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Gene Homogeneity for Aminoglycoside-Modifying Enzymes in Gram-Positive Cocci

HOURIA OUNISSI,[1] ELIANE DERLOT,[1]* CECILE CARLIER,[1] AND PATRICE COURVALIN[1,2]†

Unité des Agents Antibactériens, Unité Associée Centre National de la Recherche Scientifique 271, Institut Pasteur, 75724 Paris Cedex 15, France,[1] and Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093[2]

Received 15 March 1990/Accepted 22 August 1990

Aminoglycoside-resistant strains of *Staphylococcus* and *Enterococcus*, approximately 500 of each, were screened by dot blot hybridization for the presence of genes encoding aminoglycoside-modifying enzymes. The MICs of various aminoglycosides for the strains were determined, and the enzyme contents of the cells were inferred from the resistance phenotypes. The agreements (in percent) of the hybridization results with the deduced enzyme contents for *Staphylococcus* and *Enterococcus* species were, respectively, 80 and 87.6 for ANT(6) (aminoglycoside nucleotidyltransferase), 99.8 and 100 for both APH(3′) (aminoglycoside phosphotransferase) and APH(2″)-AAC(6′) (aminoglycoside acetyltransferase), and 100 and 100 for ANT(4′). The weak correlation obtained with the probe for ANT(6) was due to the fact that gram-positive cocci can also be streptomycin resistant by synthesis of APH(3″) or ANT(3″)(9) and by ribosomal mutation. The remaining probes appeared to be specific: they hybridized with all the resistant clinical isolates but not with the susceptible strains. These results indicate that, except for streptomycin, nucleic acid hybridization is a valid approach for the detection and characterization of aminoglycoside resistance in gram-positive cocci.

Aminoglycoside resistance in gram-positive cocci is generally due to synthesis of enzymes which modify the antibiotics (8). As in gram-negative bacteria, the enzymes can be divided into three classes depending on the reaction catalyzed (phosphorylation or adenylylation of a hydroxyl group, acetylation of an amino group) and are named according to the site they modify on the antibiotic molecule (18). The enzymes vary in their substrate ranges, which can be very broad (5).

The modifying activities detected in enterococci to date constitute a subset of the staphylococcal enzymes: enzymes with identical site specificity were previously found in staphylococci (R. Leclercq, S. Dutka-Malen, A. Brisson-Noël, C. Molinas, E. Derlot, M. Arthur, and P. Courvalin, Rev. Infect. Dis., in press) (Table 1). Comparison of expression, function, and structure of several enzymes and of the corresponding plasmid genes showed that enzymes from gram-positive cocci with identical site specificities were very similar (10, 11).

We have determined the nucleotide sequence of the structural genes for APH(3′) (aminoglycoside phosphotransferase), ANT(6) (aminoglycoside nucleotidyltransferase), and the bifunctional enzyme APH(2″)-AAC(6′) (aminoglycoside acetyltransferase) from *Enterococcus* spp. (17, 30, 36). Using intragenic probes, we studied by DNA-DNA hybridization the relationship between the resistance determinants encoding these enzymes in 1,000 clinical isolates of *Enterococcus* and *Staphylococcus*. We also included a probe specific for ANT(4′) from *Staphylococcus* spp., an enzyme which, to our knowledge, has not yet been detected in enterococci. The results obtained indicated that there is a high degree of gene homogeneity for aminoglycoside-modifying enzymes in gram-positive cocci and that, except for streptomycin, DNA probes constitute a valid approach for the detection of aminoglycoside resistance in these human pathogens.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains of *Staphylococcus* spp. and *Enterococcus* spp., approximately 500 of each, were collected in 24 French hospitals in 1985. The strains were selected for resistance to at least one of the aminoglycosides tested (Table 2). Initial identification was based on colony and microscopic morphologies and coagulase testing. Coagulase-negative staphylococci were identified at the species level with API Staph-Indent kits (API System, La Balme-les-Grottes, France). The enterococci were identified by the absence of catalase; inability to produce gas; presence of Lancefield antigen group D; and growth on 40% bile, in 6.5% sodium chloride, in 0.1% methylene blue, and at pH 9.6. Species identification (15, 31) was based on the absence of reduction of potassium tellurite and tests for acid production from 50 carbohydrates in API 50 CH galleries (API System). The staphylococcal strains were screened for aminoglycoside resistance by the disk agar diffusion technique (Diagnostics Pasteur, Marnes-la-Coquette, France). Disks containing 500 μg of streptomycin, tobramycin, and gentamicin and 1,000 μg of kanamycin (Diagnostics Pasteur) were used to screen enterococci for high-level resistance to aminoglycosides. *Enterococcus faecalis* JH2-2 harboring plasmids pJH1 (*ant6*, *aph3′*) (14) or pIP800 (*aph2″-aac6′*) (10) and BM6217 (*ant6*, *aph3′*, *aph2″-aac6′*) (7); *Staphylococcus aureus* RN450 harboring plasmids pSH2 (*aph3′*) (12), RN1956 (*aph3′*) (12), pWA1 (*aph3′*) (13), and pUB110 (*ant4′*) (21); and *S. aureus* BM3002 (*ant4′*) (23), RN450-Ag^r (*aph2″-aac6′*) (11), PALM (*aph2″-aac6′*) (24), and 80CR5 containing Tn*1545* (*aph3′*) in the chromosome (9) were included as control strains. *E. faecalis* JH2-2 (20), *S. aureus* RN450 (29) and 209P, and 10 strains from our laboratory collection belonging to the species represented in this study were used as aminoglycoside-susceptible strains.

* Corresponding author.
† Present address: Unité des Agents Antibactériens, Institut Pasteur, 75724 Paris Cedex 15, France.

TABLE 1. Classes of aminoglycoside-modifying enzymes found in gram-positive cocci[a]

Enzyme	*Staphylococcus* spp.	*Enterococcus* spp.	Probe designation
Phosphotransferases			
APH(3′)	+	+	*aph3′*
APH(2″)	+	+	*aph2″*
APH(3″)	+	−	
Nucleotidyltransferases			
ANT(6)	+	+	*ant6*
ANT(9)	+	−	
ANT(4′)	+	+[b]	*ant4′*
ANT(3″)(9)	+	−	
Acetyltransferase, AAC(6′)	+	+	*aac6′*

[a] Symbols and abbreviations: +, presence of enzyme; −, absence of enzyme; APH, aminoglycoside phosphotransferase; ANT, aminoglycoside nucleotidyltransferase; AAC, aminoglycoside acetyltransferase.
[b] Detected in this study.

Media. Brain heart infusion broth (Difco Laboratories, Detroit, Mich.) was used. Antibiotic susceptibility tests were performed on Mueller-Hinton agar (Diagnostics Pasteur, Marnes-la-Coquette, France). All incubations were done at 37°C.

Determination of MICs. The method of Steers et al. (34) was used to determine the MICs of aminoglycosides.

Preparation of DNA. Cells from 1.5 ml of an overnight broth culture were harvested, suspended in 75 μl of 0.05 M Tris hydrochloride (pH 8.0)–0.01 M EDTA–25% sucrose containing lysostaphin (1 mg/ml) for staphylococci or lysozyme (20 mg/ml) for enterococci, and incubated at 37°C for 1 h. The resulting protoplasts were lysed by phenol-chloroform extraction, and total DNA was recovered in the supernatant after centrifugation. Portions (5 μl) of the crude extracts were denatured for 10 min at 100°C and spotted onto nitrocellulose membranes. Binding of DNA to the filters was done as described previously (25).

DNA-DNA hybridization. The DNA probes (Table 1) consisted of the 530-bp *Hpa*II fragment of pJH1 (36) for *aph3′*, the 470-bp *Hpa*II fragment of pJH1 (30) for *ant6*, the 473-bp *Hin*cII fragment of pUB110 (6) for *ant4′*, and the 1,317-bp *Hin*cII-*Taq*I fragment of pIP800 (17) for *aph2″-aac6′*. DNA fragments cloned in bacteriophage M13mp18 were hybridized with the 15-bp distal primer and labeled by DNA synthesis in the presence of dGTP, dCTP, dTTP, [α-^{32}P] dATP, and DNA polymerase I (Klenow fragment) (19, 37). Dot blot hybridization under stringent conditions was done in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 42°C for 18 h followed by three washings in 2× SSC–0.1% Sarkosyl at room temperature for 15 min and by four washings in 0.2× SSC–0.1% Sarkosyl at 50°C for 15 min.

Assay for aminoglycoside-modifying enzymes. The bacterial extracts were prepared as described previously (14), and the enzymes were assayed by the phosphocellulose paper-binding technique described previously (18). The final concentration of aminoglycosides in the assay mixture was 66.7 μg/ml, and the reaction was allowed to proceed for 30 min at 30°C.

Enzymes and biochemicals. DNA polymerase I (Klenow fragment) and radioactive chemicals were purchased from Amersham International. Deoxynucleoside triphosphates were from Pharmacia-PL Biochemicals, and the M13 pentadecamer primer was from Boehringer GmbH. Lysozyme and lysostaphin were from Sigma. The antibiotics were provided as follows: gentamicins C1a, C1, C2, A, B, and complex, sisomicin, and netilmicin, Schering Corp.; kanamycins A, B, and C and amikacin, Bristol Laboratories; neomycins A and B, Upjohn Co.; paromomycin and butirosin, Parke, Davis and Co.; tobramycin and apramycin, Eli Lilly; habekacin, Roger Bellon; lividomycin A, Kowa; ribostamycin, Meiji; streptomycin and tetracycline, Pfizer Inc.; minocycline, Lederle; erythromycin, Roussel-Uclaf; rifampin, Le Petit; and fusidic acid, Leo.

TABLE 2. Break points for in vitro susceptibilities of gram-positive cocci to aminoglycosides

Aminoglycoside	MIC (μg/ml) for:	
	Staphylococcus spp.	*Enterococcus* spp.
Amikacin[a]	≥4	≥256
Gentamicin	≥4	≥128
Kanamycin	≥8	≥512
Neomycin	≥8	≥256
Netilmicin[a]	≥1	≥64
Sisomicin	≥4	≥128
Streptomycin	≥4	≥256
Tobramycin	≥4	≥128

[a] The existence of break points for amikacin and netilmicin is questionable (see the text).

RESULTS

Properties of the strains studied. A total of approximately 1,000 clinical isolates of gram-positive cocci resistant to aminoglycosides was obtained in 1985 from various hospitals in France. The ca. 500 staphylococcal strains included 80% *S. aureus* and 20% coagulase-negative staphylococci. The ca. 550 enterococci included approximately 500 *E. faecalis* and 50 *Enterococcus faecium* strains. The MICs of the aminoglycosides listed in Table 2 for the strains were determined. The values for clinical categorization chosen (Table 2) were based on the study of the distributions of the MICs for a large number of clinical isolates of gram-positive cocci that were susceptible or resistant to aminoglycosides by previously characterized mechanisms (2; Leclercq et al., in press). In this population analysis (R. Bismuth, H. Drugeon, and P. Courvalin, unpublished data), strains that were stochastically distinct from the susceptible population were considered as resistant. The enzyme contents of the strains were then inferred from the resistance phenotype, as indicated in Table 3 and described previously for gram-negative bacteria (32). Although neomycin is not used in clinical settings, it was included to detect the presence of APH(3′) or ANT(4′) when it was coresident in the same host with the bifunctional enzyme APH(2″)-AAC(6′). In a third step, the correlations between the results of hybridization experiments with specific probes (Table 1) and the deduced enzyme contents of the same strains were calculated (Tables 4 to 7). Strains representative of the various classes of discrepancies observed were examined by (i) redetermination of MICs of aminoglycosides, (ii) study of the enzyme content of the cells by the phosphocellulose paper-binding assay, and (iii) rehybridization with the set of probes.

Hybridization. (i) *ant6* probe. The 6-nucleotidyltransferase modifies streptomycin, and therefore, the corresponding probe could only differentiate strains which were susceptible or resistant to this antibiotic (Table 4). The agreement between the results of hybridization and high-level strepto-

TABLE 3. Aminoglycoside resistance phenotypes caused by modifying enzymes in gram-positive cocci[a]

Enzyme	Phenotype						
	Streptomycin	Kanamycin	Neomycin	Tobramycin	Amikacin	Gentamicin, sisomicin[b]	Netilmicin
ANT(6), APH(3″), ANT(3″)(9)	R	S	S	S	S	S	S
APH(3′)	S	R	R	S	**S/R**[c]	S	S
ANT(4′)	S	R	R/S	R	**S/R**	S	S
APH(2″)-AAC(6′)	S	R	S	R	**S/R**	R	**S/R**
APH(2″)-AAC(6′) + APH(3′)	S	R	R	R	**R/S**	R	**S/R**
APH(2″)-AAC(6′) + ANT(4′)	S	R	R/S	R	**R/S**	R	**S/R**

[a] Abbreviations: R, resistant; S, susceptible; ANT, aminoglycoside nucleotidyltransferase; APH, aminoglycoside phosphotranspherase; AAC, aminoglycoside acetyltransferase.

[b] Gentamicin and sisomicin are equivalent, except against *E. faecium*, because of the chromosomally encoded AAC(6′), which confers resistance to the latter but not to the former antibiotic (27, 38).

[c] Phenotypes of antibiotics that are bacteriostatic but not bactericidal are indicated in boldface type.

mycin resistance of the strains was rather low, 80 and 87.6% for staphylococci and enterococci, respectively. Approximately 60 strains of each group of bacteria were resistant to streptomycin and did not hybridize with the probe. The isolates were resistant by production of enzymes other than ANT(6) (Table 3) or following chromosomal mutation. The latter mechanism confers a very high level of resistance to enterococci (16), and the MIC of streptomycin for 3 of 10 strains of this group was greater than 100,000 μg/ml. More puzzling were the strains, mainly staphylococci, that were streptomycin susceptible but that hybridized with the probe. Curiously, they were all also resistant to kanamycin and structurally related aminoglycosides by production of an APH(3′) (see below). Three strains of this group of bacteria were studied further and were not found to produce a streptomycin-modifying enzyme by the phosphocellulose paper-binding assay.

(ii) *aph3′* probe. Apart from streptomycin, the set of aminoglycosides used in this study could theoretically discriminate five enzymes or enzyme combinations (Table 3). The percentages of agreement obtained with the *aph3′* probe were extremely high: 99.8 and 100% for staphylococci and enterococci, respectively (Table 5). Thirty-one staphylococcal strains were considered, on the basis of their resistance phenotype (Table 3), to encode both APH(2″)-AAC(6′) and APH(3′) but did not hybridize with the probe. All but one hybridized with the *aph2″-aac6′* and *ant4′* probes (see below) and therefore do not constitute major errors (Table 1). This result indicates that neomycin MICs do not allow the discrimination of strains that produce APH(2″)-AAC(6′) plus APH(3′) or ANT(4′). Clinical isolates that harbor these enzyme combinations were therefore assigned to the same group (Tables 5 to 7). The remaining discrepancy was a strain of *S. aureus* which hybridized with the *aph2″-aac6′* probe and for which the neomycin MIC was 8 μg/ml. This isolate was therefore, erroneously, considered as neomycin resistant.

(iii) *aph2″-aac6′* probe. The agreement observed with the *aph2″-aac6′* probe (Table 6) was identical to that obtained with the *aph3′* probe. The only discrepancy was an *S. aureus* strain that hybridized with the probe but that was considered to encode an ANT(4′) because the gentamicin MIC for this strain was only 2 μg/ml.

(iv) *ant4′* probe. The agreement obtained with the *ant4′* probe was 100% for both staphylococci and enterococci (Table 7). However, in the latter group of organisms, a single strain, *E. faecium* BM4102 (6), was found to produce an ANT(4′).

TABLE 4. Hybridization with the *ant6* probe

Resistance phenotype	No. of isolates with the indicated hybridization with the probe[a]:			
	Staphylococci (n = 490)		Enterococci (n = 550)	
	+	−	+	−
Streptomycin resistant	310	**63**	408	**65**
Streptomycin susceptible	**35**	82	**3**	74

[a] +, Positive hybridization; −, negative hybridization. There was 80% hybridization agreement for the staphylococci and 87.6% hybridization agreement for the enterococci. Discrepancies are indicated by boldface type.

DISCUSSION

We studied, by dot blot hybridization, the distribution of genes encoding modifying enzymes (Table 1) in ca. 1,000 clinical isolates of gram-positive cocci resistant to aminoglycosides (Table 2). As expected, the correlation between streptomycin resistance and hybridization with the probe for the 6-nucleotidyltransferase was rather weak (Table 4). The low percentages observed (80 and 88%) can be explained, at least in part, by the multiplicity of streptomycin-modifying enzymes (Table 1) and the existence of ribosomal mutants (16). More intriguing is the relatively important number (7%)

TABLE 5. Hybridization with the *aph3′* probe

Enzyme content[a]	No. of isolates with the indicated hybridization with the probe[b]:			
	Staphylococci (n = 492)		Enterococci (n = 542)	
	+	−	+	−
APH(3′)	100	0	375	0
APH(2″)-AAC(6′)	0	60	0	45
ANT(4′)	0	10	0	1
APH(2″)-AAC(6′) + APH(3′) and/or ANT(4′)	247	**31**[c]	60	0
Kanamycin and neomycin susceptible	0	44	0	61

[a] Enzyme content was deduced from the resistance phenotype determined by dilution in agar.

[b] +, Positive hybridization; −, negative hybridization. There was 99.8% hybridization agreement for the staphylococci and 100% hybridization agreement for the enterococci. Discrepancies are indicated by boldface type.

[c] Thirty of these strains encoded APH(2″)-AAC(6′) plus ANT(4′) and therefore do not constitute true discrepancies.

TABLE 6. Hybridization with the *aph2"-aac6'* probe

Enzyme content[a]	No. of isolates with the indicated hybridization with the probe[b]:			
	Staphylococci (n = 467)		Enterococci (n = 510)	
	+	−	+	−
APH(3')	0	98	0	357
APH(2")-AAC(6')	55	0	42	0
ANT(4')	**1**	9	0	1
APH(2")-AAC(6') + APH(3') and/or ANT(4')	264	0	55	0
Kanamycin, tobramycin, and gentamicin susceptible	0	40	0	55

[a] Enzyme content was deduced from the resistance phenotype determined by dilution in agar.

[b] +, Positive hybridization; −, negative hybridization. There was 99.8% hybridization agreement for staphylococci and 100% hybridization agreement for enterococci. Discrepancies are indicated by boldface type.

of streptomycin-susceptible staphylococci which hybridized with the probe. This type of discrepancy, which was much less frequent (0.5%) in enterococci, could result from the presence in the strains of a silent (26) or remnant *ant6* gene or from hybridization to another portion of the genome. The gene for ANT(6) was nearly always (99.8% in staphylococci and 99.6% in enterococci) associated with that encoding an APH(3'). This observation, combined with the fact that the streptomycin-susceptible strains which hybridized with the *ant6* probe were all kanamycin resistant, suggests a physical link between the two resistance genes.

The agreement obtained between the enzyme content of the strains inferred from aminoglycoside MIC determinations (Table 3) and hybridization with probes internal to the structural genes for APH(3') (Table 5), APH(2")-AAC(6') (Table 6), and ANT(4') (Table 7) was excellent.

The probes appeared to be specific, and they did not hybridize with susceptible strains. The results indicate that, as mentioned above, except for streptomycin, modifying enzymes accounted for aminoglycoside resistance in all the clinical isolates that we studied. In fact, one strain of *E. faecalis* that was resistant to gentamicin by defective uptake of the drug has been reported previously (28). Strains of *E. faecium* produce low levels of a chromosomally encoded AAC(6') (27, 38). The gene for this enzyme did not hybridize with the *aph2"-aac6'* probe, indicating that the two acetyltransferases are not closely related in structure. The conservation of genes for aminoglycoside-modifying enzymes in gram-positive cocci is in contrast to the heterogeneity observed in other resistance systems. For example, a minimum of six tetracycline resistance genes have been found in staphylococci and enterococci (3, 39), and at least three classes of *erm* determinants are responsible for resistance to macrolide-lincosamide-streptogramin B-type antibiotics (1).

TABLE 7. Hybridization with the *ant4'* probe

Enzyme content[a]	No. of isolates with the indicated hybridization with the probe[b]:			
	Staphylococci (n = 467)		Enterococci (n = 547)	
	+	−	+	−
APH(3')	0	98	0	378
APH(2")-AAC(6')	0	53	0	45
ANT(4')	12	0	1	0
APH(2")-AAC(6') + APH(3') and/or ANT(4')	43	221	0	61
Kanamycin and tobramycin susceptible	0	40	0	62

[a] Enzyme content deduced from the resistance phenotype determined by dilution in agar.

[b] +, Positive hybridization; −, negative hybridization. There was 100% hybridization agreement for both staphylococci and enterococci.

Nucleic acid hybridization therefore appears to be a promising approach for the detection of resistance to aminoglycosides, other than streptomycin, in gram-positive cocci. This technique avoids the pitfalls of in vitro susceptibility testing (Leclercq et al., in press) and allows prediction of the bacteriostatic (Table 3) and bactericidal activities of the aminoglycosides alone or combined with beta-lactams (Leclercq et al., in press).

The break points for aminoglycoside resistance used in this study (Table 2) were based on the analysis of the distribution of aminoglycoside MICs for a large number of susceptible or resistant strains. There was an excellent correlation, except for amikacin and netilmicin (Table 3), between the categories they define and the presence or absence of modifying enzymes in the cells; these values may therefore be of clinical relevance. When tested by assays used to evaluate the bacteriostatic activities of antibiotics, the presence of an enzyme confers to the host in vitro resistance to the aminoglycosides that are modified. There are, however, two major exceptions in gram-positive cocci: amikacin and netilmicin (4, 6, 8, 33, 35). Modification of these drugs by the cells apparently does not affect their bacteriostatic activities (Table 3). Nevertheless, the bactericidal activities of the two antibiotics and the bactericidal synergy they usually display when combined with beta-lactams are abolished (R. Bismuth, J. L. Pirault, H. Drugeon, and P. Courvalin, Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 705, 1989; Leclercq et al., in press). In vitro determination of the bacteriostatic activities of amikacin and netilmicin is therefore misleading and should not be performed. For the same reasons, assignment of break points for these two aminoglycosides against gram-positive cocci is not possible. Kanamycin instead of amikacin and gentamicin (or sisomicin) instead of netilmicin should be tested. In the case of resistance to kanamycin, amikacin should not be used; in the case of resistance to gentamicin and sisomicin, netilmicin (or amikacin) should not be used (Table 3).

This study of the prevalence of genes for aminoglycoside-modifying enzymes led to the detection of ANT(4') in *Enterococcus* spp. (6), an enzyme that, so far, is confined to staphylococci.

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