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Vancomycin Resistance Gene *vanC* Is Specific to *Enterococcus gallinarum*

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Nearly all strains of *Enterococcus gallinarum* are resistant to low levels of vancomycin. The glycopeptide resistance gene *vanC* from *E. gallinarum* BM4174 has recently been cloned and sequenced. A probe specific for *vanC* hybridized with a 2.7-kb *Eco*RI and a 4.5-kb *Hin*dIII fragment of total DNA from the 42 strains of *E. gallinarum* studied. No homology was detected with DNA of strains belonging to other species intrinsically resistant to vancomycin, including *Enterococcus casseliflavus*, a species that expresses a vancomycin resistance phenotype similar to that of *E. gallinarum*. No hybridization with DNA of enterococcal strains with acquired resistance to high or low levels of vancomycin was observed. The specificity of the *vanC* probe allowed us to distinguish *E. gallinarum* from 12 other species of enterococci, indicating that this probe is a useful tool for species identification within the genus *Enterococcus*.

Glycopeptide resistance in enterococci is associated with diverse resistance phenotypes (6). These various types of resistance have been provisionally assigned to three phenotypic classes, A, B, and C, on the basis of high- or low-level resistance to vancomycin, resistance or susceptibility to teicoplanin, and inducibility or constitutivity of resistance (6, 21). Class A includes *Enterococcus faecium* and *Enterococcus faecalis* isolates that are inducibly resistant to high levels of vancomycin and teicoplanin (7). Class B consists of strains of *E. faecalis* and *E. faecium* that are inducibly resistant to low levels of vancomycin and susceptible to teicoplanin (19, 21, 26). Class C is composed of strains of *Enterococcus gallinarum* (21). In *E. gallinarum*, vancomycin resistance is thought to be intrinsic, since nearly all the strains are constitutively resistant to low levels of the antibiotic (24, 25). Recently, two other types of glycopeptide resistance have been reported in enterococci. Almost all the strains of *Enterococcus casseliflavus* are intrinsically resistant to low levels of vancomycin (25), mimicking the VanC phenotype, and certain strains of *E. faecalis* and *E. faecium* are resistant to high levels of vancomycin and are susceptible to teicoplanin (20). The relationship between these various resistance phenotypes has not been extensively studied at the genetic level, since only the *vanA* gene of *E. faecium* BM4147, which is highly resistant to vancomycin and teicoplanin in an inducible fashion, has been characterized. This gene encodes for a ligase of altered specificity that is responsible for synthesis of peptidoglycan precursors with reduced affinity for vancomycin (2, 3, 8). We previously established that class A strains are phenotypically homogenous and that *vanA*-related genes are present in all the strains that are highly resistant to the glycopeptides studied (7). The glycopeptide resistance gene *vanC* from *E. gallinarum* BM4174 was recently cloned and sequenced (9). The purpose of the study described here was to examine the contribution of *vanC*-related sequences to intrinsic or acquired glycopeptide resistance in gram-positive bacteria.

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MATERIALS AND METHODS

Bacterial strains. The following enterococcal strains belonging to phenotypic or hybridization classes A, B, and C were studied. Five clinical isolates of *E. faecium* that were highly resistant to vancomycin and teicoplanin, including BM4147 and BM4152 (16), were assigned to class A (7). *E. faecalis* V583 (19), which is resistant to low levels of vancomycin and susceptible to teicoplanin, belongs to class B. *E. gallinarum* NCDO 2313; 22 strains of *E. gallinarum* from the laboratory collections of T. Horaud (27), L. Devriese, and API-System (La Balme-les-Grottes, France); and 19 strains of *E. gallinarum* isolated in 1989 and 1990 from clinical samples in five hospitals were grouped in class C.

The following strains, which display other types of resistance to glycopeptides, were included: *E. faecalis* UMH-1 (20), which is highly resistant to vancomycin (MIC, 1,024 μg/ml) and susceptible to teicoplanin (MIC, 0.5 μg/ml), 17 strains of *E. casseliflavus* from the collections of API-System and L. Devriese; and *E. casseliflavus* ATCC 25788, which is considered intrinsically resistant to low levels of vancomycin and susceptible to teicoplanin (25). *Erysipelothrix rhusiopathiae* A123 and A124 (Institut Pasteur Collection), *Lactobacillus bifermentans* ATCC 35409, *Lactobacillus brevis* ATCC 14869, *Lactobacillus casei* ATCC 393, *Lactobacillus confusus* ATCC 10881, *Lactobacillus fermentum* ATCC 9338, *Lactobacillus plantarum* ATCC 8014, *Lactobacillus reuteri* ATCC 23272, *Lactobacillus rhamnosus* ATCC 7469, *Lactobacillus salivarius* ATCC 11741, *Pediococcus acidilactici* ATCC 8042 and DSM 20284, *Leuconostoc mesenteroides*, *Leuconostoc paramesenteroides*, and *Leuconostoc dextranicus* were from our laboratory collection and are highly resistant to vancomycin and teicoplanin.

The following strains of various species of enterococci that are susceptible to glycopeptides were used as controls: *E. avium* D373, *E. columbae* BM4191, and *E. cecorum* K24, provided by L. Devriese, and *E. durans* ATCC 19432, *E. faecalis* ATCC 33186, *E. faecalis* ATCC 29212, *E. faecalis* JH2-2 (13), *E. faecium* ATCC 19434, *E. faecium* BM4107 (17), *E. faecium* BM4147-1 (16), *E. hirae* ATCC 8043, *E. malodoratus* NCDO 846, *E. mundtii* NCDO 2375, *E.*

TABLE 1. Phenotypic and genotypic classes among vancomycin-resistant gram-positive cocci

Phenotypic class	Genotypic class[a]	Species (no. of strains)	MIC (μg/ml)	
			Vancomycin	Teicoplanin
Susceptible	Susceptible	*Enterococcus* spp. (16)	0.5–2	0.25–2
A	A	*E. faecium* (5)	256–>1,000	32–500
B	NC	*E. faecalis* (1)	32	0.5
C	C	*E. gallinarum* (42)	2–32	0.5–1
NC	NC	*E. casseliflavus* (18)	2–32	0.5–1
NC	NC	*E. faecalis* (1)	1,024	0.5
NC	NC	*Lactobacillus* spp. (9)	>1,000	>1,000
NC	NC	*Leuconostoc* spp. (3)	>1,000	>1,000
NC	NC	*Pediococcus* spp. (2)	>1,000	>1,000
NC	NC	*E. rhusiopathiae* (2)	>1,000	>1,000

[a] A, hybridization with the *vanA* probe; NC, not classified; C, hybridization with the *vanC* probe.

pseudoavium NCDO 2138, *E. raffinosus* NCTC 12192, and *E. solitarius* NCTC 12193.

Identification of enterococci. Enterococci were identified as described by Facklam and Collins (10). The 30°C motility test and carbohydrate fermentations were used to distinguish *E. gallinarum* and *E. casseliflavus* from *E. faecalis* and *E. faecium*. A total of 18 of 42 strains of *E. gallinarum* and 7 of 18 strains of *E. casseliflavus* produced beta-hemolysis on Mueller-Hinton agar supplemented with 5% horse blood. Strains of *E. casseliflavus* could be distinguished from strains of *E. gallinarum* on the basis of the production of a yellow pigment (10).

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). All incubations were at 37°C.

Determination of in vitro susceptibilities to antibiotics. The diffusion test with disks containing 30 μg of vancomycin or 30 μg of teicoplanin (Diagnostics Pasteur) was used. The method of Steers et al. (23), with 10^4 CFU per spot, was used to determine the MICs of the antibiotics.

Preparation of DNA. Total DNAs of strains of *E. gallinarum* and *E. casseliflavus*, vancomycin-resistant *E. faecalis* V583 and *E. faecium* BM4147 and BM4152, and glycopeptide-susceptible *E. faecium* BM4147-1 and *E. faecalis* JH2-2 were prepared as described previously (9, 15). Total DNAs of the remaining strains were prepared as described previously (7), and aliquots (10 μl) were denatured for 10 min at 100°C and were spotted onto Nytran membranes (Schleicher & Schuell, Dassel, Germany).

DNA-DNA hybridization. The *vanA* probe consisted of the 290-bp *Bam*HI-*Rsa*I fragment internal to the *vanA* gene (7). The *vanC* probe was the 690-bp *Eco*RI-*Hin*cII fragment internal to the *vanC* gene. The DNA fragments cloned into bacteriophage M13mp18 were hybridized with the 15-bp distal primer and were labeled by DNA synthesis in the presence of dGTP, dATP, dTTP, [α-^{32}P]dCTP, and DNA polymerase (Klenow fragment) (12). Hybridization under stringent conditions was at 65°C in 0.1% sodium dodecyl sulfate (SDS)–0.7% nonfat dry milk–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) overnight (14). Washings were done at 65°C in 2× SSC–0.1% SDS.

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

RESULTS AND DISCUSSION

Intrinsic resistance of *E. gallinarum* and *E. casseliflavus* to vancomycin. The MIC of vancomycin for the reference strain *E. gallinarum* NCDO 2313 was 16 μg/ml. The MICs for the 42 other *E. gallinarum* strains ranged from 2 to 32 μg/ml (Table 1). The MICs for two strains were only 2 and 4 μg/ml (three independent determinations). The majority of strains of *E. casseliflavus* was also resistant to low levels of the antibiotic, with vancomycin MICs ranging from 8 to 16 μg/ml for 14 strains; the MIC was 16 μg/ml for *E. casseliflavus* ATCC 25788. Three strains were apparently susceptible to vancomycin (MICs, 2 to 4 μg/ml). By contrast, the MICs of vancomycin for strains belonging to the other enterococcal species studied were between 0.25 and 2 μg/ml. These results confirm that vancomycin MICs for *E. gallinarum* and *E. casseliflavus* are higher than those for other enterococcal species (24, 25). For these two species, the lower MIC breakpoint of vancomycin (4 μg/ml) (1, 18) divides the bacterial population into two unequal parts, and certain isolates are categorized as susceptible (Table 1) (25).

Distribution of the *vanC* gene. The probe specific for *vanC* hybridized to a 2.7-kb *Eco*RI fragment (Fig. 1 and data not shown) and to a 4.5-kb *Hin*dIII fragment (data not shown) of total DNA from all the strains of *E. gallinarum*. We did not observe homology with DNA from the other strains studied, in particular *E. casseliflavus*, by Southern or dot blot hybridization. As expected, homology with the *vanA* probe was detected only in the enterococci that were highly resistant to vancomycin and teicoplanin (Fig. 1 and data not shown). Intrinsic resistance to vancomycin in *Leuconostoc* spp., *Pediococcus* spp., and *Lactobacillus* spp. did not appear to be due to genes closely related to *vanC* (data not shown). This is consistent with differences in the phenotypic expression of glycopeptide resistance in these bacterial genera. The *vanC* gene is required for expression of low-level resistance to vancomycin in strain BM4174 (9). It encodes a protein, VanC, that is related to D-alanine:D-alanine ligases, which, like VanA (8), is probably a ligase with altered specificity (2, 9). In this study, *vanC*-related sequences were detected in the 42 strains of *E. gallinarum* tested, and synthesis of peptidoglycan precursors with reduced affinity for vancomycin is likely to account for the diminished vancomycin

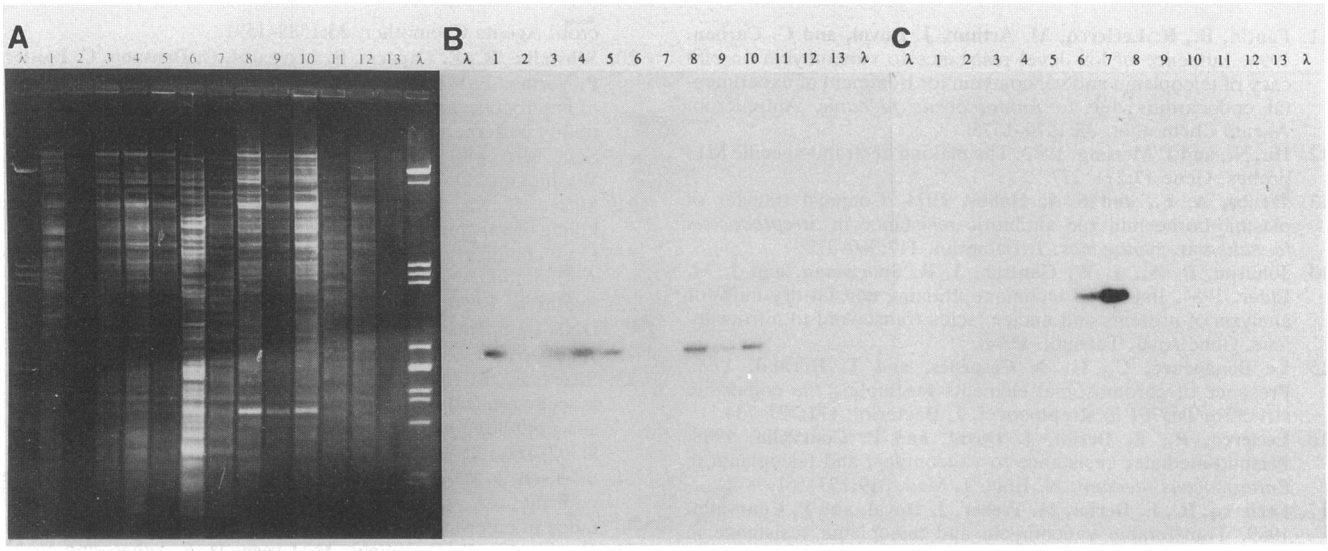

FIG. 1. Analysis of total DNA by agarose gel electrophoresis (A) and hybridization (C). Total DNA was digested with *Eco*RI; and the resulting fragments were separated by electrophoresis in a 1% agarose gel, transferred to a nylon filter (22), and hybridized to the in vitro ^{32}P-labeled *vanC* (B) and *vanA* (C) probes. Lanes 1, 3 to 5, and 8 to 10, *E. gallinarum* BM4192, 3392-86, 1-87, 2-87, BM4172, BM4173, and BM4174, respectively; lanes 2, no DNA; lanes 6 and 7, glycopeptide-resistant *E. faecium* BM4147 and BM4152, respectively; lanes 11, glycopeptide-susceptible *E. faecium* BM4147-1; lanes 12, *E. casseliflavus* BM4193; lanes 13, vancomycin-resistant *E. faecalis* V583. Fragments obtained by digestion of λ*c*I857 DNA with *Pst*I were used as molecular weight standards. Hybridization of the *vanC* probe with DNA of *E. gallinarum* 3392-86 and BM4173 (lanes 3 and 9, respectively) is barely visible in this particular analysis.

susceptibility in this species. The *vanC* resistance determinants are probably located in highly conserved regions since they are borne in all strains by *Eco*RI (Fig. 1) and *Hin*dIII (data not shown) fragments that are indistinguishable in size, despite the heterogeneity of the restriction patterns of the hosts (data not shown). Comparison of 16S rRNA sequences indicates that *E. casseliflavus* is closely related to *E. gallinarum* (5). Intrinsic low-level resistance to vancomycin is another common feature of these two species. However, the *vanC* gene is confined to strains of *E. gallinarum*.

Identification of the *E. gallinarum* species at the genetic level. Differentiation among *E. faecium*, *E. gallinarum* and *E. casseliflavus* is based on a few physiological tests, the most discriminative being motility at 30°C and production of a yellow pigment (10). However, certain strains of *E. casseliflavus* are nonpigmented, and the motility test is not totally reliable (25). The absence of these traits can lead to misidentification of enterococcal clinical isolates. The probe specific for *vanC* allowed us to rectify the identification of enterococci resistant to low levels of vancomycin (MICs, 8 μg/ml) previously considered *E. faecium*, such as strains BM4172 (11) and BM4102 (4). Physiological tests, including motility, which was not performed initially, confirmed that the strains belonged to the *E. gallinarum* species. The probe also enables the discrimination of *E. gallinarum* from 12 other species of enterococci. The recent demonstration, in an animal model of endocarditis, that low-level resistance to vancomycin can be responsible for therapeutic failure (11) emphasizes the need for accurate identification of bacterial species intrinsically resistant to this drug.

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