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Plasmid-Mediated Aminoglycoside Phosphotransferase of Broad Substrate Range That Phosphorylates Amikacin

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A plasmid-mediated aminoglycoside phosphotransferase that phosphorylates amikacin has been detected in clinical isolates of *Staphylococcus aureus*. This enzyme does not confer detectable amikacin resistance to the strains.

The discovery of aminoglycoside-modifying enzymes in antibiotic-resistant bacteria has prompted considerable research on these enzymes, their role in resistance, and their origins and on the evolution of the plasmids that code for them. Although only a small number of amino or hydroxyl groups (four to six) on aminoglycoside molecules are modified, a large number of enzymes catalyze these modifications. Thus, at least three different enzymes have been reported to acetylate the 3-amino group of deoxystreptamine (3), and three phosphotransferases have been described that modify the 3'-hydroxyl group of neomycin, kanamycin, and related antibiotics (J. Dowding and J. Davies, *Microbiology—1974*, p. 179-186, 1975). The enzymes differ in substrate range and physical properties.

We wish to describe a new 3'-phosphotransferase that differs from the aminoglycoside phosphotransferases described thus far. This 3'-phosphotransferase activity has been detected in antibiotic-resistant clinical isolates of *Staphylococcus aureus* and, in addition to neomycin and kanamycin, phosphorylates amikacin; amikacin is a derivative of kanamycin A that has been chemically altered to block modification at several sites, including phosphorylation at the 3' position. Surprisingly, although these strains possess an amikacin-modifying enzyme, they remain susceptible to the antibiotic.

MATERIALS AND METHODS

Bacterial strains and plasmids. The sources and properties of the staphylococci and their plasmids are listed in Table 1. Plasmid-free *S. aureus* strains RN450(9) and 209P were obtained from R. P. Novick and Y. A. Chabbert, respectively. Plasmid RN1956 was introduced into *S. aureus* RN450 by transduction.

Enzymatic assays. The extracts for assay were prepared as follows. A 100-ml amount of an exponential culture in broth was harvested and washed once in 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.2)-1 mM ethylenediaminetetraacetate and resuspended in 2 ml of 10 mM Tris-hydrochloride (pH 7.8)-10 mM $MgCl_2$-25 mM NH_4Cl-0.6 mM β-mercaptoethanol. The cells were treated for 30 min at 37°C with 100 μg of lysostaphin per ml (Sigma) and then subjected to two 30-s bursts from a sonicator at 60-W output. The resulting suspension was centrifuged at 105,000 × *g* for 45 min, and the supernatant was removed and used in enzyme assays as described previously (5). The time course of phosphorylation of antibiotics was determined by increasing the volume of the reaction mixture and removing samples for counting at the indicated times.

Minimal inhibitory concentrations. The method of Steers et al. (9) was used for the determination of minimal inhibitory concentrations.

Antibiotics. The antibiotics were provided by the following laboratories: kanamycins A, B, and C and amikacin, Bristol; neomycin B and spectinomycin, Upjohn; paromomycin and butirosin, Parke-Davis; lividomycin A, Kowa; ribostamycin, Meiji; tobramycin, Lilly; streptomycin, Pfizer.

RESULTS

During the course of our studies on enzyme-mediated resistance to aminoglycoside antibiotics, we have examined a large number of clinical isolates of both gram-negative and gram-positive bacteria. To our surprise, many *S. aureus* strains were found to contain an activity that catalyzed the phosphorylation of amikacin in addition to the usual substrates for aminoglycoside phosphotransferases (Fig. 1). The strains and their resistance phenotypes are shown in Table 1.

Amikacin is a derivative of kanamycin A that has a hydroxyaminobutyric acid substituent (HABA) at position 1 of the deoxystreptamine ring (Fig. 2). The presence of this substituent normally blocks modification of kanamycin at the 3'-hydroxyl, 2''-hydroxyl and 3-amino groups, thus providing an antimicrobial agent that is effective against strains containing en-

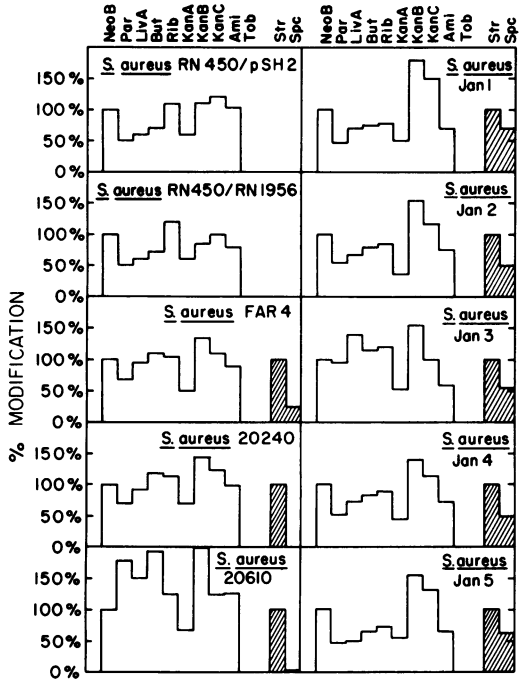

FIG. 1. *Substrate profiles of enzymes extracted from S. aureus strains. Phosphorylation (plain area) or adenylylation (hatched area) are expressed relative to neomycin B or streptomycin as 100%, respectively. Same abbreviations as in Table 2.*

zymes that modify these sites. Aminoglycoside phosphotransferases of gram-negative strains do not phosphorylate amikacin.

We assume that amikacin is modified at the 3'-hydroxyl group; none of the 3'-deoxyaminoglycosides (e.g. tobramycin, Fig. 1) is a substrate for phosphorylation by the *S. aureus* extracts. The fact that both butirosin and lividomycin A are phosphorylated (Fig. 1) indicates that the enzymes are type III aminoglycoside phosphotransferases.

In view of the fact that the *S. aureus* strains are resistant to kanamycin A and susceptible to amikacin, although the phosphotransferase modifies both antibiotics (Fig. 1), we have examined these molecules as substrates for the enzyme. Figure 3 shows kinetics of phosphorylation at different concentrations of the two drugs.

There is one other unusual property of the strains that we have examined. *S. aureus* FAR 4 and JAN 3 are highly resistant to spectinomycin and weakly resistant to streptomycin; strain FAR 4-Neo^s is highly resistant to spectinomycin and fully susceptible to streptomycin. *S. aureus* 20240 is highly resistant to streptomycin but susceptible to spectinomycin, whereas all the other strains were resistant to both drugs (Table 2). When crude extracts of these strains were examined for streptomycin and spectinomycin-modifying enzymes, with the exception of strain FAR 4-Neo^s, they were found to contain a streptomycin-adenylylating activity that was less effective against spectinomycin as substrate (Fig. 1). No phosphorylation of streptomycin has been detected in these strains.

In gram-positive bacteria such as *S. aureus*, plasmid-coded resistance to β-lactam antibiotics and chloramphenicol results from the inducible expression of modifying enzymes that detoxify all of the drug in the culture medium (2). By contrast, in gram-negative bacteria (4) and in streptococci (P. Courvalin et al., manuscript in preparation), plasmid-mediated aminoglycoside-aminocyclitol-modifying enzymes do not express resistance by gross inactivation of the antibiotic; modification of a small amount of the drug prevents further uptake of the antibiotic by the cell, by an unknown mechanism (4). When *S. aureus* strains FAR 4 and RN450/RN1956 were grown in the presence of streptomycin or neomycin B, respectively, no detectable change in the antibiotic concentration was found by microbiological methods; the antibiotic remained unchanged. Measurements of aminoglycoside-aminocyclitol-adenylylating or aminoglycoside-phosphorylating enzyme activities in strains grown with or without antibiotic revealed the presence of substantial modifying activities in all cases. Thus, the aminoglycoside-aminocyclitol-modifying enzymes in *S. aureus* (as well as in streptococci; P. Courvalin et al., manuscript in preparation), like their counterparts in gram-negative bacteria, are synthesized constitutively; however, we cannot eliminate the possibility that superinduction of the constitutive level occurs in the presence of the drug.

In *S. aureus* strains RN450/pSH2 (10), RN450/RN1956, and FAR 4 the amikacin-phosphorylating activity is plasmid mediated; the streptomycin-adenylylating enzyme in strain FAR 4 is also plasmid linked (Table 1) (P. Courvalin and M. Fiandt, manuscript in preparation). Strains 20240 and 20610 each contain at least two different plasmids and were not studied further (Table 1); it is not known if one or both of the plasmids code for the phosphotransferase and the adenylyltransferase.

DISCUSSION

A new plasmid-determined 3'-phosphotransferase has been detected in a number of inde-

TABLE 1. *Properties of the staphylococcal strains and plasmids*

S. aureus strain/plasmid	Phenotype[a]	Genotype[b]	Plasmid content		
			No.	Mol wt (Mdal)[c]	Source
RN450/pSH2	Neo	APH	1	10.1	S. Cohen (10)
RN450/RN1956	Neo	APH	1	10.6	R. P. Novick
FAR 4-Neo^s/pWA2	Em_c Su		1	1.9	R. W. Lacey (6)
FAR 4/pWA1, pWA2	Neo Str Em_i Su Fa	APH SAdT	2	11.7 1.9	R. W. Lacey (6)
20240	Neo Str Pc Tc Su	APH SAdT	≥2[c]	ND[d]	J. W. Griffith
20610	Neo Str Pc Tc Em_c Cm Su	APH SAdT	≥2[c]	ND	K. Casson
JAN 1	Neo Str Pc Mc Tc Em_c Su	APH SAdT	ND		J. Witchitz
JAN 2	Neo Str Pc Mc Tc Em_c	APH SAdT	ND		J. Witchitz
JAN 3	Neo Str Pc Em_i	APH SAdT	ND		J. Witchitz
JAN 4	Neo Str Pc Mc Tc Em_c Su	APH SAdT	ND		J. Witchitz
JAN 5	Neo Str Pc Mc Tc Em_c Cm Su Rif	APH SAdT	ND		J. Witchitz

[a] Neo, Neomycin; Str, streptomycin; Pc, penicillin; Mc, methicillin; Tc, tetracycline; Em_i, erythromycin inducible; Em_c, erythromycin constitutive; Cm, chloramphenicol; Su, sulfonamide; Rif, rifampin; Fa, fusidic acid. Susceptibility tests were performed by a standard agar diffusion method (1).

[b] APH, Aminoglycoside phosphotransferase; SAdT, streptomycin adenylyltransferase.

[c] P. Courvalin and M. Fiandt, manuscript in preparation.

[d] ND, Not done.

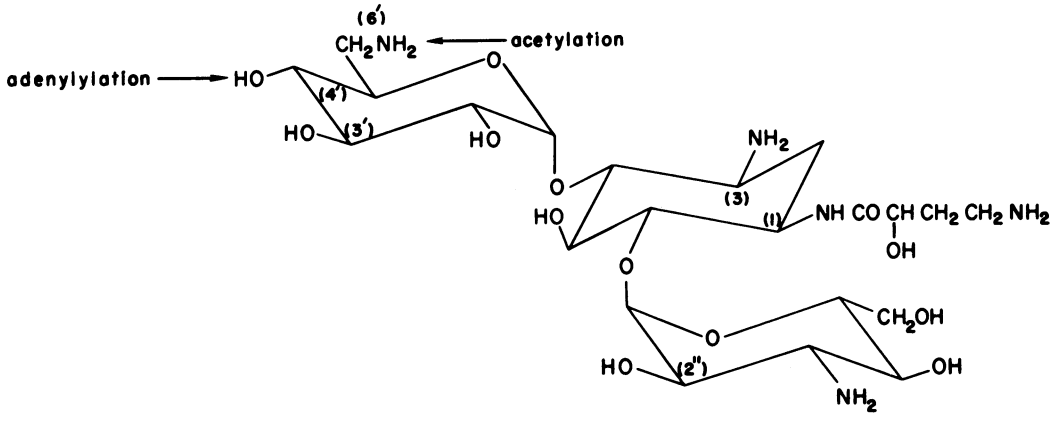

FIG. 2. *Structure of amikacin. The arrows indicate the previously known sites of modification.*

pendent clinical isolates of *S. aureus* obtained from a variety of different sources. This phosphotransferase is distinct from those previously described since it catalyzes the modification of the 3'-hydroxyl group of amikacin. Amikacin (1-*N*-HABA kanamycin A) is an important semisynthetic aminoglycoside that has a broad spectrum of activity; it is effective against most kanamycin-resistant strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *S. aureus*. The modification at the 1-amino group normally blocks phosphorylation of the 3'-hydroxyl group. The reason for the "blocking" capacity of the hydroxyaminobutyric acid side chain is not known; it presumably alters the conformation of the molecule so that it is no longer a substrate for the modifying enzymes. Until recently, no enzyme of the aminoglycoside phosphotransferase class had been found to modify amikacin. A gentamicin-phos-

phorylating enzyme (2"-phosphotransferase) that modifies amikacin weakly has been detected in gentamicin-resistant *S. aureus* strains (F. H. Kayser, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 16th, Chicago, Ill., abstr. 204, 1976; Davies et al., unpublished observation). A 3'-phosphotransferase from *S. aureus* and *S. epidermidis*, which appears to be similar to the enzyme described in this study, has been described recently (Kayser, Progr. Abstr. Intersci. Conf. Antimicrob. Agents Chemother, 16th, Chicago, Ill., abstr. 204, 1976).

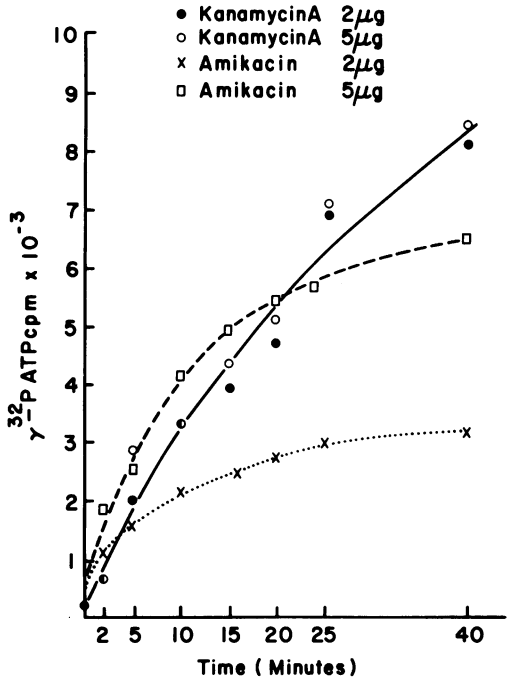

FIG. 3. *Kinetics of phosphorylation of kanamycin A and amikacin by a cell-free extract of S. aureus RN450/RN1956.*

It should be noted that the 1-*N*-HABA substitution does not block all 3'-phosphorylating enzymes, since aminoglycoside phosphotransferase II is capable of phosphorylating butirosin (which can be considered a 1-*N*-HABA ribostamycin).

The new phosphotransferase we have described has the widest substrate range of any 3'-phosphotransferase and is different from other phosphotransferases. It does not cross-react with antisera prepared against aminoglycoside phosphotransferase II (D. Smith, unpublished observation), nor is there any detectable homology at the deoxyribonucleic acid level with the aminoglycoside phosphotransferase gene(s) from the butirosin-producing *Bacillus circulans*, as studied by electron microscopy heteroduplex analysis (P. Courvalin et al., unpublished observation). It has a substrate range quite different from aminoglycoside phosphotransferases isolated from the neomycin-producing *Streptomyces fradiae* and the butirosin-producing *B. circulans* (5); the latter do not phosphorylate amikacin but can modify the HABA-substituted antibiotic butirosin. From the number of different 3'-phosphotransferase enzymes, it would appear that extensive divergent evolution has occurred.

There is one interesting and paradoxical

TABLE 2. *Minimal inhibitory concentrations (MICs) of various aminoglycoside-aminocyclitol antibiotics for S. aureus strains*

S. aureus Strain/plasmid	MIC (μg/ml)[a]											
	Neo B	Par	Liv A	But	Rib	Kan A	Kan B	Kan C	Ami[b]	Tob	Str	Spc
RN450	1	1	2	2	4	2	0.5	8	1 (1-1)	0.5	4	32
RN450/pSH2	256	512	2,048	128	4,096	2,048	512	>1,024	4 (4-4)	0.5	4	32
RN450/RN1956	512	512	2,048	512	4,096	4,096	512	>1,024	8 (8-8)	2	8	32
FAR 4-Neo^s/pWA2	≤0.5	1	2	2	2	2	0.5	8	1 (1-1)	≤0.25	1	2,048
FAR 4/pWA1, pWA2	16	512	>4,096	16	2,048	256	64	>1,024	1 (1-1)	≤0.25	128	>4,096
209P	≤0.5	≤0.5	2	1	2	2	0.5	8	2	≤0.25	1	32
20240	256	4,096	>4,096	128	4,096	1,024	512	>1,024	8	≤0.25	>4,096	32
20610	32	1,024	>4,096	32	2,048	512	128	>1,024	4	≤0.25	>4,096	2,048
JAN 1	128	4,096	>4,096	64	>4,096	1,024	512	>1,024	8	≤0.25	>4,096	>4,096
JAN 2	256	2,048	>4,096	64	>4,096	1,024	512	>1,024	8	≤0.25	>4,096	2,048
JAN 3	16	512	2,048	32	2,048	256	256	>1,024	2	≤0.25	64	>4,096
JAN 4	256	2,048	>4,096	64	>4,096	1,024	256	>1,024	8	≤0.25	>4,096	>4,096
JAN 5	256	4,096	>4,096	128	>4,096	2,048	256	>1,024	8	0.5	>4,096	2,048

[a] Neo B, Neomycin B; Par, paromomycin; Liv A, lividomycin A; But, butirosin; Rib, ribostamycin; Kan A, kanamycin A; Kan B, kanamycin B; Kan C, Kanamycin C; Ami, amikacin; Tob, tobramycin; Str, streptomycin; Spc, spectinomycin.
[b] Average of two independent determinations.

property of the new 3'-phosphotransferase of *S. aureus*. Amikacin is an excellent substrate for the enzyme (Fig. 1), but all the strains are susceptible (Table 2)! Does this mean that enzymatic modification (inactivation) of aminoglycoside antibiotics is an artifact not related to the determination of resistance? This is unlikely since the same enzyme modifies and determines resistance to high levels of neomycin B and kanamycins A, B, and C (Fig. 1, Table 2).

Enzymatic modification of aminoglycoside antibiotics determines resistance, not by inactivation of the drug but (presumably) by establishing a block to transport or entry of the aminoglycoside into the cell (4). We can, perhaps, explain the amikacin susceptibility of these *S. aureus* strains, in spite of the presence of an amikacin-modifying enzyme, in either of the following ways. (i) Amikacin, although structurally related to kanamycin, is transported into the cell by a separate transport mechanism that is not affected by the phosphorylation of amikacin that takes place inside the cell. (ii) Amikacin and kanamycin are transported by the same transport system, but 3'-phosphorylation of amikacin occurs at a slow rate that is not sufficient to affect the transport of the antibiotic; the drug still enters the cell, binds to ribosomes, and inhibits protein synthesis and growth. Modification of kanamycin and neomycin leads to the establishment of a block in the transport of these antibiotics, with concomitant resistance.

Other explanations may be possible, but we favor the latter. Consistent with this model is the fact that modification of amikacin is concentration dependent and occurs at a slower rate than that of kanamycin A (Fig. 3); these experiments, with a crude enzyme preparation, indicate that the K_m for amikacin phosphorylation is substantially higher than that for kanamycin A. This phenomenon has also been observed with several *E. coli* strains, carrying aminoglycoside phosphotransferase I, that are resistant to neomycin and kanamycin but susceptible to the related aminoglycoside seldomycin; the enzyme extracted from these strains is capable of phosphorylating seldomycin but at a slower rate than neomycin and kanamycin (D. Smith, unpublished observation). In *P. aeruginosa* gentamicin acetyltransferase I determines resistance to gentamicin and not tobramycin but is capable of modifying both drugs in extracts; kinetic studies with the pure enzyme have shown that there is a fivefold difference in K_m for gentamicin C_{1a} and tobramycin (J. W. Williams and D. B. Northrop, Fed. Proc. 35:1705, 1976). The enzymatic modification of an aminoglycoside antibiotic is not sufficient alone to establish resistance; the rate of modification, coupled with an effect on transport of the antibiotic into the cell, must determine the expression of this resistance. Another possibility is that amikacin 3'-phosphate is an inhibitor of the enzyme.

There is another unusual characteristic of the *S. aureus* strains that we have examined. They contain a streptomycin adenylyltransferase that modifies streptomycin and spectinomycin in extracts and determines various phenotypic responses to streptomycin and spectinomycin; susceptibility to streptomycin may also be due to a rate phenomenon, as described above. The mechanism of resistance to spectinomycin in these strains remains to be explained, it may be associated with *O*-adenylylation, but strain 20240, although it possesses such activity, is susceptible to this drug. Strain FAR 4-Neo^s does not possess streptomycin-adenylylating or -phosphorylating activity and is highly resistant to spectinomycin.

The discovery of this new plasmid-mediated 3'-phosphorylating enzyme shows that potential resistance mechanisms to antibiotics such as amikacin already exist in the bacterial population. A 4'-adenylylating enzyme that determines resistance to amikacin, tobramycin, and kanamycin has also been detected in *S. aureus* (7). Since it is obvious that extensive genetic interchange occurs between resistance plasmids, we must anticipate the possibility that amikacin-modifying and resistance-determining enzymes, such as the aminoglycoside phosphotransferase described here, may, in due course, appear in gram-negative bacteria and restrict the useful life of antibiotics such as amikacin.

ACKNOWLEDGMENTS

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