R Factor-Mediated Aminoglycoside Antibiotic Resistance in Pseudomonas aeruginosa: a New Aminoglycoside 6'-N-Acetyltransferase

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The newly introduced semisynthetic aminoglycoside antibiotics, i.e., 3',4'-dideoxykanamycin B (DKB), 6'-N-methyl DKB (6'-Me-DKB) and amikacin (AK) have been found to be effective against gram-negative pathogens including Pseudomonas aeruginosa, which are resistant to the known aminoglycoside antibiotics. We have demonstrated in our stock cultures two types of P. aeruginosa strains resistant to DKB, i.e., (DKB)AK•6'-Me-DKB) and (DKB•AK*•6'-Me-DKB') (where r = resistant; s = sensitive). Both groups of strains inactivate the drugs by acetylation. The acetylating enzyme was extracted from GN4925(DKB•AK*•6'-Me-DKB') and purified by affinity chromatography. Enzymatic studies of the inactivation reaction and chemical studies of the inactivated products indicate that DKB and 6'-Me-DKB were inactivated by acetylation of the 6'-amino group of the drugs. This enzyme acetylates kanamycin A (KM-A), KM-B, DKB, 6'-Me-DKB, 6'-N-methyl kanamycin B, but not KM-C, AK, and gentamicin C. The enzyme is named aminoglycoside 6'-N-acetyltransferase 3. Genetic studies of two strains resistant to DKB and 6'-Me-DKB disclosed that the enzyme catalyzing inactivation of both DKB and 6'-Me-DKB was mediated by an R factor, i.e., R\text{met1} and R\text{met2}, capable of conferring resistance to KM, DKB, and 6'-Me-DKB, in addition to resistance to gentamicin, streptomycin, and sulfanilamide, and resistance to tetracycline, chloramphenicol, streptomycin and sulfanilamide respectively.

As a result of studies of the biochemical mechanisms of aminoglycoside resistance, new semisynthetic aminoglycoside antibiotics, i.e., 3',4'-dideoxykanamycin B (DKB) (11), amikacin (AK) (5) and 6'-N-methyl DKB (6'-Me-DKB) (13), were prepared and found to be effective against various species of bacterial strains (including Pseudomonas aeruginosa) resistant to the known aminoglycoside antibiotics. Recently, we have isolated P. aeruginosa strains resistant to DKB and AK, which inactivate the drugs by an aminoglycoside 6'-N-acetyltransferase activity, as already reported in Escherichia coli (1, 9, 10) and P. aeruginosa (2, 4, 6, 8, 12, 14). Further studies have disclosed that almost all strains of P. aeruginosa resistant to both DKB and AK are susceptible to 6'-Me-DKB. Recently, we have isolated two P. aeruginosa strains, i.e., GN4925 and GM5462, resistant to DKB and 6'-Me-DKB, but susceptible to AK. This paper deals with their biochemical mechanism of resistance to DKB and 6'-Me-DKB, and a comparison with that of (DKB•AK) resistance.

MATERIALS AND METHODS

Bacterial strains. P. aeruginosa strains were isolated from clinical specimens and stocked in this laboratory. Their resistance patterns to kanamycin A (KM-A), DKB, gentamicin C complex (GM), 6'-Me-DKB, streptomycin (SM) and AK are shown in Table 1. P. aeruginosa MLA561 (leu-arg-ilv-his-rifr) and P. aeruginosa MLA344 (leu-trp-) were used as recipients of the R factor.

Antibiotics and chemicals. Sodium [14C]acetate (specific activity 58 mCi/mmol) and [14C]acetyl coenzyme A (CoA) (48.6 mCi/mmol) were purchased from the Radiochemical Center (Amersham, England). KM-A, kanamycin C (KM-C), DKB, and SM were obtained from the Meiji Seika Co., Ltd., GM from the Sionogi Seiyaku Co., Ltd., AK from the Bristol-Banyu Research Institute, tetracycline (TC) from the Pfizer Taito Co., Ltd., sulfisomidin (SA) from Dainippon Seiyaku Co., Ltd., and chloramphenicol (CM) from Sankyo Seiyaku Co., Ltd. 6'-Me-DKB and 6'-N-methyl kanamycin A (6'-Me-KM) (13) were kindly supplied from S. Umezawa, K-to University, Tokyo. Gentamicin C, (GM-C,) was kindly supplied from J. Weinstein, the Schering Laboratories (Bloomfield, N.J.).

Media. Nutrient broth containing 0.25% KNO3 was...
TABLE 1. Minimal inhibitory concentrations (MIC) of aminoglycoside antibiotics against P. aeruginosa strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg/ml)</th>
<th>KM</th>
<th>GM-C</th>
<th>DKB</th>
<th>6'-Me-DKB</th>
<th>AK</th>
<th>SM</th>
</tr>
</thead>
<tbody>
<tr>
<td>GN4925</td>
<td>200</td>
<td>50</td>
<td>50</td>
<td>100</td>
<td>3.1</td>
<td>800</td>
<td></td>
</tr>
<tr>
<td>GN5462</td>
<td>200</td>
<td>3.1</td>
<td>50</td>
<td>100</td>
<td>3.1</td>
<td>800</td>
<td></td>
</tr>
<tr>
<td>GN315</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>3.1</td>
<td>25</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>ML4561a</td>
<td>50</td>
<td>1.6</td>
<td>0.8</td>
<td>1.6</td>
<td>1.6</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>ML4344a</td>
<td>25</td>
<td>1.6</td>
<td>0.8</td>
<td>1.6</td>
<td>1.6</td>
<td>12.5</td>
<td></td>
</tr>
</tbody>
</table>

*Two strains of P. aeruginosa were used as recipients in R factor transfer.

used for liquid culture. Minimal medium was used for the transfer of the R factor from exconjugant ML4561 to ML4344.

**Determination of drug resistance.** One loopful of a 100-fold dilution (about 10^9 cells/ml) of an overnight broth culture was spotted on heart infusion agar (Eiken, Tokyo) plates containing serial twofold dilutions of each antibiotic. The minimal inhibitory concentration of each drug was scored after 18 h of incubation at 37 C.

**Preparation of the S-105 fraction and inactivation reaction.** The S-105 fraction from P. aeruginosa was prepared by a procedure described previously (2). The reaction mixture consisted of 0.2 ml of 0.2 M acetate buffer (pH 6.0), 0.05 ml of 20 mM disodium adenosine 5'-triphosphate (ATP), 0.05 ml of 1 mM trillithium CoA, 0.05 ml of 0.02 M magnesium acetate, 0.05 ml of 1 mM drug and 0.1 ml of S-105 fraction. After incubation at 37 C for 15 min, the reaction was then stopped by heating at 80 C for 3 min. Residual antibiotic activity in the reaction mixture was determined by bioassay using B. subtilis PCI 219.

**Radioisotope assay.** The enzymatic transfer of the [1-14C]-acetyl group from acetyl CoA to the antibiotics was carried out at 37 C for 30 min, and 20 µl of reaction mixture was spotted on a phosphocellulose paper (about 2 cm², Whatman P-81), washed with distilled water, and dried. Radioactivity on the paper was counted in toluene-based scintillator with a liquid scintillation counter (Packard Instrument) (2).

**Isolation of enzymatically acetylated DKB.** The enzymatic acetylation of DKB was carried out at 37 C for 3 h in the following reaction mixture: 100 µg of DKB, 78.5 mg of trillithium CoA, 2.44 g of disodium ATP, 858 mg of magnesium acetate, 128 µl of the S-105 fraction from P. aeruginosa GN4925 (50 mg of protein/ml), and 20 ml of 1 M acetate buffer (pH 6.0) in a total volume of 192 ml. The reaction mixture was diluted with 300 ml of distilled water and the reaction was stopped by heating at 100 C for 10 min. The supernatant fluid obtained by centrifugation at 30,000 x g for 30 min was passed through a column of Amberlite CG-50 (NH₄⁺ form, 50 ml), and the column was washed with 2,000 ml of distilled water. The inactivated DKB was then eluted with 0.1 N NH₄OH. The eluate fractions which gave a positive ninhydrin reaction were collected and concentrated to dryness. The powder was applied to a thin-layer chromatogram. After chromatography the spot was raked up, washed with distilled water, and then extracted with 0.3 N NH₄OH and dried. This powder was further subjected to chromatography on Amberlite CG-50- (NH₄⁺ form, 20 ml).

**Thin-layer chromatography.** Thin-layer chromatography was carried out using the following solvent system on a thin layer of silica gel (Tokyo Kasei Co., Tokyo): butanol-ethanol-CHCl₃-17% NH₄OH (4:5:2.5). The spot on a chromatogram was detected by the ninhydrin reaction.

**Conjugal transfer of resistance.** From each donor and recipient culture in nutrient broth at 37 C for 18 h, 0.5 ml was removed and inoculated into 5 ml of fresh nutrient broth and shaken at 37 C. After 2 h of incubation, the cultures (about 2 x 10^8 cells/ml) reached the late logarithmic phase of growth and were used for the conjugal transfer of resistance. One part of donor culture was mixed with four parts of recipient culture and the mixture was incubated at 37 C with gentle shaking. After 90 min of incubation, a 0.1-ml sample of the mixed culture was plated on selective plates containing rifampin (200 µg/ml) and either DKB (3.1 µg/ml) or GM (3.1 µg/ml). The colonies which developed on selective plates after 18 h of incubation at 37 C were picked and purified by three successive single colony isolations, and their drug resistance was then determined. For the transfer of resistance from exconjugant ML4561 to ML4344, the minimal medium containing DKB (or GM), leucine (50 µg/ml), and tryptophan (50 µg/ml) was used for the selection of exconjugant ML4344. The transfer frequency was expressed as the ratio of the number of donor to recipient cells which acquired drug resistance by conjugation.

**Partial purification of the activating enzyme.** Aminoglycoside acetyltransferase was purified by affinity chromatography. KM A-Sepharose 4B was prepared as follows: cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals AFB, Upsala, Sweden) was washed with 1,000 ml of 1 M HCl, 500 ml of cold water, and 25 ml of 0.1 M NaHCO₃-0.5 M NaCl, successively, and was mixed with 0.3 g of KM in 45 ml of 0.1 M NaHCO₃-0.5 M NaCl. The mixture was stirred at 4 C for 24 h. KM A-Sepharose 4B thus prepared was washed several times with 50 ml of 0.1 M acetate buffer-1.0 M NaCl (pH 4.0), and then with 50 ml of 0.1 M borate buffer-1.0 M NaCl (pH 8.0), alternately. Then the KM A-Sepharose 4B was washed with distilled water and with 20 ml acetate buffer (pH 6.0)-20% glycerin, containing 5 mM magnesium acetate and 10 mM 2-mercaptoethanol. KM A-Sepharose 4B was packed. A column (1 by 11 cm) and 4.5 ml of S-105 fraction (100 mg of protein/ml) was passed through the column and eluted with a linear gradient of NaCl from 0 to 0.7 in the above buffer at flow rate of 25 ml/h. The eluted solution was collected in 5-ml fractions.

**RESULTS**

Resistance patterns of aminoglycoside antibiotics. Epidemiological studies disclosed

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that almost all strains of *P. aeruginosa* were susceptible to the newly introduced antibiotics: DKB, AK, and 6'-Me-DKB. It should be noted however, that a few DKB-resistant *P. aeruginosa* strains existed in our stock cultures, even before the widespread clinical use of the drug. Most of the DKB-resistant *P. aeruginosa* strains were still susceptible to AK and 6'-Me-DKB. Similarly, we could isolate DKB, AK-resistant strains of *P. aeruginosa* which were susceptible to 6'-Me-DKB. Recently, we have isolated from clinical specimens two *P. aeruginosa* strains resistant to DKB and 6'-Me-DKB but susceptible to AK. The minimal inhibitory concentration levels of various aminoglycoside antibiotics toward representative strains from our stock cultures are shown in Table 1.

**Inactivation of aminoglycoside antibiotics.** We prepared the S-105 fractions from strains GN4925, GN315, ML4344, and from the ML4344 strains which had acquired resistance by conjugation with strains GN4925 and GN315, respectively, and investigated the biochemical mechanisms of resistance to aminoglycoside antibiotics. Inactivation reaction did not take place without either CoA or ATP. However ATP, CoA, and magnesium acetate could be replaced by acetyl CoA. The inactivation of DKB, 6'-Me-DKB and AK by extracts of these strains is summarized in Table 2. Extracts from strain GN4925 that is resistant to DKB and 6'-Me-DKB, but susceptible to AK, inactivated both DKB and 6'-Me-DKB, but not AK. By contrast, extracts of strain GN315 inactivated both DKB and AK, but not 6'-Me-DKB.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inactivation (%) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DKB</td>
</tr>
<tr>
<td>GN4925</td>
<td>80</td>
</tr>
<tr>
<td>ML4344 (KM, DKB, GM, SM, SA)*</td>
<td>71</td>
</tr>
<tr>
<td>GN315</td>
<td>78</td>
</tr>
<tr>
<td>ML4344</td>
<td>0</td>
</tr>
</tbody>
</table>

*The S-105 fraction was prepared from each strain as described in Materials and Methods. See Materials and Methods for details of the reaction mixture. After 15 min of incubation at 37 C, the residual antibiotic activity in the reaction mixture was determined by bioassay.

Since the inactivation reaction requires the presence of acetyl CoA, the inactivation was considered to be due to acetylation of the drugs. We next investigated the incorporation of [14C]acetate into various aminoglycoside antibiotics by the inactivation reaction using extracts of strain GN4925 (Table 3).

As shown in Table 3, the rate of inactivation almost paralleled the rate of incorporation of [14C]acetate. From these results, we conclude that strain GN4925 inactivated aminoglycoside antibiotics by acetylation. Strain GN4925 was resistant to the GM-C complex including GM-C1, but could not inactivate GM-C, under the conditions used. The mechanism of this GM resistance will be described elsewhere.

**Conjugal transfer of resistance.** Conjugal transferability of resistance in strain GN4925 and GN5462 was examined using strain ML4561 and ML4344 as a recipient. Strain GN4925 could transfer its resistance to KM, DKB, GM, SM, and SA, including 6'-Me-DKB and 6'-Me-KM. An exconjugant, strain ML4561, which had acquired these resistances, could conjugally transfer the same resistance pattern as strain GN4925. Similarly, strain GN5462 could transfer resistance to KM, DKB, TC, CM, SM, and SA. These results indicate that the genes governing resistance to these drugs were located on R plasmids, henceforth called R_{mes167} and R_{mes100}, respectively. Typical results are shown in Table 4.

**Purification of inactivating enzyme.** The S-105 fraction from GN4925 was passed through a KM A-Sepharose 4B column and eluted by linear gradient elution with NaCl from 0 to 0.7 M in 20 mM acetate buffer (pH 6.0)-20% glycine containing 5 mM magnesium acetate and 10 mM 2-mercaptoethanol. The enzyme which inactivated KM, DKB, and 6'-Me-DKB
Enzymatic elution of N-acetyl-3',4'-dideoxyneosamine from DKB using eluted fractions were almost the same. The DKB- and 6'-Me-DKB-inactivating enzyme was purified approximately 96-fold from the S-105 fraction in this way. The optimal pH for DKB and 6'-Me-DKB inactivation was about 6.0, and the pH curves for inactivation of two drugs and time course of inactivation of both were almost identical. The results are shown in Fig. 1 and 2. These results indicated that an aminoglycoside acetyltransferase from strain GN4925 could inactivate KM, DKB, and 6'-Me-DKB.

Identification of the acetylated product. DKB inactivated by extracts of GN4925 was purified and obtained as a white powder. The nuclear magnetic resonance spectra of the inactivated DKB and a synthetic sample of 6'-N-acetyl DKB were found to be similar to each other (Fig. 3). In the nuclear magnetic resonance spectrum of the inactivated DKB in D$_2$O (16.4 mg/0.25 ml), using tetramethylsilane as the external reference, one N-acetyl signal was seen at $\delta$ 2.34. Application of the double resonance technique indicated that the signal of the C-6' methylene protons at $\delta$ 3.15 in DKB shifted to $\delta$ 3.7 in the acetylated DKB. This structure was also consistent with its mass spectrum: a peak of a mono-N-acetyl DKB at m/e 494(M + 1) and an intense peak at m/e 171 attributed to the N-acetyl-3',4'-dideoxyneosamine C unit (Fig. 4). Thus, the structure of the inactivated DKB was determined to be 6'-N-acetyl DKB.

TABLE 4. Conjugal transfer of drug resistance in P. aeruginosa

<table>
<thead>
<tr>
<th>Donor strain</th>
<th>Recipient strain</th>
<th>Selective drug</th>
<th>Transfer frequency</th>
<th>Resistance pattern of exconjugants</th>
</tr>
</thead>
<tbody>
<tr>
<td>GN4925</td>
<td>ML4561</td>
<td>GM</td>
<td>4 x 10^{-4}</td>
<td>(KM.DKB.GM.SM.SA)</td>
</tr>
<tr>
<td>ML4561 (KM.DKB. GM.SM.SA)*</td>
<td>ML4344</td>
<td>DKB</td>
<td>3 x 10^{-4}</td>
<td>(KM.DKB.GM.SM.SA)</td>
</tr>
<tr>
<td></td>
<td>GL4561</td>
<td>GM</td>
<td>5 x 10^{-4}</td>
<td>(KM.DKB.GM.SM.SA)</td>
</tr>
<tr>
<td></td>
<td>ML4561 (KM.DKB TC. CM.SM.SA)*</td>
<td>DKB</td>
<td>9 x 10^{-4}</td>
<td>(KM.DKB.GM.SM.SA)</td>
</tr>
</tbody>
</table>

* Exconjugants ML4561 which had conjugally acquired (KM.DKB.GM.SM.SA) resistance from strain GN4925 and (KM.DKB.TC.CM.SM.SA) resistance from the strain GN5462 were used as the donors of resistance. Details, see Materials and Methods.

FIG. 1. Effect of pH on DKB and 6'-Me-DKB inactivation. The reaction mixture consisted of 30 µl of 0.2 M of each buffer, 10 µl of 0.5 mM of DKB (or 6'-Me-DKB), 20 µl of enzyme purified by affinity chromatography (200 µg/ml), 10 µl of distilled water, and 10 µl of 20 µCi/ml of [14C]acetyl CoA (specific activity 48.6 mCi/mmole). Incorporation of isotope into DKB in acetate buffer (O) and tris(hydroxymethyl)aminomethane malate buffer (\(\Delta\)), and incorporation of isotope into 6'-Me-DKB in acetate buffer (○) and tris(hydroxymethyl)aminomethane malate buffer (\(\Delta\)).
Amino group of the deoxystreptamine moiety in KM-A could protect AK from acetylation by the enzyme AAC(6')-3. This fact suggests the disturbance of the formation of enzyme-substrate complex by 1-N-acylation of KM-A. The finding that enzymes capable of acetylating the 6' of aminoglycoside antibiotics can be divided into two groups depending on whether they are capable of inactivation of 6'-N-methyl aminoglycoside antibiotics and AK or not will be useful in studies of the chemical modification of aminoglycoside antibiotics.

Fig. 2. The enzymatic acetylation of aminoglycoside antibiotics by a partially purified enzyme preparation of GN4925. The reaction mixture consisted of 0.1 ml of enzyme purified by affinity chromatography (100 μg of protein/ml), 0.05 ml of 0.5 mM of drug, 0.05 ml of 0.5 mM acetyl CoA, 0.2 ml of 0.2 M acetate buffer (pH 6.0), and 0.1 ml of distilled water. Details, see Materials and Methods. (O) 6'-Me-DKB; (●) DKB; (▲) AK; (△) GM-C1.

Fig. 3. The nuclear magnetic resonance spectra of DKB (I), synthetic 6'-N-acetyl DKB (II) and the enzymatically inactivated DKB (III).

Fig. 4. The mass spectrum of the inactivated DKB by strain GN4925.
NEW AMINOGLYCOSIDE 6'-N-ACETYLTRANSFERASE

LITERATURE CITED


