ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2006, p. 3622–3630 Vol. 50, No. 11
0066-4804/06/$08.00+0 doi:10.1128/AAC.00410-06

Synergism between β-Lactams and Glycopeptides against VanA-Type Methicillin-Resistant *Staphylococcus aureus* and Heterologous Expression of the *vanA* Operon▽

Bruno Périchon* and Patrice Courvalin

Unité des Agents Antibactériens, Institut Pasteur, 75724 Paris Cedex 15, France

Received 3 April 2006/Returned for modification 3 June 2006/Accepted 29 August 2006

Vancomycin resistance of *Staphylococcus aureus* NY-VRSA and VRSA-5 is due to acquisition of a *vanA* operon located in a Tn*1546*-like element. The *vanA* gene cluster of NY-VRSA contained one copy of insertion sequences IS*1251* and IS*1216V* relative to that of VRSA-5. As evidenced by the nature of the late peptidoglycan precursors and by quantification of D,D-peptidase activities, the vancomycin resistance genes were efficiently expressed in both strains. Study of the stability and inducibility of glycopeptide resistance suggested that low-level glycopeptide resistance of NY-VRSA was most probably due to plasmid instability combined with a long delay for resistance induction. The activity of combinations of vancomycin or teicoplanin with oxacillin against the four VanA-type *S. aureus* strains already reported was tested by single and double disk diffusion, E-test on agar alone or supplemented with antibiotics, the checkerboard technique, and by determining time-kill curves. A strong synergism against the four clinical isolates, with fractional inhibitory concentration indexes from 0.008 to 0.024, was reproducibly observed between the two antibiotics by all methods. These observations indicate that cell wall inhibitors of the β-lactam and glycopeptide classes exert strong and mutual antagonistic effects on resistance to each other against VanA-type methicillin-resistant *S. aureus*.

Staphylococcus aureus is a major cause of hospital- and community-acquired infections. Methicillin-resistant *S. aureus* (MRSA) strains, which are usually multidrug resistant, have spread worldwide, and glycopeptides are often used for treating infections caused by MRSA.

Methicillin resistance in MRSA is due to the acquisition of the *mecA* gene, which encodes a penicillin-binding protein (PBP), PBP2a, that exhibits low β-lactam affinity (15). PBPs are membrane-bound D,D-peptidases that catalyze the transpeptidation and/or transglycosylation reaction that cross-links the peptidoglycan of the bacterial cell wall.

Glycopeptide antibiotics vancomycin and teicoplanin bind to the C-terminal D-alanyl-D-alanine (D-Ala-D-Ala) of externally oriented pentapeptide peptidoglycan precursors and block the transglycosylation and transpeptidation reactions (26). VanA-type glycopeptide resistance in enterococci is due to synthesis of modified precursors terminating in D-Ala-D-lactate (D-Ala-D-Lac) in place of D-Ala-D-Ala (6) and is mediated by Tn*1546* or closely related genetic elements (3, 13).

In 2002, the first two MSRA and vancomycin-resistant *S. aureus* (VRSA) strains, MI-VRSA and PA-VRSA, were isolated in the United States (20, 31). Although they both harbor a plasmid-borne Tn*1546* element (33, 34), the two strains exhibit distinct phenotypes: MI-VRSA has a high level of resistance (HLR) to both glycopeptides, whereas PA-VRSA presents a low level of resistance (LLR) to the drugs (7, 32). We have shown that low-level resistance of the PA-VRSA strain is due to loss, at high frequency, of the *vanA* operon and to a longer lag phase before induction of resistance (22).

In 2004, a third VanA-type MRSA clinical isolate, NY-VRSA, with an LLR phenotype, was detected in New York (17). As already observed for PA-VRSA, automated susceptibility testing methods failed at detecting vancomycin resistance in this strain (17, 33). Since then, a fourth HLR VanA-type MRSA was isolated and called VRSA-5 (Michigan Department of Community Health; posting date, 3 March 2005; www.michigan.gov/mdchlab).

Synergistic activity of the combination of vancomycin and oxacillin against MRSA strain COL, in which a *vanA*-carrying plasmid was transferred, has been demonstrated (27). We have studied the organization and extent of heterologous expression of the *vanA* gene cluster in strains NY-VRSA and VRSA-5. We have also evaluated the outcome of combinations of β-lactams and glycopeptides on these two strains and on the two other available clinical isolates of VanA-type MRSA.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* strains were obtained through the Network on Antimicrobial Resistance in *Staphylococcus aureus*. Methicillin- and vancomycin-resistant *S. aureus* NY-VRSA (17) was isolated from the urine of a resident in a long-term care facility. VRSA-5 was isolated from a small toe wound developed on the foot of a 78-year-old patient previously treated with vancomycin for an aortic valve replacement. MICs were determined by agar dilution on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) and by E-test as described by the manufacturer (AB Biodisk, Solna, Sweden). The strains were grown in BHI broth or agar at 37°C. Spontaneous glycopeptide-susceptible derivatives of NY-VRSA and VRSA-5 were obtained as previously described (22).

Filter mating. Transfer of vancomycin resistance from NY-VRSA to *Enterococcus faecalis* JH2-2 and *E. faecium* BM4105 was attempted by filter mating (11) with selection on agar containing rifampin (20 μg/ml), fusidic acid (10 μg/ml), and one of the following: vancomycin (6 μg/ml), gentamicin (32 μg/ml), kanamycin (500 μg/ml), or erythromycin (10 μg/ml).

* Corresponding author. Mailing address: Unité des Agents Antibactériens, Institut Pasteur, 25, rue du Docteur Roux, 75724 Paris Cedex 15, France. Phone: (33) (1) 45 68 83 18. Fax: (33) (1) 45 68 83 19. E-mail: brunoper@pasteur.fr.

▽ Published ahead of print on 5 September 2006.

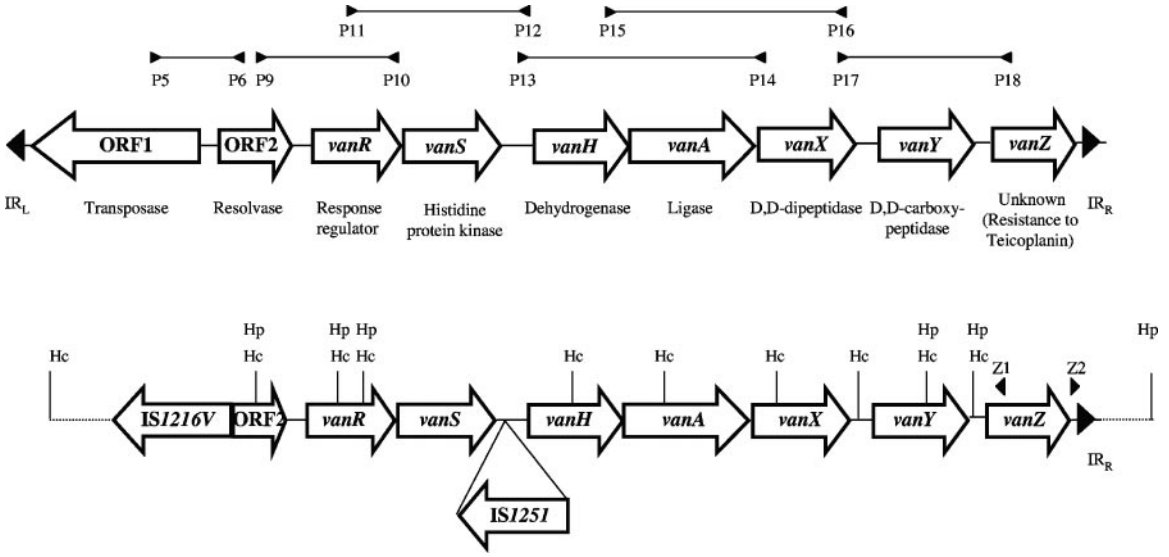

FIG. 1. Comparison of Tn*1546* elements. (Top) PCR map of Tn*1546*. Arrowheads indicate the direction of DNA synthesis with primers P1 to P18 (3). Horizontal bars depict the PCR products. IR_L and IR_R, inverted repeat left and right, respectively. (Bottom) Organization of the Tn*1546* element in NY-VRSA. Primers Z1 and Z2 were used for inverse PCR. Hc, HinCII; Hp, HpaI. Open arrows represent coding sequences and indicate the direction of transcription.

Growth rate studies. Induction of resistance by vancomycin was studied by determination of growth rates under various conditions. NY-VRSA and VRSA-5 were grown overnight at 37°C in BHI broth with or without vancomycin (6 μg/ml). The cultures were diluted 1:20 into 20 ml of BHI with or without vancomycin (6 μg/ml) and then grown at 37°C with shaking, and the optical density at 600 nm was monitored.

PCR analysis. Glycopeptide resistance of the *vanA* genotype was confirmed by PCR as described previously (10). Glycopeptide-susceptible derivatives of NY-VRSA and VRSA-5 as well as the parental strains were identified as *S. aureus* by amplification of an internal portion of the thermonuclease *nuc* gene, using total DNA as a template and specific primers (5). PCR mapping of the *vanA* operon of NY-VRSA and VRSA-5 was performed by using primers specific for every gene of the cluster (3) (Fig. 1).

Analysis of peptidoglycan precursors. Extraction and analysis by high-performance liquid chromatography of peptidoglycan precursors of NY-VRSA and VRSA-5 strains grown in BHI broth in the presence (4 μg/ml) or absence of glycopeptide were performed as described previously (19, 22).

D,D-peptidase activities. The D,D-peptidase enzymatic activities were assayed in the absence or presence of glycopeptide (vancomycin or teicoplanin at 4 μg/ml) as described previously (2, 22).

Contour-clamped homogeneous electric field gel electrophoresis. Genomic DNA of *S. aureus* NY-VRSA and VRSA-5 embedded in agarose plugs was digested with SmaI, and the resulting fragments were separated by pulsed-field gel electrophoresis as described previously (9).

Synergy testing. Three methods were used to assess synergism between glycopeptides and oxacillin, and three independent experiments were performed with each method.

(i) Disk diffusion and MIC determination by E-test. Vancomycin (30 μg), teicoplanin (30 μg), and oxacillin (5 μg) disks were placed on plates containing either vancomycin (8 μg/ml) or oxacillin (8 μg/ml for MI-VRSA, NY-VRSA, and VRSA-5 and 2 μg/ml for PA-VRSA) or no antibiotic and were inoculated with the VRSA strains. After incubation for 24 h at 30°C or 37°C, the inhibition zone diameters were measured. Disks containing glycopeptides and oxacillin were also deposited on plates inoculated with each strain at distances corresponding approximately to the sum of the radii of the inhibition zones of the drugs when tested alone, and the patterns obtained were analyzed after overnight incubation at 30°C or 37°C (23). Vancomycin and oxacillin MICs were determined at 30°C and 37°C by E-test on BHI agar or on agar containing oxacillin (8 μg/ml, except for PA-VRSA, which received 2 μg/ml) or vancomycin (8 μg/ml).

(ii) Checkerboard. Combinations of glycopeptides and oxacillin were tested by the checkerboard method against the four VRSA isolates in BHI broth. The concentration of glycopeptides and oxacillin ranged from 0.25 μg/ml to 1× MIC and from 0.06 μg/ml to 1× MIC, respectively. The final inoculum was approximately 10^7 CFU/ml. Incubations were performed at 30°C and 37°C for 24 h in a final volume of 2 ml. The fractional inhibitory concentration (FIC) index for each antibiotic in every combination was calculated with the following equation: FIC of drug A (vancomycin or teicoplanin) + FIC of drug B (oxacillin) = (MIC of drug A in combination with oxacillin/MIC of drug A alone) + (MIC of oxacillin in combination with drug A/MIC of oxacillin). Synergism was defined as an FIC

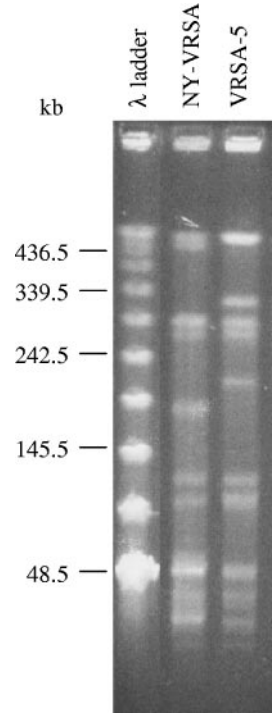

FIG. 2. Analysis of SmaI-digested genomic DNA of VanA-type *S. aureus* NY-VRSA and VRSA-5. Bacteriophage λ concatemers were used as molecular size markers (Biolabs), and the sizes are indicated at the left.

TABLE 1. Cytoplasmic peptidoglycan precursors and D,D-peptidase (VanX and VanY) activities in extracts from NY-VRSA and VRSA-5 strains[a]

Strain and drug treatment	% of peptidoglycan precursor:			Activity[b] (nmol/min/mg protein) of:	
	UDP-MurNAc-tetrapeptide	UDP-MurNAc-pentapeptide (D-Ala)	UDP-MurNAc-pentapeptide (D-Lac)	D,D-dipeptidase (cytoplasmic fraction)	D,D-carboxypeptidase (membrane fraction)
NY-VRSA					
Uninduced	5	95	ND	24 ± 6	3 ± 1
VAN (6 μg/ml)	18	17	65	502 ± 15	55 ± 8
TEC (4 μg/ml)	15	16	69	294 ± 16	26 ± 9
VRSA-5					
Uninduced	4	96	ND	5 ± 1	ND
VAN (6 μg/ml)	14	ND	86	860 ± 55	66 ± 4
TEC (4 μg/ml)	16	ND	84	480 ± 39	38 ± 3

[a] The cytoplasmic peptidoglycan precursors of NY-VRSA and VRSA-5 grown with or without glycopeptides were analyzed as described previously (19). Enzyme assays were performed with the S100 and C100 fractions of uninduced or induced cultures. VAN, vancomycin; TEC, teicoplanin. ND, not detectable.
[b] Values are means ± standard deviations.

index of ≤0.5, indifference as an FIC index between 0.5 and 2, and antagonism as an FIC index of >2 (23).

(iii) Time-kill curves. Time-kill curves were determined for every strain with an inoculum of 10^7 CFU/ml and antibiotic concentrations following a double-fold dilution from 1/2× to 1/64× MIC (from 1 to 32 μg/ml for glycopeptides and from 0.5 to 32 μg/ml for oxacillin). Bacterial counts were taken at 0, 3, 4, 5, 6, and 24 h by plating 0.1-ml aliquots (diluted or undiluted) onto BHI agar. The plates were incubated for 24 h at 30°C or 37°C. Bactericidal activity was defined as a reduction of at least 1,000-fold in the bacterial counts relative to the inoculum.

RESULTS AND DISCUSSION

Glycopeptide resistance of NY-VRSA and VRSA-5. The SmaI patterns of the two strains differed by a minimum of three bands (Fig. 2), and the isolates should therefore be considered distinct. The two clinical isolates also differed in their resistance phenotypes: NY-VRSA has a low level of resistance to vancomycin and teicoplanin (MIC, 64 μg/ml) (12), whereas VRSA-5 was resistant to high levels of both glycopeptides (MIC, 256 μg/ml). Attempts to transfer vancomycin resistance from *S. aureus* NY-VRSA and VRSA-5, as well as from MI-VRSA and PA-VRSA, to *E. faecalis* JH2-2 or *E. faecium* BM4105 by filter mating were unsuccessful.

Characterization of the *vanA* operons. The organization of the *vanA* gene cluster in NY-VRSA and VRSA-5 was determined by PCR mapping (Fig. 1). Pairs of primers, specific for each gene of the operon, were used in PCR experiments (3). For NY-VRSA, the PCRs gave fragments with the expected sizes, except for the P11-P12 pair, which gave a larger amplification product (3,100 bp instead of 1,600 bp), and for P5-P6, where no PCR product was obtained. For VRSA-5, all the PCR fragments had the expected sizes. These results indicate that the *vanR*, *vanS*, *vanH*, *vanA*, *vanX*, *vanY*, and *vanZ* genes constituting the *vanA* operon were present in both strains and in the same order as in Tn*1546*.

Sequencing of the PCR product obtained with the P11-P12 primers and total DNA of NY-VRSA as a template revealed the presence of a copy of IS*1251* (1,499 bp) (14) inserted in the *vanS-vanH* intergenic region at the same position (position 5820 of Tn*1546*), in the same orientation, and flanked by the same ATAATTTT 8-bp duplication of target DNA as in PA-VRSA (Fig. 1) (8).

To determine the sequence upstream from the P9 primer located in *orf2*, fragments obtained by digestion of total NY-VRSA DNA with HinCII were cloned in pUC18, and the recombinant plasmids were subjected to PCR amplification using M13 reverse sequencing and the P6 primers. A fragment of 1,749 bp was obtained, and its sequence was determined. An open reading frame of 809 bp, corresponding to IS*1216V* (13), was found, but it was in opposite orientation relative to the transposon (Fig. 1). This insertion sequence is also present in PA-VRSA but at a different position. A 184-bp sequence, corresponding to a fragment of *orf2* including the portion complementary to the P6 primer, was found downstream from IS*1216V*, whereas 756 bp without significant homology with known sequences were present upstream from IS*1216V*. The presence of this unknown sequence in NY-VRSA was confirmed by PCR using a primer (NY-3, 5′TCTCTGCAACTG GAATGCCT) specific for the 756-bp fragment and P6. The sequence downstream from *vanZ* was determined by inverse PCR. Total DNA of NY-VRSA was digested with HpaI, the resulting fragments were ligated with T4 DNA ligase, and inverse PCR was performed using primers Z1 (5′AAGCTAG CAATCCTCTAGA) and Z2 (5′AGTGCTGAGGAATTGG TCT) (Fig. 1). A 700-bp fragment, with no homology with known sequences, was obtained. The presence of the latter sequence in NY-VRSA was also confirmed by PCR with a primer (NY-5, 5′GTAATTACATTGTACGCT) specific for the 700-bp fragment and Z2 (Fig. 1).

Expression of the *vanA* gene cluster in NY-VRSA and VRSA-5. The cytoplasmic peptidoglycan precursors of *S. aureus* NY-VRSA and VRSA-5 grown with 4 μg/ml of vancomycin or teicoplanin or without antibiotic were analyzed as described previously (19, 22). The late precursors synthesized by NY-VRSA and VRSA-5 were qualitatively similar to those of MI-VRSA and PA-VRSA, but the relative proportions obtained with NY-VRSA were slightly different from those found with the three other strains after induction with vancomycin (22). Although the relative proportions of peptidoglycan precursors synthesized by PA-VRSA and NY-VRSA differed, both strains exhibited a similar low level of resistance to glycopeptides (Table 1). Thus, a smaller proportion of precursors ending in D-Lac are synthesized by NY-VRSA in the presence of vancomycin, in comparison with that of PA-VRSA as well as

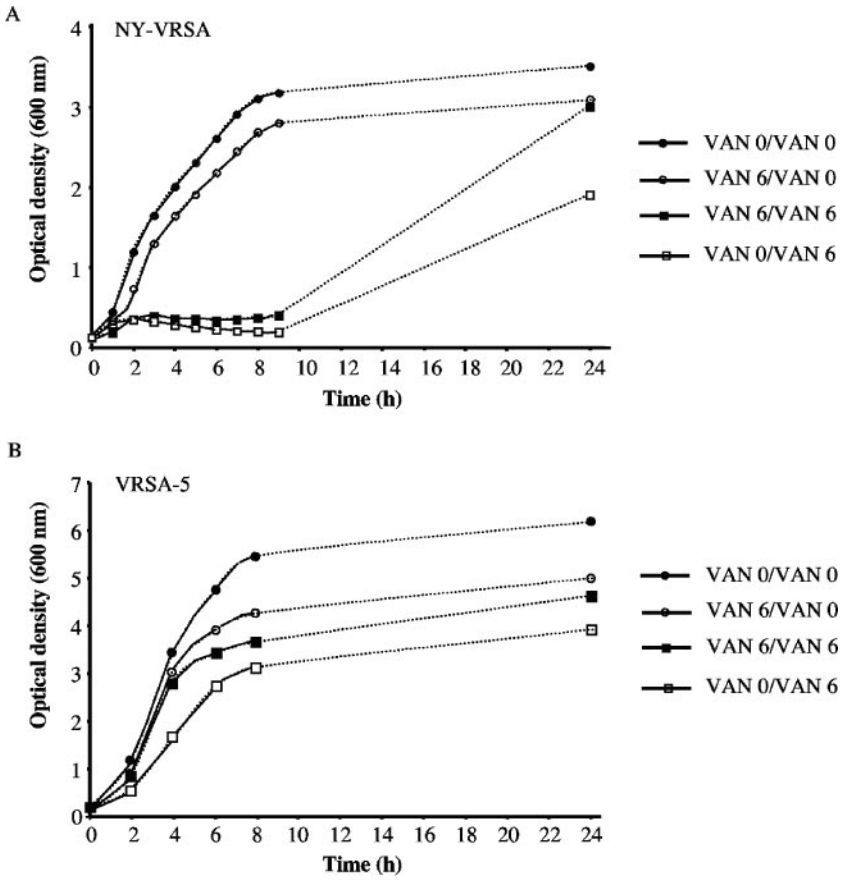

FIG. 3. Growth of *S. aureus* NY-VRSA (A) and VRSA-5 (B) in the absence (VAN 0) or in the presence of 6 μg/ml vancomycin (VAN 6) after overnight culture with or without vancomycin (6 μg/ml). The lines indicate ratio of the vancomycin (VAN) concentration in the overnight culture/vancomycin concentration in the culture medium.

those of the two other MRSA strains (22), is not reflected in the level of resistance to glycopeptides. As already reported for PA-VRSA (22), low-level resistance of NY-VRSA could be related to loss, at a high frequency, of the *van* operon (see below).

As observed with the two other VanA-type MRSA isolates (22), very weak D,D-peptidase activities were found in the cytoplasmic extracts from uninduced cells of NY-VRSA and VRSA-5 (Table 1), whereas substantial activities were detected when the cells were incubated in the presence of vancomycin or teicoplanin. However, the amounts of D,D-carboxypeptidase (VanY) and D,D-dipeptidase (VanX) activities in extracts from NY-VRSA were higher and lower, respectively, than those of the three other vancomycin-resistant *S. aureus* strains. These results are in agreement with the peptidoglycan precursors synthesized by NY-VRSA in which the proportions of UDP–*N*-acetyl-muramyl (MurNAc)–tetrapeptide and UDP-MurNAc-pentapeptide and those of UDP-MurNAc-pentadepsipeptide are higher and lower than in MI-VRSA or PA-VRSA, respectively.

Analysis of the *vanA* gene cluster, of the peptidoglycan precursors, and of the VanX and VanY activities associated with glycopeptide resistance indicated that the *vanA* operon was fully functional in both strains. Thus, the difference in phenotypic expression of glycopeptide resistance in the two isolates cannot be accounted for by differences in the expression of the resistance genes.

Stability of glycopeptide resistance. Inheritance of the *vanA* operon in NY-VRSA and VRSA-5 was tested by replica plating. NY-VRSA derivatives susceptible to vancomycin (MIC, 2 μg/ml) were obtained in three independent experiments at high frequencies, ranging from 45% to 48%, similar to those observed with PA-VRSA (22), whereas a single glycopeptide-susceptible clone (0.3%) was obtained with VRSA-5. Amplification of the *vanA* gene with specific primers was positive for parental NY-VRSA and VRSA-5 and negative for susceptible derivatives (24 and 1 colonies tested, respectively). Loss of glycopeptide resistance in VRSA-5 at very low frequency could be due to transposon or plasmid instability, whereas loss of resistance at a high rate in NY-VRSA is most likely due to plasmid segregation by inefficient replication. The Tn*1546*-like element in the latter strain has been stabilized by deletion of *orf1* and part of *orf2* that are required for the movements of the element. The apparent glycopeptide phenotypic susceptibility of two of the VanA-type MRSA strains, PA-VRSA and NY-VRSA, represents a significant public health problem; since resistance is inducible, it can confidently be predicted that treatment of an infection due to such a strain with vancomycin or teicoplanin will lead to clinical failure. In addition, lack of

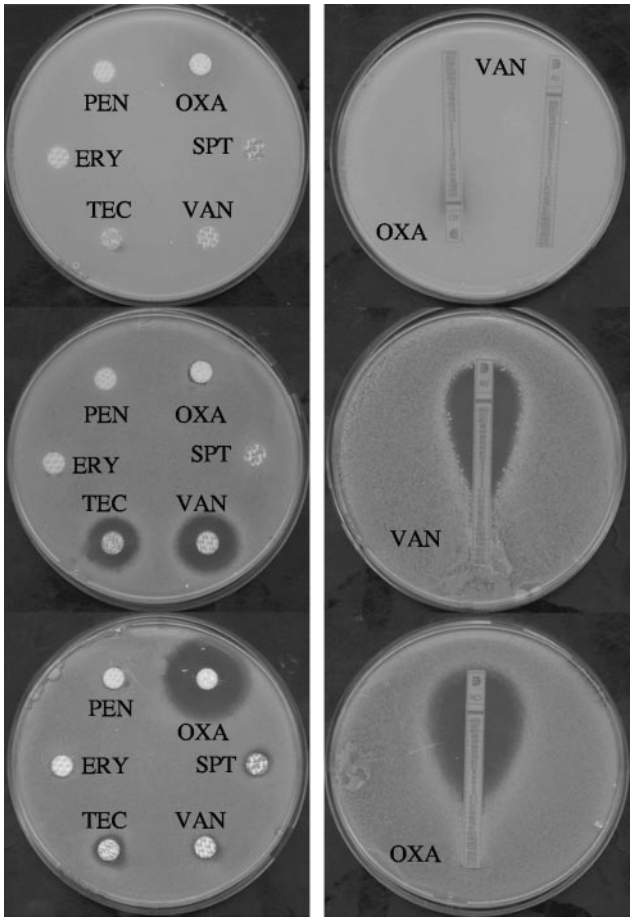

FIG. 4. Oxacillin-glycopeptide synergism against VRSA-5 as tested by (left) disk diffusion and (right) E-test. Top panel, BHI agar; middle panel, BHI plus oxacillin (8 μg/ml); bottom panel, BHI plus vancomycin (8 μg/ml). OXA, oxacillin (5 μg); VAN, vancomycin (30 μg); TEC, teicoplanin (30 μg); PEN, penicillin (6 μg); ERY, erythromycin (15 μg); SPT, spectinomycin (100 μg).

detection will delay implementation of the required hygiene measures to avoid spread of these isolates.

Inducibility of vancomycin resistance. Inducibility of resistance in NY-VRSA and VRSA-5 was studied in liquid medium. After overnight culture at 37°C in BHI with or without vancomycin (6 μg/ml), growth of a subculture in the presence of vancomycin resulted in a long lag phase of at least 8 h for NY-VRSA that was not observed with VRSA-5 (Fig. 3).

This long delay for induction of resistance by vancomycin is consistent with the fact that NY-VRSA escaped detection by automated systems (17). Combination of a high rate of spontaneous loss of the *vanA* cluster and a longer delay in induction of resistance, which could be due to differences between the host bacteria, may explain the difference in the level of glycopeptide resistance of NY-VRSA and VRSA-5.

Oxacillin-glycopeptide synergism. Marked synergy between oxacillin and vancomycin has been reported for MRSA COLVA, a derivative of strain COL harboring a *vanA*-bearing staphylococcal plasmid (27). Laboratory strain COL does not contain any chromosome- or plasmid-borne regulatory gene that controls transcription of the methicillin resistance structural *mecA* gene for PBP2a in most clinical isolates (35). It was therefore of interest to test if synergy between glycopeptide and β-lactam-resistant strains occurs in all the available VanA-type MRSA strains. Several in vitro techniques, such as checkerboard, time-kill curve, E-test, and double disk diffusion, have been described to study the synergism between antibiotics. However, the results obtained by the first two methods were not always consistent (18). Thus, the activity of combinations of glycopeptides and oxacillin against the four VRSA isolates was tested by the four methods and, in each case, in three independent experiments.

Drug interaction was first assessed by placing a vancomycin disk or an E-test strip on agar containing oxacillin at a subinhibitory concentration (8 μg/ml for MI-VRSA, NY-VRSA, and VRSA-5 and 2 μg/ml for PA-VRSA) inoculated with a VRSA strain. After incubation at 37°C, a major size increase of the inhibition zone around the vancomycin disk was observed (Fig. 4, left column), and a decrease in vancomycin resistance of more than 128 times was observed by E-test (Fig. 4, right column; Table 2). Similar results were obtained with teicoplanin (data not shown). In the reverse experiment, a major decrease in resistance to oxacillin (>128 times) was observed when an E-test strip was laid onto BHI agar containing glycopeptide concentrations easily achievable in patients (vancomycin, 8 μg/ml [Fig. 4, right column; Table 2]; teicoplanin, 8 μg/ml [data not shown]). Synergism was also demonstrated when disks impregnated with glycopeptides or oxacillin were placed at an appropriate distance (23); enhancement at the junction of the inhibition zones was observed with the four strains (Fig. 5).

Synergism was also tested by diffusion with disks impregnated with various β-lactams on plates containing vancomycin or teicoplanin (8 μg/ml). A synergy was observed with cefoxitin, cefotaxime, and ceftriaxone against the low-level resistant strains and with oxacillin and imipenem for all strains (data not shown).

Synergistic activity of vancomycin (Fig. 6) or teicoplanin (data not shown) and oxacillin was then studied by the checkerboard technique. The FIC indexes ranged from 0.008 to 0.024 (synergism is defined as a FIC index of ≤0.5). A bacteriostatic synergism was observed for vancomycin and oxacillin concentrations equal to or greater than 4 μg/ml for the LLR strains and 8 μg/ml for the HLR strains, concentrations that are easily achievable in the sera of patients. Curiously, and in contrast with our data, lack of synergism was observed for strain MI-

TABLE 2. MICs of vancomycin and oxacillin for VRSA strains determined by E-test

Strain	MIC (mg/ml) of vancomycin on:		MIC (mg/ml) of oxacillin on:	
	BHI	BHI plus oxacillin[a]	BHI	BHI plus vancomycin[b]
MI-VRSA	>256	2	>256	2
PA-VRSA	24–32	0.015–0.03	4–8	0.015–0.03
NY-VRSA	64	0.25	64	0.25
VRSA-5	256	1	128	1

[a] Oxacillin, 8 mg/ml for MI-VRSA, NY-VRSA, and VRSA-5 and 2 mg/ml for PA-VRSA.
[b] Vancomycin, 8 mg/ml.

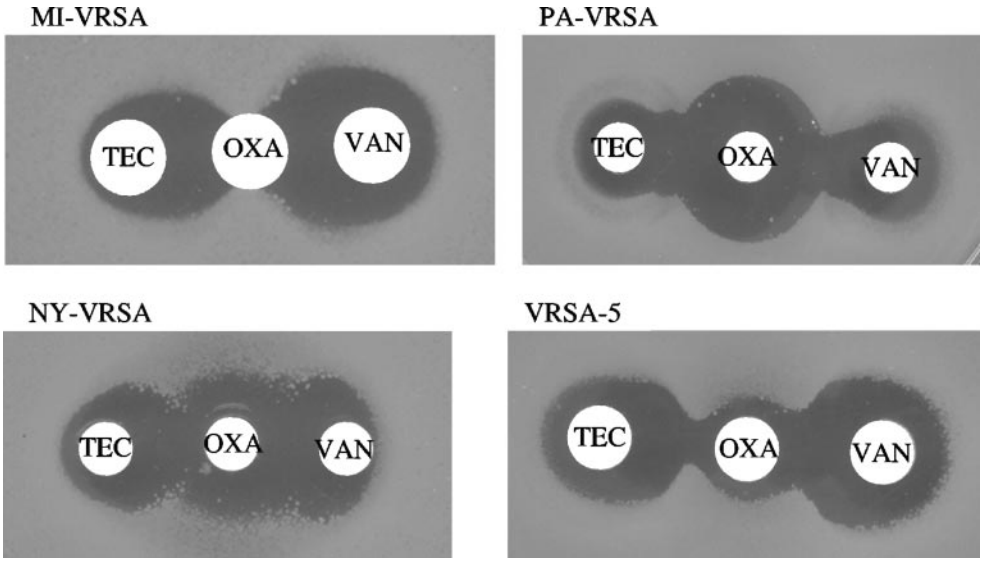

FIG. 5. Oxacillin-glycopeptide synergism in VRSA tested by disk diffusion. OXA, oxacillin (5 μg); VAN, vancomycin (30 μg); TEC, teicoplanin (30 μg). The name of the strain is indicated above the assay.

VRSA by the checkerboard technique when performed on solid medium (16).

Lastly, time-kill curves were performed with all VRSA strains. Vancomycin and oxacillin were tested alone or in combination from 1/64× to 1/2× MIC (from 1 to 32 μg/ml and from 0.5 to 32 μg/ml for glycopeptides and oxacillin, respectively). As expected, no bactericidal activity was obtained with the antibiotics alone. No bactericidal activity was observed at 3 h and 6 h against all strains (Fig. 7) (data not shown). By contrast, a reduction of bacterial counts of at least 3 $\log_{10}$

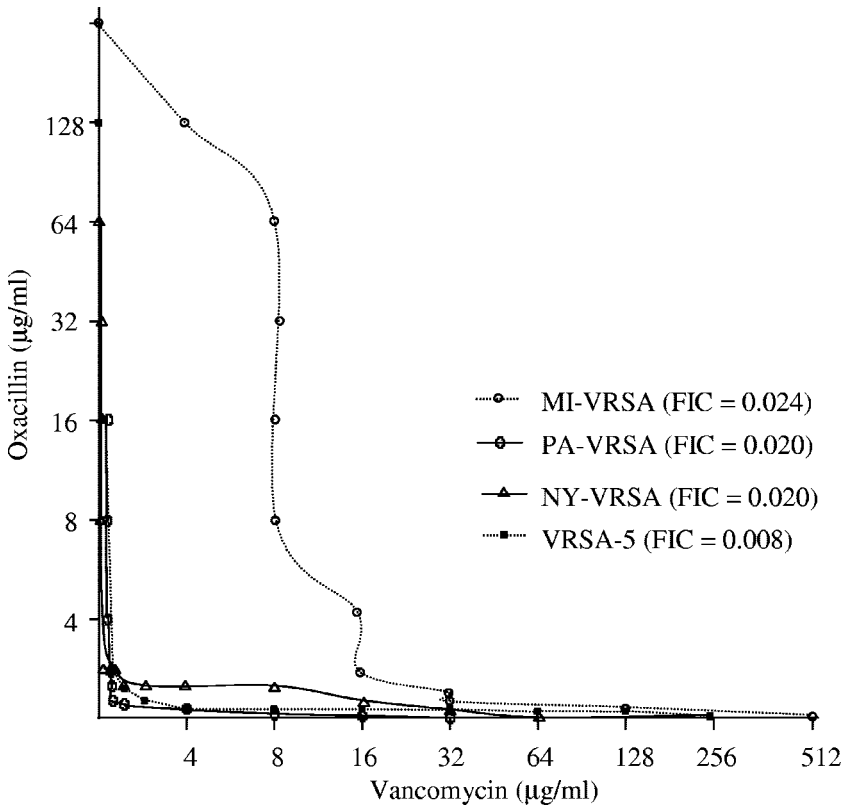

FIG. 6. Study of vancomycin and oxacillin combinations by the checkerboard method. Isobolograms plotted on an arithmetic scale indicate synergism between the antibiotics.

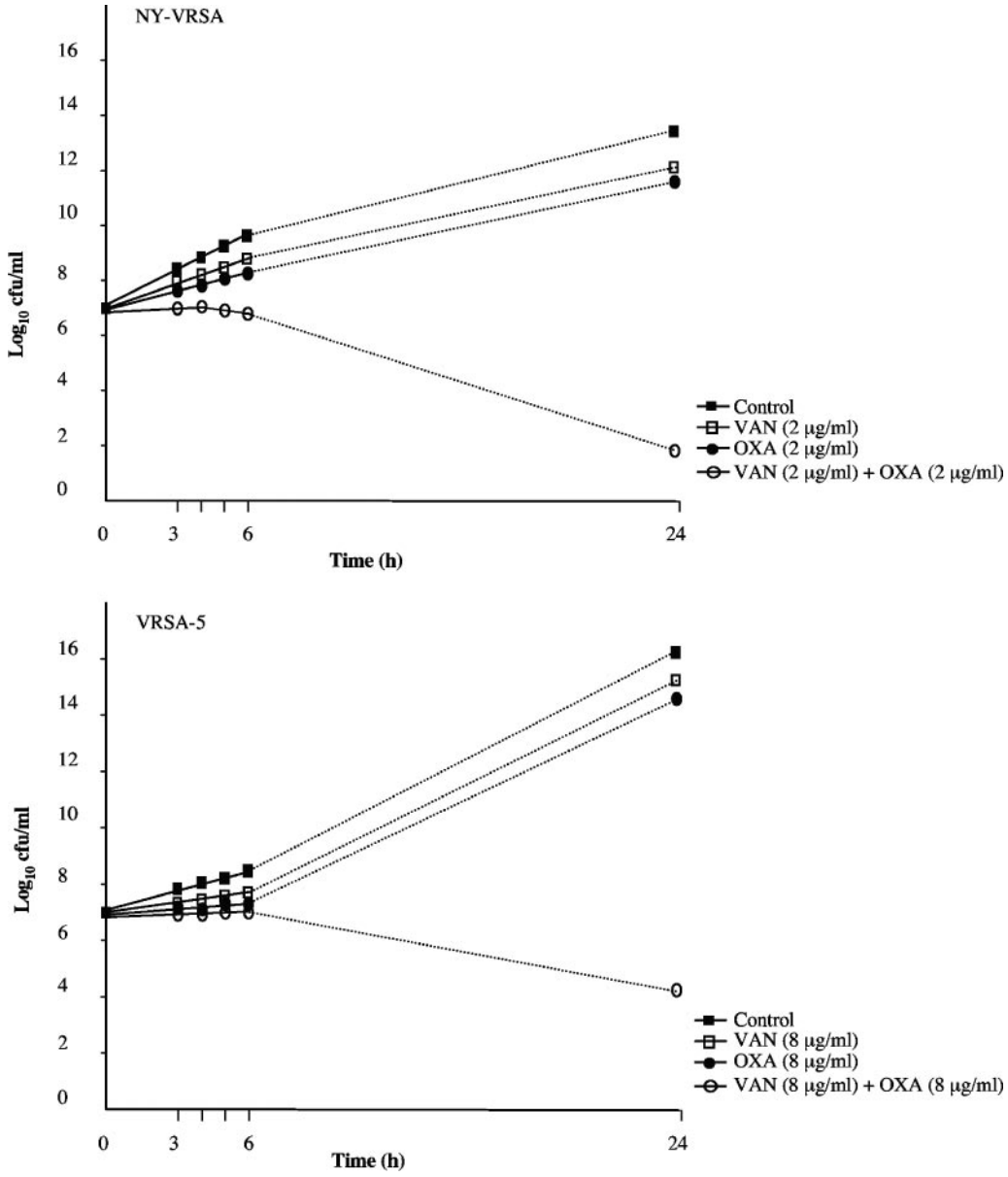

FIG. 7. Time-kill curves for NY-VRSA and VRSA-5 strains. OXA, oxacillin; VAN, vancomycin.

CFU/ml was seen after 24 h of incubation for combinations of vancomycin (≥2 μg/ml for LLR strains and ≥8 μg/ml for HLR strains) with oxacillin (≥2 μg/ml for LLR strains and ≥8 μg/ml for HLR strains) (Table 3). The antibiotic concentrations that had a synergistic effect are achievable in therapy, and higher concentrations did not lead to an increase in the bactericidal effect.

These results confirm that, against each strain, vancomycin and oxacillin had a strong synergistic effect in combination. Similar bactericidal effects were obtained with combinations of teicoplanin and oxacillin (data not shown).

Effect of growth conditions on methicillin resistance expression. Expression of methicillin resistance in *S. aureus* depends on environmental factors such as temperature and osmolarity (4). Determination of MICs and all synergy experiments were repeated at 30°C and/or by incorporating NaCl into the growth medium. As previously reported, better phenotypic expression of oxacillin resistance was observed at 30°C or after addition of NaCl (4%) (data not shown) to the culture medium. Vancomycin resistance was not affected at 30°C and was weakly reduced (by 1 dilution) when NaCl was added. Synergism studies by the time-kill and checkerboard methods gave results at 30°C similar to those at 37°C (data not shown). However, detection of the synergy by disk diffusion at 30°C was less obvious (data not shown).

Strong synergistic activity of glycopeptides and oxacillin, when associated, was consistently observed against the four clinical isolates. PBP2a has transpeptidase activity and exhibits low affinity for all β-lactams. High-level resistance to methicillin in MRSA requires the cooperativity of PBP2a and PBP2

TABLE 3. Bactericidal activity of combinations of vancomycin and oxacillin against NY-VRSA and VRSA-5 strains

Concn (μg/ml) of:		Decrease of bacterial count (in $\log_{10}$ CFU/ml) after 24 h for strain[a]:	
Vancomycin	Oxacillin	NY-VRSA	VRSA-5
1	1	2.5 ± 0.17	1.4 ± 0.10
	2	2.9 ± 0.05	1.5 ± 0.30
	4	3.6 ± 0.32	1.9 ± 0.18
	8	4.3 ± 0.12	2.1 ± 0.08
2	0.5	2.6 ± 0.30	1.6 ± 0.16
	1	2.9 ± 0.02	2.1 ± 0.05
	2	4.4 ± 0.21	2.2 ± 0.06
	4	4.2 ± 0.13	2.5 ± 0.06
	8	4.8 ± 0.15	2.7 ± 0.27
4	0.5	3.5 ± 0.16	1.4 ± 0.10
	1	3.7 ± 0.24	1.3 ± 0.16
	2	4.3 ± 0.10	1.6 ± 0.31
	4	4.3 ± 0.19	2.1 ± 0.16
	8	4.7 ± 0.26	2.9 ± 0.03
8	0.5	3.9 ± 0.05	1.5 ± 0.36
	1	4.3 ± 0.07	1.4 ± 0.13
	2	4.4 ± 0.55	2.1 ± 0.10
	4	4.8 ± 0.08	2.1 ± 0.09
	8	5.4 ± 0.15	3.0 ± 0.03
16	0.5	3.5 ± 0.04	3.1 ± 0.09
	1	4.5 ± 0.04	3.2 ± 0.11
	2	4.2 ± 0.10	3.2 ± 0.06
	4	4.8 ± 0.11	3.5 ± 0.16
	8	5.3 ± 0.22	4.5 ± 0.07
32	0.5	3.6 ± 0.26	3.2 ± 0.04
	1	4.4 ± 0.55	3.2 ± 0.08
	2	4.5 ± 0.47	3.4 ± 0.21
	4	4.9 ± 0.07	4.3 ± 0.53
	8	5.4 ± 0.09	4.4 ± 0.15

[a] Shown are means (±standard deviations) of three independent experiments.

(24, 25), which is a bifunctional *S. aureus* PBP with both transpeptidase and transglycosylase activities (21). In the presence of β-lactam in the culture medium, the cooperation between the transpeptidase activity of PBP2a and the penicillin-insensitive transglycosylase activity of PBP2 appears essential for growth and cell wall biosynthesis (24). PBP2 is required for expression of high-level vancomycin resistance in the COLVA strain (28). In order to account for the loss of oxacillin resistance in the presence of sub-MIC concentrations of vancomycin in this strain, it has been proposed that the pentadepsipeptide cell wall precursors are not substrates for PBP2A, which is the transpeptidase that remains active in the presence of oxacillin (27). Our data indicate that this apparently holds true for the MI-VRSA, PA-VRSA, and VRSA-5 strains that synthesize large amounts of pentadepsipeptide precursors in the presence of glycopeptides in the culture medium (Table 1) (22). In NY-VRSA exhibiting a low level of resistance to vancomycin for a VanA-type strain, the dramatic consequences on cell survival of addition of a glycopeptide and of oxacillin to the growth medium could be the consequence of two phenomena: PBP2a cannot process late peptidoglycan precursors ending in D-Ala-D-Lac, and the binding of vancomycin to the remaining (ca. 20%) precursors terminating in D-Ala-D-Ala prevents the transglycosylation reaction. It has also been demonstrated that vancomycin could (i) induce a decrease in transcription of PBP2A and (ii) be associated with inactivation or deletion of the *mecA* gene (1, 29, 30).

Our data confirm efficient heterologous expression of the glycopeptide resistance genes from enterococci in *S. aureus*. They also suggest, most surprisingly, that combination of a glycopeptide with a β-lactam might be useful for the treatment of infections due to VanA-type MRSA strains that are resistant to both low and high levels of both drug classes. However, this hypothesis remains to be confirmed in vivo in an animal model.

ACKNOWLEDGMENTS

We thank R. Leclercq and P. E. Reynolds for critical reading of the manuscript. *S. aureus* strains were obtained through the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) Program, supported under NIAID/NIH contract no. N01-AI-95359.

REFERENCES

1. **Adhikari, R. P., G. C. Scales, K. Kobayashi, J. M. B. Smith, B. Berger-Bachi, and G. M. Cook.** 2004. Vancomycin-induced deletion of the methicillin resistance gene *mecA* in *Staphylococcus aureus*. J. Antimicrob. Chemother. **54:**360–363.
2. **Arthur, M., F. Depardieu, P. Reynolds, and P. Courvalin.** 1996. Quantitative analysis of the metabolism of soluble cytoplasmic peptidoglycan precursors of glycopeptide-resistant enterococci. Mol. Microbiol. **21:**33–44.
3. **Arthur, M., C. Molinas, F. Depardieu, and P. Courvalin.** 1993. Characterization of Tn*1546*, a Tn*3*-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J. Bacteriol. **175:**117–127.
4. **Berger-Bachi, B., and S. Rohrer.** 2002. Factors influencing methicillin resistance in staphylococci. Arch. Microbiol. **178:**165–171.
5. **Brakstad, O. G., K. Aasbakk, and J. A. Maeland.** 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. J. Clin. Microbiol. **30:**1654–1660.
6. **Bugg, T. D. H., G. D. Wright, S. Dutka-Malen, M. Arthur, P. Courvalin, and C. T. Walsh.** 1991. Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. Biochemistry **30:**10408–10415.
7. **Centers for Disease Control and Prevention.** 1997. Interim guidelines for prevention and control of staphylococcal infection associated with reduced susceptibility to vancomycin. Morb. Mortal. Wkly. Rep. **46:**626–636.
8. **Clark, N. C., L. M. Weigel, J. B. Patel, and F. C. Tenover.** 2005. Comparison of Tn*1546*-like elements in vancomycin-resistant *Staphylococcus aureus* isolates from Michigan and Pennsylvania. Antimicrob. Agents Chemother. **49:**470–472.
9. **Depardieu, F., M. G. Bonora, P. E. Reynolds, and P. Courvalin.** 2003. The *vanG* glycopeptide resistance operon from *Enterococcus faecalis* revisited. Mol. Microbiol. **50:**931–948.
10. **Dutka-Malen, S., S. Evers, and P. Courvalin.** 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. **33:**24–27.
11. **Dutka-Malen, S., R. Leclercq, V. Coutant, J. Duval, and P. Courvalin.** 1990. Phenotypic and genotypic heterogeneity of glycopeptide resistance determinants in gram-positive bacteria. Antimicrob. Agents Chemother. **34:**1875–1879.
12. **Girard-Blanc, C., and P. Courvalin.** 2005. Evaluation of techniques for phenotypic detection of VanA-type *Staphylococcus aureus*. Eur. J. Clin. Microbiol. Infect. Dis. **24:**562–565.
13. **Handwerger, S., and J. Skoble.** 1995. Identification of chromosomal mobile element conferring high-level vancomycin resistance in *Enterococcus faecium*. Antimicrob. Agents Chemother. **39:**2446–2453.
14. **Handwerger, S., J. Skoble, L. F. Discotto, and M. J. Pucci.** 1995. Heterogeneity of the *vanA* gene cluster in clinical isolates of enterococci from the northeastern United States. Antimicrob. Agents Chemother. **39:**362–368.
15. **Hartman, B. J., and A. Tomasz.** 1984. Low-affinity penicillin-binding protein associated with β-lactam resistance in *Staphylococcus aureus*. J. Bacteriol. **158:**513–516.
16. **Hiramatsu, K., L. Z. Cui, and K. Kuwahara-Arai.** 2004. Has vancomycin-resistant *Staphylococcus aureus* started going it alone? Lancet **364:**565–566.
17. **Kacica, M., and L. C. McDonald.** 2004. Vancomycin-resistant *Staphylococcus aureus* - New York, 2004. Morb. Mortal. Wkly. Rep. **53:**322–323.

18. **Lozniewski, A., C. Lion, F. Mory, and M. Weber.** 2001. *In vitro* synergy between cefepime and vancomycin against methicillin-susceptible and -resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. J. Antimicrob. Chemother. **47:**83–86.
19. **Messer, J., and P. E. Reynolds.** 1992. Modified peptidoglycan precursors produced by glycopeptide-resistant enterococci. FEMS Microbiol. Lett. **73:**195–200.
20. **Miller, D., V. Urdaneta, A. Weltman, and S. Park.** 2002. Vancomycin-resistant *Staphylococcus aureus*. Morb. Mortal. Wkly. Rep. **51:**902.
21. **Murakami, K., T. Fujimura, and M. Doi.** 1994. Nucleotide sequence of the structural gene for the penicillin-binding protein 2 of *Staphylococcus aureus* and the presence of a homologous gene in other staphylococci. FEMS Microbiol. Lett. **117:**131–136.
22. **Périchon, B., and P. Courvalin.** 2004. Heterologous expression of the enterococcal *vanA* operon in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **48:**4281–4285.
23. **Pillai, S. K., R. C. Moellering, and G. M. Eliopoulos.** 2005. Antimicrobial combinations, p. 365–440. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 5th ed. The Lippincott Williams & Wilkins Co., Philadelphia, Pa.
24. **Pinho, M. G., H. De Lencastre, and A. Tomasz.** 2001. An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant and drug-susceptible staphylococci. Proc. Natl. Acad. Sci. USA **98:**10886–10891.
25. **Pinho, M. G., S. R. Filipe, H. De Lencastre, and A. Tomasz.** 2001. Complementation of the essential peptidoglycan transpeptidase function of penicillin-binding protein 2 (PBP2) by the drug resistance protein PBP2A in *Staphylococcus aureus*. J. Bacteriol. **183:**6525–6531.
26. **Reynolds, P. E.** 1989. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. Eur. J. Clin. Microbiol. Infect. Dis. **8:**943–950.
27. **Severin, A., K. Tabei, F. Tenover, M. Chung, N. Clarke, and A. Tomasz.** 2004. High level oxacillin and vancomycin resistance and altered cell wall composition in *Staphylococcus aureus* carrying the staphylococcal *mecA* and the enterococcal *vanA* gene complex. J. Biol. Chem. **279:**3398–3407.
28. **Severin, A., S. W. Wu, K. Tabei, and A. Tomasz.** 2004. Penicillin-binding protein 2 is essential for expression of high-level vancomycin resistance and cell wall synthesis in vancomycin-resistant *Staphylococcus aureus* carrying the enterococcal *vanA* gene complex. Antimicrob. Agents Chemother. **48:**4566–4573.
29. **Sieradzki, K., and A. Tomasz.** 1999. Gradual alterations in cell wall structure and metabolism in vancomycin-resistant mutants of *Staphylococcus aureus*. J. Bacteriol. **181:**7566–7570.
30. **Sieradzki, K., S. W. Wu, and A. Tomasz.** 1999. Inactivation of the methicillin resistance gene *mecA* in vancomycin-resistant *Staphylococcus aureus*. Microb. Drug Resist. **5:**253–257.
31. **Sievert, D. M., M. L. Boulton, G. Stolman, D. Johnson, M. G. Stobierski, F. P. Downes, P. A. Somsel, J. T. Rudrik, W. Brown, W. Hafeez, T. Lundstrom, E. Flanagan, R. Johnson, J. Mitchell, and S. Chang.** 2002. *Staphylococcus aureus* resistant to vancomycin. Morb. Mortal. Wkly. Rep. **51:**565–567.
32. **Tenover, F. C., M. V. Lancaster, B. C. Hill, C. D. Steward, S. A. Stocker, G. A. Hancock, C. M. O'Hara, S. K. McAllister, N. C. Clark, and K. Hiramatsu.** 1998. Characterization of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. J. Clin. Microbiol. **36:**1020–1027.
33. **Tenover, F. C., L. M. Weigel, P. C. Appelbaum, L. K. McDougal, J. Chaitram, S. McAllister, N. Clark, G. Killgore, C. M. O'Hara, L. Jevitt, J. B. Patel, and B. Bozdogan.** 2004. Vancomycin-resistant *Staphylococcus aureus* isolate from a patient in Pennsylvania. Antimicrob. Agents Chemother. **48:**275–280.
34. **Weigel, L. M., D. B. Clewell, S. R. Gill, N. C. Clark, L. K. McDougal, S. E. Flannagan, J. F. Kolonay, J. Shetty, G. E. Killgore, and F. C. Tenover.** 2003. Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. Science **302:**1569–1571.
35. **Weller, T. M. A.** 1999. The distribution of *mecA*, *mecR1* and *mecI* and sequence analysis of *mecI* and the *mec* promoter region in staphylococci expressing resistance to methicillin. J. Antimicrob. Chemother. **43:**15–22.