ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1990, p. 1875–1879
0066-4804/90/101875-05$02.00/0

Vol. 34, No. 10

Phenotypic and Genotypic Heterogeneity of Glycopeptide Resistance Determinants in Gram-Positive Bacteria

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Received 5 April 1990/Accepted 31 July 1990

Gram-positive glycopeptide-resistant bacteria isolated in various hospitals in Europe and in the United States between 1986 and 1988 were collected. Three resistance phenotypes could be distinguished. Thirty-one enterococci were highly resistant to vancomycin and teicoplanin. Resistance was transferable to other enterococci by conjugation for 16 of the 22 isolates that were tested. Homology was detected by hybridization between a probe specific for the *vanA* gene, which encodes an inducible high-level glycopeptide resistance protein in *Enterococcus faecium* BM4147, and DNA of the 31 clinical isolates and the 16 corresponding transconjugants. This indicates that a single class of resistance determinants accounts for high-level glycopeptide resistance in enterococci. The strains differed in their biotypes and resistance phenotypes and harbored resistance plasmids of various sizes, suggesting that spread of this resistance phenotype is due to dissemination of a gene rather than of a bacterial clone or of a single plasmid. Four enterococcal isolates were resistant to low levels of vancomycin and susceptible to teicoplanin. Twenty-three coagulase-negative staphylococcal isolates were resistant to teicoplanin and susceptible to vancomycin. These two groups of strains did not hybridize with the *vanA* probe and did not transfer resistance at a detectable frequency. The *vanA* gene was not detected in the glycopeptide-producing strains of *Amycolatopsis orientalis* (vancomycin) and *Actinoplanes teichomyceticus* (teicoplanin) or in various gram-positive bacteria intrinsically resistant to glycopeptides.

Glycopeptide antibiotics, vancomycin and teicoplanin, are active against most gram-positive bacteria and are increasingly used in the treatment of infections due to staphylococci, streptococci, and enterococci. *Pediococcus* spp., *Leuconostoc* spp., and certain species of *Lactobacillus* are intrinsically resistant to these antibiotics (5, 6). Acquired resistance to glycopeptides is still uncommon, despite the use of vancomycin in human therapy for over three decades. However, several recent reports emphasize the emergence and spread of resistance to these drugs in *Staphylococcus* spp. and *Enterococcus* spp.

In staphylococci, vancomycin resistance was first described in a strain of *Staphylococcus haemolyticus* recovered after treatment with this antibiotic (20). Coagulase-negative staphylococci, mainly *S. haemolyticus* strains, resistant to teicoplanin and susceptible to vancomycin have also been reported (2, 9, 28).

In enterococci, acquired resistance to glycopeptides is inducible by these antibiotics and two phenotypes can be distinguished: (i) low-level resistance to vancomycin only (13, 19, 27) and (ii) high-level resistance to vancomycin and teicoplanin (15, 16, 18, 21). In both phenotypes, induction of resistance is associated with inducible synthesis of a protein of ca. 40 kilodaltons (18, 21, 27).

We previously established that in four clinical isolates of *Enterococcus faecium*, the determinants for high-level resistance to glycopeptides were plasmid borne (15, 16). Three of the plasmids were self-transferable to other enterococci (16). In the remaining strain, *E. faecium* BM4147, the genetic information for glycopeptide resistance was carried by the nonconjugative plasmid pIP816 (4, 15). The resistance determinant of pIP816 was cloned into *Escherichia coli* (4), and the nucleotide sequence of the *vanA* gene encoding the inducible resistance protein was determined (S. Dutka-Malen, C. Molinas, M. Arthur, and P. Courvalin, Mol. Gen. Genet., in press). In an attempt to elucidate the origin of *vanA* and to study its distribution, we have tested for the presence of *vanA*-related sequences in 75 bacteria with intrinsic or acquired resistance to glycopeptides.

(This study was presented in part at the 29th Interscience Conference on Antimicrobial Agents and Chemotherapy, Houston, Tex., 17 to 20 September 1989 [R. Leclercq, V. Coutant, S. Dutka-Malen, J. Duval, and P. Courvalin, Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 273, 1989].)

MATERIALS AND METHODS

Bacterial strains. Thirty-one enterococcal strains resistant to high levels of glycopeptides were collected from 10 hospitals in France, Spain, and the United Kingdom between 1986 and 1988. *E. faecium* BM4147, BM4152, BM4165, and BM4178 (15, 16) and four glycopeptide-resistant strains representative of the biotypes found in the English outbreak (A. H. C. Uttley, C. H. Collins, J. Naidoo, and R. C. George, Letter, Lancet, i:57–58, 1988) (*Enterococcus faecalis* NCTC 12201 and NCTC 12202 and *E. faecium* NCTC 12203 and NCTC 12204) were included. *E. faecium* isolates were recovered from stool samples of neutropenic patients (19 strains), pus (2 strains), urine (3

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strains), and blood cultures (1 strain). *E. faecalis* strains were isolated from blood cultures (one strain) and pus (one strain). The origins of two *E. faecalis* and two *E. faecium* strains were not specified. Enterococci were identified by Gram staining; 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 was based on the tests for reduction of potassium tellurite and for acid production from 50 carbohydrates in API 50 CH galleries (API System, la Balme-les-Grottes, France). Four enterococcal isolates resistant to low levels of vancomycin and detected in the United States in 1988 were studied: *E. faecalis* V583 (19), isolated from blood cultures, and three strains of *Enterococcus gallinarum* (13) isolated from pus (two strains) and blood cultures (one strain). The 23 staphylococcal strains (15 *Staphylococcus epidermidis* and 8 *S. haemolyticus*) resistant to teicoplanin and susceptible to vancomycin were isolated in 1987 at Henri Mondor Hospital (Créteil, France) and were identified by the method of Kloos and Schleifer (14); the strains appeared unrelated since they had different antibiotic resistance phenotypes. *E. faecalis* JH2-2 (11); *E. faecium* BM4107, BM4147-1, BM4165-1, and BM4178-1 (15, 16); *Staphylococcus aureus* 80CR5 (8); and *S. epidermidis* BM4625 from our laboratory collection were used as susceptible control strains.

Intrinsically resistant *Leuconostoc dextranicum* (1 strain), *Leuconostoc mesenteroides* (1 strain), *Leuconostoc paramesenteroides* (1 strain), *Leuconostoc lactis* (1 strain), *Leuconostoc pseudomesenteroides* (1 strain), *Leuconostoc citreum* (1 strain), *Lactobacillus confusus* (2 strains), and *Pediococcus* spp. (11 strains) were from the collections of R. R. Facklam and of our laboratory. *Amycolatopsis orientalis* ATCC 19795, which produces vancomycin, was provided by N. Allen. DNA of *Actinoplanes teichomyceticus* ATCC 31211, which produces teicoplanin, was provided by M. Denaro.

Media. Brain heart infusion broth and agar (Difco Laboratories, Detroit, Mich.) were used. Susceptibility tests were performed on Mueller-Hinton agar (Diagnostics Pasteur, Marnes-la-Coquette, France) supplemented with 5% horse blood. All incubations were at 37°C.

Determination of in vitro susceptibility to antibiotics. The diffusion test with disks containing 30 μg of vancomycin or teicoplanin (Diagnostics Pasteur) was used. Disks containing 500 μg of streptomycin or gentamicin and 1,000 μg of kanamycin were used to screen enterococci for high-level resistance to aminoglycosides. β-Lactamase production was assessed with Cefinase disks (bioMérieux, Marcy l'Etoile, France). The method of Steers et al. (22) with 10^4 CFU per spot was used to determine the MICs of antibiotics.

Transfer of antibiotic resistance traits. Matings on filters were performed as previously described (23). *E. faecium* BM4107 (16) and *E. faecalis* JH2-2 (11), resistant to fusidic acid and rifampin, were used as recipients in mating experiments with enterococci, lactobacilli, leuconostocs, and pediococci. Transconjugants from mating between enterococci were selected on plates containing 50 μg of rifampin per ml and 20 μg of fusidic acid per ml plus each of the following: erythromycin, 10 μg/ml; gentamicin, 100 μg/ml; kanamycin or streptomycin, 400 μg/ml; tetracycline, 8 μg/ml; and vancomycin, 10 μg/ml. The antibiotic resistance of five transconjugants from every selective plate was determined. In matings with lactobacilli, leuconostocs, and pediococci as donors, only transfer of vancomycin resistance (10 μg/ml) was selected for. Antibiotic concentrations for selection of transfer of teicoplanin resistance from coagulase-negative staphylococci to *S. aureus* 80CR5 and *S. epidermidis* BM4625 were as follows: rifampin, 50 μg/ml; fusidic acid, 20 μg/ml; and teicoplanin, 8 μg/ml. Transfer frequencies were expressed relative to the number of donor CFU after the mating period.

Preparation of DNA. Cells from 1.5 ml of an overnight culture were harvested; suspended in 100 μl of a solution containing 0.05 M Tris hydrochloride (pH 8.0), 0.01 M EDTA, and 25% sucrose containing either lysostaphin (0.25 mg/ml) (for staphylococci) or lysozyme (5 mg/ml) (for enterococci, *Leuconostoc* spp., and *Pediococcus* spp.); 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. Presence of DNA was monitored by agarose gel electrophoresis. Portions (10 μl) were denatured for 10 min at 100°C and spotted on Nytran membranes (Amersham Corp., Amersham, England). Plasmid DNA from transconjugants was purified by alkaline lysis as described previously (7, 24).

DNA-DNA hybridization. The DNA probe consisted of the 290-base-pair *Bam*HI-*Rsa*I fragment internal to the *vanA* gene. The DNA fragment cloned in bacteriophage M13mp18 was hybridized with the 15-base-pair distal primer and labeled by DNA synthesis in the presence of dGTP, dATP, dTTP, [α-^{32}P]dCTP, and DNA polymerase (Klenow fragment) (10). Hybridization in stringent conditions was at 65°C in a solution containing 0.1% sodium dodecyl sulfate, 0.7% nonfat dry milk, 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) overnight (12). Washings were at 65°C in 2× SSC–0.1% sodium dodecyl sulfate. Hybridization in moderately stringent conditions was at 60°C overnight, and washings were at 45°C.

Enzymes and reagents. Lysostaphin and lysozyme were from Sigma Chemical Co. (St. Louis, Mo.). RNase A (bovine pancreas) and proteinase K were from Calbiochem Corp. (San Diego, Calif.), and [α-^{32}P]dCTP-triethylammonium salt (specific activity, 400 Ci/mmol) was obtained from the Radiochemical Centre (Amersham Corp.). Ramoplanin and teicoplanin were provided by Gruppo Lepetit (Milan, Italy); vancomycin and daptomycin were from Eli Lilly & Co. (Indianapolis, Ind.).

RESULTS

Glycopeptide resistance phenotypes. The clinical isolates could be assigned to three groups (designated 1, 2, and 3) of glycopeptide resistance phenotypes (Table 1). Group 1 consisted of 27 strains of *E. faecium* and 4 strains of *E. faecalis*, originating from France, Spain, and the United Kingdom, that were highly resistant to glycopeptides. The MICs of vancomycin and teicoplanin against these strains were greater than or equal to 64 and 16 μg/ml, respectively (greater than 256 and 64 μg/ml, respectively, for 24 of the 31 strains). When tested by the disk-agar diffusion technique, the cultures grew at the point of contact of disks containing 30 μg of vancomycin or teicoplanin. The *E. faecium* strains were all resistant to penicillin G (MICs ≥ 16 μg/ml) but did not produce a β-lactamase. Glycopeptide resistance was associated with resistance to macrolides-lincosamides-streptogramin B-type antibiotics (27 strains), tetracycline (28 strains), streptomycin and kanamycin (18 strains), and chloramphenicol (8 strains). Eight combinations of resistance characters could be distinguished among the 31 isolates.

The second group was composed of *E. faecalis* V583 (19) and of three strains of *E. gallinarum* (13), isolated in the

TABLE 1. Distribution of strains according to their susceptibility to glycopeptides, lipopeptides, and glycolipodepsipeptides and hybridization with the *vanA* probe

Group[a] and organism (no. of strains)	MIC range (μg/ml)[b]				Hybridization with *vanA*[c]
	Vancomycin	Teicoplanin	Daptomycin	Ramoplanin	
1					
E. faecium (27)	64–1,000	16–500	0.5–2	0.5–2	+
E. faecalis (4)	256–1,000	128–500	0.5	0.5–2	+
2					
E. gallinarum (3)	16	0.5–1	0.5	1	–
E. faecalis (1)	32	0.5	0.5	1	–
3					
S. epidermidis (15)	2–4	8–32	ND	ND	–
S. haemolyticus (8)	2–4	8–32	ND	ND	–
4					
E. faecium (4)	0.5–1	0.25–0.5	0.5–1	1	–
E. faecalis JH2-2	1	0.25	1	1	–
S. aureus 80CR5	1	0.5	ND	ND	–
S. epidermidis BM4625	1	1	ND	ND	–
5					
Leuconostoc spp. (6)	>1,000	>1,000	ND	ND	–
Pediococcus spp. (11)	>1,000	>1,000	ND	ND	–
L. confusus (2)	>1,000	>1,000	ND	ND	–
A. teichomyceticus ATCC 31211	ND	ND	ND	ND	–
A. orientalis ATCC 19795	ND	ND	ND	ND	–

[a] Group 1, Enterococci highly resistant to vancomycin and teicoplanin; group 2, enterococci resistant to vancomycin and susceptible to teicoplanin; group 3, coagulase-negative staphylococci resistant to teicoplanin; group 4, control strains susceptible to glycopeptides; group 5, gram-positive bacteria naturally resistant to glycopeptides.
[b] ND, Not determined.
[c] +, Positive hybridization; –, negative hybridization.

United States and resistant to low levels of vancomycin (MICs of 16 or 32 μg/ml) but susceptible to teicoplanin (MICs of 0.5 or 1 μg/ml). As already noted (19), this resistance phenotype is difficult to detect by the disk diffusion assay. The inhibition zone diameters around the vancomycin disks (15 to 17 mm) were only slightly smaller than those (20 to 21 mm) of susceptible strains *E. faecalis* JH2-2 and *E. faecium* BM4107. However, for resistant strains, minute colonies were present at the edge of the vancomycin inhibition zone after an incubation period of 24 h; they were more visible after incubation for 48 h. The strains of groups 1 and 2 remained susceptible to lipopeptide (daptomycin) and glycolipodepsipeptide (ramoplanin) antibiotics.

The coagulase-negative staphylococci in group 3 were resistant to teicoplanin (MICs, 8 to 32 μg/ml) and susceptible to vancomycin (MICs, 2 to 4 μg/ml). By regression analysis, a very poor correlation ($r = -0.6$) between the inhibition zone diameters with 30-μg disks and the MICs of teicoplanin was obtained. Similar results have already been reported (9).

Transfer of glycopeptide resistance. Transfer of glycopeptide resistance by filter mating was obtained with enterococci highly resistant to glycopeptides (group 1) but not with strains belonging to the other groups (2 to 5). Of 22 isolates tested, 16 transferred their resistance to other enterococcal cells: 11 strains conjugated to both *E. faecalis* JH2-2 and *E. faecium* BM4107, at frequencies ranging from 10^{-2} to 10^{-8}, and 5 strains transferred glycopeptide resistance to *E. faecium* BM4107 at frequencies ranging from 10^{-4} to 10^{-8} but not to *E. faecalis* JH2-2 (frequency, $<10^{-9}$). Transfer frequencies depended upon the species of both donor and recipient strains. In three independent experiments, we did not detect transfer to either recipient from the six remaining strains. The MICs of vancomycin and teicoplanin for the transconjugants were similar to those for the wild strains (data not shown). Nine strains cotransferred resistance to one or several antibiotics (i.e., macrolides-lincosamides-streptogramin B, kanamycin, streptomycin, and tetracycline) and glycopeptide resistance, leading to different combinations of resistance markers in the recipients. The five resistance phenotypes obtained after selection with vancomycin are shown in Table 2.

Plasmid DNA was extracted by the alkaline lysis procedure (24) from 10 of the 16 transconjugants obtained. The plasmids varied in size from approximately 40 to 50 kilobases, as determined by agarose gel electrophoresis of crude bacterial lysates (data not shown). Because of the sensitivity of the technique used, the apparent lack of extrachromosomal DNA in the remaining strains does not necessarily imply absence of plasmid.

TABLE 2. Transferable resistance in enterococci highly resistant to glycopeptides

Resistance characters transferred[a]	No. of strains	*Enterococcus* recipient
None	6	
Te, Vm	2	BM4107
Te, Vm	5	BM4107, JH2-2
Em, Te, Vm	4	BM4107, JH2-2
Km, Sm, Te, Vm	1	BM4107, JH2-2
Em, Km, Sm, Te, Vm	1	BM4107
Em, Km, Sm, Te, Vm	1	BM4107, JH2-2
Em, Km, Sm, Tc, Te, Vm	2	BM4107

[a] Em, Erythromycin resistance; Km, kanamycin resistance; Sm, streptomycin resistance; Te, teicoplanin resistance; Tc, tetracycline resistance; Vm, vancomycin resistance.

Analysis of DNA by hybridization. Homology was detected, by dot blot hybridization, between the probe specific for *vanA* and DNA of the 31 strains of enterococci and of the 16 transconjugants highly resistant to glycopeptides (Table 1). We did not observe homology with the other strains, including the cured derivatives, even under moderately stringent hybridization conditions.

DISCUSSION

Reports on emergence of glycopeptide resistance in gram-positive cocci are recent, in spite of more than three decades of therapeutical use of vancomycin (26). The long delay between the initial use of glycopeptides and emergence of resistance is rather surprising when compared with the situation in other antibiotic families. Acquired resistance to glycopeptides was observed in both staphylococci and enterococci. Review of the literature indicates that different resistance phenotypes can be distinguished in these bacterial genera. However, comparative analyses of the resistance phenotypes are limited to a few clinical isolates, and the resistance determinants were not compared. We have gathered 35 glycopeptide-resistant enterococci previously described or prospectively collected in 10 hospitals in France, Spain, the United Kingdom, and the United States. The enterococcal isolates can be considered representative of glycopeptide-resistant strains from various geographical areas that have emerged since 1986. We also studied strains of coagulase-negative staphylococci from our hospital that are resistant to teicoplanin, and we included gram-positive bacteria that are naturally resistant to glycopeptides from other laboratory collections. The strains with acquired resistance could be assigned to three groups, each corresponding to a glycopeptide resistance phenotype (Table 1).

The 31 *E. faecium* and *E. faecalis* strains in group 1 and the corresponding transconjugants were resistant to high levels of vancomycin and teicoplanin. All the strains grew at the point of contact of the vancomycin and teicoplanin disks and hybridized with the *vanA* probe. These results indicate that a single class of *vanA*-related genes is responsible for high-level resistance in enterococci. The wide range of glycopeptide MICs against these strains indicated phenotypic heterogeneity for *vanA* resistance determinants.

The *vanA* gene was detected in two species of enterococci, and within a species, the strains could be distinguished on the basis of their biotypes (data not shown) and their resistance phenotypes. We previously established that in four *E. faecium* strains, the glycopeptide resistance determinants were borne by one mobilizable and three self-transferable plasmids (15, 16). Comparison of the restriction and hybridization profiles indicated that these plasmids were distinct, although related. In this study, transfer of high-level glycopeptide resistance to enterococcal recipients was observed for 16 of 22 strains, and plasmid DNA could be detected in 10 transconjugants. The *vanA* gene appeared, therefore, to be located in most cases on self-transferable plasmids that differ in their resistance markers, their transfer characteristics, and their size. These results suggest that dissemination of high-level glycopeptide resistance in enterococci is due to spread of a gene rather than of a strain or of a plasmid. Alternatively, an epidemic plasmid could have acquired other resistance characters and undergone rearrangements in the course of conjugative transfers.

The four isolates in group 2 were resistant to low levels of vancomycin but susceptible to teicoplanin. The apparent absence of transfer could explain the limited dissemination of this resistance phenotype. However, its frequency could be underestimated because of difficulty of detection. No homology with the *vanA* probe was found in isolates of this group, even under moderately stringent conditions, indicating that the determinants for low- and high-level glycopeptide resistance belong to distinct gene classes. The fact that the inducible proteins associated with the two types of resistance are antigenically distinct (S. Al Obeid, L. Gutmann, D. Shlaes, and E. Collatz, 29th ICAAC, abstr. no. 274, 1989) is consistent with this notion. However, these results do not exclude the possibility that heterologous genes encode functionally related proteins. Studies on the mechanism of glycopeptide resistance suggest that these proteins may have similar functions (1).

Twenty-three staphylococcal strains in group 3 were resistant to low levels of teicoplanin but susceptible to vancomycin. The probe did not hybridize with DNA of these isolates, indicating that the teicoplanin resistance determinants in *Staphylococcus* spp. are distinct from *vanA*. To our knowledge, glycopeptide resistance determinants from staphylococci and enterococci had not yet been compared.

The plasmid location of *vanA* and the lack of hybridization between the probe and the chromosome of glycopeptide-susceptible strains indicated an exogenous origin for this gene in *Enterococcus* spp. In several families of antibiotics, similarities have been detected between the resistance mechanisms in human pathogens and the mechanisms by which antibiotic-producing bacteria avoid self-destruction. It has been proposed that this analogy might indicate that the resistance genes found in human pathogens originated in antibiotic producers (3, 25). Since then, accumulation of data on the amino acid sequences of resistance proteins from both groups of organisms has neither dismissed nor confirmed this hypothesis. The structural genes for the proteins were found to be related, but horizontal gene transfer could not be inferred because of substantial sequence divergences. We did not detect homology between the *vanA* probe and genomic DNA of *Amycolatopsis orientalis* ATCC 19795, which produces vancomycin, nor with genomic DNA of *Actinoplanes teichomyceticus* ATCC 31211, which produces teicoplanin. This observation indicates that emergence of high-level glycopeptide resistance in enterococci is not due to recent acquisition of a gene from one of these antibiotic producers. However, a lack of relationship between resistance determinants or a difference in resistance mechanisms cannot be inferred from negative hybridization data. Similarly, *vanA* apparently does not originate in *Lactobacillus*, *Pediococcus*, or *Leuconostoc* spp., which are intrinsically resistant to glycopeptide antibiotics; antibodies against the protein associated with high-level resistance in enterococci also did not cross-react with members of these bacterial genera (17).

ACKNOWLEDGMENTS

We thank N. Allen, F. Baquero, M. Bartoli, E. Bingen, C. Branger, R. R. Facklam, J. Fleurette, T. Fosse, T. Lambert, D. Mathieu, C. Morel, G. Prats, D. S. Sahm, and M. Weber for the gifts of bacterial strains; M. Denaro, B. Goldstein, and F. Parenti for the gift of DNA from *Actinoplanes teichomyceticus* ATCC 31211; and M. Arthur for help with writing the manuscript.

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