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Resistance of *Pseudomonas aeruginosa* to Gentamicin and Related Aminoglycoside Antibiotics

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This study was undertaken to investigate biochemical, genetic, and epidemiological aspects of resistance to aminoglycoside antibiotics among 650 consecutive isolates of *Pseudomonas aeruginosa* from Parkland Memorial Hospital, Dallas, Tex. In 364 strains, minimal inhibitory concentrations were 25 μg/ml or greater for gentamicin (G), tobramycin (T) or kanamycin (K). Four patterns of resistance were noted: (A) G, T, K (four strains), (B) G, K (23 strains), (C) T, K (one strain), and (D) K (336 strains). Gentamicin acetyltransferase (GAT) activities were associated with resistance to gentamicin in strains of groups A and B, whereas kanamycin phosphotransferase activity was found in strains of group D. The GAT from group B strains acetylates both gentamicin and tobramycin. Resistance to gentamicin and susceptibility to tobramycin may reflect the fact that the K_m's for tobramycin (25 to 44 μg/ml) of GAT activities in these group B strains are much greater than the K_m's for gentamicin (1.9 to 2.7 μg/ml) and exceed the minimal inhibitory concentrations for tobramycin (1.25 to 7.5 μg/ml). GAT from strains of group A was associated with resistance to G, T, and K. Gentamicin acetyltransferases can be distinguished by their specificities for aminoglycoside substrates. The substrate specificity of GAT from group B strains is similar to that reported for GAT_I, but the specificity of GAT from group A strains differs from those described for GAT_I and GAT_{II}. Conjugal transfer of gentamicin or tobramycin resistance from our strains of *P. aeruginosa* to various potential recipient strains was not observed. Pyocin typing showed that many, but not all, of the strains resistant to gentamicin were similar, and retrospective epidemiological investigation revealed that these strains were isolated almost exclusively from patients in the adult and pediatric burn intensive care units and geographically continguous areas of the hospital.

Pseudomonas aeruginosa is a cause of serious opportunistic infections and is often resistant to many antibiotics (12). Gentamicin and certain other aminoglycoside antibiotics are used frequently for the treatment of such infections, but strains of *P. aeruginosa* and of other bacterial species resistant to gentamicin have now been reported from several parts of the world (5, 12, 19, 24, 26). The mechanisms of bacterial resistance to aminoglycoside antibiotics have been studied in detail. In clinical isolates, resistance to an aminoglycoside is usually controlled by enzymatic inactivation of the antibiotic, and nine different enzymes that catalyze the phosphorylation, acetylation, or adenylylation of aminoglycosides have now been identified in bacteria (3). The synthesis of aminoglycoside-modifying enzymes is often controlled by R factors, permitting rapid spread of resistance among genetically compatible populations of bacteria exposed to appropriate selective conditions (5, 11). An independent mechanism for resistance to aminoglycosides, impermeability of the cell to the antibiotic, has also been reported in some cases of resistance to gentamicin in *P. aeruginosa* (L. E. Bryan, Prog. Abstr. Intersci. Conf. Antimicrob. Ag. Chemother., 13th, Washington, D.C., Abstr. 185, 1973).

Few data are available to correlate the incidence and patterns of aminoglycoside resistance, the enzymatic mechanisms of resistance, and the relatedness of resistant strains among large numbers of clinical isolates of *P. aeruginosa*. In the present study, 650 consecutive isolates of *P. aeruginosa* from Parkland Memorial Hospital, Dallas, Tex., were tested in vitro for susceptibility to three aminoglycosides, gentamicin, tobramycin, and kanamycin.

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Twenty-eight strains were found to have a high level of resistance to gentamicin, to tobramycin, or to both and were characterized further to determine their pyocin types and to identify the mechanisms responsible for resistance to these aminoglycosides. The charts of patients infected with gentamicin-resistant strains of *P. aeruginosa* were also examined to identify epidemiological factors determining the distribution of gentamicin-resistant bacteria in the hospital environment. The results of these studies are the subject of this report.

(Preliminary reports of this work have been presented at the 13th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, D.C., 1973, and at the Symposium on Pseudomonas Infections, Walter Reed Army Institute of Research, Washington, D.C., 1973.)

MATERIALS AND METHODS

Bacterial strains. Strains of *P. aeruginosa* obtained from clinical specimens at Parkland Memorial Hospital were designated by five-digit numbers. The prefixes A, B, C, and D used with these numbers indicate patterns of resistance to the aminoglycosides gentamicin, tobramycin and kanamycin, and will be described in detail later. Nomenclature, properties, sources, and references to previous descriptions of other bacterial strains used in this study are summarized in Table 1. Strains BHM103 and BHM105 in Table 1 were selected in our laboratory by the following methods. Rifampin-resistant mutants grew as isolated colonies from a large bacterial inoculum plated on Trypticase soy agar (Baltimore Biological Laboratories, Cockeysville, Md.) containing 100 μg of rifampin per ml. Treatment of bacterial cultures with the chemical mutagen, ethyl methanesulfonate, and subsequent isolation and characterization of auxotrophic bacterial mutants were carried out by methods described previously (10, 18). The minimal medium used for *P. aeruginosa* was described by Bryan et al. (6). After mutagenesis, cultures were grown in Trypticase soy broth (BBL) without glucose. In addition, a set of 18 strains of *P. aeruginosa*, designated ALA strains, were used as indicators for pyocin typing and were provided by Evan T. Thomas and J. J. Farmer (14). Eighteen strains of *P. aeruginosa* isolated at Parkland Memorial Hospital (PMH strains) were also used as indicators for pyocin typing and were numbered as follows: #1, D06519; #2, D06237; #3, D08717; #4, D04336; #5, D03580; #6, D08442; #7, D09875; #8, D59493; #9, D04176; #10, D01369; #11, D17259; #12, D56196; #13, D59628; #14, A06530; #15, A08034; #16, B01017; #17, B15353; #18, B07910. Newly isolated strains were cloned by repeated single-colony isolations. All strains were maintained as lyophilized cultures.

Bacterial cultures. Unless otherwise noted, all bacteria were grown in Trypticase soy broth, aerated by rotary shaking at 240 rpm in a model G76 water-bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, N.J.), and incubated at 37 C overnight. Routine streaking for isolation of single bacterial colonies was performed on Trypticase soy agar.

Antibiotic susceptibility testing. Minimal inhibitory concentrations (MICs) were determined by a previously described plate dilution method in plastic petri dishes containing antibiotics at concentrations of 0.312, 0.625, 1.25, 2.5, 5, 10, 12.5, 25, 50, and 100 μg/ml in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) solidified with agar (Difco) (9).

Antibiotics and chemicals. Gentamicin complex, gentamicin A, gentamicin C1, gentamicin C1a, gentamicin C2, and sisomicin were provided by Gerald H. Wagman (Schering Corporation, Bloomfield, N.J.). Tobramycin was the gift of H. R. Black (Lilly Laboratory for Clinical Research, Indianapolis, Ind.). Kanamycin, kanamycin A, kanamycin B, and amikacin (BB-K8) were provided by Kenneth E. Price (Bristol Laboratories, Syracuse, N.Y.). Butirosin was supplied by Ronald E. Keeney (Parke, Davis and Co., Ann Arbor, Mich.). Rifampin and reduced dithiothreitol were purchased from Calbiochem, San Diego,

TABLE 1. *Summary of bacterial strains*

Strain	Properties[a]	Source	Reference
P. aeruginosa Capetown 18	Transferrable R factor, GAT_1	G. A. Jacoby	25
P. aeruginosa Stone 130	Transferrable R factor, GAT_1	G. A. Jacoby	25
P. aeruginosa PU 21	*ilv*$^-$ *leu*$^-$ *str*^{r} *rif*^{r}, derived from OT 47	G. A. Jacoby	16
P. aeruginosa PAT 904 *rif*^{r}	*rif*^{r} derivative of PAT 904 (*his*$^-$ *str*^{r} FP*)	G. A. Jacoby	21
P. aeruginosa 280 *met*$^-$ *rif*^{r}	*met*$^-$ *rif*^{r} derivative of 280	L. E. Bryan	6
P. aeruginosa 130	GAT_1	J. Davies	7
P. aeruginosa 209	GAT_1	J. Davies	7
P. aeruginosa BHM 103	*rif*^{r} *met*$^-$ derivative of D03459	Isolated	
P. aeruginosa BHM 105	*rif*^{r} derivative of D04336, *arg*$^-$ or *leu*$^-$	Isolated	
E. coli RC 703	*E. coli* K-12 F$^-$ derived from F$^+$ wild type by curing with acridine orange	R. Clowes	Personal communication
E. coli RC 703 *rif*^{r}	Derived from RC 703	Isolated	

[a] Abbreviations: gentamicin acetyltransferase I (GAT_1); resistant to rifampin (*rif*^{r}) or to streptomycin (*str*^{r}); nutritional requirement for isoleucine and valine (*ilv*$^-$), leucine (*leu*$^-$), histidine (*his*$^-$), methionine (*met*$^-$), arginine (*arg*$^-$); *Pseudomonas* sex factor (FP).

Calif. All antibiotics were obtained as dry samples, except tobramycin which was supplied as a liquid standard. In this paper, the concentration of all aminoglycoside antibiotics is expressed as free base (μg/ml or mM). Ethyl methanesulfonate was from Eastman Kodak Co., Rochester, N.Y.

[γ-^{32}P]adenosine 5'-triphosphate (ATP) was obtained from New England Nuclear, Boston, Mass. [8-^{14}C]ATP and [1-^{14}C]acetyl coenzyme A were from Amersham/Searle, Arlington Heights, Ill. Non-radioactive ATP was from Schwarz/Mann, Orangeburg, N.Y. Two samples of [1-^{14}C]acetyl coenzyme A were used as supplied and had specific radioactivities of 5.8 and 3.7 mCi/mmol. [8-^{14}C]ATP and [γ-^{32}P]ATP were mixed with non-radioactive ATP and used at specific activities of 10 mCi/mmol. All other chemicals were reagent grade.

Preparation of bacterial extracts. The osmotic shock procedure of Nossal and Heppel (20), as previously modified in our laboratory (13), was scaled down for 40-ml cultures of bacteria and was used to prepare crude extracts of bacterial cells.

Enzymatic assays. All assays for enzymes that modify aminoglycoside antibiotics were based on phosphocellulose paper binding assays developed by Davies and collaborators (1, 4, 22). The assays used in the current study were modified slightly from procedures previously used in our laboratory (13).

The following method was used to test for the presence or absence of acetylating, phosphorylating, or adenylylating activities in extracts from aminoglycoside-resistant strains of *P. aeruginosa*. All reaction mixtures of 60-μliter volumes were incubated 30 min at 37 C and contained 50 mM tris(hydroxymethyl)aminomethane-chloride buffer, 8 mM magnesium chloride, and 1 mM dithiothreitol. Assays for adenylylation or phosphorylation of aminoglycoside antibiotics were at pH 8.1, and assays for acetylation were at pH 7.6. Radioactive substrates were present at 0.1 mM as follows: [8-^{14}C]ATP in assays for adenylylation; [γ-^{32}P]ATP in assays for phosphorylation; and [1-^{14}C]acetyl coenzyme A in assays for acetylation. Aminoglycoside substrates were present at 50 μg/ml, and separate assays with each extract were performed with gentamicin, kanamycin, and tobramycin. Reaction mixtures contained 25-μliter samples of crude bacterial extract as the source of enzymes. Duplicate 25-μliter samples were withdrawn from the reaction mixtures and applied to phosphocellulose paper that was washed, dried, and counted in a scintillation counter as described previously (13). In assays without an aminoglycoside substrate, the background radioactivity was usually less than 100 counts per min per 25 μliters. Extracts were considered to contain the enzymatic activity tested (+) if the radioactivity exceeded background by more than 100 counts per min per 25 μliters. Assays were scored as equivocal (+/−) if lower counts were observed and negative (−) if the counts were not significantly different from background.

The substrate specificities of gentamicin acetyltransferase (GAT) or kanamycin phosphotransferase activities were tested with extracts prepared from the selected strains of *P. aeruginosa*. The volume of each extract used in the reaction mixtures was selected to give no more than 15% conversion of the radioactive substrate to product in 30 min. Aminoglycoside substrates were present at concentrations of 100 μg/ml. Under these conditions the amount of product formed is proportional to the initial velocity of the reaction. The results were expressed as relative reaction rates, with the acetylation of gentamicin complex or the phosphorylation of kanamycin assigned a value of 100%.

For kinetic experiments, initial velocities (v) were determined in the following manner. The volumes of reaction mixtures were increased to 360 μliters, the concentrations of the selected aminoglycoside substrates were varied from 2 to 100 μg/ml, and the volumes of crude extract used as sources of GAT activities were selected to give no more than 20% conversion of the radioactive substrate to product in 30 min. Duplicate 25-μliter samples were withdrawn at 0, 10, 20, and 30 min, when the initial concentration of the aminoglycoside substrate was 10 to 100 μg/ml. Samples were withdrawn at 0, 2. 4, 6, and 8 min, when the initial concentration of the aminoglycoside substrate was less than 10 μg/ml. Initial velocities were determined, and the reciprocals of the initial velocities were plotted against the reciprocals of the substrate concentrations as described by Lineweaver and Burk (15). The K_m of each enzymatic activity for the aminoglycoside substrate tested was calculated from the slope (K_m/V_{max}) and from the ordinate intercept ($1/V_{max}$) obtained in the Lineweaver-Burk plot.

Pyocin typing. The ability of selected strains of *P. aeruginosa* to produce pyocins was tested with the two sets of indicator strains designated ALA and PMH. The procedures for growth of pyocin-producing and indicator strains of *P. aeruginosa*, preparation and spotting of the indicator lawns, reading of results, and recording of pyocin production patterns were carried out essentially by the methods of Jones et al. (14). Medium 81 (Trypticase soy broth without glucose, containing 10 g of potassium nitrate per liter) was used for the preparation of indicator lawns and for pyocin production. After spotting of the indicator lawns, plates were incubated overnight for approximately 18 h at 32 C. Reactions scored as positive included both strong and weak clearing of the indicator lawns or the presence of bacteriophage plaques. The 18 plus or minus reactions with each set of indicator strains were converted to a six-digit number representing the pyocin production pattern or type.

Conjugal transfer of R factors. Matings in liquid media were performed as described by Bryan et al. (6). Equal volumes of exponentially growing donor and recipient strains in Trypticase soy broth without glucose were mixed and incubated for 4 h at 37 C. Selective media was either Trypticase soy agar or minimal agar containing Casamino Acids (20 μg/ml) (6), rifampin (100 μg/ml), and gentamicin at concentrations appropriate for the recipient strain and medium used. Concentrations of gentamicin ranged from 1 to 25 μg/ml.

Matings on membrane filters (6, 17) with *P. aeruginosa* donors and *Escherichia coli* RC703 *rif*^{r} recipient were incubated for 20 h on Trypticase soy

agar at 37 C. The mating mixture was resuspended from the filter in Trypticase soy broth without glucose and plated on MacConkey agar (BBL) containing rifampin (100 μg/ml) and gentamicin (10 μg/ml).

RESULTS

Among 650 strains of *P. aeruginosa* isolated at Parkland Memorial Hospital from December 1972 through March 1973 and tested for susceptibility to aminoglycoside antibiotics, 364 were selected for further study because the MIC was at least 20 μg of kanamycin or 5 μg of gentamicin or tobramycin per ml. Figure 1 shows the cumulative susceptibility curves to kanamycin, gentamicin, and tobramycin obtained with these strains. The MIC of kanamycin was 25 μg/ml or greater in all strains and 100 μg/ml or greater in 350 strains. In contrast to the high frequency of kanamycin resistance, an MIC of 10 μg/ml or greater was found for gentamicin in 58 strains and for tobramycin in only seven strains. An MIC for gentamicin or tobramycin of 25 μg/ml or greater was observed in 28 strains and will be designated as high-level resistance to these aminoglycosides.

The 364 selected strains of *P. aeruginosa* were separated into four groups differing in their relative susceptibilities to gentamicin, tobramycin, and kanamycin (Table 2). Two of four strains in group A and 20 of 23 strains in group B were inhibited by gentamicin only at concentrations of 100 μg/ml or greater. Among the 27 strains of *P. aeruginosa* with high-level resistance to gentamicin (groups A and B), 23 of 27 were susceptible to tobramycin. All strains in subgroups A, B, and C were subsequently tested for susceptibility to amikacin (BB-K8) and butirosin. MICs of amikacin were 25 μg/ml or greater for all 28 strains and 100 μg/ml or greater for 23 strains. MICs of butirosin were

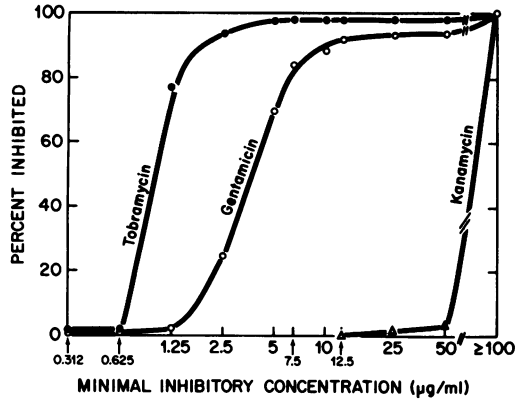

FIG. 1. *Susceptibility of 364 selected strains of P. aeruginosa to tobramycin, gentamicin, and kanamycin.*

TABLE 2. *Patterns of resistance to aminoglycoside antibiotics in 364 selected strains of P. aeruginosa*

Resistance group	No. of strains	MIC (μg/ml)		
		Gentamicin	Tobramycin	Kanamycin
A	4	≥25	≥100	≥100
B	23	≥25	≤7.5	≥100
C	1	2.5	50	>100
D	336	≤12.5	≤12.5	≥25

100 μg/ml or greater for all strains, except one with an MIC of 50 μg/ml. Only one isolate (group C) was resistant to tobramycin but susceptible to gentamicin. Aminoglycoside resistance in some strains in groups A, B, and C was unstable. Although the original patterns of resistance to aminoglycosides were confirmed on two separate occasions, some of these strains became susceptible to gentamicin or tobramycin after extended periods of laboratory passage. The instability of resistance in these strains will be discussed subsequently.

All strains of groups A, B, and C plus six strains of group D were examined for the presence of enzymes that acetylate, phosphorylate, or adenylylate gentamicin, tobramycin, or kanamycin. The results are summarized in Table 3. GAT activity was demonstrated in most strains of groups A and B. GAT from group A strains acetylated gentamicin, tobramycin, and kanamycin. In contrast, GAT from group B strains acetylated gentamicin and tobramycin but not kanamycin. Kanamycin phosphotransferase activity was observed in two strains of group D, and equivocal activities of kanamycin phosphotransferase were detected in some strains of groups A and B.

Representative strains from groups A, B, and D were selected for further studies of the substrate specificity of their GAT or kanamycin phosphotransferase activities. For comparison, assays were performed with extracts of *P. aeruginosa* strains 130 and 209 (Table 1), since these strains are known to contain GAT_1 activity. The data in Table 4 show that the GAT activity from strain B08606 closely resembles GAT_1 with all substrates tested except tobramycin. Strains B08606, 130, and 209 (Table 4) are all susceptible to tobramycin, but under the conditions of these assays the GAT activity from strain B08606 acetylates tobramycin almost as well as gentamicin. Group B strains and GAT_1-containing strains are susceptible to tobramycin, but only group B strains contain a GAT activity that acetylates tobramycin at a high rate in vitro. This property of the GAT from strain B08606 will be discussed in detail

TABLE 3. *Presence of aminoglycoside-modifying enzymes in selected strains of P. aeruginosa*

Resistance group	Enzyme activity[a]		
	Acetyl-transferase	Phospho-transferase	Adenylyl-transferase
A	+ (4/4)	+/−	−
B	+ (17/23)	+/−	−
C	− (0/1)	−	−
D	−	+ (2/6)	−

[a] Symbols: +, enzyme present (number positive/number tested); +/−, low levels of enzyme in some strains; −, enzyme not detected. See text for discussion of the substrate specificities of these enzymes.

subsequently. Like GAT_I, the GAT from strain B08606 does not acetylate kanamycin A, the major component of injectable kanamycin used clinically, but does acetylate kanamycin B. The GAT activity from strain A08034 has a broad substrate specificity and acetylates gentamicin A, all three components of the gentamicin C complex, tobramycin, and kanamycin, correlating well with the resistance of group A strains to gentamicin, tobramycin, and kanamycin (Table 2).

Kinetic parameters (K_m and V_{max}) of GAT from selected strains were determined with various aminoglycoside substrates in an effort to clarify the relationships between antimicrobial susceptibility patterns and the presence of specific GAT activities (Fig. 2). The maximal rates of acetylation of gentamicin C1 and tobramycin by GAT from strain B08606 were identical. The K_m for gentamicin C1 was 2.7 μg/ml. In contrast, the K_m for tobramycin was 44 μg/ml (Fig. 2A). With GAT_I from *P. aeruginosa* strain 130, the K_m for gentamicin C1 was 1.9 μg/ml, whereas the K_m for tobramycin was 25 μg/ml. The maximal rate of acetylation of tobramycin was only one-fourth the maximal rate for acetylation of gentamicin C1 (Fig. 2B). The MICs of tobramycin for strains of group B vary from 1.25 to 7.5 μg/ml, and most are 2.5 μg/ml. These data suggest that strains with GAT_I or closely related GATs are susceptible to tobramycin, because the MIC is significantly below the K_m for tobramycin. Under these conditions, the activity of GAT against tobramycin may be insufficient to confer resistance. The relative values of the maximal velocity of GAT with gentamicin and tobramycin do not correlate with susceptibility and resistance to these antibiotics (Fig. 2A and B). In contrast, the GAT from strain A08034 has a K_m of less than 1 μg/ml both for gentamicin and for tobramycin (Fig. 2C), and strains of group A are resistant to both of these aminoglycosides. The upward deflections of the curves in Fig. 2C at high concentrations of substrate suggest substrate inhibition of GAT activity by tobramycin and possibly by gentamicin.

The 28 strains in groups A, B, and C were also examined by pyocin typing to provide additional information about the possible relatedness of the individual isolates. Two different sets of indicator strains, designated ALA and PMH, were used to compare their ability to discriminate between these strains of *P. aeruginosa*. The data are summarized in Table 5. Among the four strains of group A, three different pyocin types were observed. Among the 23 strains of group B, 14 belonged to a single pyocin type. Other pyocin types among group B strains were each represented only once or twice. One group A strain was indistinguishable by pyocin typing from the most common type among group B strains. One pair of strains gave identical pyocin types with PMH indicators but different types with ALA indicators. With this one exception, the results obtained with the two sets of indicator strains were comparable. Nineteen kanamycin-resistant strains of group D were also typed with the ALA indicators, and 12 different pyocin types were identified (data not shown). These included four isolates similar to

TABLE 4. *Substrate specificities of aminoglycoside-modifying enzymes from selected strains of P. aeruginosa*

Substrates	Relative reaction rates[a]				
	GAT from strain A08034	GAT from strain B08606	GAT_I from strain 130	GAT_I from strain 209	KPT from strain D06519
Gentamicin	100	100	100	100	11
Gentamicin A	65	16	23	14	198
Gentamicin C1	33	99	103	95	0.3
Gentamicin C1a	110	78	138	136	0.4
Gentamicin C2	140	77	112	100	0.4
Sisomicin	NT	114	132	88	NT
Tobramycin	82	93	31	21	1.0
Kanamycin	58	1.2	0	0	100
Kanamycin A	NT	1.1	0.2	1.0	NT
Kanamycin B	NT	54	29	29	NT
Amikacin (BB-K8)	6	0.7	8.5	0	5
Butirosin	6	1.5	0.1	0.4	218

[a] Abbreviations: Gentamicin acetyltransferase (GAT); kanamycin phosphotransferase (KPT). A rate of 100 was assigned for acetylation of gentamicin and for phosphorylation of kanamycin. All other rates are expressed as relative rates. NT indicates not tested. The amount of product formed in 30 min per 60 μliters of reaction mixture in the assay assigned a rate of 100 was: A08034, 242 pmol; B08606, 1027 pmol; strain 130, 773 pmol; strain 209, 1062 pmol; and D06519, 650 pmol.

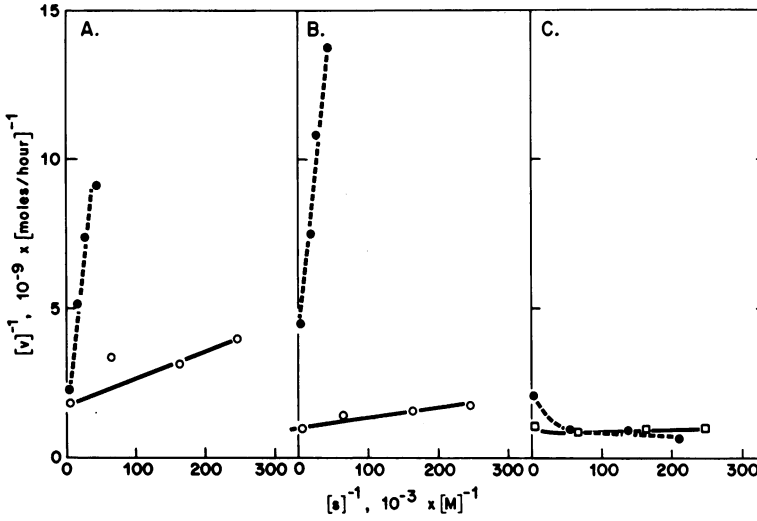

FIG. 2. *Kinetic properties of GAT activities from selected strains of P. aeruginosa. Reciprocal of the initial velocity is plotted on the ordinate, and reciprocal of substrate concentration is on the abscissa. Measurement of initial velocity and calculation of K_m were as described in Materials and Methods. Aminoglycoside substrate was gentamicin C1 (○), gentamicin C2 (□), or tobramycin (●). (A) GAT from strain B08606. K_m was 2.7 μg/ml for gentamicin C1 and 44 μg/ml for tobramycin. (B) GAT$_I$ from strain 130. K_m was 1.9 μg/ml for gentamicin C1 and 25 μg/ml for tobramycin. (C) GAT from strain A08034. K_m was less than 1 μg/ml both for gentamicin C2 and for tobramycin. Substrate inhibition was observed with tobramycin.*

the group C strain, one isolate like one of the miscellaneous types in group B, and 10 types not previously recognized.

Additional correlations between pyocin type, aminoglycoside resistance patterns, stability of gentamicin resistance, and demonstration of GAT activity in these strains are summarized in Table 6. Among 21 strains with stable resistance to gentamicin and demonstrable GAT activity, 19 were accounted for by only three pyocin types. In contrast, six strains of group B of diverse pyocin types became susceptible to gentamicin during passage in the laboratory and had no detectable GAT activity when subsequently tested. Gentamicin-resistant colonies were isolated from each of these six strains after plating on Trypticase soy agar containing 10 μg of gentamicin per ml and had MICs of gentamicin at 25 μg/ml (one strain) or 50 μg/ml (10 strains). Three of the newly isolated resistant strains were tested and all three also lacked GAT activity. Among the original 23 isolates of group B, only four had MICs less than 100 μg of gentamicin per ml, and in all four the resistance patterns were unstable. These observations indicate heterogeneity in the properties of the original 27 isolates of *P. aeruginosa* with a high level of resistance to gentamicin.

A large number of experiments has been performed in an attempt to demonstrate conjugal transfer of resistance to gentamicin in matings between gentamicin-resistant isolates of *P. aeruginosa* from Parkland Memorial Hospital and several gentamicin-susceptible recipient strains, including *P. aeruginosa* 280, PU 21, PAT 904, BHM 103, BHM 105, and *E. coli* RC 703 *rif*^{r} (Table 1). Rifampin was used for coun-

TABLE 5. *Pyocin typing of aminoglycoside-resistant strains of P. aeruginosa with two sets of indicator strains*[a]

Resistance group	Pyocin type	
	ALA indicator strains	PMH indicator strains
A (4)	11 1411 (2)	44 7656 (2)
	46 5873	88 8586
	18 3111	27 7156
B (23)	18 3111 (14)	27 7156 (14)
	16 3111 (2)	24 7156 (2)
	61 1111, 66 3111	44 4457 (2)
	68 5883	27 7556
	37 4122	85 5478
	64 1111	44 8488
	61 1131	44 6458
	88 8888	88 8888
C	62 1421	46 7656

[a] Numbers in parentheses designate the number of strains of the type indicated. If only one strain of a type was identified, parentheses are omitted.

TABLE 6. *Correlations between stability of high-level gentamicin resistance and other properties of P. aeruginosa*

Resistance group	No. of strains	Resistance stable	Acetylating enzyme demonstrated	Pyocin types[a]
A	4	Yes	Yes	11 1411 (2) 46 5873 18 3111
B	17	Yes	Yes	18 3111 (14) 16 3111 (2) 68 5883
B	6	No	No	61 1111 37 4122 66 3111 64 1111 61 1131 88 8888

[a] ALA indicator strains; numbers in parentheses designate the number of strains of the type indicated.

terselection, and potential donor and recipient strains were tested for compatibility of pyocin types. No conjugal transfer of gentamicin resistance was observed among 35 different matings with our gentamicin-resistant strains of *P. aeruginosa* as donors. In contrast, transfer of gentamicin resistance from *P. aeruginosa* strain Capetown #18 or Stone #130 to strain PU 21 occurred at high frequency, as previously reported (G. A. Jacoby, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1973, G 248, p. 67).

A retrospective examination of charts from patients infected with these 28 gentamicin- or tobramycin-resistant strains of *P. aeruginosa* was performed to identify common factors potentially relevant to the epidemiology of infection with resistant strains in this hospital (Table 7). The 28 strains selected for study on the basis of resistance patterns were isolated from 18 different patients. Two or more isolates were obtained from each of seven patients. In all cases, except one (R.S.), only one pyocin type was isolated from each patient. The 14 group B strains with similar pyocin types were isolated from eight different patients, and the dates of isolation were distributed throughout the period of the study. Strains A12584, B15165, and B15450, with different susceptibility patterns but similar pyocin types, were all isolated from the urinary tract of a single patient (F.J.) in the adult burn unit. The most striking finding is that almost all of the isolates originate from patients in the adult and pediatric burn intensive care units. Patients F.E. and R.S. were surgical patients who occupied rooms adjacent to the adult burn unit. The only medical patient (J.W.-1) is a quadruplegic who had had an indwelling Foley catheter for 2 years and had had multiple hospital admissions prior to the time that he acquired a urinary tract infection with a gentamicin-resistant strain of *P. aeruginosa*.

DISCUSSION

The emergence and dissemination of resistant bacterial strains is a major problem limiting the effectiveness of antibiotics as chemotherapeutic agents. A previous bacteriological study of gentamicin and tobramycin from Parkland Memorial Hospital included 141 isolates of *P. aeruginosa*, among which 4% were resistant to gentamicin and 1% to tobramycin at 10 μg/ml (9). In the present study, 650 isolates were collected 1 year later. The incidence of resistance at 10 μg/ml was 8.9% for gentamicin and 1.1% for tobramycin. Fifty-six per cent of the isolates were resistant to 25 μg or more of kanamycin per ml. The incidence of resistance to these aminoglycoside antibiotics appears to be directly proportional to the duration of their use in the hospital environment. At the time of this study, tobramycin was an investigational drug with restricted usage. Most gentamicin-resistant strains of *P. aeruginosa* in this institution are susceptible to tobramycin, but a small reservoir of tobramycin-resistant strains is already present. Amikacin (BB-K8) is reported to be active against most strains of *P. aeruginosa* (23), but none of the present strains with a high level of resistance to gentamicin were susceptible to amikacin. These highly resistant strains were also resistant to butirosin.

The data in Tables 2, 3, 4, and Fig. 2 show that the pattern of resistance to aminoglycoside antibiotics can be correlated with the presence of specific enzymes that modify them. However, modification of an aminoglycoside does not necessarily destroy its antibacterial activity (1, 3). The presence of a particular enzyme may therefore confer resistance to aminoglycosides that are inactivated by a specific modification, whereas full or partial susceptibility may persist if the aminoglycosides are not modified or retain antibiotic activity after modification. In addition, the data presented here suggest that kinetic properties of an inactivating enzyme may determine susceptibility or resistance to particular aminoglycosides. For example, both gentamicin and tobramycin are substrates for the GAT activities from *P. aeruginosa* strains of

TABLE 7. *Epidemiological data about aminoglycoside-resistant strains of P. aeruginosa from Parkland Memorial Hospital*

Strain[a]	Pyocin type[b]	Date isolated	Specimen cultured	Patient	Age (years)	Sex	Hospital service[c]
A 06516	11 1411	2-6-73	Foley catheter	JW-1	20	M	Medicine
A 08034	11 1411	2-15-73	Foley catheter	JW-1	20	M	Medicine
A 06530	46 5873	2-6-73	Foley catheter	BD	55	F	ABICU
A 12584	18 3111	3-12-73	Foley catheter	FJ	29	M	ABICU
B 15166	18 3111	3-24-73	Foley catheter	FJ	29	M	ABICU
B 15450	18 3111	3-26-73	Foley catheter	FJ	29	M	ABICU
B 01017	18 3111	1-5-73	Urine	DJ	10	F	PBICU
B 01265	18 3111	1-8-73	Sputum	DJ	10	F	PBICU
B 01228	18 3111	1-8-73	Sputum	DJ	10	F	PBICU
B 08606	18 3111	2-17-73	Foley catheter	TV	1½	F	PBICU
B 09680	18 3111	2-24-73	Foley catheter	TV	1½	F	PBICU
B 02224	18 3111	1-12-73	Urine	CB	61	F	ABICU
B 03564	18 3111	1-20-73	Intravenous catheter	CB	61	F	ABICU
B 04802	18 3111	1-26-73	Saphenous vein	CB	61	F	ABICU
B 03594	18 3111	1-20-73	Sputum	HP	57	M	ABICU
B 06306	18 3111	2-8-73	Urine	FE	65	M	Surgery
B 05637	18 3111	1-31-73	Blood (autopsy)	CL	57	M	ABICU
B 07667	18 3111	2-13-73	Wound (burn)	RS	64	M	Surgery
B 07911	16 3111	2-14-73	Sputum	RS	64	M	Surgery
B 04361	16 3111	1-24-73	Sputum	KB	61	F	ABICU
B 12261	61 1111	3-9-73	Foley catheter	JD	34	M	ABICU
B 13474	66 3111	3-16-73	Pus	JD	34	M	ABICU
B 07910	68 5883	2-14-73	Tracheostomy	CJ	52	M	ABICU
B 15353	64 1111	3-26-73	Blood	JW-2	8	M	PBICU
B 03288	37 4122	1-18-73	Sputum	VM	59	M	MICU, SICU
B 16517	61 1131	4-2-73	Pustule	MC	5	F	PBICU
B 05312	88 8888	1-30-73	Sputum	WC	78	M	SICU
C 08893	62 1421	2-30-73	Sputum	JM	41	M	SICU

[a] Prefix A, B, or C designates resistance group (see Table 1).

[b] With ALA indicator strains.

[c] Abbreviations: Adult burn intensive care unit (ABICU); pediatric burn intensive care unit (PBICU); surgical and medical intensive care units (SICU and MICU).

groups A and B (Table 4), although group B strains are susceptible to tobramycin. This may be explained by the observation (Fig. 2) that the GAT from strain B08606 has a K_m for tobramycin that is much greater than the MIC of tobramycin for this strain.

The nomenclature and properties of nine enzymes that modify aminoglycosides by acetylation, phosphorylation, or adenylylation have been summarized by Benveniste and Davies (3). The kanamycin phosphotransferase activity found in strain D06515 resembles neomycin-kanamycin phosphotransferase II in substrate specificity. As shown in Table 4, the GAT activity from strain B08606 resembles GAT_I with all substrates tested except tobramycin. With GAT from strain B08606 the V_{max} is identical for tobramycin and for gentamicin C1, but with GAT_I from strain 130 the V_{max} for tobramycin is much smaller than for gentamicin C1 (Fig. 2). In contrast, the GAT activity from strain A08034 acetylates gentamicin A, all three gentamicin C components, tobramycin, and kanamycin, but not amikacin (BB-K8) or butirosin. This GAT activity differs from KAT, GAT_I, or GAT_{II}. Acetylation of gentamicin A, but not amikacin or butirosin, excludes the combination of kanamycin acetyltransferase with GAT_I or GAT_{II} in strain A08034. A GAT with similar properties has been observed independently by Julian Davies (ASM Conference on Extrachromosomal Elements in Bacteria, Amer. Soc. Microb., New Orleans, La., January 1974) and has been tentatively designated GAT_{III}. Purification and detailed characterization of GAT_{III} were not reported and have not yet been published. Future studies on immunological relationships, amino acid sequences, and the effects of mutations on substrate specificity and kinetic properties of these acetylating enzymes may clarify the possible evolutionary relationships between them.

Conjugal transfer of resistance to gentamicin or tobramycin from our strains of *P. aeruginosa* was not observed in our experiments, but indirect evidence suggests the possibility that R factors determine gentamicin and tobramycin resistance in our strains. The GAT from strain B08606 closely resembles GAT_1 which is controlled by R factors in *P. aeruginosa* strains Capetown 18 and Stone 130. Resistance to gentamicin and tobramycin in some of our isolates was unstable, consistent with loss of a plasmid. Lack of conjugal transfer of resistance could be explained by the presence of an R factor incapable of self transfer, by lack of an appropriate recipient strain, by frequency of transfer below the level detectable by our methods ($\leq 10^{-7}$ per donor), or by chromosomal localization of the genes responsible for synthesis of the GAT enzymes. Physical studies to confirm the presence or absence of plasmids in our isolates of *P. aeruginosa* have not yet been carried out.

Epidemiological data concerning our strains of *P. aeruginosa* highly resistant to gentamicin or tobramycin are summarized in Table 7. Although the present study included all strains isolated during a defined period of time from the hospital laboratory, the majority of resistant strains were from the adult and pediatric intensive care burn units or from geographically contiguous areas of the hospital. Most of the isolates were from urine and sputum; few were from burn wound cultures. During this study, most burn wound cultures were handled by a separate laboratory, and antibiotic susceptibility testing was performed by a disk diffusion method different from our plate dilution method. It is not clear why gentamicin- and tobramycin-resistant strains of *P. aeruginosa* were not detected in cultures from burn wounds of these same patients. In the burn units at Parkland Memorial Hospital gentamicin has been used extensively, including subeschar administration of gentamicin by clysis, but topical gentamicin therapy has been used infrequently for burn wounds. It is of interest that strains A12584 and B15166 with similar pyocin types but different patterns of resistance to aminoglycosides were both isolated from the urinary tract of the same patient (Table 7). Tobramycin has been used as an investigational drug in the adult burn unit, and it is interesting to speculate that strains A12584 and B15166 might be derived from one another by a mutation that altered the substrate specificity of GAT.

A large number of different enzymes capable of inactivating aminoglycoside antibiotics have become disseminated among various bacterial species. R factors control the production of some of these enzymes, but the possibility that others may be controlled by chromosomal genes cannot be excluded at the present time. Individual strains of aminoglycoside-resistant bacteria can contain more than one aminoglycoside-modifying enzyme (2, 23), and one or more R factors controlling aminoglycoside resistance may be present simultaneously (8). In addition, it appears that resistance to gentamicin in clinical isolates of *P. aeruginosa* can be determined by other mechanisms, such as impermeability to the antibiotic (L. E. Bryan, Prog. Abstr. Intersci. Conf. Antimicrob. Ag. Chemother. 13th, Washington, D.C., Abstr. 185, 1973). For these reasons, caution must be used in inferring mechanisms of resistance directly from the patterns of susceptibility to a spectrum of aminoglycosides. As each new aminoglycoside has been introduced, resistant bacterial strains have emerged and become disseminated under the selective pressure of antibiotic usage. The data discussed here illustrate this trend in *P. aeruginosa* with the antibiotics kanamycin, gentamicin, and tobramycin. It seems likely that similar principles will apply to newer aminoglycoside agents currently under investigation. If the value of these new and potent aminoglycoside antibiotics is to be optimized for use in the chemotherapy of human infections, it seems mandatory that they be employed only for specific indications and that indiscriminate usage that favors the emergence and selection of resistant bacteria be avoided.

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