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Transferrable Resistance to Tobramycin in *Klebsiella pneumoniae* and *Enterobacter cloacae* Associated with Enzymatic Acetylation of Tobramycin

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Among gram-negative bacilli isolated from burn wound cultures, some strains of *Enterobacteriaceae* were resistant to tobramycin (minimal inhibitory concentration [MIC] $\geq$ 20 μg/ml) but susceptible to gentamicin (MIC $\leq$ 5 μg/ml). One *Klebsiella pneumoniae* and two *Enterobacter cloacae* strains were selected for studies on their mechanisms of resistance to aminoglycoside antibiotics. Resistance to high concentrations of tobramycin (MICs of 25 to 50 μg/ml) was conjugally transferred to a susceptible *Escherichia coli* strain at rates of 1.2×10^{-4} to 2.8 to 10^{-4} per donor cell, suggesting that resistance is controlled by R factors. Resistances to tobramycin, kanamycin, and neomycin were cotransferred. Enzymatic activities were present that acetylated tobramycin, gentamicin, and kanamycin in osmotic lysates from the donor and transcipient strains. Enzymatic adenylylation of these aminoglycosides was not observed. The aminoglycoside-acetylating activities from *K. pneumoniae* and *E. cloacae* resembled kanamycin acetyltransferase (KAT) in their specificity for aminoglycoside substrates. Not all isolates of bacteria that produce KAT are resistant to tobramycin, but the factors that determine susceptibility or resistance to tobramycin in KAT-producing bacteria have not yet been established.

A number of recent studies have attested to the broad spectrum of antibacterial activity for tobramycin, a new aminoglycoside antibiotic (5, 7, 9). Its effectiveness in vitro is comparable with that of gentamicin, a chemically related aminoglycoside, against *Staphylococcus aureus* and certain species of *Enterobacteriaceae*, but most susceptible strains of *Pseudomonas aeruginosa* are inhibited by a lower concentration of tobramycin than gentamicin. Although resistance both to gentamicin and to tobramycin occurs in some bacterial strains, other isolates are either resistant to gentamicin and susceptible to tobramycin or resistant to tobramycin and susceptible to gentamicin (5-9, 15). Resistance to aminoglycoside antibiotics in clinical isolates of bacteria is generally determined by R factors, although other mechanisms of resistance have been described. Some R factors have been shown to control the synthesis of enzymes that modify and inactivate clinically useful aminoglycosides (3).

In this study we report transferable resistance to tobramycin associated with enzymatic acetylation of tobramycin and other aminoglycosides in *Klebsiella pneumoniae* and *Enterobacter cloacae* isolated from burn wounds. The acetylating activities from these strains resemble kanamycin acetyltransferase (KAT) in their specificities for aminoglycoside substrates.

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MATERIALS AND METHODS

Bacterial strains. *K. pneumoniae* 355 (Kp355) and *E. cloacae* strains 359 (Ec359) and 264 (Ec264) were isolated from burn wound cultures at Parkland Memorial Hospital, Dallas, Tex. RC703, obtained from R. C. Clowes, is an F^- *Escherichia coli* K-12 strain derived from an F^+ strain by elimination of F with acridine orange. A rifampin-resistant mutant of RC703 selected in our laboratory and designated RC703 *rif*^{r} was used as the recipient in conjugal transfer experiments. All strains were maintained as lyophilized cultures. Working stocks were grown on slants of tryptone soy agar (Oxoid Consolidated Laboratories, Inc., Chicago Heights, Ill.).

Antibiotics and chemicals. Tobramycin as a standard solution was provided by H. R. Black (Lilly Laboratory for Clinical Research, Indianapolis, Ind.). All other aminoglycosides were obtained as dry samples. Gentamicin complex, gentamicin A, gentamicin C1, gentamicin C1a, gentamicin C2, and sisomicin were supplied by G. H. Wagman (Schering Corp., Bloomfield, N.J.), kanamycin, kanamycin A, kana-

mycin B, and amikacin (BB-K8) by K. E. Price (Bristol Laboratories, Syracuse, N.Y.), and butirosin by R. E. Keeney (Parke-Davis and Co., Ann Arbor, Mich.). The concentration of all aminoglycoside antibiotics is expressed as free base (micrograms per milliliter or millimolar). Rifampin was from Calbiochem (La Jolla, Calif.). Tetracycline (Tetracyn, Pfizer Inc., New York, N.Y.) was obtained commercially. Antibiotic disks for susceptibility testing were products of Baltimore Biological Laboratories. [8-^{14}C]adenosine 5'-triphosphate and [1-^{14}C]acetyl coenzyme A were from Amersham/Searle, Arlington Heights, Ill. All other chemicals were of reagent grade.

Antibiotic susceptibility testing. Minimal inhibitory concentrations (MICs) of aminoglycoside antibiotics were determined by a plate dilution method with antibiotic concentrations of 0.312, 1.25, 2.5, 5, 7.5, 10, 12.5, 25, 50, and 100 μg/ml in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) solidified with agar (Difco) (7). Cultures for susceptibility testing were grown in Trypticase soy broth (BBL, Cockeysville, Md.). Resistance or susceptibility to other antibiotics was determined by the disk diffusion method of Bauer et al. (1).

Bacterial extracts and enzymatic assays. Osmotic lysates of bacterial cells were prepared by the procedure of Nossal and Heppel (14), modified in our laboratory as previously described (11).

Assays for the acetylation or adenylylation of aminoglycoside antibiotics used radioisotopically labeled acetyl coenzyme A or adenosine 5'-triphosphate as substrate and were modified from the phosphocellulose paper binding methods of Benveniste et al. (2, 4). Qualitative assays to determine the presence or absence of acetylating or adenylylating enzymes in osmotic lysates of bacterial cells and quantitative methods for determining relative reaction rates of such enzymes with various aminoglycoside substrates have been described in detail elsewhere (10).

Conjugal transfer of resistance to antibiotics. Cultures of donor and recipient strains were grown overnight in Trypticase soy broth without glucose enriched with 0.5% yeast extract (BBL). Diluted cultures were incubated at 35 C with shaking until the cell densities were between 6×10^7 and 1×10^8 colony-forming units per ml for donor strains and 5×10^8 colony-forming units per ml for the recipient RC703 *rif*^{r}. Equal volumes (0.5 ml) of donor and recipient strains were mixed and incubated without agitation for 1 h at 35 C in horizontal tubes (16 by 100 ml) to provide a larger surface for aeration. Volumes (0.1 ml) of 10^{-1} and 10^{-2} dilutions of the mating mixtures and 0.1 ml of 10^{-1} dilutions of individual parental strains as controls were plated on Mueller-Hinton broth (BBL) solidified with 1.5% Noble agar (Difco) (designated MBHA medium) containing rifampin at 100 μg/ml and tobramycin at 1 μg/ml. In addition to testing for antibiotic susceptibility patterns, the purified transcipient cultures were identified as *E. coli* by routine biochemical tests (13).

RESULTS

A retrospective survey of resistance to gentamicin and tobramycin among strains of *Enterobacteriaceae* from 1,133 burn wound cultures obtained from patients at Parkland Memorial Hospital from March 1973 to February 1974 is presented in Table 1. *P. aeruginosa* was the organism most frequently isolated from burn wounds. Among *K. pneumoniae*, *Entero-*

TABLE 1. *Tobramycin and gentamicin resistance among Enterobacteriaceae[a] from burn wounds*

Date	No. of patients on burn service	No. of cultures	No. of isolates resistant[b] to tobramycin or gentamicin or both[c]								
			Klebsiella pneumoniae			*Enterobacter cloacae*[d]			*Escherichia coli*		
			T	G	T & G	T	G	T & G	T	G	T & G
26-31 March 1973	36	36	4	0	1	1	0	0	0	0	0
April 1973	51	146	9	0	0	1	0	0	4	0	1
May 1973	46	108	2	0	0	0	0	0	1	0	1
June 1973	41	71	0	0	0	0	0	0	0	0	0
July 1973	37	110	3	0	1	1	3	0	2	0	0
Aug. 1973	31	110	1	0	10	0	0	0	1	0	1
Sept. 1973	33	81	1	0	9	0	0	0	0	0	0
Oct. 1973	30	67	3	0	0	0	0	0	0	0	0
Nov. 1973	28	109	0	0	0	0	0	0	0	0	0
Dec. 1973	29	106	2	0	0	19	0	0	0	0	0
Jan. 1974	50	137	2	0	2	36	0	5	2	0	2
1-16 Feb. 1974	53	52	0	0	0	9	0	4	0	0	1

[a] One *Providencia stuartii* resistant to both tobramycin and gentamicin was also isolated.

[b] Strains with an MIC of 20 μg/ml or greater were considered resistant, and those with an MIC of 5 μg/ml or less were considered susceptible.

[c] Abbreviations: T, tobramycin only; G, gentamicin only; T & G, tobramycin and gentamicin.

[d] Included are one *Enterobacter aerogenes* resistant to tobramycin only and one resistant to gentamicin only.

bacter cloacae, and *Escherichia coli*, a number of isolates were resistant to gentamicin or tobramycin. *K. pneumoniae* and *E. coli* resistant to tobramycin but susceptible to gentamicin were isolated principally in March and April of 1973 but less frequently thereafter. In contrast, multiple isolates of *E. cloacae* with this resistance pattern first appeared in December 1973 and were subsequently observed until the end of the survey in February 1974. These tobramycin-resistant, gentamicin-susceptible strains of *E. cloacae* were isolated from six patients in December 1973, from 13 patients in January 1974, and from 8 patients in February 1974.

Strains Kp355, Ec359, and Ec264 were isolated from burn wound cultures. By disk diffusion susceptibility testing, these strains were resistant to ampicillin, neomycin, and streptomycin but susceptible to chloramphenicol. Ec359 and Ec264 but not Kp355 were resistant to tetracycline. Conjugal transfer of resistance to tobramycin from these strains to *E. coli* RC703 *rif*^{r} occurred at rates of 1.2×10^{-4} to 2.8×10^{-4} per donor cell. Each cross was performed at least four times, and the highest frequency of transfer was reported (Table 2). The differences in the frequency of transfer by the various donor strains were not significant. Transduction of tobramycin resistance was ruled out by demonstrating that no resistant colonies could be isolated when bacteria-free filtrates of exponential-phase cultures of the donors were incubated with RC703 *rif*^{r} in the same manner as described for conjugation. Tobramycin-resistant transcipient colonies from the crosses in Table 2 were replica plated to MHBA containing kanamycin (30 μg/ml) and to MHBA containing tetracycline (20 μg/ml). All tobramycin-resistant transcipient colonies of RC703 *rif*^{r} tested grew in the presence of 30 μg of kanamycin per ml. Tetracycline resistance was observed in 1 of 299 transcipient colonies tested from a mating of Ec359 × RC703 *rif*^{r} and in 1 of 263 transcipients tested from a mating of Ec264 × RC703 *rif*^{r}. Five transcipients from each of the three crosses in Table 2 were tested for susceptibility to other antibiotics by disk diffusion. All 15 transcipient colonies tested were resistant to neomycin, resistant or indeterminant to streptomycin and ampicillin, and susceptible to tetracycline and chloramphenicol. The recipient RC703 *rif*^{r} was susceptible to all these antibiotics.

TABLE 2. *Transfer of tobramycin resistance to Escherichia coli RC703 rif*^{r}

Donor strain	Frequency of transfer[a]
K. pneumoniae 355	1.2×10^{-4}
E. cloacae 359	2.8×10^{-4}
E. cloacae 264	1.2×10^{-4}

[a] Based on total number of donor cells in the mixture at the initiation of mating. The selective medium contained 100 μg of rifampin and 1 μg of tobramycin per ml in Mueller-Hinton broth solidified with Noble agar.

TABLE 3. *MICs of tobramycin, gentamicin, and kanamycin for parental and transcipient strains*

Bacterial strain	MIC (μg)/ml)		
	Tobramycin	Gentamicin	Kanamycin
Recipient			
Escherichia coli RC703 *rif*^{r}	0.312	1.25	5
Donor			
Klebsiella pneumoniae 355	50	7.5	>100
Enterobacter cloacae 359	25	5.0	>100
E. cloacae 264	50	7.5	>100
Transcipient			
E. coli RC703 *rif*^{r} (R355)[a]	25–50	2.5–5.0	>100
E. coli RC703 *rif*^{r} (R359)	25–50	2.5–5.0	>100
E. coli RC703 *rif*^{r} (R264)	25–50	2.5–5.0	>100

[a] The strain number of the donor is shown in parenthesis.

The MICs of tobramycin, gentamicin, and kanamycin were determined for the *K. pneumoniae* and *E. cloacae* donor strains, the *E. coli* recipient strain, and five transcipients from each of three mating experiments (Table 3). The MICs for tobramycin and kanamycin were comparable in all of the donor and transcipient strains, but the MICs for gentamicin were slightly lower in the transcipients. Although the donor and transcipient strains were both susceptible to gentamicin (observed MIC, 2.5 to 7.5 μg/ml), they were two- to sixfold more resistant to gentamicin than was the recipient (MIC, 1.25 μg/ml). When the susceptibility of these strains to the purified components gentamicin C1, gentamicin C1a, and gentamicin C2 was compared, most of the transcipients were significantly more resistant than the recipient strain but more susceptible than the donor strains. The MICs for the recipient strain were 1.25 μg/ml for gentamicins C1 and C1a and 0.312 μg/ml for gentamicin C2. The MICs for the three donor strains were 10 to 12.5 μg of gentamicin C1 per ml, 25 to 50 μg of gentamicin C1a

per ml, and 10 to 25 μg of gentamicin C2 per ml. In contrast, the MICs for the transcipients were 1.25 to 7.5 μg/ml for gentamicin C1, 2.5 to 12.5 μg/ml for gentamicin C1a, and 1.25 to 7.5 μg/ml for gentamicin C2.

Enzymatic acetylation of tobramycin, gentamicin, and kanamycin was observed with osmotic lysates of the three donor strains, Kp355, Ec359, and Ec264, and of three transcipient strains, RC703 *rif*ʳ (R355), RC703 *rif*ʳ (R359), and RC703 *rif*ʳ (R264). The R followed by a three digit number indicates that resistance to tobramycin was conjugally transferred from the donor strain identified by the same number. Adenylylation of gentamicin, tobramycin, or kanamycin was not detected with osmotic lysates of Kp355, Ec359, and Ec264.

The acetylation of various aminoglycoside antibiotics was assayed with osmotic lysates of strains Kp355 and Ec264 to determine the substrate specificities of the acetylating activities (Table 4). Under the conditions of these assays, the acetyltransferases were active against kanamycins A and B, tobramycin, gentamicins C1a and C2, amikacin (BB-K8), sisomicin, and butirosin, but gentamicins A and C1 were not acetylated. The significance of the differences observed between the two enzyme preparations in the relative reaction rates with these aminoglycoside substrates has not been established. These results do indicate that the acetyltransferases from our strains resemble KAT. The properties of KAT have recently been reviewed by Benveniste and Davies (3).

TABLE 4. *Substrate specificities of aminoglycoside-acetylating activities from Klebsiella pneumoniae and Enterobacter cloacae*

Substrate	Relative reaction rate[a]	
	K. pneumoniae 355	*E. coli* 264
Gentamicin	44	30
Gentamicin A	0	0
Gentamicin C1	0	1
Gentamicin C1a	88	53
Gentamicin C2	71	31
Tobramycin	95	76
Kanamycin	100	100
Kanamycin A	96	99
Kanamycin B	115	77
Sisomicin	17	20
Amikacin (BB-K8)	38	9
Butirosin	8	16

[a] A rate of 100 was assigned for the acetylation of kanamycin. Other rates are expressed as relative values. The amount of product formed in 30 min per 60-μliter reaction mixture in the kanamycin assay was 97.7 pmol for Kp355 and 885.3 pmol for Ec264. Under the conditions used, the reaction is linear for at least 30 min and the amount of acetylated product formed is proportional to the rate of the acetylation reaction.

DISCUSSION

The strains of *K. pneumoniae*, *Escherichia coli*, and *Enterobacter cloacae* listed in Table 1 were isolated from wound cultures of patients in the adult burn intensive care unit or in an adjacent hospital area. Detection of tobramycin-resistant, gentamicin-susceptible enteric bacteria from these cultures stimulated the present study. During the period of this study, tobramycin was used as an investigational drug only in patients with burns over more than 40% of the total body surface area who exhibited persistent signs of sepsis after a therapeutic trial with a combination of gentamicin and carbenicillin. Tobramycin at a total dose of 4 mg/kg per day was administered intramuscularly in divided doses.

One *K. pneumoniae* and two *E. cloacae* strains with similar antibiotic resistance patterns were isolated after the retrospective review and were studied to clarify the mechanisms of resistance to tobramycin and to determine whether tobramycin resistance might be determined by R factors. Resistances to tobramycin, kanamycin, and neomycin were cotransferred by conjugation from these resistant strains to *E. coli* (Table 2), and the transcipient strains were shown to be resistant to high concentrations of tobramycin and kanamycin (Table 3). Acetylation of tobramycin, gentamicin, and kanamycin was observed with osmotic lysates from the donor strains Kp355, Ec359, and Ec264 and from representative transcipients from the matings in Table 2. These results suggest that tobramycin resistance in our strains of *K. pneumoniae* and *E. cloacae* is controlled by R factors, but this has not yet been confirmed by characterization of R-factor deoxyribonucleic acid from these strains by physical methods.

Aminoglycoside antibiotics may be enzymatically modified by acetylation, adenylylation, or phosphorylation. Nine bacterial enzymes that catalyze these reactions have been characterized (3). Three distinct enzymes have been reported to acetylate aminoglycosides: KAT, gentamicin acetyltransferase I (GAT_I), and gentamicin acetyltransferase II (GAT_{II}). In addition, a third gentamicin acetyltransferase tentatively designated GAT_{III} has recently been reported independently by J. Davies (American

Society for Microbiology Conference on Extrachromosomal Elements in Bacteria, 9–11 January 1974, New Orleans, La.) and by Holmes et al. (10). The aminoglycoside-acetylating enzymes KAT, GAT_{I}, GAT_{II}, and GAT_{III} can be distinguished by their specificities for aminoglycoside substrates. KAT is reported to acetylate kanamycin A, kanamycin B, neomycin, gentamicin C1a, gentamicin C2, butirosin, vistamycin, sisomicin, tobramycin, and amikacin (BB-K8) but not gentamicin A, gentamicin C1, streptomycin, or spectinomycin (3). The acetyltransferases from strains Kp355 and Ec264 resemble KAT in their specificity for the aminoglycoside substrates tested (Table 4).

Although the aminoglycoside-acetylating activity in our strains appeared to be KAT, both our donor strains and our *E. coli* transcipients were resistant to high concentrations of tobramycin (MIC, 25 to 50 μg/ml). In addition, the transcipients were more resistant to gentamicin complex, to gentamicin C1, to gentamicin C1a, and to gentamicin C2 than was the recipient strain. In contrast, Benveniste and Davies studied *E. coli* carrying R factors that determined the production of KAT and resistance to kanamycin, but the R^+ strain was susceptible to tobramycin (MIC, 7 μg/ml) (2). They also reported that the acetylated derivative of tobramycin formed by KAT retained some antibiotic activity (3). Among aminoglycoside-resistant bacteria, Price et al. (15) reported the possible presence of KAT in only one isolate of *E. coli*, and that strain was resistant to tobramycin. For treatment of experimental infections in animals, Price et al. (15) found that tobramycin, gentamicin, kanamycin, and amikacin (BB-K8) were more effective against *E. coli* than *P. aeruginosa*, although both strains produced KAT. Thus, the presence of KAT in bacteria can be associated either with resistance or with susceptibility to tobramycin.

Although reasons for variability of resistance to tobramycin in KAT-producing bacterial strains have not been clarified, two types of mechanisms should be considered: differences in the activity of KAT among KAT-producing bacteria, and the presence or absence of other resistance determinants in addition to the determinant for KAT. Since the acetylated derivative of tobramycin formed by KAT is active as an antibiotic, the first explanation seems unlikely. Resistance to aminoglycosides that is not determined by enzymatic inactivation of the antibiotic has been described by several investigators, and such mechanisms could be relevant to the altered susceptibility to tobramycin and gentamicin in our KAT-producing strains. Streptomycin resistance due to mutational alterations in ribosomal proteins has been studied in detail in *E. coli* (3). Bryan has described gentamicin-resistant strains of *P. aeruginosa* that do not produce gentamicin-modifying enzymes and has shown that they are less permeable to gentamicin than are susceptible strains of *P. aeruginosa* (R. Haraphongse and L. E. Bryan, Prog. Abstr. Intersci. Conf. Antimicrob. Ag. Chemother., 13th, Washington, D.C., Abstr. 185, 1973). Lundbäck and Norström (12) have recently studied mutants of *E. coli* carrying R factors that determine enzymatically mediated resistance to low concentrations of streptomycin and have shown that a chromosomal mutation can act synergistically with the R factors to produce high-level streptomycin resistance. Although it is well established that R factors can confer simultaneous resistance to multiple antibiotics, the possibility that individual R factors may be capable of determining several mechanisms of resistance to a single antibiotic agent deserves further investigation. Additional studies should reveal whether or not the synergistic interaction of multiple-resistance mechanisms is important in determining resistance to very high concentrations of some antibiotics in clinical isolates of bacteria.

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