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Inhibition of Cell Wall Turnover and Autolysis by Vancomycin in a Highly Vancomycin-Resistant Mutant of *Staphylococcus aureus*

KRZYSZTOF SIERADZKI, AND ALEXANDER TOMASZ*

The Rockefeller University, New York, New York 10021

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A highly vancomycin-resistant mutant (MIC = 100 μg/ml) of *Staphylococcus aureus*, mutant VM, which was isolated in the laboratory by a step-pressure procedure, continued to grow and synthesize peptidoglycan in the presence of vancomycin (50 μg/ml) in the medium, but the antibiotic completely inhibited cell wall turnover and autolysis, resulting in the accumulation of cell wall material at the cell surface and inhibition of daughter cell separation. Cultures of mutant VM removed vancomycin from the growth medium through binding the antibiotic to the cell walls, from which the antibiotic could be quantitatively recovered in biologically active form. Vancomycin blocked the in vitro hydrolysis of cell walls by autolytic enzyme extracts, lysostaphin and mutanolysin. Analysis of UDP-linked peptidoglycan precursors showed no evidence for the presence of D-lactate-terminating muropeptides. While there was no significant difference in the composition of muropeptide units of mutant and parental cell walls, the peptidoglycan of VM had a significantly lower degree of cross-linkage. These observations and the results of vancomycin-binding studies suggest alterations in the structural organization of the mutant cell walls such that access of the vancomycin molecules to the sites of wall biosynthesis is blocked.

Multidrug-resistant strains of methicillin-resistant *Staphylococcus aureus* (MRSA) in which the therapeutic choice is often reduced to a small number of antibiotics, primarily vancomycin, have spread worldwide during the late 1980s and mid-1990s. The appearance of vancomycin resistance among clinical isolates of enterococci has raised concern about transfer of the resistance genes to highly virulent strains of MRSA with obvious dire implications for chemotherapy. While current concern is directed primarily to interspecific transfer of enterococcal resistance genes, attention has also been paid to other, alternative vancomycin resistance mechanisms that may emerge among *S. aureus* and coagulase-negative strains of nosocomial staphylococci as a consequence of the extensive use of vancomycin in the hospital environment worldwide (for a review, see reference 42). Moderately increased vancomycin (and teicoplanin) MICs have indeed been noted among some clinical isolates of coagulase-negative staphylococci (3, 6, 20, 31, 32, 40), and glycopeptide-resistant variants or mutants of staphylococci have also been isolated in several laboratories by the usual step-pressure procedures (5, 7, 8, 16, 18, 40).

While studying the effect of cell wall synthesis inhibitors on the expression of methicillin resistance in *S. aureus*, we observed rare staphylococcal cells that were able to form colonies on agar containing 6 and even 12 μg of vancomycin per ml. Serial passages of such colonies in vancomycin-containing media (step-pressure procedure) resulted in the emergence of stable variants (mutants) with even higher levels of vancomycin resistance. One mutant for which the vancomycin MIC was 100 μg/ml had the unique capacity to quantitatively remove vancomycin from the surrounding medium (35). A key feature of the resistance mechanism in this mutant appears to be some alteration of cell wall structure such that it allows "capture" of the glycopeptide molecules at the periphery of the cells distant from sites of cell wall biosynthesis, which thus become protected from the antibiotic.

* Corresponding author. Mailing address: The Rockefeller University, 1230 York Ave., New York, NY 10021. Phone: (212) 327-8277. Fax: (212) 327-8688.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. All *S. aureus* strains were grown in tryptic soy broth (TSB) (Difco, Detroit, Mich.) at 37°C with aeration. Strains of *Enterococcus faecalis*, *E. faecium*, and *Leuconostoc mesenteroides* were grown in brain heart infusion broth (Difco) at 37°C without aeration, and *Streptococcus pneumoniae* R36A was grown in a casein-based semisynthetic medium (33). For each experiment, the overnight cultures were diluted 10,000-fold into prewarmed TSB to allow exponential growth conditions. Growth was observed by monitoring the optical density at 620 nm (OD_{620}) with a spectrophotometer (Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden). Viable titers and antibiotic resistance levels (population analysis) were determined by plating diluted cultures on tryptic soy agar (Difco) as described previously (39).

Isolation of vancomycin-resistant mutant VM. A highly vancomycin-resistant mutant of *S. aureus* was isolated by step-pressure, as described in a recent communication (35).

Electron microscopy. Cells suspended in growth medium were fixed with an equal volume of 5% glutaraldehyde and stored for at least 24 h at 4°C until they were further processed for electron microscopy, as described previously (38).

Cell wall turnover. Cells were labeled for six generations in TSB containing 2 μCi and 5 μg of tritium-labeled *N*-acetylglucosamine ([^{3}H]GlcNAc) per ml (Amersham, Arlington Heights, Ill.) and then grown in isotope-free medium (supplemented with 5 mM nonradioactive GlcNAc), during which time the rate of release of radioactive cell wall components was measured as described previously (10). In some experiments, the radioactive labeling followed by monitoring of cell wall turnover was carried out in mutant VM in the presence of vancomycin (50 μg/ml).

Autolysis assay. Triton X-100-stimulated autolysis in glycine buffer (pH 8.0) was measured as previously described (10). Cells were grown exponentially to an OD_{620} of about 0.3. The cultures were then rapidly chilled, and the cells were washed once with ice-cold distilled water and suspended to an OD_{620} of 1.0 in 50 mM glycine–0.01% Tris X-100 buffer. In some cases, the autolysis buffer contained vancomycin (50 μg/ml). Autolysis was measured during incubation at 37°C as a decrease in optical density at OD_{620} by using a model 340 spectrophotometer (Sequoia-Turner Corp., Mountain View, Calif.).

Cell wall hydrolysis in vitro. Purified cell walls were suspended in the appropriate buffer (50 mM Tris-Cl [pH 7.5] for lysostaphin and for crude lytic enzyme extracts, and 25 mM phosphate buffer [pH 5.5] for muramidase) to an initial OD_{620} of 1.0. Prior to this step, some samples were preincubated with vancomycin at saturating concentrations (1,000 μg/mg of cell wall) in 0.15 M NaCl buffered with 0.7 mM phosphate buffer (pH 7.2). After incubation at 37°C for 120

TABLE 1. Strains used in this study

Strain	Relevant phenotype[a]	Source
S. aureus		
COL[b]	Mc^r Vm^s	RU collection
VM[b]	Mc^r Vm^r	This study
RN-450	Vm^s	RU collection
E. faecalis		
JH2-2[b]	Vm^s	S. Handwerger collection
221[b]	Vm^r (VanA)	S. Handwerger collection
E. faecium		
22850[b]	Vm^r (VanA)	S. Handwerger collection
Copes	Vm^r (VanA)	S. Handwerger collection
EFSK-2	Vm^r (VanB)	RU collection
EFSK-33[c]	Vm^r (VanA)	RU collection
EFSK-80[c]	Vm^r (VanB)	RU collection
L. mesenteroides		
VR1	Vm^r	S. Handwerger collection
S. pneumoniae		
R36A[b]	Pc^r	RU collection

[a] Abbreviations: Mc, methicillin; Pc, penicillin; Vm, vancomycin.
[b] Cell walls of the strains used in the binding assay.
[c] Strains used as controls in the DNA hybridization procedure.

min, the wall suspension was washed twice with distilled water to remove unbound drug. Lysis was measured as a decrease in OD_{620} during incubation of wall samples with crude lytic enzyme extracts (7 μg of protein/ml), lysostaphin (10 μg/ml), or muramidase (5 μg/ml) at 37°C.

Crude autolytic enzyme extracts. Crude autolytic extracts were prepared similarly to the method described previously (37). Bacterial cultures were grown to mid-exponential phase in 250 ml of TSB (the resistant mutant was also grown in the presence of 50 μg vancomycin per ml) at 37°C with aeration, chilled rapidly, harvested by centrifugation, washed once in ice-cold 50 mM Tris-Cl (pH 7.5), and extracted with 250 μl of 4% sodium dodecyl sulfate (SDS) at room temperature for 30 min with stirring. Supernatants were used as autolytic extracts.

Protein determination. Protein concentrations were determined with the bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.) with bovine serum albumin as a standard.

Heat-inactivated cells. Bacteria were grown to the mid-exponential phase (occasionally in the presence of 50 μg of vancomycin per ml) at 37°C with aeration, chilled rapidly, harvested by centrifugation, washed once in cold 1.0 M NaCl (buffered with 50 mM Tris-Cl [pH 7.5]), resuspended in the above solution, and autoclaved for 30 min. After sterilization, the samples were washed twice in distilled water and lyophilized.

Polyacrylamide gel electrophoresis (PAGE). Proteins were separated by the technique of Laemmli (19). Resolving gels were composed of 10% acrylamide and 0.27% bisacrylamide or 7.5% acrylamide and 0.2% bisacrylamide. The samples were run at a constant current of 20 mA at room temperature until the blue dye reached the bottom of the separation gel. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, Calif.).

Bacteriolytic enzyme profiles after SDS-PAGE. Resolving gels (7.5% acrylamide, 0.2% bisacrylamide) contained heat-inactivated cells (2 mg [dry weight] per ml). Bacteriolytic enzymes were visualized as described previously (13) with some modifications. The gels were initially washed in distilled water for 1 h with changes of water every 15 min and then washed in buffer composed of 50 mM Tris-Cl (pH 7.5), 0.1% Triton X-100, 10 mM $CaCl_2$, and 10 mM $MgCl_2$; finally, they were incubated for 24 h at 37°C with gentle agitation in the same buffer as above.

Preparation of the peptidoglycan. Cell wall peptidoglycan was prepared, and enzymatic cell wall hydrolysates were analyzed by reversed-phase high-performance liquid chromatography (HPLC) as described previously (9), except that the alkaline phosphatase step was omitted.

Preparation of the UDP-linked precursors and analysis by HPLC. Cytoplasmic pools of the UDP-linked peptidoglycan precursors were extracted by a modification of the technique of Mengin-Lecreulx et al. (21). The cells were grown to mid-exponential phase in TSB (in some experiments, the resistant strain, VM, was grown in the presence of vancomycin at 50 μg/ml) at 37°C with aeration, chilled rapidly, harvested by centrifugation, washed in 0.9% saline, and extracted with cold trichloroacetic acid (final concentration, 5%) for 30 min at 4°C. The extract containing cell wall precursors was separated by gel filtration on a Sephadex G-25 column eluted with water. Hexosamine-containing fractions identified by the assay of Ghuysen et al. (14) were combined and lyophilized. The muropeptides were separated by HPLC essentially by the method of Flouret et al. (12) with some modifications. Samples were applied to a 3.9- by 300-mm reversed-phase column (μBondapack C_{18}; Millipore Corp., Waters Chromatography, Milford, Mass.) guarded by a 15- by 3.2-mm Perisorb RP-18 precolumn (Pierce). The column was operated under isocratic elution at 40°C with 50 mM ammonium phosphate (pH 4.5) at a flow rate of 0.5 ml/min, which was changed at 26 min to 2.0 ml/min. Eluted compounds were detected by their absorption at 254 nm (Spectroflow 757; Kratos Analytical, Ramsey, N.J.).

Amino acid analysis. Amino acid analysis was done as described previously (2).

DNA manipulations. Preparation of chromosomal DNA and plasmid DNA, conventional and pulsed-field gel electrophoresis, blotting of DNA, and hybridization were performed as described previously (11).

Peptidoglycan synthesis. Peptidoglycan synthesis was measured by labeling portions of the cultures with [^{3}H]GlcNAc (5 μCi and 1 μg per ml; Amersham), as described previously (15).

Vancomycin binding studies. (i) Binding in vivo. The vancomycin-resistant strains of *E. faecalis* 221, *E. faecium* 22850, Copes, and EFSK-2, *L. mesenteroides* VR1, and the vancomycin-resistant *S. aureus* mutant VM were grown in the presence of 50 μg of vancomycin per ml until late stationary phase. Bacteria were removed by centrifugation, the supernatants were sterilized through 0.45-μm-pore-size sterile filters (Uniflo-25; Schleicher & Schuell, Inc., Keene, N.H.), and the amounts of vancomycin remaining in the supernatants were determined by bioassay with *S. aureus* RN-450 as the indicator organism. Turbidity was read after 24 h of incubation at 37°C in TSB. The concentration of vancomycin was also determined by HPLC (17).

(ii) Binding in vitro. Purified cell walls of bacterial strains (listed in Table 1) were incubated with vancomycin (250 to 1,000 μg of drug per mg of walls) in 0.15 M NaCl (buffered with 0.7 mM phosphate buffer [pH 7.2]) at 37°C for 2 h. After incubation, the cell walls were separated by centrifugation and supernatants were analyzed for free vancomycin. Cell walls of the mutant VM and parental strain COL were additionally digested with muramidase or treated with hydrofluoric acid, incubated with vancomycin (0 to 250 μg of antibiotic per mg of cell wall equivalent), and subjected to determination of the "free" antibiotic concentration, as described above.

RESULTS

Isolation of the vancomycin-resistant mutant VM. The isolation of mutant VM was prompted by an accidental observation made during experiments testing the effect of sub-MIC levels of various cell wall synthesis inhibitors on the expression of methicillin resistance in the highly and homogeneously methicillin-resistant strain *S. aureus* COL (36). Vancomycin included at one-quarter of the MIC in the agar plates with the various concentrations of methicillin was found to reduce the homogeneous methicillin MIC for strain COL from 800 μg/ml to a heterogeneous 25 to 50 μg/ml (Fig. 1A). The rare bacterial colonies that retained their capacity to grow in a high concentration of methicillin (400 μg/ml) plus 0.4 μg of vancomycin per ml were picked from the agar plate (arrow in Fig. 1A), grown to dense liquid cultures in TSB, and then tested for their vancomycin resistance by population analysis. The majority of cells in such a culture were associated with a vancomycin MIC of about 1.5 to 3 μg/ml, but the culture also included bacterial colonies that could grow in the presence of 6 μg of vancomycin per ml. After several serial passages of such a colony in increasing concentrations of vancomycin, a mutant strain, VM, with a vancomycin MIC of 100 μg/ml was isolated (Fig. 1B). Mutant VM was also associated with an increased (and heterogeneous) MIC of teicoplanin (Fig. 1C) but a drastically reduced (and heterogeneous) MIC of methicillin (800 μg/ml for the parent strain, COL, reduced to 1.5 μg/ml for the majority of the cells of mutant VM) (Fig. 1D). Resistance to other β-lactam antibiotics and, to a lesser degree, to D-cycloserine and phosphonomycin, was also decreased (Table 2).

Mechanism of resistance: physiological tests. (i) Effect on cell division and growth and removal of vancomycin from the medium. Mutant VM had slower growth in TSB (doubling time, about 1 h) than did the parental culture COL (doubling time, about 30 min).

Upon addition of vancomycin to an exponentially growing

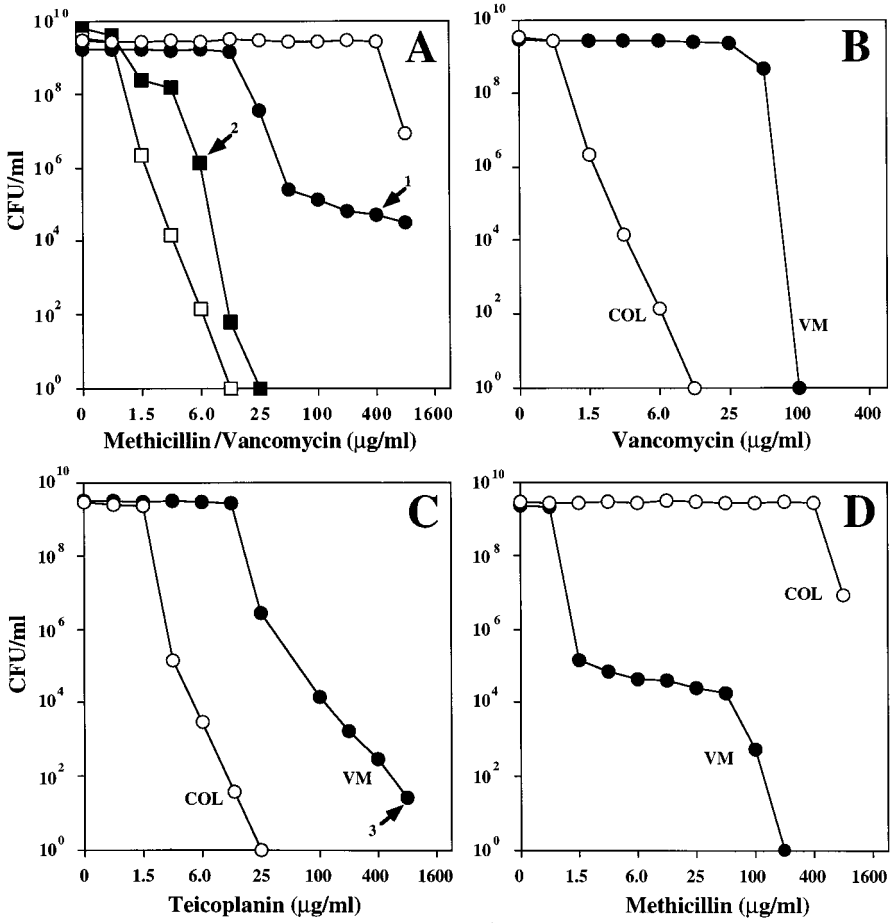

FIG. 1. Origin of the *S. aureus* mutant VM. (A) Methicillin (○) and vancomycin (□) susceptibility of the parental strain COL, tested by population analysis. Inclusion of one-quarter of the MIC of vancomycin in the methicillin-containing agar plates resulted in the reduction of methicillin resistance (●). A colony growing on the agar plate containing 400 μg of methicillin per ml plus one-quarter of the MIC of vancomycin was picked (arrow 1), grown to dense liquid culture, and retested for its vancomycin susceptibility (■). A colony capable of growing on agar containing 6 μg of vancomycin per ml (arrow 2) was picked and was the origin of mutant VM, which was generated by serial subcultures of these bacteria in increasing concentrations of vancomycin. (B) Documentation of the vancomycin resistance of mutant VM. Population analysis on vancomycin-containing plates was done as described in Materials and Methods for VM and parental strain COL. (C) Teicoplanin resistance of mutant VM and parental strain COL determined by population analysis. Arrow 3 indicates the presence of highly teicoplanin-resistant cells for which the MIC was >1,600 μg/ml. (D) Increased methicillin susceptibility and heterogeneous expression of methicillin resistance in mutant VM. Population analysis profiles for mutant VM and parental strain COL are shown.

culture of VM, the OD of the culture continued to increase (with a rate about half the drug-free rate) but cell division (increase in viable titer) was inhibited (Fig. 2). The concentration of antibiotic in the medium (assayed by a bioassay [vertical bars in Fig. 2]) began to decline and eventually dropped to an undetectable level. At about the time (10 to 12 h) when the free drug concentration was reduced to a few micrograms per milliliter, there was a burst in the viable titer of the culture (Fig. 2).

(ii) Abnormal morphology. Cells of VM grown in antibiotic-free medium showed little, if any, difference from parental cells in electron microscopic thin sections. On the other hand, growth of VM in the presence of vancomycin resulted in the formation of multicellular clusters surrounded by massive amounts of extracellular debris which had the appearance of cell wall material (Fig. 3).

Mechanism of resistance: biochemical tests. (i) Inhibition of cell wall turnover. The morphological appearance of VM grown in the presence of vancomycin suggested that the antibiotic also inhibited cell wall turnover of the bacteria. To test

TABLE 2. Antibiotic susceptibility profile of the *S. aureus* mutant VM

Compound	MIC (μg/ml) for:	
	Parental COL	VM
Vancomycin	1.5	100
Teicoplanin	3.0	25
Methicillin	800	1.5
Imipenem	50	<0.75
Cefotaxime	800	<0.75
Cephradine	200	1.5
Cefoxitin	400	3.0
Bacitracin	50	100
D-Cycloserine	100	50
Phosphonomycin	50	12
Chloramphenicol	6.0	3.0
Erythromycin	0.4	0.4
Gentamycin	0.4	3.0
Tetracycline	100	100
Temafloxacin	0.2	0.4

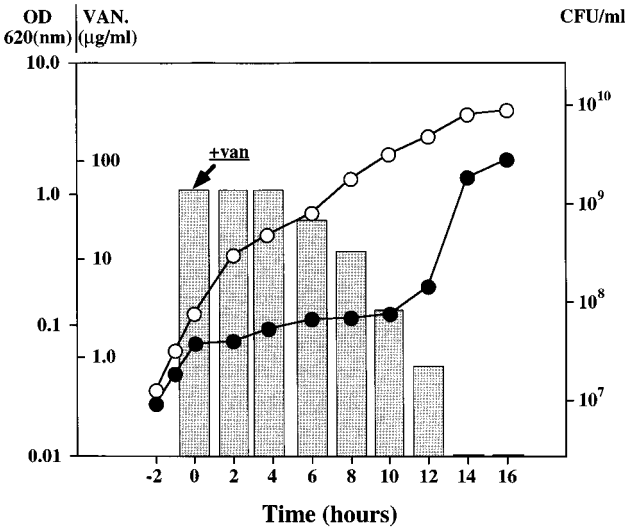

FIG. 2. Inhibition of cell division and removal of vancomycin (van) from the culture medium during growth of mutant VM. Mutant VM was grown in TSB at 37°C. The OD_{620} was monitored (○), and viable titers were determined at intervals (●). At 0 h ($OD_{620} \approx 0.1$) of the experiment, vancomycin (50 μg/ml) was added to the growth medium. At various times, sterile filtrates of the culture were used to determine the titer of vancomycin in the supernatant medium by the bioassay (solid bars), as described in Materials and Methods.

this, cultures of the parent strain COL and mutant VM were grown in TSB supplemented with [^{3}H]GlcNAc to label the cell wall peptidoglycan (phase 1 of the experiment). An additional culture of VM was also grown under the same conditions but in the presence of vancomycin (50 μg/ml). After growth in the presence of the radioactive tracer for six generations, the cells were washed and back-diluted into isotope-free medium, and the rate of release of radioactivity from the cells was monitored as a function of cell generation (phase 2 of the experiment). Both parent and mutant had comparable rates of wall turnover, while in the mutant radiolabeled in the presence of vancomycin, release of wall material was significantly accelerated (Fig. 4A). However, addition of vancomycin to the phase 2 medium invariably inhibited cell wall turnover (Fig. 4A).

(ii) Inhibition of autolysis. Cultures of COL, VM, and VM grown in the presence of vancomycin were resuspended in glycine buffer containing Triton X-100 to trigger autolysis. Autolysis proceeded with comparable rates in the suspensions of both parental and mutant cells. However, when vancomycin was added to the growth medium of VM or to the Triton X-100-containing buffer, autolysis was completely inhibited (Fig. 4B).

(iii) Inhibition by vancomycin of in vitro cell wall hydrolysis, catalyzed by autolytic enzyme extracts from mutant VM. Crude autolytic enzyme extracts prepared from VM degraded parental and mutant walls at virtually equal rates (Fig. 4C). Walls prepared from VM grown in the presence of the antibiotic were degraded significantly more slowly. Saturation of cell walls with vancomycin prior to addition of the autolytic enzyme preparation completely blocked cell wall hydrolysis (Fig. 4C). Similar results were obtained with extracts prepared from the parental strain COL and VM grown in the presence of vancomycin (data not shown).

(iv) Inhibition of the hydrolytic action of lysostaphin and mutanolysin. The same wall preparations used in the experiment illustrated in Fig. 4C were also tested for their susceptibility to in vitro degradation by lysostaphin and mutanolysin. Preincubation of cell walls with vancomycin caused substantial (in strain COL) or almost complete (in mutant VM) inhibition of cell wall hydrolysis by mutanolysin (Fig. 4D) and complete inhibition of lysis by lysostaphin (data not shown).

(v) Mechanism of inhibition of autolysis and wall hydrolysis. The results of the experiments illustrated in Fig. 4 suggested that the mechanism by which vancomycin inhibited autolysis and in vitro wall hydrolysis involved interaction of the antibiotic with the cell wall. Nevertheless, to exclude additional interference with the production and/or activity of the autolytic enzymes, the profiles of autolysins were compared in strains COL, VM, and VM grown in the presence of vancomycin, using the method of zymogen gels. No significant differences could be detected in the number of hydrolytic bands (Fig. 5). However, the highest molecular size hydrolytic band and also another (low molecular size) lytic band appeared to be present in larger relative quantities in the mutant. The significance of this observation is currently under investigation.

(vi) Peptidoglycan structure of mutant VM and recovery of vancomycin, in biologically active form, from cells grown in the presence of the antibiotic. Enzymatic hydrolysates of cell wall peptidoglycan prepared from cultures of COL and VM were analyzed for muropeptide composition by HPLC (Fig. 6A and B). Except for the greatly decreased amounts of muropeptide 1 in the mutant, the patterns of peaks in the elution profiles were superimposable. However, the relative amount of highly cross-linked muropeptide species representing about 60% of muropeptide material in the parental cell was reduced in the mutant to about 40% of the total muropeptide material and was further decreased to about 20% in the peptidoglycan of VM grown in the presence of vancomycin. Peptidoglycan prepared from VM grown in the presence of vancomycin contained an unusual large peak with retention time of about 122 min on the HPLC column (Fig. 6C, component X). Subsequent analysis of component X showed that it was a degradation product of vancomycin generated during the HF treatment (data not shown).

A culture of VM was grown from small inocula for eight to nine cell generations in the presence (or absence) of vancomycin. After harvesting the bacteria from early exponential phase, the purified cell walls (still containing both peptidoglycan and wall teichoic acids) were hydrolyzed by mutanolysin, and the hydrolysates were analyzed by the same HPLC method used for the peptidoglycan. Identical elution profiles were obtained for cell wall hydrolysates prepared from cells grown with or without antibiotic in the growth medium, except for a single large and sharp peak (X) that was present only in hydrolysates of vancomycin-grown cells. This peak had a retention time of about 80 min, indistinguishable from the retention time of pure vancomycin (inset in Fig. 7). Amino acid analysis and bioassay of the peak recovered at the 80-min retention time from the HPLC column indicated that the recovered material was biologically active vancomycin (data not shown).

(vii) Vancomycin binding capacity of the mutant cell wall. Purified cell walls (0.2 mg/ml) of strains COL and VM were incubated in buffer with concentrations of vancomycin that saturated the binding capacity of the wall preparations. Wall suspensions of VM were able to bind 500 μg of vancomycin per mg, in contrast to 235 μg bound per mg of the parental cell walls. This more than twofold difference between binding capacities became marginal when the peptidoglycan fractions (HF-treated walls) of the two bacterial strains were compared

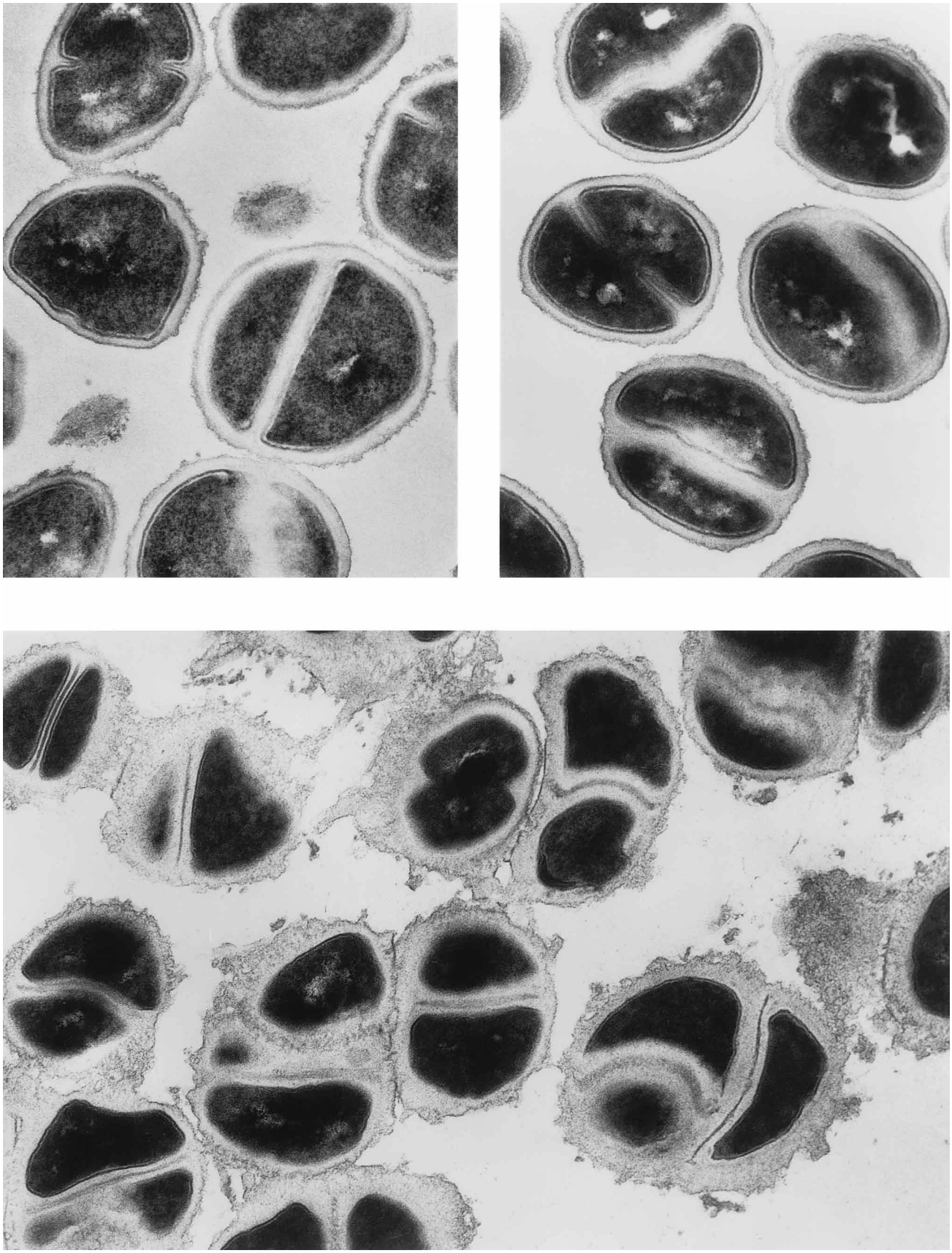

FIG. 3. Morphological abnormality of mutant VM grown in the presence of vancomycin. Cultures of parental strain COL (top left) and mutant VM (top right) were grown in TSB. A culture of mutant VM was also grown in the presence of 50 μg of vancomycin per ml (bottom). At an OD of approximately 0.5, the cultures were harvested and prepared for transmission electron microscopy. Electron microscopic thin sections stained with uranyl acetate and lead citrate were photographed. Magnification, ×44,000.

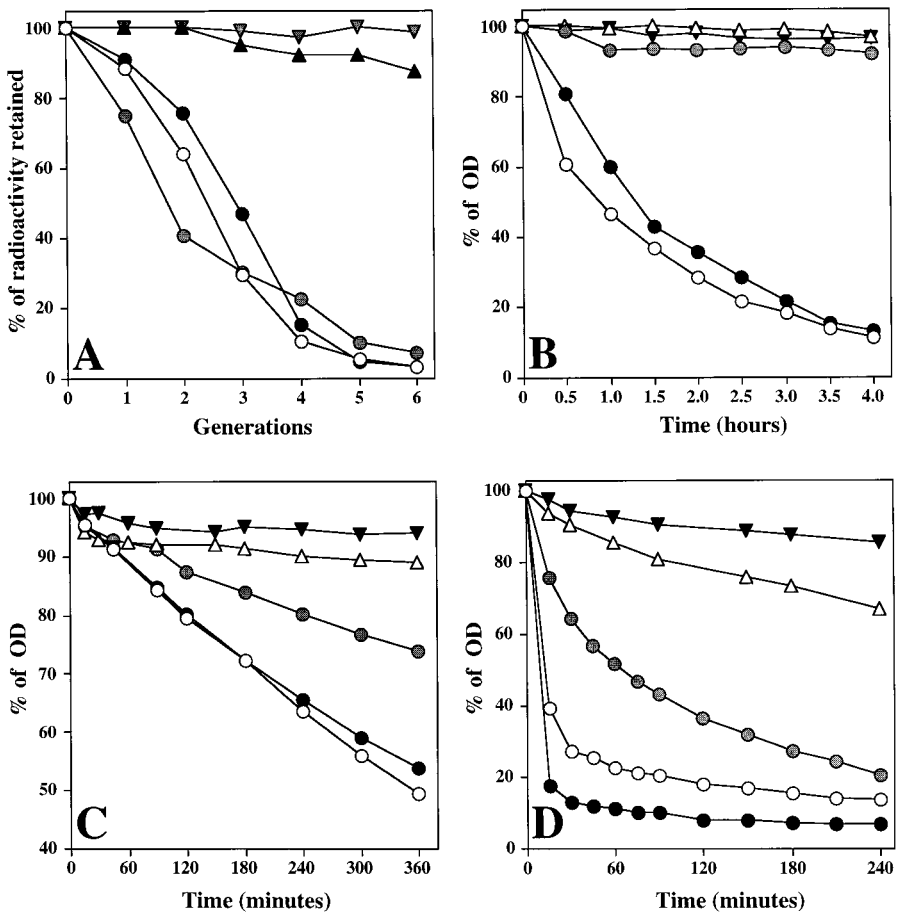

FIG. 4. Effect of vancomycin on cell wall turnover, autolysis, and susceptibility of cell walls to mutanolysin. (A) Cultures of parental strain COL (○) and mutant VM (●) prelabeled with [^{3}H]GlcNAc in their cell walls were used to determine the rate of cell wall turnover by the technique described in Materials and Methods. Mutant VM was also labeled with the radioactive precursor while growing in the presence of vancomycin, and the cell wall turnover of this culture (in drug-free medium) is shown (◍). The virtually complete inhibition of cell wall turnover in the presence of vancomycin (50 μg/ml) is also shown for bacteria prelabeled in the absence (▲) and presence (▼) of vancomycin. (B) Cultures of strain COL (open symbols) and mutant VM (solid symbols) were suspended in lysis buffer to an initial OD of ≈1.0, and the rates of autolysis were monitored as described in Materials and Methods, in lysis buffer without vancomycin (○, ●) or in buffer containing vancomycin at 50 μg/ml (△, ▼). Inhibition of autolysis in a culture of VM, which was grown in the presence of vancomycin and was subsequently tested for autolysis in the antibiotic-free lysis buffer, is also shown (◍). (C) Crude autolytic extract prepared from mutant VM (see Materials and Methods for procedure) was used to test the susceptibility of cell walls for autolytic degradation in vitro, both in the absence and in the presence of vancomycin (50 μg/ml) in the in vitro assay mixture. Cell walls prepared from strain COL were tested in the absence (○) and presence (△) of vancomycin. Cell walls prepared from mutant VM were tested in the absence (●) or presence (▼) of vancomycin. The degradation of cell walls prepared from VM grown in the presence of vancomycin is also indicated (◍). (D) The same cell wall preparations used in panel C were tested for their susceptibility to in vitro degradation by mutanolysin (see Materials and Methods). Symbols are as in panel C.

or when the cell walls of the two strains were first solubilized by digestion with mutanolysin (Table 3).

(viii) Effect of vancomycin on the rate of cell wall synthesis. Exponentially growing COL and VM cells were pulse-labeled with [^{3}H]GlcNAc at intervals before and after addition of vancomycin (50 μg/ml) to measure the effect of the antibiotic on the rate of cell wall incorporation. Figure 8 shows that addition of vancomycin caused a rapid initial drop in the rate of wall synthesis in mutant VM, after which wall synthesis continued at the lower but constant rate adjusted to the lower growth rate of the bacteria, as measured by the rate of increase of OD. Addition of vancomycin to the antibiotic-susceptible parent culture caused a rapid, substantial (500-fold) drop in the rate of wall synthesis followed by a continued, somewhat slower decline in the rate of wall incorporation (Fig. 8).

Comparison to enterococcal vancomycin resistance. Genomic DNAs were prepared from VM (and from the control strains *E. faecium* EFSK33 [*vanA*] and *E. faecium* EFSK80 [*vanB*]), digested with *Sma*I, and separated by pulsed-field gel electrophoresis. In contrast to the control strains, Southern hybridization with DNA probes specific for the enterococcal *vanA* and *vanB* genes produced no detectable signals in VM (data not shown).

Vancomycin-resistant enterococci accumulate the abnormal cell wall precursor containing a D-lactyl group at the carboxy-terminal residue of the UDP-linked muramyl peptide (for a review, see reference 42). Analysis of the wall precursor pool fraction of VM by an HPLC technique showed no detectable material in the position characteristic of the retention time of the D-lactyl peptide (data not shown).

Cultures of the highly vancomycin-resistant strains *E. faecalis* 221, *E. faecium* Copes and 22850, each carrying the *vanA* gene, *E. faecium* EFSK-2 (*vanB*), and a strain of highly and intrinsically vancomycin-resistant *L. mesenteroides* (VR1) were grown in the presence of 50 μg vancomycin per ml in the growth medium, from small inocula (10^4 CFU/ml) to dense stationary-phase cultures, and the concentration of vancomycin in the medium was determined by a bioassay after over-

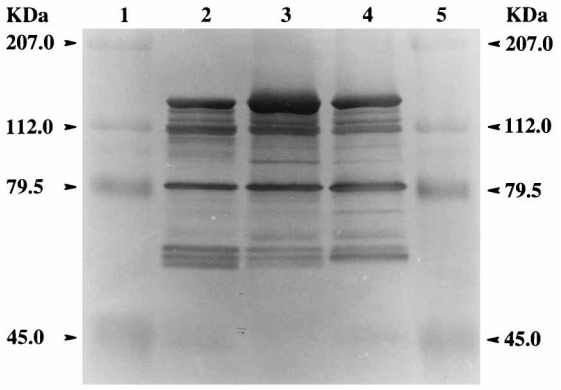

FIG. 5. Autolytic enzymes of *S. aureus* COL and VM. Autolytic extracts were prepared and assayed by the SDS-PAGE zymogen method for the number of lytic bands, as described in Materials and Methods. Lanes 1 and 5 represent prestained molecular size markers. The central three lanes represent zymogen fingerprints of parental strain COL (lane 2), mutant VM (lane 3), and VM grown in the presence of vancomycin (lane 4), with heat-inactivated COL cells as the substrate (2 mg of dry cells per ml of gel). Bands with lytic activity were observed as clear zones in the opaque gel.

night incubation of stationary-phase cultures. No decline in vancomycin titer could be detected (data not shown).

DISCUSSION

The mechanism of vancomycin resistance described in the staphylococcal mutant VM appears to be different from the well-studied glycopeptide resistance in enterococci: there was no evidence for the presence of D-lactate-terminating wall precursors and no reactivity with the DNA probes for the *vanA* and *vanB* genes. Highly vancomycin-resistant strains of *E. faecium*, *E. faecalis*, and *L. mesenteroides* did not remove vancomycin from the culture medium.

The vancomycin that disappeared from the culture medium of the resistant staphylococcal mutant VM was recovered in a biologically active and physically unaltered form and virtually quantitatively from the cell wall fraction of the bacteria.

Our results suggest that the inhibition of cell wall turnover by vancomycin is in great part related to the blocking of access of the relevant hydrolytic enzymes to their cell wall substrate at the outer surface of the bacterium due, presumably, to steric hindrance by the bound glycopeptide molecules. Crude autolytic enzyme extracts prepared from either mutant or parental cells could not hydrolyze cell wall preparations saturated with vancomycin in vitro. Such cell wall preparations were also resistant to the hydrolytic action of lysostaphin and mutanolysin at enzyme concentrations that rapidly lysed untreated cell walls.

Our data do not allow us to propose a clear-cut and fully satisfying mechanism for the vancomycin resistance of mutant VM. Analysis of parental and mutant peptidoglycans by high-resolution HPLC showed virtually identical profiles for all the well-resolved muropeptide species (except for the decrease in the linear muropeptide monomer [peak 1] in the mutant peptidoglycan). On the other hand, representation of the highly cross-linked muropeptide species eluting from the column with long retention times was substantially reduced in the mutant. For instance, while about 30% of all muropeptides are present as highly cross-linked species (eluting at retention times longer than 110 min) in the parental strain, only 15% of muropeptides are in this fraction in mutant VM. As a consequence of this, the relative proportion of muropeptides carrying intact D-ala-

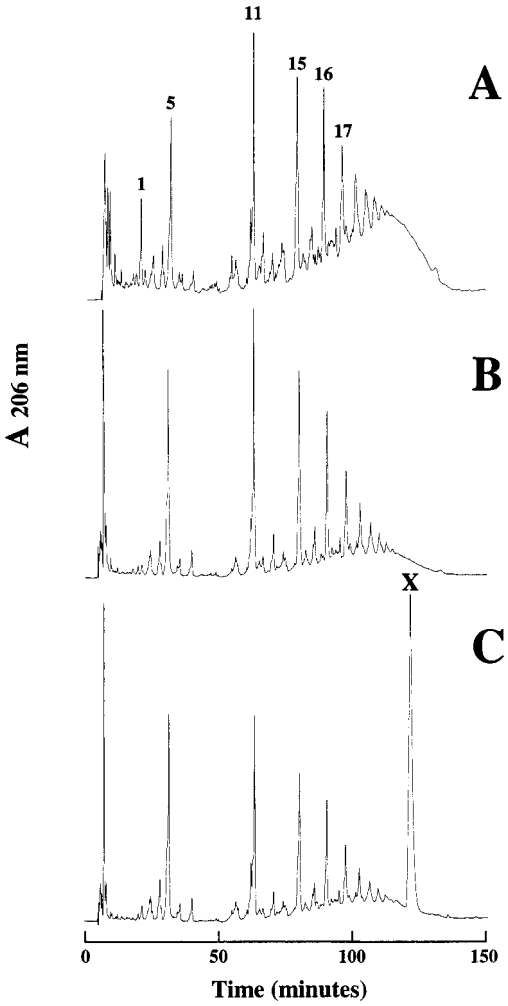

FIG. 6. Analysis of peptidoglycan prepared from *S. aureus* COL, VM, and VM grown in the presence of vancomycin. Cell wall peptidoglycans were prepared from strains COL (A) and VM (B) and from VM grown in the presence of 50 μg of vancomycin per ml (C) and analyzed by HPLC, as described in Materials and Methods. Numbers identify major muropeptide components (9). Component X is a degradation product of vancomycin (see the text). A 206 nm, absorbance at 206 nm.

nyl-D-alanine carboxy termini capable of binding vancomycin has increased substantially in the mutant peptidoglycan. For instance, the percentage of the monomeric (peptides) and dimeric (peptides) muropeptides carrying free D-Ala-D-Ala termini increased in strain VM over their representation in strain COL, from 3.1 to 7.4% (peak 5) and from 5.6 to 11.2% (peak 11), respectively. Nevertheless, this compositional shift does not correlate well with the large difference in the vancomycin MICs for the strains, since the amounts of drug bound by parental and mutant peptidoglycans differed only marginally (Table 3). In fact, most of the increased (2.5-fold) binding capacity of mutant cell walls was lost upon treatment with hydrofluoric acid or mutanolysin, suggesting that the relevant difference between mutant and parental walls may include either the teichoic acid and/or the secondary structure of the peptidoglycan.

Such organizational changes in the cell wall material structure, together with the increase in free D-Ala-D-Ala residues, may form a barrier to the penetration of vancomycin molecules to sites of cell wall synthesis. This model is similar to models

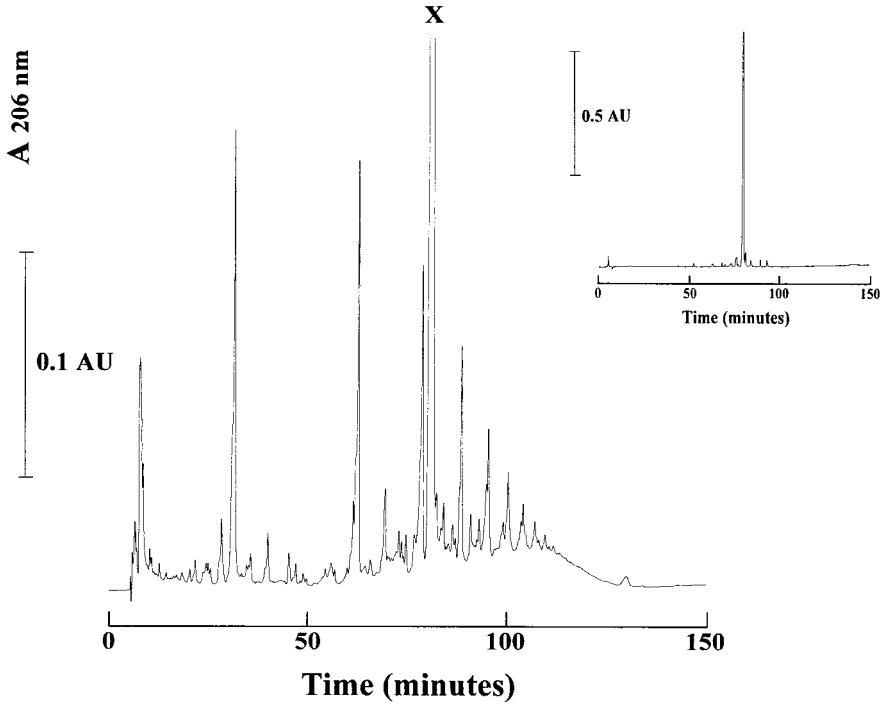

FIG. 7. Cell wall HPLC profile of mutant VM grown in the presence of vancomycin. Mutant VM grown in the presence of 50 μg of vancomycin per ml was harvested, and cell walls (purified as described in Materials and Methods) were hydrolyzed by mutanolysin without prior removal of teichoic acid. The hydrolysate was passed through the same reverse-phase HPLC separation as used for the analysis of peptidoglycan. The inset shows the elution profile of pure vancomycin on the same HPLC column. A 206 nm, absorbance at 206 nm; AU, absorbance units.

predicted by Reynolds in 1989 (28), i.e., protection of wall synthetic sites through "drug capture" at the periphery of the target cell.

Several of our observations suggest that the efficacy of such a resistance mechanism may improve when vancomycin is added to the growth medium of the mutant cultures. Virtually immediately after addition of the antibiotic, cell wall turnover came to a halt, resulting in the retention of cell wall material at the outer surface of the bacterium and inhibition of the separation of daughter cells at the end of cell division. We envision that these phenomena progressively contribute to the deposition of increasing amounts of cell wall material capable of entrapping vancomycin at the periphery of the cells and thus hindering or preventing access of the drug molecules to the cell wall synthesis sites close to the plasma membrane. It is conceivable that the initial drop in the rate of peptidoglycan incorporation observed immediately upon the addition of vancomycin to the culture medium of the resistant mutant represents the initial relative inefficiency of this drug capture mechanism. The subsequent slower peptidoglycan incorporation (adjusted for the decreased rate of bacterial growth) may be the result of the activation of a regulatory circuit that slows cell wall synthesis to compensate for the lack of cell wall turnover in the bacteria.

Some of the properties of the highly vancomycin-resistant *S. aureus* mutant VM described in this communication have also been seen in other bacteria, such as binding of vancomycin to bacterial cell walls (1, 4, 24, 27), abnormal morphology and the appearance of amorphous material on the surface of glycopeptide-treated bacteria (29, 41), and the capacity of some low-level vancomycin-resistant laboratory mutants and/or clinical isolates of coagulase-negative staphylococci to remove vancomycin from the growth medium (25, 30). Whether the mechanism of resistance of these low-level vancomycin-resistant staphylococci is similar to the mechanism we propose for the highly vancomycin-resistant *S. aureus* described in this communication remains to be tested.

Similarly to observations described for some laboratory or clinical isolates of moderately glycopeptide-resistant mutants of various coagulase-negative staphylococci and *S. aureus* (6, 22–25, 34), the highly vancomycin-resistant mutant of *S. aureus*, VM, also showed changes in several protein bands when analyzed by SDS-PAGE. Some of these proteins showed increased amounts in the mutant (e.g., two proteins with the highest molecular masses of 130 and 110 kDa and the proteins in the molecular mass range of 31.5, 29, 21, 18, and 17.5 kDa), while some others appeared to have decreased representation

TABLE 3. Vancomycin binding by cell walls and wall fractions prepared from parental and mutant cells

Strain	Amt of vancomycin (μg) bound or neutralized[a] by cell fraction:		
	Intact cell walls[b]	Peptidoglycan[c]	Mutanolysin hydrolysate of intact cell walls[d]
Parental strain COL	235	190	112
Resistant mutant VM	500	235	120

[a] Vancomycin was titrated in the supernatants of cell wall or peptidoglycan or in the mutanolysin hydrolysate by bioassay, as described in Materials and Methods.

[b] Purified cell walls were used at 0.2 mg/ml, and all data are expressed as the amounts of vancomycin bound per milligram of cell wall.

[c] Peptidoglycan equivalent of 1 mg of cell wall.

[d] Vancomycin neutralized by the mutanolysin hydrolysate of 1 mg of cell walls.

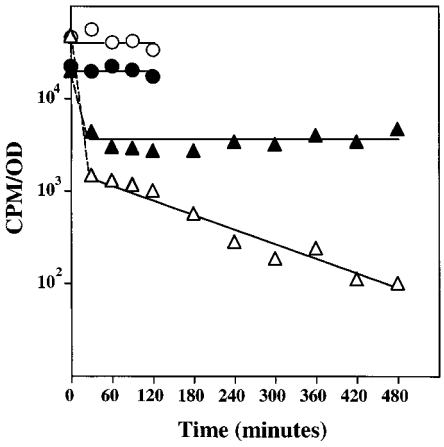

FIG. 8. Effect of vancomycin on the rate of peptidoglycan synthesis. Cultures of parental strain COL and mutant VM were grown in TSB. Optical densities were recorded, and the rate of incorporation of [^{3}H]GlcNAc into the cell wall material during 5-min pulses was determined as described in Materials and Methods. The specific rates of incorporation (the counts per minute of radioactive label associated with the cell wall divided by OD) are plotted as a function of time. Specific rates of peptidoglycan synthesis in COL (○) or VM (●) grown in the absence of antibiotic are shown. The addition of vancomycin at 0 h resulted in a rapid decline in the specific rate of incorporation in mutant VM, which then became adjusted to a new steady-state rate (▲). The addition of vancomycin to a culture of parental strain COL resulted in a rapid initial drop followed by progressive decline in the specific rates of incorporation (△). The turbidity of the culture of strain COL remained constant after the addition of vancomycin.

in the mutant (e.g., the protein at the bottom of the gel with a molecular mass of about 16 kDa). Growth in the presence of vancomycin caused decrease in the amounts of some of these proteins and increase in some others (Fig. 9). In view of the mechanism we propose for this particular form of resistance, it

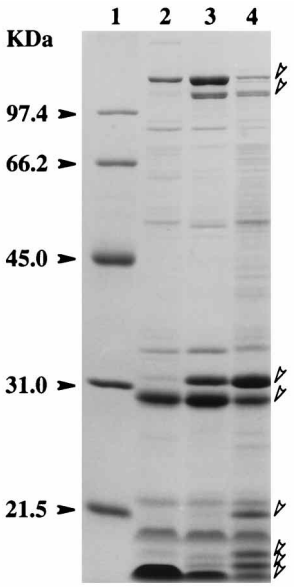

FIG. 9. SDS-PAGE analysis of protein extracts from *S. aureus* COL and mutant VM. SDS extracts prepared from COL, VM, and VM grown in the presence of vancomycin were analyzed by SDS-PAGE, as described in Materials and Methods. Lane 1 contains molecular size markers. The next three lanes contain extracts of strain COL (lane 2), mutant VM (lane 3), and mutant VM grown in the presence of vancomycin (lane 4). Arrowheads indicate bands with altered intensity in the mutant.

is possible that the increased protein bands are related to the overproduction of polypeptides involved with cell wall biosynthesis or its regulation. The biochemical nature of these proteins and their relationship to the mechanism of vancomycin resistance remains to be determined.

One of the striking phenotypes of the vancomycin-resistant mutant VM was the reduction of β-lactam resistance in parallel with the increasing MICs of the glycopeptides. The mechanism of this "see-saw" effect and the altered MICs of some other cell wall inhibitors are not known. It is possible that the profound disturbance of cell wall metabolism associated with the vancomycin resistance of this *S. aureus* mutant creates conditions analogous to those observed in some of the auxiliary mutants of MRSA in which quantitative and/or qualitative distortions in the cell wall precursor pool appear to be responsible for the suppression of the methicillin resistance level of the bacteria (11, 26).

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