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Gentamicin-Adenylyltransferase Activity as a Cause of Gentamicin Resistance in Clinical Isolates of *Pseudomonas aeruginosa*

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Gentamicin adenylyltransferase activity was found in extracts of clinical isolates of gentamicin-resistant *Pseudomonas aeruginosa*. Extracts of one of these isolates, *P. aeruginosa* POW, inactivated gentamicin in the presence of adenosine 5'-triphosphate. Extracts of strain POW catalyzed the binding of radioactivity from [^{14}C]adenine adenosine 5'-triphosphate to gentamicin components, tobramycin, sisomicin, kanamycin A and B and, to a variable degree, streptomycin and spectinomycin. The substrate profile with these agents and other aminocyclitols was similar to that obtained with R factor-mediated gentamicin adenylyltransferase found in *Enterobacteriaceae*. Adenylylating activity was absent in gentamicin-susceptible mutants of strain POW. Adenylylation may be added to acetylation as an enzymatic mechanism responsible for gentamicin resistance among strains of *P. aeruginosa*.

Resistance to aminocyclitol antibiotics in *Pseudomonas aeruginosa* is often based upon enzymatic inactivation of the antibiotic, as is the case in most other gram-negative bacilli (4). Resistance to gentamicin in the *Enterobacteriaceae* may be mediated by adenylylation or acetylation. In clinical isolates of gentamicin-resistant *P. aeruginosa*, the only well-documented enzymatic mechanism demonstrated heretofore has been acetylation (7). We report the detection of gentamicin adenylyltransferase activity in some gentamicin-resistant isolates of *P. aeruginosa*.

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MATERIALS AND METHODS

Organisms. Clinical isolates of *P. aeruginosa* were obtained from urine specimens submitted to the Microbiology Laboratory, Michael Reese Hospital and Medical Center, Chicago, Ill. Antibiotic susceptibility was determined qualitatively by the disk test according to standard Kirby-Bauer criteria (2). Results were confirmed when necessary by determining the minimal inhibitory concentration (MIC) of the antibiotic diluted in Mueller-Hinton agar. For this purpose, inocula obtained from a 10^{-2} dilution of an overnight Mueller-Hinton broth culture were deposited by means of a Steers-Foltz replicator. The MIC was the concentration that completely inhibited growth after 24 h of incubation at 37 C. Most studies were made with *P. aeruginosa* POW, resistant by the disk test to gentamicin, tobramycin, kanamycin, neomycin, streptomycin, spectinomycin, tetracycline, chloramphenicol, carbenicillin, cephalothin, and sulfonamide. *Escherichia coli* strain WIL was isolated from a urine specimen at the Michael Reese Hospital. It carried an *fi*$^+$ R factor mediating gentamicin resistance (10). Strain JR66/W677, obtained from J. Davies, was *E. coli* K-12 bearing a R factor that mediated gentamicin resistance by adenylyltransferase action (3).

Preparation of sonic extracts. Cultures of *P. aeruginosa* in the exponential phase of growth, in 300 ml of brain heart infusion broth (Difco), were centrifuged at 4 C and washed twice with 0.125 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.0) containing 0.02 M $MgCl_2$. The cells were then suspended in 10 ml of this buffer containing 7.5 mM dithiothreitol and were broken in a Biosonik oscillator. Cellular debris was spun down at 30,000 rpm for 60 min in a Spinco ultracentrifuge. The supernatant was dialyzed overnight against buffer containing dithiothreitol and stored at −20 C. Extracts of *Pseudomonas* strains contained 2 to 5 mg of protein per ml (11). Extracts of *E. coli* were prepared similarly, except that the bacteria were grown in double-strength Penassay broth (Difco) and the concentration of dithiothreitol employed was 2.5 mM.

Antibiotics and reagents. The aminocyclitols used in these studies were laboratory standard preparations with the exception of streptomycin, which was a medicinal preparation of Pfizer Laboratories. Gen-

tamicin (a mixture of approximately equal parts of gentamicin C_1, C_{1a}, and C_2), pure preparations of gentamicins C_1, C_{1a}, and C_2, and sisomicin were supplied by J. A. Waitz of the Schering Corp.; tobramycin was from R. S. Griffith of the Eli Lilly Co.; kanamycin A and B and amikacin (BB-K8), a semisynthetic kanamycin derivative, were from Bristol Laboratories; spectinomycin and neomycin B were from the Upjohn Co. [8-^{14}C]adenosine 5'-triphosphate (ATP; 50 mCi/mmol), [1-^{14}C]acetylcoenzyme A (52.8 mCi/mmol), and [γ-^{32}P]ATP (6.9 Ci/mmol) were products of the New England Nuclear Corp.

Enzymatic assays. The assay for adenylyltransferase was performed by the method of Benveniste and Davies (3, 5). The standard assay contained 20 μliters of bacterial extract (40 to 100 μg of protein), 10 nmol of antibiotic, 20 nmol of labeled ATP (4 μCi/μmol), and 20 μliters of 0.125 M Tris-hydrochloride-0.02 M $MgCl_2$ buffer (pH 8.0) containing 150 nmol of dithiothreitol, all in a final volume of 60 μliters. The reaction mixture was usually incubated for 20 min at 30 C. Duplicate volumes of 10 μliters each were spotted on phosphocellulose paper, washed, and counted in a Packard scintillation spectrometer. Acetylation and phosphorylation of gentamicin were tested by published methods (7, 13).

Inactivation of gentamicin and other antibiotics was also demonstrated by the method of Benveniste and Davies (3). The reaction mixture (100 μliters) contained 20 nmol of antibiotic, 240 nmol of ATP, 5 μmol of Tris (pH 8.0), 0.8 μmol of $MgCl_2$, 300 nmol of dithiothreitol, and variable amounts of enzyme, up to 50 μliters. The mixture was incubated at 30 C for 5 to 22 h and then tested after 0-fold to 40-fold dilution with Tris-hydrochloride buffer containing dithiothreitol. Residual antibiotic activity was assayed by spotting 20-μliter volumes on filter paper disks placed on petri plates of Sensitivity Test Agar (Colab) inoculated with *Bacillus subtilis* spores (15). The diameters of the zones of inhibition were measured after incubation for 18 h at 37 C. Standards containing known amounts of antibiotics similarly incubated either without ATP or with extracts of gentamicin-susceptible organisms were employed. The residual amounts of antibiotic in the test samples were calculated by comparison with the standards.

Conjugation studies. Attempts to transfer antibiotic resistance by conjugation were performed by conventional techniques reported previously (6, 10).

Curing and mutagenesis. Published techniques using acriflavine and ultraviolet irradiation were employed for attempts at elimination of R factors (16). Mutagenic treatment was performed with ethyl methane sulfonate (EMS) (5). Mutants were obtained after exposure to EMS for 4 min, followed by washing, growth in broth for 3 h, plating on heart infusion agar to obtain individual colony-forming units, and replica plating to heart infusion agar containing 10 μg of gentamicin per ml.

RESULTS

Among 13 isolates of gentamicin-resistant *P. aeruginosa*, extracts of four strains caused the binding of radioactivity from [8-^{14}C]ATP to gentamicin C_1. In the standard assay with extracts of these four strains, gentamicin C_1 bound from 100 to 765 counts/min. Two gentamicin-susceptible strains gave 0 counts/min, whereas the remaining gentamicin-resistant strains gave low counts ranging from 20 to 40 counts/min.

Most of our observations were made with *P. aeruginosa* POW. Table 1 shows that the aminocyclitol substrate specificity of adenylyltransferase in extracts of strain POW resembled that of the R factor-mediated enzyme in strains WIL and JR66/W677. The degree of adenylylation of gentamicin C_1 increased linearly with the amount of POW extract within the range of 5 to 30 μliters (data not shown). The activity of POW extracts per milligram of protein was similar to that of strain WIL and about 21% of strain JR66/W677. Similar experiments with [1-^{14}C]acetylcoenzyme A showed no evidence of enzymatic attack on gentamicin C_1 or C_{1a} at pH 5.8 and 7.6. Also, in experiments with [γ-^{32}P]ATP there was no evidence of enzymatic

TABLE 1. *Substrate specificity of gentamicin adenylyltransferases*

Substrates	Adenylylation relative to gentamicin C_1 (%)[a]		
	POW[b]	WIL[b]	JR66/W677[b]
Gentamicin C_1	100[c]	100[c]	100
Sisomicin	72	90	57
Tobramycin	40	44	30
Kanamycin A	33	47	41
Kanamycin B	35	47	44
Gentamicin C_2	30	36	35
Gentamicin C_{1a}	26	29	27
BB-K8	25	58	16
Neomycin B	10	22	5
Streptomycin	5–104[d]	9	2
Spectinomycin	5–69[d]	5	1

[a] Results are means of from two to five assays per substrate. Counts per minute bound by gentamicin C_1 per milligram of protein of bacterial extract in a 20-min assay were 38,200 for POW, 42,000 for WIL, and 185,000 for JR66/W677. Adenylylation of 1 nmol of substrate is equivalent to 6,600 counts/min.

[b] Extracts from *P. aeruginosa* POW, *E. coli* WIL, and *E. coli* K12 JR66/W677.

[c] Results are based on assays that gave about 400 to 600 counts/min. Percent adenylylation values of 10% or less reflect net experimental counts equal to or less than background (40 to 50 counts/min) and therefore signify little or no enzymatic activity.

[d] Adenylylation by extracts of two clones was 52 to 104% of that with gentamicin C_1, and for three clones was 5 to 17%.

reaction with gentamicin C_1 at pH 8. Thus, there were no signs of acetylation or phosphorylation of the substrates tested.

Extracts of cultures made from randomly chosen colonies of strain POW gave similar results with the substrates listed in Table 1, except for streptomycin and spectinomycin. With these compounds the degree of adenylylation varied widely among extracts made from different colonies, suggesting the possible presence of a separate streptomycin-spectinomycin adenylyltransferase that may be missing or variably expressed in some colonies. The mechanism of resistance to these agents in strain POW will require additional study.

Incubation of the gentamicins with strain POW extracts in the presence of ATP for 22 h inactivated gentamicin C_1 completely but reduced the activity of gentamicin C_{1a} and C_2 only partially (Table 2). Control experiments with the omission of ATP or with the substitution of ATP by acetylocoenzyme A, or with an extract of a gentamicin-susceptible strain of *P. aeruginosa*, gave no inactivation. Generally similar results were obtained with extracts of strain WIL, but strain JR66/W677 extracts inactivated gentamicin C_{1a} and C_2 and the commercial gentamicin mixture to a greater extent. Strain JR66/W677 extract was used in these experiments in amounts that adenylylated gentamicin C_1 five times as rapidly as did those of POW and WIL extracts. Thus, the greater inactivation of gentamicin C_{1a} and C_2 may be due to the greater amount of JR66/W677 enzymatic activity employed. Differences in inactivation may also be attributed in part to greater stability of the enzyme from extracts of strain JR66/W677. Extracts of JR66/W677 incubated for 5 h in the presence of gentamicin C_1 but without ATP retained 75% of their gentamicin C_1 adenylyltransferase activity. Under the same conditions, strain POW extracts retained 41% and strain WIL retained 50% of their activity.

TABLE 2. *Inactivation of aminocyclitols by adenylylating enzymes*

Substrate	Inactivation[a] (%)					
	POW[b]		WIL[b]		JR66/W677[b]	
	5 h	22 h	5 h	22 h	5 h	22 h
Gentamicin C_1	75	100	62	82	100	100
Gentamicin C_{1a}	10	20	10	30	20	70
Gentamicin C_2	0	10	22	22	10	68
Gentamicin[c]	19	33	42	45	57	80
Tobramycin	15	28	15	28	38	54
Sisomicin	30	39	31	64	100	100
Kanamycin A	31	43	100	100	100	100

[a] Means of two assays or duplicate assays. Reaction mixture contained initially 20 nmol of antibiotic in 100 μliters.

[b] Extracts from *P. aeruginosa* POW, *E. coli* WIL, and *E. coli* K12 JR66/L677.

[c] Laboratory standard preparation of medicinal product consisting of approximately one-third each of gentamicin C_1, C_{1a}, and C_2.

Sisomicin and tobramycin were inactivated by the three extracts to a degree generally intermediate between that of gentamicin C_1 and C_{1a}.

Inactivation of kanamycin A by POW extract was distinctly less than that by WIL extract even though they were adenylylated similarly. It is possible that strain WIL may inactivate kanamycin by an additional mechanism, e.g., kanamycin phosphotransferase, as does strain JR66/W677 (3). The likelihood of the occurrence of this enzyme in WIL is supported by the complete resistance of WIL to neomycin in the disk test.

Six independent gentamicin-susceptible mutants of strain POW were isolated after treatment with EMS. Four of these mutants, strains 1401, 1406, 1412, and 1416, were obtained from a mutant parental strain of POW bearing a tryptophan auxotrophic marker. The gentamicin-susceptible mutants were also auxotrophic for tryptophan and, hence, were authentic mutants. Table 3 shows that mutants 1401 and 1406 were susceptible to low concentrations of gentamicin, tobramycin, and sisomicin, with MICs similar to those of eight wild-type gentamicin-susceptible strains of *Pseudomonas*. Concomitantly, strains 1401 and 1406 lost adenylyltransferase activity for the three gentamicins, tobramycin, and sisomicin. Extracts of these mutants were no longer capable of inactivating these antibiotics. The remaining four mutants, including 1412 and 1416, were studied in less detail. They failed to adenylylate gentamicin C_1 or tobramycin and were susceptible to gentamicin and tobramycin. Thus, resistance to gentamicin, tobramycin, and sisomicin in *P. aeruginosa* POW appeared to be due to enzymatic inactivation of these antibiotics by a single adenylyltransferase. Attempts to obtain adenylyltransferase-positive revertants from these mutants were unsuccessful owing to the large number of apparently chromosomally mediated resistant mutants produced.

The adenylyltransferase activity for kanamycin A and BB-K8 present in strain POW was not found in the mutants. Nevertheless, the MICs

TABLE 3. *Relation of loss of aminocyclitol resistance to adenylyltransferase activity*

Antibiotics	MIC (μg/ml)			Adenylylation relative to gentamicin C_1 by *P. aeruginosa* POW[a] (%)		
	POW[b]	1401[b]	1406[b]	POW[c]	1401[c]	1406[c]
Gentamicin	200	3.1	3.1			
Gentamicin C_1	400	12.5	12.5	100	0.2	0.6
Gentamicin C_{1a}	200	6.2	3.1	21	0.0	0.0
Gentamicin C_2	200	6.2	6.2	24	0.0	0.0
Tobramycin	20	2.5	2.5	35	0.0	0.0
Sisomicin	50	3.1	3.1	84	0.5	1.0
Kanamycin A	200	200.0	200.0	30	0.4	0.9
BB-K8	20	20.0	20.0	23	0.9	3.0
Neomycin B	50	25.0	25.0	10	0.0	0.0
Streptomycin	800	400.0	400.0	5	2.0	2.0
Spectinomycin	>800	>800.0	>800.0	5	0.2	2.0

[a] Percent adenylylation values of 10 or less reflect net experimental counts equal to or less than background and therefore signify little or no enzymatic activity. Results are means of two to seven assays.
[b] Strains *P. aeruginosa* POW and its mutants 1401 and 1406.
[c] Extracts of strains *P. aeruginosa* POW and its mutants 1401 and 1406.

of these antibiotics for the mutant strains were not reduced from that of the parental POW strain. It was unlikely that strain POW possessed acetyltransferase activity for kanamycin in view of its lack of this activity for gentamicin C_{1a} and C_2 (4). Extracts of strains 1401 and 1406 did not inactivate kanamycin A in the presence of ATP, a finding that argued against any significant phosphotransferase activity. The cause for the sustained kanamycin resistance in the gentamicin-susceptible mutants of POW remains undetermined.

As mentioned previously, we obtained variable degrees of streptomycin-spectinomycin adenylyltransferase activity from different extracts of strain POW. Whether this difference in activity is clonally heritable remains to be investigated. In any case, this variation in enzymatic activity of the parental strain made it impossible to draw conclusions for the present from the low levels of streptomycin-spectinomycin adenylyltransferase activity found in mutants 1401 and 1406 (Table 3). Evidently the mutants had not lost their major mechanism for streptomycin resistance since their MICs were not significantly different from the parental POW strain.

Mutants 1401 and 1412 had lost their resistance to carbenicillin and sulfadiazine in addition to gentamicin. Among possible explanations may be mutation at multiple sites, deletion of linked markers or curing of an R factor, or a polar or pleiotropic effect of a single mutation.

We have been unable to transfer, by conjugation, antibiotic resistance from *P. aeruginosa* POW to *E. coli* K-12, *Chromobacter violaceum* ATCC 12472, or to a methionine auxotroph of *P. aeruginosa* 280 (6). Attempts at elimination of gentamicin resistance from POW by treatment with acriflavine and ultraviolet irradiation were similarly unsuccessful. Despite our own failure to transfer resistance to other strains of *P. aeruginosa*, we have been informed that gentamicin resistance in strain POW is conjugally transmissible to some strains of *P. aeruginosa* and, therefore, is also associated with an R factor (L. E. Bryan, personal communication). Resistance to tobramycin, carbenicillin, streptomycin, and sulfadiazine was concomitantly transferred.

DISCUSSION

In general, clinical isolates of *P. aeruginosa* mediate resistance to aminocyclitol antibiotics either by acetylation or phosphorylation of the antibiotic (4). Additionally, some resistant isolates display no evidence of enzymatic inactivation of the antibiotic (R. Haraphongse and L. E. Bryan, presented at the 13th Interscience Conference on Antimicrobial Agents and Chemotherapy). In the case of gentamicin, acetylation by gentamicin acetyltransferase I is the only enzymatically mediated resistance mechanism in *P. aeruginosa* that is established by biochemical evidence (7). This enzyme does not attack kanamycin A and has little effect on tobramycin. An enzyme with similar properties has been demonstrated in *E. coli* and *Klebsiella pneumoniae* (4). In these organisms, resistance to gentamicin may be caused by another enzyme, gentamicin adenylyltransferase, that also attacks and inactivates kanamycin A and tobramycin (3). The genetic determinant for gen-

tamicin adenylyltransferase is usually situated on an R factor (10, 12). In one strain of *P. aeruginosa*, Witchitz and Chabbert found an R factor that was transmissible to *E. coli* and that mediated resistance both to gentamicin and kanamycin (17). They inferred that the resistance of the strain of *P. aeruginosa* was caused by adenylyltransferase, as was the case in their strains of *Klebsiella* and *E. coli*.

Our experiments offer biochemical and genetic evidence that gentamicin resistance in *P. aeruginosa* mediated by adenylyltransferase is not a rare event, at least in our hospital. The relative substrate specificity of the enzyme in crude extracts of strain POW resembled that of the gentamicin adenylyltransferase in *Klebsiella* and *E. coli*. The enzyme in *P. aeruginosa* may, therefore, be very similar to that in the *Enterobacteriaceae*. However, resistance to gentamicin was not transmissible from strain POW to *E. coli*. Evidently, therefore, the resistance determinant in strain POW was not associated with transfer genes similar to those present in *Enterobacteriaceae* and in Witchitz and Chabbert's gentamicin-resistant strain of *P. aeruginosa*.

A number of possibilities may be entertained for the source of the gentamicin adenylyltransferase in *P. aeruginosa*. Some strains may have acquired the genetic determinant for the enzyme by transfer of an intact R factor from *Enterobacteriaceae*, as Witchitz and Chabbert suggested for their strain. Evidence for the natural occurrence of intergeneric transfer of R factors to *P. aeruginosa* has presented (9, 14). In some intergeneric transfer of R factors, the resistance determinants, but not the transfer genes, are transmitted to the recipient (1). A resistance determinant, transferred in this way, might be coupled subsequently to conjugative plasmids which are known to occur in *Pseudomonas* (8). An event of this kind could give rise to an R factor with the restricted transmissibility typical of some pseudomonal R factors (6) but bearing enterobacterial genes for adenylyltransferase. A history of this type could account for the properties of the gentamicin resistance factor in strain POW. Alternatively, the enzyme may have evolved separately in *P. aeruginosa* or have been acquired from still another bacterial species unrelated to *Enterobacteriaceae*. Isolation and comparison of the enzymes from both *Pseudomonas* and *Enterobacteriaceae* would be desirable to investigate this question.

Enterobacteriaceae harboring R factors that mediate resistance to gentamicin owing to adenylyltransferase are now fairly common in our hospital (S. Kabins, C. Nathan, and S. Cohen, unpublished data). Therefore the possibility exists that resistance of this type may be gradually transferred from *Enterobacteriaceae* to *P. aeruginosa*, thereby adding to the difficulty of treating infections with this refractory organism.

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