to the antibiotics neomycin, streptomycin, and spectinomycin was in the same range for both organisms, which argues against the existence of a nonspecific resistance mechanism in *M. purpurea*, e.g., a general permeability barrier to aminoglycosides. There was no difference in the growth response when plates were inoculated with cells from the exponential or stationary growth phase, which means that the resistance of *M. purpurea* is a permanent property and is not acquired solely during the phase of antibiotic production.

The effect of the addition of aminoglycosides on protein synthesis was also tested in liquid cultures. With concentrations of gentamicin up to 1 mg/ml, there was no detectable influence on the time course of incorporation of [3H]leucine into protein (data not shown).

Analysis of aminoglycoside-modifying enzymes.

During a search for aminoglycoside-modifying enzymes in antibiotic producer organisms, Benveniste and Davies (3) found that such enzymes were absent in an industrial strain of *M. purpurea*. We repeated this analysis for acetylating, adenylylating, and phosphorylating enzymes with *M. purpurea* DSM43036. Adenylyltransferase and phosphotransferase activity could not be detected; there was, however, acetyltransferase activity present (Table 2) in both *M. purpurea* and *M. melanosporea*. Extracts from *M. purpurea* transferred the same amount of acetyl groups to compounds binding to phosphocellulose paper regardless of whether gentamicin was present. Those from *M. melanosporea* clearly showed some activity specific for the acetylation of gentamicin. The acetylation reaction products were separated on thin-layer plates. Whereas the enzyme from the reference strain, *K. pneumoniae*, exhibited high specificity for gentamicin, this was not the case for those from *M. purpurea* and *M. melanosporea*, since extracts from both species transferred acetyl groups from acetyl-coenzyme A to many compounds in addition to a minor amount transferred to gentamicin (data not shown).

Ribosomal resistance. The effect of aminoglycosides on polypeptide synthesis in a system containing ribosomes from *Micromonospora* and 100,000 × g supernatant from *E. coli* was determined. This heterologous system was chosen because of high protease activity in streptomyces extracts (25), which drastically limits incorporation, and because of the absence of any aminoglycoside-modifying activity in extracts of *E. coli* A19. Figure 1 gives the results obtained with 70S ribosomes. Polyuridylic acid-dependent polyphenylalanine synthesis was unaffected by gentamicin, kanamycin, lividomycin, and tobramycin up to concentrations of 100 µg/ml. It was highly sensitive to neomycin and somewhat less sensitive to paromomycin.

To localize the site of gentamicin action, the effect of this compound was tested on polyphenylalanine synthesis by homologous and heterologous reconstituted ribosomes (Fig. 2 and 3). Ribosomes reconstituted from the 30S subunit from the susceptible strain *M. melanosporea* and the 50S subunit from *M. purpurea* were as susceptible as ribosomes reconstituted from 30S and 50S subunits from *M. melanosporea*. Those from *M. purpurea* were resistant to concentra-

| Extract from: | pH 5.8 | pH 7.5 |
|---------------|--------|--------|
|               | +Gm    | -Gm    | +Gm    | -Gm    |
| *K. pneumoniae* (AAC 31I) | −70,000 | 150 | 35,000 | 100 |
| *M. purpurea* | 5,000  | 3,700  | 11,000 | 12,000 |
| *M. melanosporea* | 20,300 | 200 | 5,800  | 100 |

* +Gm, Gentamicin sulfate (50 µg/ml) was present; −Gm, no drug.
FIG. 1. Effect of gentamicin ($\Delta$), neomycin (●), and paromomycin (○) on polyuridylic acid-dependent polyphenylalanine synthesis by 70S ribosomes from M. purpurea. Kanamycin, tobramycin, and lividomycin yielded the same result as gentamicin (100% = 15,500 cpm).

tions up to 200 μg/ml. When the 50S subunit came from the susceptible strain and the 30S subunit came from the producer, there was even a stimulation of incorporation, which could be reduced by increasing the Mg$^{2+}$ concentration from 8 to 12 mM.

The same experiments were performed to localize the ribosomal site at which gentamicin induces misincorporation of amino acids. Figure 3 shows that polyuridylic acid-dependent incorporation of isoleucine, tyrosine, and serine was obligatorily connected with the 30S subunit. When these were derived from the producer strain M. purpurea, no misreading could be induced.

The results presented in Fig. 2, 3, and 4 show that the main target of gentamicin is the 30S ribosomal subunit; in the presence of antibiotic concentrations of higher than 50 μg/ml, gentami-
cin stimulated phenylalanine incorporation on ribosomes which contained 30S subunits from M. purpurea and 50S subunits from the susceptible M. melanospora (Fig. 2 and 3). Since this stimulation was not observed when both subunits were from the producer organism (M. purpurea), one has to conclude that the 50S subunit from the susceptible strain interacts with gentamicin at high concentrations and that the large subunit from M. purpurea is altered in such a way that this interaction cannot take place. Identical results were obtained when 30S subunits from E. coli were paired with 50S subunits from either M. purpurea or M. melanospora.

We do not know, however, whether this interaction of gentamicin with the 50S ribosomal subunit, which has previously been demonstrated by genetic and biochemical means (5, 19), contributes to the resistance of M. purpurea.

DISCUSSION

Comparison of the in vitro and in vivo resistance patterns of M. purpurea to different aminoglycosides revealed an excellent correspondence in that those compounds which do not inhibit growth up to concentrations of 1 mg/ml are also unable to affect polyuridylic acid-dependent polyphenylalanine synthesis in vitro. This resistance is confined to the kanamycin group of aminoglycosides and also to lvidomycin; there is therefore no close correspondence with the chemical type of aminoglycoside, since the kanamycin group of compounds contains a 4,6-substituted 2-deoxystreptamine residue, whereas lvidomycin is 4,5 substituted, similar to neomycin or paromomycin.

The following evidence supports the notion that resistance to gentamicin in the producer organism M. purpurea is ribosomal in nature. (i) 70S ribosomes from M. purpurea are highly resistant in vitro; polypeptide synthesis is not inhibited, nor does gentamicin induce misreading. (ii) The nonproducing organism M. melanospora shows about equal susceptibilities to those aminoglycosides which are inhibitory for M. purpurea. This indicates that M. purpurea does not contain a general permeation barrier against aminoglycosides. (iii) In agreement with the results of Benveniste and Davies (3), we could not find any specific gentamicin-modifying enzyme in extracts of M. purpurea; the acetyltransferase activity present accepts substrates other than gentamicin. (iv) The pattern of resistance does not correspond to the substrate profile of any of the known aminoglycoside-modifying enzymes. M. purpurea, therefore, in addition to the istamycin-producing organism (26), is another example of an antibiotic producer which possesses ribosomes structurally altered in such a way that the organism is no longer inhibited by its product.

The possibility of reassociating ribosomes from gentamicin-"resistant" and -"sensitive" subunits allowed the identification of the major target of the drug. Clearly, the 30S subunit must be altered to prevent both inhibition of translation and induction of misreading. In agreement with genetic (5) and biochemical (18) studies, however, is the finding that the 50S subunit also seems to interact with the antibiotic. It must be emphasized that all our conclusions drawn from in vitro studies depend on the results from a pure polypeptide elongation (polyuridylic acid) system. No statements on the effect of gentamicin on the initiation step can be made because of the lack of a suitable test system.

M. purpurea and M. melanospora are closely related organisms since they exhibit a high degree of ribosomal protein homology; separation of 30S and 50S subunit proteins revealed only a few electrophoretic differences, namely, in about four proteins from the 30S and three proteins from the 50S subunit (data not shown). Also, heterologously reassociated ribosomes are as active as homologously associated ones. It is interesting to ask which structural alterations of the ribosome led to the high degree of resistance observed, and whether the few differences in ribosomal proteins or some change in the RNA moiety are involved.

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