ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1978, p. 641-644
0066-4804/78/0013-0641$02.00/0

Vol. 13, No. 4
Printed in U.S.A.

Aminoglycoside Modification by Gentamicin-Resistant Isolates of *Staphylococcus aureus*

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Received for publication 15 November 1977

Three clinical isolates of *Staphylococcus aureus*, which were previously shown to contain a 50S plasmid conferring resistance to several aminoglycosides, were examined for modifying enzymes. Both the wild-type and heat-cured derivatives of the isolates were screened for acetyl-, adenylyl-, and phosphotransferase activities. The substrates were gentamicin, amikacin, and netilmicin; the results indicated that even though all three activites were present, the phosphotransferase reaction was most responsible for resistance to these antibiotics. The absence of any of the modifying activites in cured derivatives of the three isolates supports the conclusion that aminoglycoside resistance in these strains is conferred by a plasmid.

Gentamicin resistance in clinical isolates of *Staphylococcus aureus* has been reported in Europe (4, 7, 9, 10) and the United States (11). Recent studies in this laboratory identified a 50S plasmid in two strains as the genetic element responsible for this resistance (11). The organisms studied were recovered from an outbreak of infections in our neonatal intensive care unit (Talmadge Memorial Hospital, Augusta, Ga.) and were of different phage types. Speller et al. (10) reported an outbreak in three hospitals by a single strain of gentamicin-resistant *S. aureus*. In France, Le Goffic et al. (7) have studied a gentamicin-resistant strain isolated from a blood culture.

Since gentamicin resistance in *S. aureus* has only recently been noted in clinical isolates, we undertook the biochemical characterization of our strains in an effort to determine which of several possible modifying enzymes could be involved in this resistance. We now describe the relative susceptibility of gentamicin, amikacin, and netilmicin to acetyltransferase (AAC) adenylyltransferase (ANT), and phosphotransferase (APT) modification by crude cell-free extracts of our *S. aureus* strains.

MATERIALS AND METHODS

Bacterial strains. Each of the *S. aureus* strains used in this study was isolated from blood cultures of patients in the neonatal intensive care unit of Talmadge Hospital, Augusta, Ga. The isolates have been designated Gordon, Spratlin, and Williamson and were identified by standard procedures. The 50S plasmid was eliminated from each strain by growth at 44°C as reported previously (11).

Susceptibility tests. The minimum inhibitory concentration (MIC) of gentamicin, netilmicin (both provided by Schering Laboratories, Bloomfield, N.J.), and amikacin (Bristol Laboratories, Syracuse, N.Y.) for each strain was determined using twofold dilutions in Penassay broth (Difco Laboratories, Detroit, Mich.). An initial inoculum of 10^5 colony-forming units was used for these assays.

Preparation of cell-free extracts. Cells were grown in tryptic soy broth to the late-log phase and harvested by centrifugation. The pellets were washed twice with 0.85% NaCl and suspended in buffer I (10 mM tris(hydroxymethyl)aminomethene-[Tris]-chloride 10 mM $MgCl_2$-25 mM NH_4Cl-0.6 mM β-mercaptoethanol [pH 7.8]), lysostaphin (Schwarz/Mann) was added to a final concentration of 50 μg/ml. After 30 min at 37°C, unlysed cells and cell debris were removed by centrifugation at 30,000 × *g* for 30 min. The supernatant fraction was retained and dialyzed against 500 volumes of buffer I (overnight at 4°C). This crude extract was either used immediately or kept at −70°C.

Enzyme assays. Aminoglycoside modification enzymes were assayed by the phosphocellulose paper binding procedure as described by Haas and Dowding (5). Unless otherwise indicated, reaction mixtures contained the following in a total volume of 35 μl: ANT, 3 μmol of potassium citrate (pH 6.0), 0.3 μmol of magnesium acetate, 0.1 μmol of dithiothreitol, 6.7 nmol of [^{14}C]acetyl coenzyme A containing ~10^5 cpm, 7 nmol of antibiotic, and 10 μl of crude enzyme extract containing 40 to 60 μg of protein; ANT, 6.7 μmol of Tris maleate (pH 7.1), 417 nmol of $MgCl_2$, 4 μmol of NH_4Cl, 16 nmol of dithiothreitol, 100 nmol of [^{3}H]-adenosine 5'-triphosphate (ATP) containing ~1.7 × 10^5 cpm, 7 nmol of antibiotic, and 10 μl of crude enzyme extract; and APT, 6.7 μmol of Tris maleate

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(pH 7.8), 417 nmol of $MgCl_2$, 4 μmol of NH_4Cl, 16 nmol of dithiothreitol, 50 nmol of [γ-^{32}P]ATP containing ~1.7×10^5 cpm, 7 nmol of antibiotic, and 10 μl of crude enzyme extract. Reactions were initiated by the addition of enzyme and carried out for 30 min at 30°C. Each transferase reaction was linear with respect to a protein concentration of up to 150 μg of protein.

Chemicals. [^{3}H] ATP was obtained from Schwartz/Mann. [^{14}C]acetyl coenzyme A and [γ-^{32}P]-ATP were obtained from New England Nuclear. Unlabeled substrates were obtained from Sigma Chemical Co., and the other chemicals were of reagent grade.

RESULTS

Each of the *S. aureus* isolates studied was highly resistant to amikacin, gentamicin, and netilmicin (Table 1). Elimination of the 50S plasmid by growth of the cells at 44°C resulted in the sensitization of all three strains to the three aminoglycosides. The MICs of the cured strains were all below 0.5 μg/ml. This is comparable to that generally reported for gentamicin-susceptible strains of *S. aureus*. For example, Phillips et al. (8) screened over 120 strains and found that the MIC range for susceptible strains was 0.03 to 4.0 μg/ml for gentamicin. The data in Table 2 support the susceptibility tests in that extracts from the cured strains had no detectable gentamicin-modifying activity. Extracts from the original, wild-type isolates had appreciable levels of all three transferase activities for gentamicin.

Since the MIC of gentamicin exceeded that of amikacin two- to fourfold in these strains (Table 1), these two antibiotics and netilmicin were compared for their relative susceptibility to modification by the cell-free extracts. The results are summarized in Table 3. Each of the extracts had comparable AAC and ANT activity for gentamicin and amikacin. However, netilmicin was only poorly acetylated and not adenylylated. All three of the aminoglycosides tested were substrates for APT. Gentamicin and netilmicin were phosphorylated to about the same extent, whereas amikacin was modified only one-fourth to one-third as well as the other drugs (Table 3).

When the antibiotic dependence for each of the modification reactions was determined for amikacin and gentamicin, plots of the degree of acetylation and adenylylation against amikacin or gentamicin concentration were superimposable (data not shown). However, a marked difference was found with respect to the substrate dependence for phosphorylation (Fig. 1). Linear transformations of these data yielded apparent K_m values of 4 and 400 μM for gentamicin and amikacin, respectively, for the *S. aureus* Gordon APT.

DISCUSSION

Since gentamicin resistance in clinical isolates of *S. aureus* has only recently been observed on

TABLE 1. *Aminoglycoside susceptibility of S. aureus strains studied*

S. aureus strain	MIC (μ/ml)[a]		
	Amikacin	Gentamicin	Netilmicin
Gordon	25 (0.19)	>100 (0.39)	>100 (0.19)
Spratlin	25 (0.39)	>100 (0.39)	>100 (0.39)
Williamson	50 (0.39)	>100 (0.19)	>100 (0.19)

[a] The MIC of each wild-type strain (cured derivative in parentheses) was determined from twofold serial dilutions in Penassay broth.

TABLE 2. *Gentamicin-modifying activity in cell-free extracts of S. aureus*

S. aureus strain	Phenotype	Modification (cpm)[a]		
		Acetylation	Adenylylation	Phosphorylation
Gordon	Wild-type	441 ± 38	233 ± 30	11,501 ± 370
	Cured	51 ± 4	42 ± 6	331 ± 51
Spratlin	Wild-type	1,509 ± 70	200 ± 10	10,466 ± 814
	Cured	54 ± 4	34 ± 2	290 ± 10
Williamson	Wild-type	1,478 ± 68	153 ± 8	19,282 ± 236
	Cured	52 ± 2	31 ± 1	340 ± 25
Boiled extracts		50 ± 3	30 ± 2	280 ± 24

[a] Values listed are the means ± standard error from triplicate assays with [^{14}C]acetyl coenzyme A, [^{3}H]ATP, and [γ-^{32}P]ATP.

TABLE 3. *Comparison of amikacin, gentamicin, and netilmicin as substrates for modifying enzymes from three S. aureus strains*

Activity measured	Substrate	Relative modification (%)		
		Gordon	Spratlin	Williamson
AAC	Gentamicin	100	100	100
	Amikacin	79	88	85
	Netilmicin	0	3	5
ANT	Gentamicin	100	100	100
	Amikacin	129	123	101
	Netilmicin	0	0	0
APT	Gentamicin	100	100	100
	Amikacin	25	22	31
	Netilmicin	85	86	86

[a] Results are expressed relative to gentamicin (100%). All figures are averages of duplicate determinations.

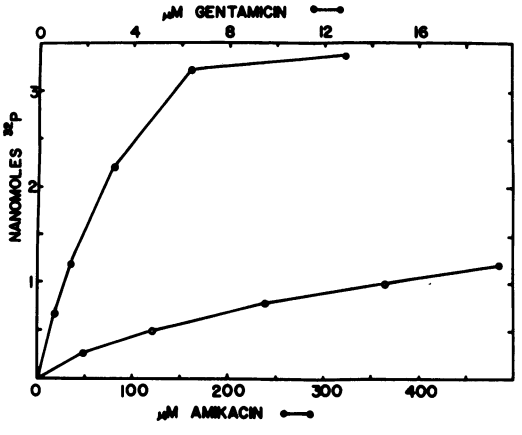

FIG. 1. *Aminoglycoside dependence of the APT enzyme present in a cell-free extract of S. aureus Gordon.*

a widespread basis, we were interested in characterizing the strains involved in a recent outbreak in our neonatal intensive care unit to establish how similar our strains are to those in Europe and elsewhere in the United States. Based on published studies to date, our strains are apparently different from other gentamicin-resistant *S. aureus* isolates.

Speller et al. (10) reported that extracts of their gentamicin-resistant strain, which is amikacin susceptible, possessed both ANT and AAC activities toward gentamicin but not toward amikacin. Those workers apparently did not test for APT activity.

Brown et al. (1) tested strain 5532, which was isolated by Porthouse et al. (9), and found APT activity with gentamicin, but not with amikacin. Both gentamicin and amikacin were acetylated, but no adenylylation of the aminoglycosides was reported.

Phillips et al. (8) examined several gentamicin-resistant strains and found that most produced an AAC that did not confer resistance and a APT that modifies gentamicin but not amikacin. No mention was made of nucleotidylation by their staphylococcal strains.

The strains studied by Le Goffic and co-workers (6, 7) are also apparently different from our strains. Their Ap01 strain produced an ANT(4′) that modified several aminoglycosides but not the gentamicin components (6). More recently, these workers (7) reported *S. aureus* R. Palm to possess an AAC(6′) and an APT(2″), but apparently this strain did not produce an ANT with activity for the gentamicins.

The Cambridge strains studied by Dowding (4) also possess AAC(6′) and two APT activities apparently acting on the 3′ and the 2″ hydroxyl groups of aminoglycosides.

Because of differences in methods and approaches to the study of the organisms, comparisons of strains are very difficult. However, our finding that the apparent K_m for amikacin is two orders of magnitude higher than that for the gentamicin complex suggests that previous reports indicating an absence of APT activity with amikacin may have resulted from the use of too low a concentration of amikacin in the assays (1, 8). It should be noted that Courvalin and Davies (2) report that amikacin is also a poor substrate for the *S. aureus* APT(3′) enzyme.

As judged by the observed substrate specificities, our strains possess both the AAC(6′) and the APT(2″) activities reported for the European strains (4, 7). In addition an ANT activity is elaborated in our strains. Although this finding confirms the report of Speller et al. (10) that gentamicin may be adenylylated, our ANT differs from theirs in that it also adenylylated amikacin. The site of adenylylation remains to be determined.

Bacterial resistance to aminoglycosides is generally considered to require the enzymic modification of the antibiotic in the outer layers of

the cell, and this modification results in an inhibition of the uptake of the antibiotic rather than in its inactivation (3). In theory, gentamicin resistance in *S. aureus* could be explained by the possession of any one of the three modification enzymes. However, a comparison of the MICs and the degrees of enzymatic modification found for the aminoglycosides used in the present report and in that of LeGoffic et al. (7) reveals that the MICs of the aminoglycosides are most closely related to their susceptibilities to modification by APT(2″). In addition, all gentamicin-resistant strains of *S. aureus* examined to date have APT activity. Finally, we find that netilmicin is not adenylylated, and only poorly acetylated, but it is appreciably phosphorylated. Since our strains are also resistant to this aminoglycoside, we conclude that the APT(2″) enzyme is most responsible for conferring resistance to these three aminoglycosides in our *S. aureus* strains.

ACKNOWLEDGMENTS

This project was supported by Public Health Service grant FR-S365 from the Division of Research Facilities and Resources and by a grant from the Georgia Heart Association (to G.K.B.).

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