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Tobramycin Adenylyltransferase: A New Aminoglycoside-Inactivating Enzyme from *Staphylococcus epidermidis*

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Certain strains of *Staphylococcus epidermidis* resistant to the aminoglycoside antibiotics were shown to contain an enzyme that inactivates the kanamycins, neomycins, butirosins, paromomycin, gentamicin A, amikacin, and tobramycin by adenylylation. Tobramycin adenylyltransferase, as this enzyme is called, was found to be optimally active at pH 5.5. With paromomycin or neomycin B and C as substrates, however, two pH values (5.5 and 9.0) for optimal activity were observed. The enzyme requires Mg^{++} for activity and is stabilized significantly by dithiothreitol. It is probable that the 4'-hydroxyl group of ring I of the antibiotics is adenylylated. Those aminoglycosides that are not substrates for the enzyme lack a hydroxyl group in the corresponding position.

An epidemiological study of the frequency of resistance of staphylococci to aminoglycosides showed that 2% of isolates of *Staphylococcus epidermidis* were inhibited by $\geqslant 12.5$ μg of tobramycin/ml. Such tobramycin-resistant (Tm^R) strains were also resistant to kanamycin A, paromomycin, butirosin, and gentamycin A and intermediately resistant to neomycin and amikacin. In a genetic study of Tm^R in such strains, it was found that resistance was conferred by a small plasmid (mol wt, 2.7×10^6 daltons) present in multiple copies [1]. Loss of this plasmid resulted in tobramycin-susceptible variants, which were also susceptible to the other aminoglycosides except streptomycin.

We now report that the mechanism of resistance to aminoglycosides in these staphylococci can be explained by the production of an adenylylating enzyme. We propose to call this enzyme by the trivial name tobramycin adenylyltransferase, because it was detected in staphylococci unusually resistant to tobramycin, and tobramycin was found to be one of the best substrates for it.

Materials and Methods

Antibiotics and chemicals. Tobramycin was supplied by Eli Lilly and Company (Indianapolis, Ind.); the kanamycins A, B, and C, amikacin (BB-K8), and butirosin by Bristol Laboratories (Syracuse, N.Y.); neomycins B and C and spectinomycin by the Upjohn Co. (Kalamazoo, Mich.); paromomycin by Parke, Davis and Co. (Detroit, Mich.); and the gentamicins C_1, C_2, C_{1a}, and A and sisomicin by Schering Corp. (Bloomfield, N.J.). The other antimicrobial agents were provided by the pharmacy of the University Hospital (Zurich, Switzerland).

Radiolabeled chemicals were bought from the Radiochemical Centre (Amersham, England). Dithiothreitol and adenosine triphosphate (ATP) were obtained from Calbiochem (Los Angeles, Calif.). All other inorganic salts of analytic grade were obtained from Merck (Darmstadt, Federal Republic of Germany).

Organism. The representative strain used for our studies was *S. epidermidis* strain 109, which was isolated from clinical material obtained in Zurich. In addition to tobramycin, this strain is resistant to penicillin (Pen^R), chloramphenicol (Cm^R), tetracycline (Tc^R), streptomycin (Sm^R), and sulfamethoxazole (SMZ^R) and is moderately resistant to methicillin ($Meth^R$). The organism was maintained on brain-heart infusion agar (Difco, Detroit, Mich.) containing 20 μg of neomycin/ml. For production of enzyme, the strain was subcultured on brain-heart infusion agar containing 10 μg of neomycin B/ml. The culture was inoculated into 2 ml of a medium composed of Bacto-tryptone (Difco), 10 g; Bacto-yeast extract

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(Difco), 50 g; NaCl, 10 g; glucose, 4 g; and an amount of water sufficient to make 1 liter. The pH was adjusted to 7.2. For growth of the inoculum, this medium contained 10 μg of neomycin B/ml. After overnight incubation, 1 ml of the inoculum was inoculated into 100 ml of fresh medium, which was incubated in a shaking water bath at 37 C. At the beginning of the exponential phase of growth, 1 ml of neomycin B (400 μg/ml) was added to the culture. When the culture reached an OD of about 1 at 550 nm, cells were harvested by centrifugation at 11,000 *g* for 10 min at 4 C and were washed twice with 40 ml of a cold buffer containing 0.01 M Tris and 0.03 M NaCl (pH 7.8).

Production of enzyme. The osmotic shock method [2], slightly modified, was used. The washed cells were suspended in 10 ml of 0.033 M Tris-HCl buffer (pH 7.3) containing 0.003 M EDTA and 20% sucrose. After it stood at room temperature (about 20 C) for 30 min, the suspension was stirred with a magnetic stirrer for 5 min and centrifuged for 15 min at 12,000 *g* and 4 C. The supernatant was discarded, and the remaining sucrose was removed carefully from the wall of the centrifuge tubes with cotton swabs. The pellet of cells was suspended in 5 ml of an ice-cold solution of 0.0005 M $MgCl_2$, and the suspension was aspirated forcefully about 20 times with a 5-ml pipette. After stirring for 5 min and centrifugation for 15 min (22,000 *g*) at 4 C, the supernatant was carefully decanted to a flask containing 150 μl of a 0.04 M solution of dithiothreitol.

For preparation of extracts by sonification, the washed cells were suspended in an ice-cold solution of 0.0005 M $MgCl_2$, frozen at — 70 C, thawed rapidly, and sonified for 3 min at 55 W (Branson sonifier B-12; Branson, Danbury, Conn.). The procedure was repeated once, after which the suspension was centrifuged for 15 min at 22,000 *g* at 4 C. The supernatant was decanted and stabilized with dithiothreitol.

For preparation of lysates, cells were lysed by lysostaphin according to procedures described by Shaw and Brodsky [3].

Enzymatic assay. The methods used were similar to those described by Benveniste et al. [4] and to those by Davies et al. [5]. The assay for adenylylation consisted of 20 μl of cell extract (0.5–1.0 mg of protein/ml), 1.5 μmol of buffer, 0.5–0.7 μmol of $MgCl_2$, 55 nmol of dithiothreitol, 20 nmol of [2-^{3}H]ATP (specific activity, 60 μCi/μmol), and 0.6–0.8 nmol of drug base (total volume, 65 μl). After incubation at 35 C for various periods, 50-μl samples were pipetted onto a 2-cm^2 phosphocellulose paper (Whatman P-81; Balsbon, Maidstone, England), which was washed twice with double-distilled water and dried at 55 C. Radioactivity was counted in a Packard Tri-Carb scintillation spectrometer (Packard Instruments, Downers Grove, Ill.) in 10 ml of a toluene-based scintillation fluid. Reaction mixtures devoid of either the enzyme or the drug were used as controls for nonspecific binding of [2-^{3}H]ATP. When adenylylation had to be measured before completion of the reaction, the tubes containing the reaction mixtures were chilled in ice-water for termination of the reaction. The amount of drug used in all experiments was within the linear range of a quantitative assay.

Acetylating and phosphorylating activities in the cell extract were checked according to methods described by Haas and Davies [6] and Benveniste and Davies [7].

Assays for inactivation of drugs. After completion of the reaction, 50 μl of the reaction mixture containing the ingredients mentioned above were placed in wells punched in antibiotic agar no. 3 (Difco) containing 10^4 spores of *Bacillus subtilis* (ATCC 6633)/ml as the test organism, and inactivation of drugs was checked. After incubation at 30 C for 24 hr, the degree of inactivation was measured by comparison of the zones of inhibition with appropriate controls.

MIC of drugs. The MIC of antibiotics was determined in Mueller-Hinton broth (Baltimore Biological Laboratories, Baltimore, Md.) [8].

Protein assay. The protein content of cell extracts was measured by the method of Lowry et al. [9].

Results

Enzymatic inactivation of aminoglycosides. Natural resistance to aminoglycosides in gram-positive and gram-negative bacteria is usually the result of a chemical modification and the consequent inactivation of the antibiotics by certain enzymes [10]. Since the known aminoglycoside-

inactivating enzymes are periplasmic enzymes and are released from R-factor-containing gram-negative bacteria by osmotic shock [2], the same procedure was used for extraction of the enzyme from *S. epidermidis* strain 109.

Those drugs that are substrates for the enzyme were inactivated when the reaction mixture contained ATP. No inactivation was observed when the reaction mixture contained either acetyl coenzyme A or cell extracts from tobramycin-susceptible variants. All of the drugs except gentamicins C_{1a}, C_2, and C_3, sisomycin, streptomycin, and spectinomycin were inactivated (table 1).

Adenylylation of aminoglycosides. Since ATP was required for inactivation of the enzyme, we assumed that the antibiotics were modified either by O-adenylylation or O-phosphorylation. No transfer of radioactivity from [γ-^{32}P]ATP to the aminoglycosides was detectable in the enzymatic assay. Radioactivity was, however, transferred to the drugs from [2-^{3}H]ATP. The optimal pH for enzymatic reaction with tobramycin, kanamycin A, B, and C, the butirosins, and gentamicin A was 5.5; with amikacin it was 6.0, although adenylylation occurred over a broad range of pH (4.5–9.5). Adenylylation of paromomycin, neomycin B, and neomycin C had two pH optima, 5.5 and 9.0 (figure 1).

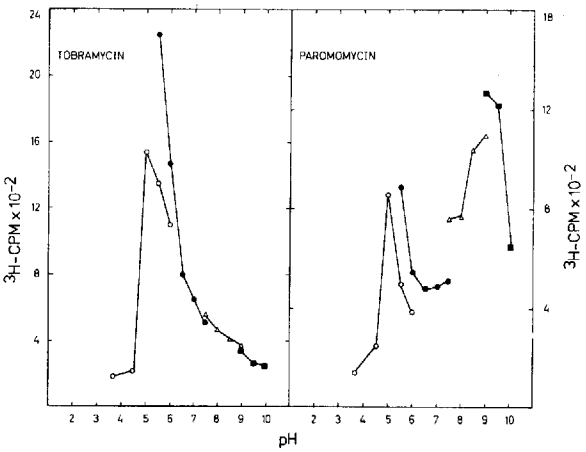

Figure 1. pH dependence of adenylylation reaction with tobramycin adenylyltransferase. Reaction mixtures were prepared at the indicated pH values with use of appropriate buffers. (○——○) = citrate-phosphate buffer, pH 3.8–6.0; (●——●) = Tris-maleate buffer, pH 5.5–7.5; (△——△) = barbital buffer, pH 7.5–9.0; (■——■) = glycine-NaOH buffer, pH 9.0–10.0. Incubation at 35 C lasted for 2.5 min with tobramycin and for 9 min with paromomycin. These periods of incubation were in the linear range of assay at the indicated pH values.

Table 1. Effects of adenosine triphosphate (ATP) and acetyl coenzyme A (acetyl CoA) on inactivation of aminoglycoside antibiotics by lysates of osmotically shocked *Staphylococcus epidermidis* strain 109.

	Strain 109 plus			Strain 109 (Tm^S) plus ATP†
Antibiotic	ATP	Acetyl CoA	Buffer*	
Tobramycin	+	—	—	—
Neomycin B	+	—	—	—
Butirosin	+	—	—	—
Kanamycin A	+	—	—	—
Gentamicin A	+	—	—	—
Paromomycin	+	—	—	—
Amikacin	+	—	—	—
Gentamicins C_{1a}, C_1, C_2	—	—	—	—
Sisomicin	—	—	—	—
Streptomycin	—	—	—	—
Spectinomycin	—	—	—	—

NOTE. Inactivation was determined by the agar diffusion test as described in Materials and Methods. A plus sign indicates that the antibiotics were inactivated; a minus sign indicates no inactivation in comparison with results in appropriate controls. Unlabeled ATP (20 nmol) or unlabeled acetyl CoA (10 nmol) was used in each assay.

* The buffer used was Tris-maleate (pH 5.5).

† Strain 109 (Tm^S) is a spontaneous, tobramycin-susceptible variant of strain 109.

The efficiencies of adenylylation of the different aminoglycosides by tobramycin adenylytransferase at pH 5.5 are listed in table 2. It can be seen that this enzyme is different from both streptomycin-spectinomycin adenylyltransferase and gentamicin adenylyltransferase, since streptomycin and spectinomycin, the gentamicins C_{1a}, C_1, and C_2, and sisomicin are not adenylylated [10].

At pH 5.5, tobramycin is one of the best substrates, whereas amikacin is a rather poor one. In general, the efficiency data reflect the phenotypic resistance of strain 109, except in the case of neomycin B, which is readily adenylylated but weakly inhibitory to the strain. Adenylylation therefore may inactivate the drug incompletely, an effect resulting in a low MIC value.

When the kinetics of adenylylation were examined, it was found that some antibiotics were

Table 2. Adenylylation of aminoglycoside antibiotics by cell-free extracts of *Staphylococcus epidermidis* strain 109.

Antibiotic	[2-^{3}H]ATP incorporated (cpm)*	Percentage of ATP incorporated	MIC (μg/ml)†
Kanamycin B	3,750	127	200
Neomycin C	3,625	123	100
Tobramycin	2,950	100	100
Neomycin B	2,425	82	6.25
Kanamycin C	1,650	56	400
Butirosin	1,500	51	200
Kanamycin A	1,450	49	100
Gentamicin A	1,200	41	400
Paromomycin	1,000	34	100
Amikacin	425	14	6.25
Gentamicin C_{1a}	237	0	<0.047
Gentamicin C_2	168	0	<0.047
Gentamicin C_1	223	0	<0.047
Sisomicin	172	0	<0.047
Streptomycin	203	0	>400
Spectinomycin	222	0	25
None	230	0	...

* ATP = adenosine triphosphate. The assays were performed as described in Materials and Methods. Samples were incubated for 6 min with all substrates to ensure an assay in the linear range. Efficiency of adenylylation was calculated with reference to radioactivity of the adenylylated tobramycin. A count of 2,950 cpm/min was equated with 100% efficiency. Controls devoid of either enzyme or drug had an average value of 230 cpm.

† MICs of drugs were determined in Mueller-Hinton broth [8].

adenylylated at approximately the same rate but not to the same extent (figure 2). A possible explanation for this behavior is that the end products differ slightly in ability to inhibit the enzymatic reaction. However, such inhibition does not seem to be a major factor in determining levels of resistance in intact bacteria (table 2).

Properties of tobramycin adenylyltransferase. Of the three methods tested, osmotic shock yields a preparation with the highest enzymatic activity. Like other aminoglycoside-inactivating enzymes, the enzyme has an absolute requirement for Mg^{++} ions. Dithiothreitol stabilizes the enzyme significantly. The transferase is stable for at least 60 days when stored at −40 C in the presence of dithiothreitol. Nearly 90% of activity was lost after storage for 60 days at 4 C and for one week at 22 C (table 3).

Discussion

In recent years it has become apparent that, under some circumstances, *S. epidermidis* can be considered to be as pathogenic as *Staphylococcus aureus*. Certain infections, such as endocarditis, urinary tract infections, and colonization of ventriculoatrial shunts are typically caused by *S. epidermidis* [11–14]. In addition, this organism can sometimes be isolated in pure culture from pyogenic material. The isolated bacteria often prove to be quite resistant to antibiotics [15–17]. Resistance to all β-lactam antibiotics, to the tetracyclines, to erythromycin, streptomycin, and the sulfonamides, and often also to kanamycin, neomycin, and chloramphenicol has been demonstrated [18]. Thus the new aminoglycoside antibiotics may become significant aids in the treatment of staphylococcal infections.

However, we have found that some *S. epidermidis* strains are resistant to multiple drugs, including many of the new aminoglycoside antibiotics. A previous genetic study revealed that the determinants of resistance to aminoglycosides in representative strains of these staphylococci are plasmid-borne and can be transduced between

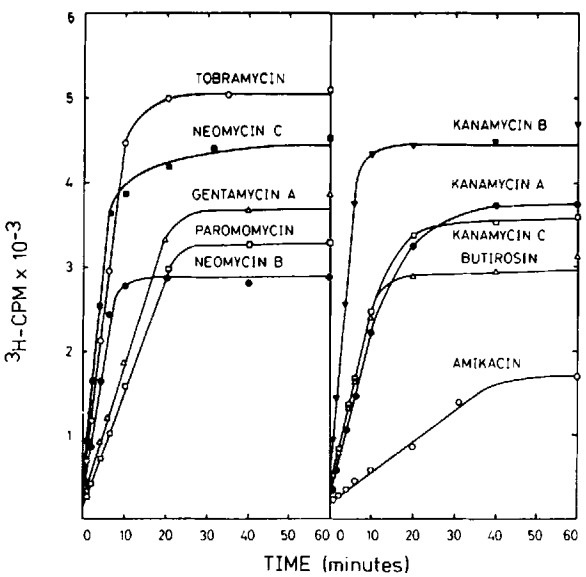

Figure 2. Kinetics of adenylylation of various aminoglycoside antibiotics. Samples were incubated at 35 C for various periods, after which 50-μl portions were pipetted onto a phosphocellulose paper which was washed and counted for radioactivity.

Table 3. Properties of tobramycin adenylyltransferase.

Property	Adenylylation (%)*
Preparation of enzyme†	
Osmotic lysate (0.5 mg of protein/ml)	100
Sonified extract (1.0 mg of protein/ml)	54
Lysed extract (2.9 mg of protein/ml)	11
Mg++ requirement (per assay)‡	
None	4
0.05 μmol	33
0.1 μmol	64
0.3 μmol	88
0.5 μmol	97
0.7 μmol	100
1.0 μmol	95
2.0 μmol	91
Dithiothreitol requirement (per assay)§	
None	44
55 nmol	100
Stability (days)†	
0	100, 100, 100
1	100, 100, 87
6	100, 100, 61
7	100, 100, 11
20	100, 87, 0
30	100, 80, 0
60	100, 9, 0

* Adenylylation values listed in relation to the property of stability were obtained at −40 C, 4 C, and 22 C, respectively.

† Activity was measured after incubation for 30 min at 35 C, with neomycin B as substrate, in Tris-HCl buffer (pH 8.0). A count of 3,658/min was equated with 100% activity.

‡ Activity was measured after incubation for 15 min at 35 C, with tobramycin as substrate, in Tris-maleate buffer (pH 5.5). A count of 5,276/min was equated with 100% activity.

§ Activity was measured after incubation for 6 min at 35 C, with neomycin B as substrate, in Tris-maleate buffer (pH 5.5). A count of 3,884/min was equated with 100% activity.

strains with rather high frequency [1]. The size and other characteristics of this extrachromosomal element are not unusual for staphylococci [1]. However, the plasmid is unusual, insofar as it carries the determinants for production of an aminoglycoside-inactivating enzyme with a broad range of activity. To our knowledge, an enzyme with the properties of tobramycin adenylyltransferase has not been found in either gram-positive or gram-negative bacteria.

Although the end products of enzymatic reaction are yet to be isolated and identified, the substrate profile strongly suggests that adenylylation occurs at the 4′-hydroxyl group of ring I of these antibiotics. The kanamycins, the neomycins, the butirosins, paromomycin, amikacin, gentamicin A, and tobramycin all contain such a group in this position, whereas sisomicin and gentamicins C_{1a}, C_1, and C_2 do not (figure 3). Except for butirosin, all of the substrates have yet another hydroxyl group that can be adenylylated (i.e., the 4″-hydroxyl group of ring III of tobramycin, gentamicin A, the kanamycins, and amikacin, and the 4‴-hydroxyl group of ring IV of the neomycins and paromomycin. Whether or not this second hydroxyl group is also adenylylated remains to be determined after analysis of the adenylylated antibiotics.

The demonstration of a tobramycin adenylyltransferase that inactivates the classical aminoglycosides as well as some of the newer antibiotics of this group implies that no guarantees of freedom from resistance to antibiotics of the future can be given. Thus, although resistance to gentamicin has not been found in staphylococci isolated in our area despite use of the drug for a decade, it is perhaps only a matter of time before resistant strains appear. If such resistance is plasmid-borne, the widespread intraspecies and perhaps also interspecies distribution [19] of this plasmid under appropriate selective conditions in vivo might result. Therefore, an important aspect of antistaphylococcal chemotherapy in the future should be a restriction of the use of the new, highly potent antibiotics to cases in which these drugs are absolutely necessary.

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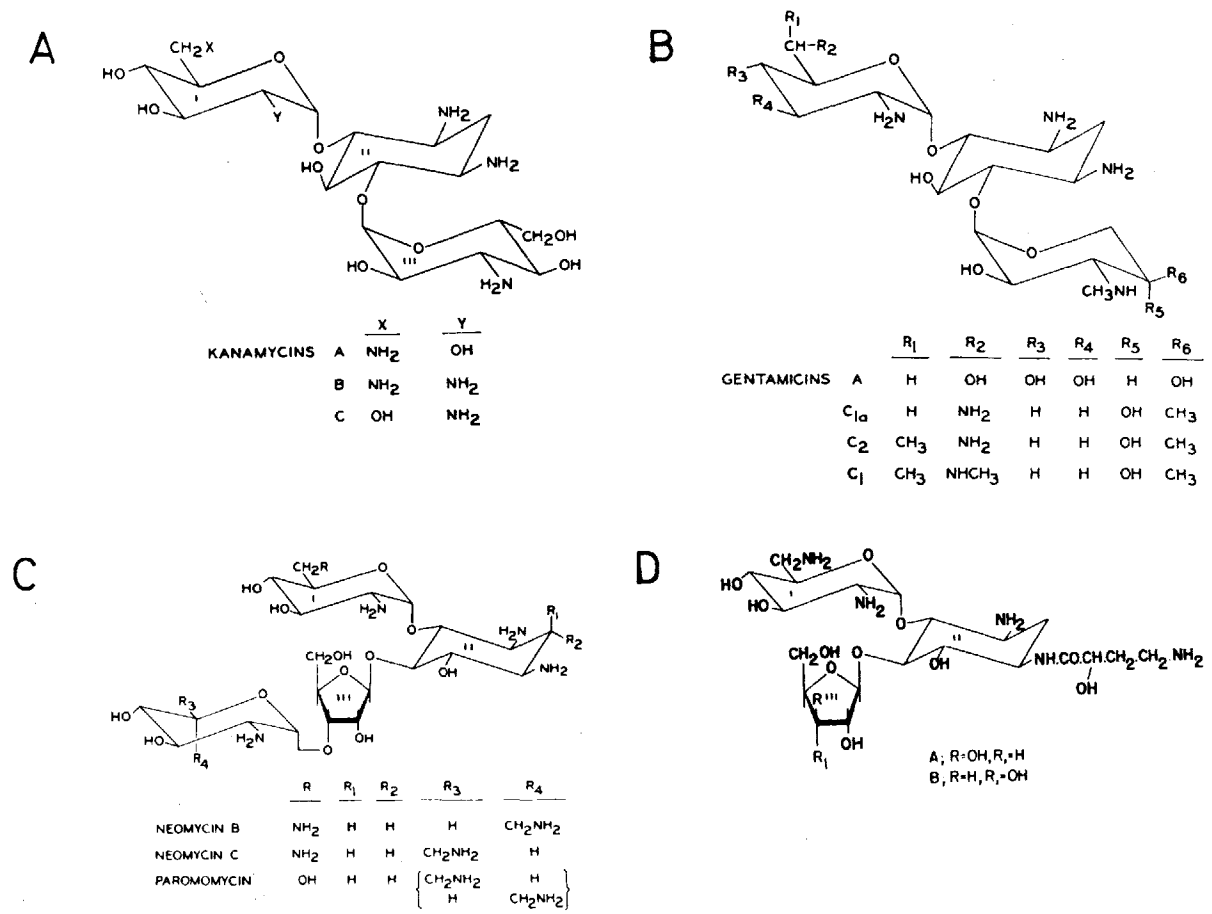

Figure 3. *A*, structure of kanamycins. Positions in ring I are numbered 1′–6′; in ring II, 1–6; and in ring III, 1″–6″. Position γ is the 2′ position. Tobramycin is 3′-deoxykanamycin B. Amikacin is 1-hydroxyamino-butyryl-kanamycin A. *B*, structure of gentamicins. Sisomicin is 4′, 5′-dehydrogentamicin C_{1a}. *C*, structure of neomycins. *D*, structure of butirosins.

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