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Constitutively Vancomycin-Resistant *Enterococcus faecium* Resistant to Synergistic β-Lactam Combinations

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Vancomycin resistance among enterococci has recently been recognized. Synergy between vancomycin and penicillin has been shown in vitro for isolates of *Enterococcus faecium* resistant to both of these antibiotics. We describe three isolates of vancomycin-resistant *E. faecium* which demonstrate unique phenotypic characteristics. The isolates exhibited high-level resistance to both vancomycin and teicoplanin, consistent with the VanA phenotype. However, resistance in these isolates could not be induced or cured, and mating experiments failed to detect a transfer of resistance. The combination of vancomycin and penicillin did not significantly change the MIC of penicillin for any of the three isolates. Immunoblotting with polyclonal anti-VanB antibody showed no reaction with the cellular proteins of these strains. Probing with a *vanA* oligonucleotide revealed hybridization with chromosomal but not plasmid DNA. The mechanism of constitutive resistance of those strains remains unclear. A second mutational change, perhaps involving PBP 5, may explain the presence of resistance to synergistic combination penicillin-vancomycin therapy. In vitro evaluation of penicillin-vancomycin should be carried out in all clinical cases where this therapeutic regimen is being considered.

Vancomycin resistance among enterococci has recently been recognized (4, 5, 13, 15, 24, 27). Although *Enterococcus faecium* may be resistant to both vancomycin and penicillin, synergy between these two antibiotics in vitro has been shown previously (14, 23). This combined regimen may offer a treatment for patients infected with these organisms and for whom there are no other therapeutic options. This is a report of three isolates of constitutively vancomycin-resistant *E. faecium* resistant to synergistic beta-lactam combinations. These phenotypic characteristics differ substantially from those described previously (14, 15, 23).

MATERIALS AND METHODS

Bacterial isolates. Clinical isolates of *E. faecium* obtained from three different patients (two adult liver transplant recipients and one adult patient residing in the surgical intensive care unit) at Presbyterian University Hospital during an outbreak of infection with vancomycin-resistant *E. faecium* which began in April 1991 were evaluated. The sources of the isolates were blood (X34044), intra-abdominal tissue (W61804), and pancreatic drainage fluid (H80721).

Isolates were identified by the scheme recently proposed by Facklam and colleagues (8, 9). Morphology was confirmed by Gram staining of overnight cultures grown in thioglycolate broth. The key characteristics examined were the lack of production of gas from glucose, pyroladonylarylamidase activity (Wellcome Diagnostics), hydrolysis of esculin, growth in 6.5% NaCl, growth at 10 and 45°C, the presence of group D antigen (Streptex; Wellcome Diagnostics), motility at 30°C, pigment production, hydrolysis of arginine, and fermentation of arabinose.

Susceptibility testing. Initial susceptibility testing of the clinical isolates was performed by standardized disk diffusion methods (18). Tube broth micro- and macrodilutions and agar dilution testing were used to establish the MICs of some antibiotics (19). β-Lactamase testing was performed with nitrocefin disks (Cefinase; BBL Microbiology Systems, Cockeysville, Md.).

Inducibility of resistance. Experiments to test for the presence of inducible vancomycin resistance were performed (22). Isolates were grown overnight in brain heart infusion (BHI) broth with and without 80 μg of vancomycin per ml. An aliquot of each isolate from overnight cultures with and without vancomycin was diluted in BHI broth containing 0 or 80 μg of vancomycin per ml to achieve an optical density at 650 nm (OD_{650}) of about 0.02. Serial spectrophotometer measurements were then recorded over a 12-h period. *E. faecium* BM 4178 (kindly provided by R. LeClercq) was used as a control (16).

Confirmation of constitutive nature. The constitutive nature of the resistance was confirmed by measuring the inhibition of peptidoglycan synthesis in pulse-labeling experiments (22). Strains were grown overnight at 37°C in BHI broth with and without 64 μg of vancomycin per ml and were then diluted with fresh BHI broth with and without vancomycin to an OD_{650} of 0.05 in the morning. They were incubated at 37°C until an OD_{650} of 0.2 was reached. The cells were then harvested by centrifugation at 2,900 × *g* at 20°C and were washed twice in phosphate buffer (0.05 M; pH 7.5) at 20°C. The cell pellets were resuspended to a final OD_{650} of 0.2 and were incubated at 37°C for 30 min in the presence of various concentrations of vancomycin and 0.5 μCi of [^{3}H]*N*-acetylglucosamine (5.79 Ci/mmol; Amersham, Amersham, United Kingdom). Cells were harvested on Whatman GFA filters, precipitated with 5% cold trichloroacetic acid (TCA), and washed with ethanol, and the TCA-precipitable radioactivity on the filters was counted by liquid scintigraphy.

Curing procedures. Novobiocin curing was performed in

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duplicate on all three isolates and on strain BM 4178 (as a control) in an effort to eliminate plasmid-mediated resistance as described previously (17).

Mating conditions. Transference of resistance was attempted by filter and cross-streak mating. Filter mating was performed between vancomycin-resistant enterococcal isolates and *Enterococcus faecalis* JH2-2 (25). Donor and recipient strains were grown in BHI broth with gentle shaking for 3 to 5 h at 37°C. The mating procedure was performed with and without preincubation of recipient strains at 45°C for 20 min prior to mating. Donor and recipient strains were mated by mixing 0.1-ml aliquots of each strain onto a sterile filter (type HAEP; pore size, 0.45 μm; diameter, 47 mm; Millipore Corp., Bedford, Mass.) which was then placed on a 5% horse serum–BHI agar plate. After overnight incubation at 37°C, the filters were removed from the agar and washed several times with BHI broth. Dilutions of the washed broth were plated onto both blood agar and VRGPC agar (11) and were incubated at 37°C overnight.

Cross-streak mating was performed with *E. faecium* D61Rif, *E. faecalis* JH2-2 (rifampin resistant, fusidic acid resistant), and *E. faecalis* JH2-7 (rifampin resistant, fusidic acid resistant). Donor strains were grown overnight on selective medium. Donor strains and then recipient strains were cross streaked onto mating plates containing only BHI agar and were incubated overnight at 37°C. All growth was scraped from the plates and was resuspended in 200 μl of BHI. One microliter of the resultant sludge was diluted 1:1,000 three times (i.e., 10^{-3}, 10^{-6}, 10^{-9}); 100 μl of 10^{-6} and 10^{-9} dilutions was spread onto BHI plates as colony count controls. The remainder of the 200 μl of the original suspension was plated onto selective medium (64 μg of vancomycin per ml, 100 μg of rifampin per ml, and, when JH2-2 or JH2-7 was the recipient, 25 μg of fusidic acid per ml) and incubated at 37°C for 5 days.

Synergy experiments. The ability of vancomycin to induce penicillin susceptibility in the isolates was tested as recently described by Williamson et al. (28). Briefly, isolates were grown overnight in BHI broth containing 80 μg of vancomycin per ml. On the day of testing the culture was subcultured into the same medium and incubated until sufficient turbidity developed to standardize the culture against a McFarland 0.5 standard. BHI broth containing serial dilutions of vancomycin alone (1,024 to 0.5 μg/ml), penicillin alone (1,024 to 0.5 μg/ml), and penicillin (1,024 to 0.5 μg/ml) in the presence of 80 μg of vancomycin per ml was inoculated with 5×10^5 CFU/ml. The density of the organisms was verified by making serial dilutions from a similarly inoculated control culture without antibiotics. These dilutions were plated in duplicate onto commercial blood agar plates.

Screening for the presence of *vanA* and VanB. Anti-VanB protein antibody was obtained as described previously (1). Protein from whole-cell lysates was prepared for the control VanA, VanB, and the test strains, from both cells grown in the presence of (induced) and absence (uninduced) of subinhibitory concentrations of vancomycin. One milliliter of the overnight culture was centrifuged and lysed with 10 μl of 1 mg of mutanolysin (Sigma Chemical Co., St. Louis, Mo.) per ml and 10 μl of 40 mg of lysozyme (Sigma) per ml for 30 min at 37°C. Sodium dodecyl sulfate (SDS)-Blue and mercaptoethanol were added, and the lysate was boiled and centrifuged to remove cellular debris. The lysate was subsequently loaded onto a polyacrylamide gel containing 10% SDS.

Western blotting (immunoblotting) was performed by using nitrocellulose membranes (Millipore Corp.) by using a Transblot SD semidry electrophoretic transfer cell under the conditions recommended by the manufacturer (Bio-Rad, Richmond, Calif.). The VanB protein was then detected by using an Immuno-Blot Assay Kit (Bio-Rad) by using the purified rabbit anti-VanB antibody diluted 1:5,000 in antibody buffer as the first antibody and goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase as the second antibody (1).

TABLE 1. MICs of penicillin and vancomycin for *E. faecium* isolates previously incubated in the presence of 80 μg of vancomycin per ml

Isolate	Gentamicin MIC screen (2,000 μg/ml)[a]	MIC (μg/ml)		
		Vancomycin	Penicillin	Penicillin with vancomycin (80 μg/ml)
BM 4178	ND	256	32	<0.5
W61804	S	512	256	64
X34044	S	1,024	512	512
H80721	R	1,024	1,024	1,024

[a] Screening for the presence of high-level gentamicin resistance at 2,000 μg/ml. ND, not done; S, susceptible; R, resistant.

An oligonucleotide based on the previously published sequence of the *vanA* gene (5′-GAT AGG GTG GCA GCT AC-3′) was labeled at the 5′ end with [^{32}P]ATP (Amersham Nuclear, Amersham, United Kingdom) and polynucleotide kinase (Promega Corp., Madison, Wis.). Plasmid DNA was extracted from *E. faecium* X34044, W61804, and H80721 and from control strains D366 (*vanB*) and D399 (*vanA*) as described by Ehrenfeld and Clewell (7). Whole-cell DNA was extracted from the strains after lysis by mutanolysin and lysozyme as described previously (2). The purified DNA was cut with *Eco*RI (Boeringher-Mannheim, Indianapolis, Ind.) and electrophoresed through 0.8% agarose gels containing 0.5 μg of ethidium bromide per ml. The DNA fragments were transferred to nylon membranes (S&S Nytran; Schleicher & Schuell, Keene, N.H.), and Southern hybridization with labeled oligonucleotide was performed. Conditions for hybridization were 48°C in 6× SSC buffer (1× SSC buffer is 0.15 M NaCl plus 0.015 M sodium citrate). The membranes were washed three times for 15 min each time at room temperature and once at 42°C in the same buffer and were then dried and autoradiographed.

RESULTS

All three isolates were identified as *E. faecium* and were initially identified as resistant to vancomycin by disk diffusion methods. MIC quantification by tube macrodilution testing of the three isolates revealed high-level vancomycin resistance (range, 512 to 1,024 μg/ml) (Table 1). The organisms were also found to be resistant to ampicillin and penicillin by microdilution MIC testing and to daptomycin and teicoplanin by both disk diffusion and tube macrodilution susceptibility testing. None of the isolates produced β-lactamase.

Results of attempts to induce vancomycin resistance are shown in Fig. 1. Pregrowth in subinhibitory concentrations of vancomycin did not shorten the lag time for subsequent growth in vancomycin for any of the three isolates. In contrast, pregrowth in vancomycin did shorten the lag time for subsequent growth for BM 4178, an isolate known to demonstrate the inducible VanA phenotype.

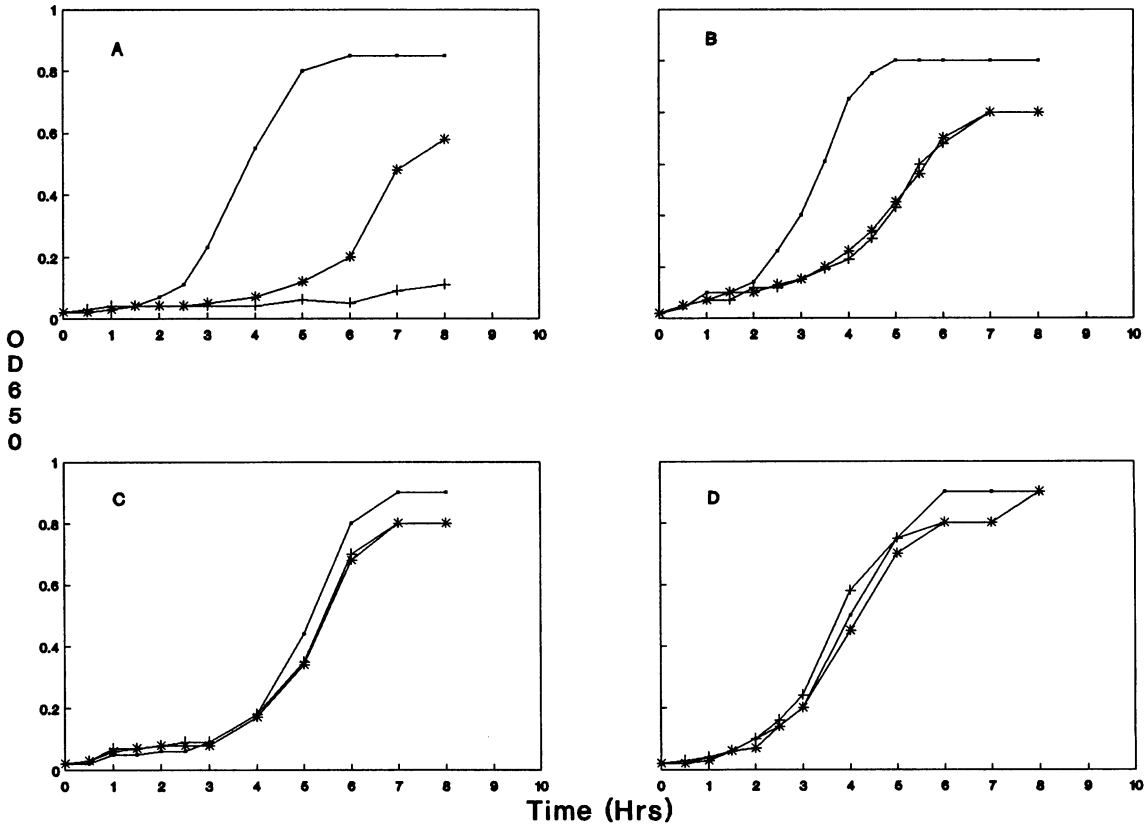

FIG. 1. Induction of vancomycin resistance. Control growth is indicated by the curve on the left of each panel (•). For each strain tested, the asterisk represents growth after pregrowth with vancomycin and the plus sign represents growth after pregrowth without vancomycin. (A) *E. faecium* BM4178. (B) *E. faecium* W61804. (C) *E. faecium* X34044. (D) *E. faecium* H80721.

Results of pulse-labeling experiments revealed no difference in resistance to inhibition of peptidoglycan synthesis between cells preexposed to subinhibitory concentrations of vancomycin and those not preexposed to vancomycin, thus confirming the constitutive nature of vancomycin resistance in these isolates.

Curing experiments with novobiocin were performed for all three isolates of *E. faecium* as well as for *E. faecium* BM 4178. No change was seen in the MICs for the three isolates identified in our outbreak. In contrast, the MIC for *E. faecium* BM 4178 fell from 512 to 2 μg/ml after curing.

Mating experiments failed to detect a transfer of vancomycin resistance by either filter or cross-streak mating (frequency, $<10^{-8}$ per donor CFU).

The results of synergy experiments measuring the ability of vancomycin to induce penicillin susceptibility in the isolates are shown in Table 1. Preincubation with vancomycin on dual exposure to vancomycin and penicillin during susceptibility testing did not significantly change the MIC of penicillin for two of the three isolates. The MIC of W61804 did decrease fourfold in the presence of vancomycin; however, the combination did not reduce the MIC to the extent usually seen. In contrast, the MIC for *E. faecalis* BM 4178 decreased from 32 to <0.5 μg/ml when tested under identical conditions.

Immunoblotting with the anti-VanB antibody showed no reaction with the cellular proteins of *E. faecium* X34044, W61804, and H80721, although positive reactions with a 39.5-kDa protein were observed for the *E. faecium* D366 control (data not shown). *vanA* oligonucleotide probing, however, demonstrated the presence of a chromosomal *Eco*RI fragment of 3 kb which hybridized with the *vanA* probe. No hybridization was observed with plasmid DNA (Fig. 2). Chromosomal DNA from D366 (VanB) did not hybridize with the probe, whereas hybridization with our *vanA* control strain, D399, was positive (data not shown). The fact that the plasmid digestion profiles, DNA digestion profiles, and chromosomal localization of the *vanA* gene were all virtually identical suggests, in addition, that these strains are closely related.

DISCUSSION

Since the first reports of vancomycin-resistant enterococci in 1988 (15, 26), much has been learned regarding the mechanism and control of expression of this resistance. Strains exhibiting inducible, high levels of resistance to both vancomycin and teicoplanin have been designated VanA (23). VanB strains express variable levels of resistance to vancomycin but remain susceptible to teicoplanin (20, 28). Expression of vancomycin resistance is associated with an increased susceptibility to penicillin in at least some VanA and VanB strains (14, 23). Some workers have been able to take advantage of this association of vancomycin resistance and penicillin hypersusceptibility to achieve killing of vancomycin-resistant enterococci by synergistic combinations of penicillin and vancomycin with or without gentamicin (14, 23).

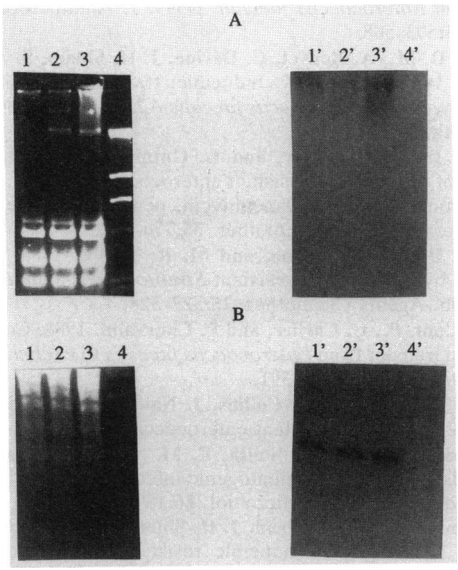

FIG. 2. (A) Plasmid DNAs of *E. faecium* strains digested with *Eco*RI. Lane 1, H80721; lane 2, W61804; lane 3, X34044; lane 4, bacteriophage lambda DNA cut with *Hin*dIII as a molecular size marker. Lanes 1′ to 4′ are the result of probing with the *vanA* oligonucleotide. (B) Whole-cell DNAs of *E. faecium* strains digested with *Eco*RI. Lane 1, H80721; lane 2, W61804; lane 3, X34044; lane 4, bacteriophage lambda DNA cut with *Hin*dIII as a molecular size marker (fragments of 2.0, 2.3, 4.4, and 6.6 kb are shown). Lanes 1′ to 4′ are the result of probing with the *vanA* oligonucleotide.

The *vanA* gene has been cloned and sequenced (3), and its gene product has been characterized (4, 6). The gene codes for a D-alanine ligase which forms a depsipeptide precursor of D-alanine-D-2-hydroxycarboxylic acid, possibly lactic acid (6). It is thought that the muramyl-depsipeptide cannot be further metabolized by PBP 5 of *E. faecium*, which is the low-affinity penicillin-binding protein (PBP) that is responsible for penicillin resistance in these strains (29, 30). This would then leave the penicillin-susceptible PBPs 1 to 3 available to form a cross-linked cell wall from the depsipeptide precursor.

The isolates identified during the outbreak described here demonstrated a unique phenotypic resistance pattern. They were resistant to high levels of both vancomycin and teicoplanin, and the resistance was expressed constitutively. Additionally, the strains remained resistant to the synergistic combination penicillin-vancomycin. Immunoblotting of cellular proteins with a polyclonal anti-VanB antibody showed that the strains did not produce the VanB protein. Probing of whole-cell DNA with an oligonucleotide derived from the published gene sequence of *vanA* confirmed the presence of the *vanA* gene localized on chromosomal DNA. This result confirms the previously proposed chromosomal location of the *vanA* gene in some isolates on the basis of the absence of plasmid DNA in donor or transcipient cells despite their transferability on filters (21, 22). Synergy-resistant VanA isolates have also recently been reported in the absence of constitutive resistance (10, 12).

This phenotype is probably a result of two separate mutational events. One led to the constitutive expression of VanA. It is possible that some such mutants are not stable, leading to selective pressure for either the loss of vancomycin resistance or a change in PBPs. We postulate that the mutants have undergone another mutation resulting in an alteration in the expression of PBP 5 or a change in its affinity for the depsipeptide precursor.

The isolation of the constitutive phenotype raises several important therapeutic concerns. The existence of these constitutive mutants makes the development of noninducing classes of glycopeptides for clinical use against infections with vancomycin-resistant enterococci less tenable than ever. In addition, the isolation of strains of enterococci resistant to the synergistic combination of drugs suggests the necessity for in vitro evaluation of the penicillin-vancomycin combination in all clinical cases.

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