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Rapid Depletion of Free Vancomycin in Medium in the Presence of β-Lactam Antibiotics and Growth Restoration in *Staphylococcus aureus* Strains with β-Lactam-Induced Vancomycin Resistance▽

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A class of methicillin-resistant *Staphylococcus aureus* strains shows vancomycin resistance in the presence of β-lactam antibiotics (β-lactam-induced VAN-resistant methicillin-resistant *S. aureus* [BIVR]). Two possible explanations may be offered: (i) vancomycin in culture medium is depleted, and (ii) the D-Ala–D-Ala terminal of the peptidoglycan network is replaced with D-Ala–D-lactate. We tested these hypotheses by quantifying free vancomycin in the medium through the course of cell growth and by PCR amplification of the *van* genes. Growth of the BIVR cells to an absorption level of ~0.3 at 578 nm required about 24 h in the presence of vancomycin alone at the MIC (4.0 μg/ml). However, growth was achieved in only about 10 h when 1/1,000 to 1/2,000 the MIC of β-lactam antibiotic was added 2 h prior to the addition of vancomycin, suggesting that the β-lactams shortened the time to recovery from vancomycin-mediated growth inhibition. Free vancomycin in the culture medium decreased to 2.3 μg/ml in the first 8 h in the culture containing vancomycin alone, yet cell growth was undetectable. When the vancomycin concentration dropped below ~1.5 μg/ml at 24 h, the cells began to grow. In the culture supplemented with the β-lactam 2 h prior to the addition of vancomycin, the drug concentration continuously dropped from 4 to 0.5 μg/ml in the first 8 h, and the cells began to grow at a vancomycin concentration of ~1.7 μg/ml or at 4 h of incubation. The gene encoding the enzyme involved in D-Ala–D-lactate synthesis was undetectable. Based on these results, we concluded that BIVR is attributable mainly to a rapid depletion of vancomycin in the medium triggered or promoted by β-lactam antibiotics.

Infection by methicillin-resistant *Staphylococcus aureus* (MRSA) is a serious problem in hospitals and, more recently, in the community as well (6, 28). The isolation frequency of MRSA among *S. aureus* infections is very high in many countries, ranging, for example, from 60 to 80% in Japan (23, 25). A serious problem associated with MRSA infection is that the organism shows resistance to structurally and functionally diverse antibiotics (18, 19). Due to the multiantibiotic-resistant nature of this organism, only limited chemotherapeutic agents can be used, the most preferable of which is vancomycin (VAN) (10). Patients with MRSA infection have often been found to have mixed infection with gram-negative bacteria such as *Pseudomonas aeruginosa* (23). These patients may be treated with a combination of VAN and β-lactam antibiotics like imipenem (IPM) because gram-negative bacteria are intrinsically resistant to VAN. Thus, the combination of VAN and β-lactam antibiotics has been recommended for the therapy of MRSA infection and has been in wide use in Japan for several years (23, 27). In fact, a synergistic effect of these drugs was previously reported (9, 22). However, the combination therapy of VAN and a β-lactam antibiotic caused the emergence of VAN-resistant MRSA (15, 26), designated β-lactam-induced VAN-resistant MRSA (BIVR) (12, 14). Currently, about 20% of MRSA strains isolated from septicemia patients have been reported to be BIVR (13). A stereochemical specificity of β-lactam antibiotics for the BIVR induction was not found, as 46 species of β-lactam antibiotics have been tested so far (13).

The mode of VAN action is understood to be that glycopeptide VAN specifically binds to the un-cross-linked D-Ala–D-Ala terminal of the peptidoglycan network and peptidoglycan precursor, consequently blocking access of peptidoglycan transpeptidases or penicillin binding proteins (1, 20, 21). Several classes of VAN-resistant bacteria have been reported. First, a particular type of *Enterococcus* produces the VanA, VanB, and VanD enzymes, which are involved in the synthesis of D-Ala–D-lactate in place of D-Ala–D-Ala (5), preventing the access of VAN. Three cases of *vanA*-positive MRSA have been reported (2–4), and some more have been reported in meeting abstracts. Proteins encoded by *vanC*, *vanE*, and *vanG* are involved in the synthesis of D-Ala–D-Ser and were found only in *Enterococcus* species (5). Second, a fraction of MRSA strains showed different degrees of VAN resistance, such as strain Mu50 (designated as vancomycin-intermediate-resistant *S. aureus*), which showed elevated peptidoglycan synthesis and a thickened cell wall (11). Accordingly, it was explained that the thickened peptidoglycan layers trap the free VAN that is clog-

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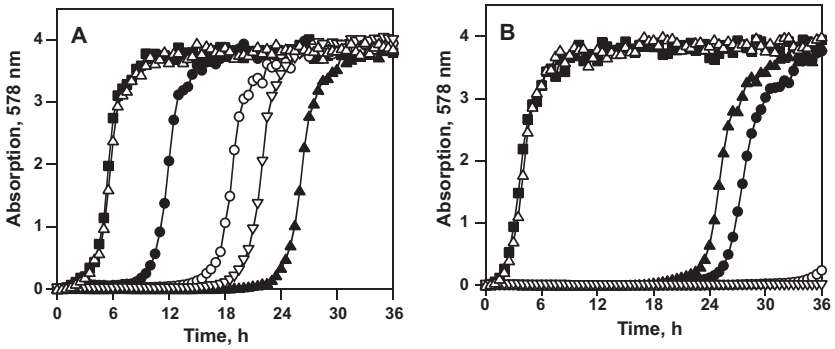

FIG. 1. Effect of β-lactam antibiotic on the growth of BIVR and non-BIVR cells in the presence and absence of VAN. A preculture grown overnight was diluted with fresh BHIB, adjusting the cell number to 10^7 CFU/ml (A_{578} = 0.03), and the growth of the cells was monitored spectrophotometrically at the A_{578} at 30-min intervals. (A) BIVR cells, strain K744. (B) Non-BIVR MRSA cells, strain S602. BHIB was supplemented with either 4 μg/ml of VAN, 1 μg/ml of ZOX, or 4 μg/ml of VAN plus 1 μg/ml of ZOX. ■, drug free; △, ZOX was added at time zero; ●, ZOX was added at time zero and VAN was added at 2 h of incubation; ○, both ZOX and VAN were added at time zero; ▽, VAN was added at time zero and ZOX was added at 2 h of incubation; ▲, VAN was added at time zero.

ging the peptidoglycan mesh and consequently blocking the penetration of VAN through peptidoglycan layers into the cell (7, 8). However, the vancomycin-intermediate-resistant *S. aureus*-type VAN resistance is irrelevant to β-lactam antibiotics. We hypothesized that VAN resistance in BIVR cells is likely to be attributable to a rapid depletion of free VAN from the culture medium that was somehow promoted or triggered by β-lactam antibiotics. Thus, we quantified free VAN in the medium through the course of cell growth in the presence and absence of β-lactam antibiotics and found that the decrease in free VAN was linked to growth restoration.

MATERIALS AND METHODS

Bacterial strain, culture medium, and antimicrobial agents. BIVR and non-BIVR MRSA strains were isolated from clinical materials according to a method described elsewhere previously using Mu3 agar (Nippon Becton-Dickinson, Tokyo, Japan) (13). Among these, a representative BIVR strain, K744, showed stable VAN resistance in the presence of 1 μg/ml of ceftizoxime (ZOX). A representative non-BIVR MRSA strain used in the experiment was strain S602. The MIC of VAN for both K744 and S602 was 1 μg/ml in the ZOX-free medium as determined by the CLSI-recommended agar dilution method. The MICs of ZOX for K744 and S602 were 4,096 and 2,048 μg/ml, respectively, and those of IPM were 128 and 32 μg/ml, respectively. The MIC of VAN in the presence of 1 μg/ml of ZOX in both K744 and S602 was 4 μg/ml, as determined by the broth dilution method, in brain heart infusion broth (BHIB) (Nippon Becton-Dickinson, Tokyo, Japan), conditions identical to those of the growth experiment (see below). The culture medium used throughout this study was BHIB. VAN, ZOX, and IPM were purchased from Sigma-Aldrich, Astellas Pharma Inc. (Tokyo, Japan), and Merck Ltd. (Tokyo, Japan), respectively.

Growth assay in the presence of antibiotics. Cells were grown in BHIB at 35°C for 18 h, and the absorption at 578 nm was adjusted to 0.03 (about 10^7 CFU/ml). The culture medium was supplemented with the following concentrations of antibiotics: (i) 4 μg/ml of VAN, (ii) 1 μg/ml of ZOX, and (iii) 4 μg/ml of VAN plus 1 μg/ml of ZOX. The tubes were aerated by shaking at 20 strokes at 35°C, and the cell density was monitored every 30 min by use of a Bio-Photorecorder (Advantec, Japan) at 578 nm.

Quantitative determination of VAN. The amount of free VAN in the culture medium was quantified by the newly developed competitive enzyme-linked immunosorbent assay method. Briefly, wells of a 96-well microtiter plate were coated with 0.075 mg of VAN conjugated with bovine serum albumin (BSA) in 10 mM sodium carbonate buffer (pH 9.3) overnight. Unbound VAN-BSA conjugate was removed. Wells were treated with the blocking buffer containing 0.5% BSA–10 mM sodium carbonate buffer (pH 9.3) at 24°C for 60 min. After removal of the blocking buffer, 25 μl of the sample to be tested (diluted with half-strength BHIB with buffer consisting of 50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$, 0.1% Triton X-100 [pH 7.0], and 1% of BSA) and 25 μl of anti-VAN antibody (Kanto Chemical Co., Tokyo, Japan) were added sequentially. The plate was incubated at 24°C for 60 min and washed four times with wash buffer containing 50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$, and 0.1% Triton X-100 (pH 7.0), after which 50 μl of biotinylated goat anti-mouse immunoglobulin G antibody (Vector Laboratories UK) was added, and the solution was incubated at 24°C for 60 min. The plate was washed four times with wash buffer, 50 μl of streptavidin-horseradish peroxidase conjugate (GE Healthcare, United Kingdom) was added, and the solution was incubated at 24°C for 60 min. The plate was washed four times with wash buffer. The complex consisting of VAN, antibodies, streptavidin, and horseradish peroxidase was developed by adding 50 μl of the TMB Microwell peroxidase substrate system (Kirkegaard & Perry Laboratories, Inc.), and the reaction was terminated by adding 50 μl of 1.0 M phosphoric acid to the mixture. The absorption at 450 nm was recorded with a plate reader (EL808 Ultra microplate reader; Biotek Instruments), and the absorption at 630 nm (background) was then subtracted. A calibration curve was drawn using reagent-grade VAN, and the curve appeared linear in a range of 30 to 300 ng VAN/ml. The correlation index between the absorption (450 to 630 nm) and the VAN concentration appeared to be 0.986.

Electron microscopy. Cells in the desired growth phase were harvested by centrifugation at 8,000 × *g* for 15 min, and the pellet was washed three times with 50 mM phosphate buffer (pH 7.2). The cells were fixed with 1% of glutaraldehyde at 4°C overnight, dehydrated with gradient concentrations of ethanol, and embedded in EPOK812 (Ohken, Tokyo, Japan). Ultrathin sections were stained with uranyl acetate, lead acetate, and lead citrate consecutively and then examined under a TEM-1200EX transmission electron microscope (Jeol, Tokyo, Japan).

RESULTS

Effect of β-lactam antibiotics on growth restoration of BIVR and non-BIVR MRSA isolates from VAN-mediated growth inhibition. BIVR cells were inoculated in BHIB after adjusting the absorption at 578 nm to ~0.03. The cells entered early log phase (absorption ~0.3) after 3.5 h of incubation in medium free of antibiotics and medium containing 1 μg/ml of ZOX (Fig. 1A). Once the cell density reached the absorption level of ~0.3, the cells continued to grow exponentially. When 4 μg/ml of VAN was present, the cell density reached ~0.3 in about 24 h, indicating that cell growth was largely retarded in the presence of VAN. When ZOX was added at −2, 0, and +2 h of incubation relative to 4 μg/ml of VAN, the absorption at 578 nm reached ~0.3 at 10, 16.5, and 19.5 h, indicating that advanced β-lactam treatment significantly shortened the time of recovery from VAN-mediated growth inhibition (Fig. 1A).

In the control experiment, non-BIVR cells were tested for

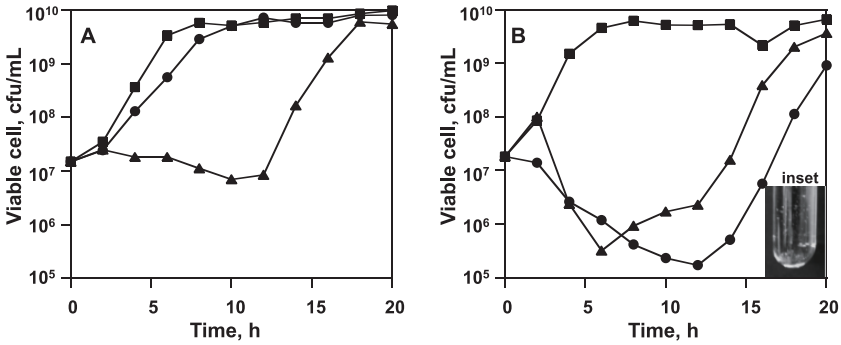

FIG. 2. Viability of BIVR and non-BIVR cells in the presence and absence of VAN and ZOX. A cell suspension of strains K744 and S602 was prepared as described in the legend of Fig. 1, adjusting the viable number to about 10^7 CFU/ml, and incubated in the presence or absence of antibiotics. (A) BIVR cells, strain K744. (B) Non-BIVR MRSA cells, strain S602. The culture was supplemented with either 4 μg/ml of VAN or 4 μg/ml of VAN plus 1 μg/ml of ZOX. A fraction of the cell suspension was withdrawn every 2 h, diluted, and plated onto agar plates, and a viable cell number was counted as CFU/ml after 24 h of incubation at 37°C. ■, drug free; ●, ZOX was added at time zero and VAN was added at 2 h of incubation; ▲, VAN was added at 2 h of incubation. (Inset) Photography of the culture containing VAN at 6 h of incubation.

the effect of β-lactam antibiotics. In the presence of 1 μg/ml of ZOX, the cells began to grow to an absorption level of ~0.3 after about 2 h of incubation, and the curve was nearly superimposable with that for drug-free culture (Fig. 1B). In medium containing 4 μg/ml of VAN alone, cells grew to a density of ~0.3 at 23 h, which was consistent with the effect of VAN on BIVR cells. As 1 μg/ml of ZOX was added at −2 h and time zero relative to 4 μg/ml of VAN, the cells began to grow to the absorption level of ~0.3 at 25.5 and 36.5 h, respectively. When ZOX was added at +2 h, cell growth was undetectable even at 70 h of incubation (Fig. 1B). These results clearly demonstrated that a combination of VAN and ZOX exerted a strong synergistic effect on the growth inhibition of non-BIVR MRSA, in marked contrast to the effect of VAN plus ZOX on BIVR cells.

Viability of BIVR cells in the presence of VAN and ZOX. Turbidity measurements showed that the presence of both VAN and ZOX caused early growth restoration of the BIVR K744 cells compared with that in the presence of VAN only. However, this early growth restoration does not necessarily mean that the viable cell number had increased. Thus, we carried out the viable cell count experiment under conditions similar to those of the growth experiments shown in Fig. 1. In the drug-free culture of K744, the cell number increased exponentially from ~10^7 CFU/ml to 3 × 10^9 CFU/ml in the first 5 h, and thereafter, the rate of increase slowed down, reaching ~10^{10} CFU/ml at ~20 h of incubation (Fig. 2A). When 4 μg/ml of VAN was added at 2 h of incubation, the viable number stayed at ~10^7 CFU/ml for about 12 h and then entered the exponential growth phase, reaching 10^{10} CFU/ml at 20 h (Fig. 2A). In contrast, the growth curve entered the exponential phase soon after 2 h of incubation when ZOX was added at time zero, followed by VAN at 2 h of incubation, reaching the stationary phase at about 10 h (Fig. 2A). The trace of this curve was close to that of BIVR cells in the drug-free culture except that the curve lagged about 2 h behind that of the drug-free culture. These results are in good agreement with the turbidity measurement shown in Fig. 1A. Note that there is a time gap between the curves of viable number and turbidity due mainly to the fact that turbidity measurements at low cell numbers cannot be as accurate as the viable count.

When non-BIVR S602 cells were subjected to a similar ex-

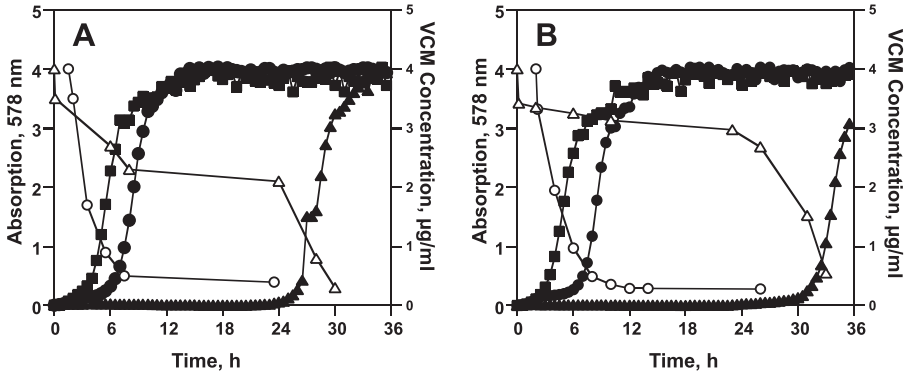

FIG. 3. Quantitative determination of VAN in the culture of BIVR cells in the presence and absence of β-lactam antibiotic. The BIVR cells, strain K744, were diluted with fresh BHIB as described in the legend of Fig. 1, and growth was monitored at A_{578}. Free VAN in the culture medium was quantified along the course of incubation by the competitive enzyme-linked immunosorbent assay method. The culture was supplemented with 4 μg/ml of VAN, 4 μg/ml of VAN plus 1 μg/ml of ZOX, or 4 μg/ml of VAN plus 0.003 μg/ml of IPM. (A) ZOX plus VAN. (B) IPM plus VAN. Closed and open symbols represent the absorption at 578 nm and VAN concentration, respectively. ■, drug free; ● and ○, ZOX or IPM was added at time zero and VAN was added at 2 h of incubation; ▲ and △, VAN was added at time zero.

periment, viable cells in the culture containing ZOX and VAN showed largely delayed growth restoration (Fig. 2B), confirming the result of the turbidity measurement experiment. The culture containing VAN only showed growth restoration about 3 h earlier than the culture containing ZOX plus VAN (Fig. 2B). This is again consistent with the result of the turbidity measurement experiment. A large difference in the VAN-containing cultures of S602 cells compared to K744 cells is that the apparent viable number decreased roughly 100 times at 6 to 12 h. This apparent viability drop is due mainly to the fact that S602 cells tend to aggregate in the presence of VAN, forming large clumps visible by the naked eye (Fig. 2B, inset). The reason for this aggregation is not known. Therefore, the drop of the curves is unlikely to be due to an actual decrease in the number of viable cells.

Quantitative determination of free VAN along the course of cell growth. In another set of experiments, the effects of VAN only and VAN plus ZOX on the growth of K744 cells were monitored. As shown in Fig. 3A, turbidity in the culture containing 4 μg/ml of VAN stayed undetectably low for the first 24 h, confirming the above-described results. The turbidity reached ~0.3 at 26 h and thereafter quickly entered the exponential growth phase. The concentration of VAN in the same culture dropped from 4 to 3.5 μg/ml immediately after VAN was added, suggesting that free VAN was trapped by the preexisting cells and then gradually decreased to ~2.1 μg/ml in the first 24 h. Growth of the cells measured by a turbidity meter was undetectable up to this point. Subsequently, the VAN concentration quickly dropped to 0.8 and 0.3 μg/ml at 28 and 30 h, respectively, as the cells grew to absorption levels of 1.60 and 3.24, respectively. These results clearly demonstrated that the growth restoration of BIVR cells was tightly coupled with the decrease in the free-VAN concentration below the critical level of about 1.5 μg/ml (Fig. 3A). The point of intersection of the absorption and the VAN concentration curves appeared to be 27 h of incubation.

When 1 μg/ml of ZOX was added 2 h prior to the addition of 4 μg/ml of VAN, the turbidity stayed below 0.1 for the first 2 h and thereafter gradually increased to 0.139, 0.327, and 1.44 at 4, 6, and 8 h, respectively. The concentration of free VAN in the medium consistently dropped from 4 μg/ml at time zero to 3.5, 2.7, 1.7, 0.9, and 0.5 μg/ml at absorption levels of 0.06, 0.062, 0.139, 0.327, and 1.44 or that in time scale immediately after, at 2.0, 4.0, 6.0, and 8.0 h, respectively, after the addition of VAN (Fig. 3A). Again, the cells began to grow at a VAN concentration below about 1 μg/ml. The times needed to reach the absorption level of ~0.3 were 6 h in the presence of ZOX plus VAN and 26 h in the presence of VAN only. The point of intersection of the absorption curve at 578 nm and the VAN concentration curve appeared to be 7 h of incubation in the presence of both 4 μg/ml of VAN and 1 μg/ml of ZOX. These results again showed that the BIVR cells began to grow as free VAN in the medium dropped below the threshold concentration of around 1.0 to 1.5 μg/ml.

Similar experiments were carried out in the presence of 4 μg/ml of VAN and 0.003 μg/ml of IPM or 1 μg/ml of piperacillin to test whether or not other β-lactam antibiotics also exert the same effect as ZOX. The result clearly demonstrated that these β-lactams caused a rapid depletion of VAN and early growth restoration (Fig. 3B; data for piperacillin are not shown). Thus, the β-lactam antibiotic-mediated rapid depletion of VAN and early growth restoration of BIVR are not unique to a VAN and ZOX combination but are a general phenomenon associated with VAN plus β-lactam antibiotics.

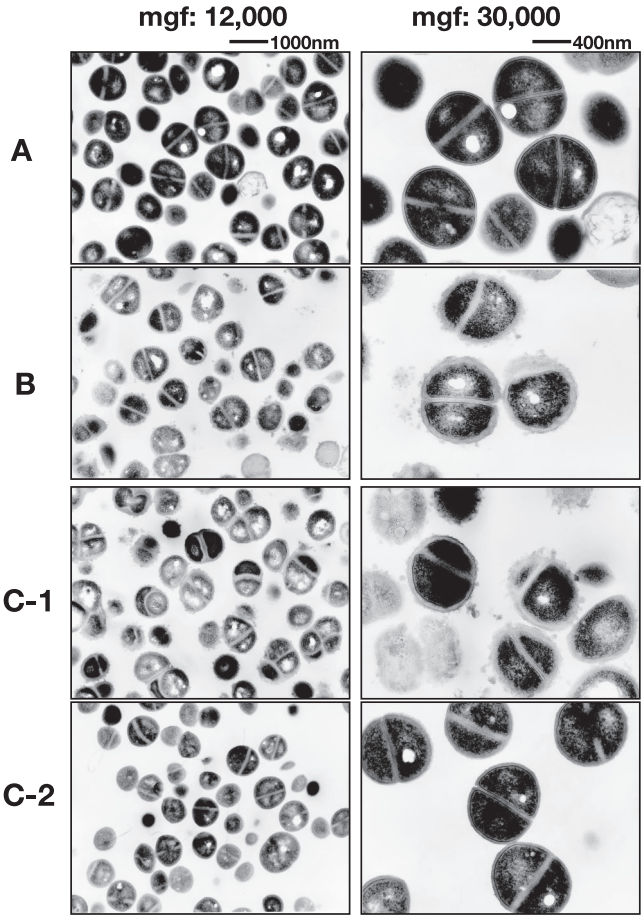

FIG. 4. Electron micrographs of BIVR cells at various stages of cell growth in the presence of antibiotics. A thin section of the BIVR cell line, strain K744, was prepared as described above and was observed under an electron microscope. Magnifications were 12,000- and 30,000-fold. The bar is a measure of the magnification. Growth conditions and sampling times are as follows. (A) Drug-free culture at an A_{578} of ~0.2; (B) 4 μg/ml of VAN at an A_{578} of ~0.2; (C-1) 4 μg/ml of VAN and 1 μg/ml of ZOX at an A_{578} of ~0.2; (C-2) 4 μg/ml of VAN and 1 μg/ml of ZOX at an A_{578} of ~1.3.

Electron micrograph of BIVR cells in the presence and absence of antibiotics. The above-described data showed that growth inhibition caused by VAN was canceled as the VAN concentration in the medium dropped below a critical level, and thereafter, the cells grew as if they were in a drug-free medium. The presence of β-lactam antibiotics seemed to shorten the time needed for the depletion of VAN in the medium. This finding led to the question of what happened to the cells whose growth was inhibited by VAN and then recovered from VAN-mediated growth inhibition. We observed the morphological appearance of the cell surface in thin sections by electron microscopy.

First of all, we observed the surface structure of K744 cells grown in drug-free culture at absorption levels of ~0.2 (Fig. 4A) and ~1.8. The surface of the BIVR cells under these

conditions showed the typical *S. aureus* cell wall with a tightly packed and condensed structure and a thickness of about 25 nm. The structure in the latent-period cells was indistinguishable from that in late-log-phase cells. K744 cells in the presence of 4 μg/ml of VAN at an absorption level of ~0.2 (Fig. 4B) showed a swollen and thickened surface structure with a thickness of about 40 nm. In addition, the cell surface appeared to be rough and irregular. The same batch of cells grown to an absorption level of ~1.5 to 2 showed a tightly and densely packed cell wall and a smooth surface that was fully comparable with the structure of the cells in the drug-free culture shown in Fig. 4A.

Cells in the presence of both 1 μg/ml of ZOX and 4 μg/ml of VAN at the absorption level of ~0.2 showed a thickened and irregular cell surface (Fig. 4C-1) that was comparable with the structure of the cells in 4 μg/ml of VAN alone at an absorption level of ~0.2. The cell wall and surface structure of such cells grown to an absorption level of ~1.3 were indistinguishable from those of cells grown in the drug-free culture (Fig. 4C-2). Cells in the culture containing 1 μg/ml of ZOX showed a structure indistinguishable from that in the drug-free culture regardless of cell density or sampling time (data not shown). These results suggested that the cell wall of BIVR cells under VAN-mediated growth inhibition became thickened and irregular. ZOX itself exerted no noticeable change in the morphological appearance of the BIVR cells and led only to a shortened time to recovery from VAN inhibition.

DISCUSSION

Hospital patients whose immune activity is lowered for some reason often acquire a mixed infection of MRSA and gram-negative bacteria such as *P. aeruginosa* (23). Antibiotics available for the treatment of such patients may be a combination of VAN and β-lactam antibiotics. We reported recently that VAN-resistant MRSA emerged as a result of the simultaneous use of VAN and β-lactam antibiotics (15, 26). A hospital outbreak of BIVR infection was reported previously (16). However, it is not known whether or not the mechanism of VAN resistance in the BIVR cells is similar to those of previously reported VAN-resistant MRSA isolates such as Mu50. In this study, we investigated this challenging topic by the quantitative determination of free VAN in the medium throughout the course of cell growth.

BIVR cells recovered from VAN-mediated growth inhibition in the presence of β-lactam antibiotics much earlier than in medium containing VAN alone. We found that growth restoration monitored by turbidity measurement was essentially consistent with that measured by the viable cell count. It must be stressed that the non-BIVR cells never showed earlier growth recovery in the presence of both VAN and ZOX than in the culture containing VAN only. Quantitative determination of VAN revealed that the growth of BIVR cells in the presence of VAN was inhibited when the VAN concentration in the medium was higher than ~2 μg/ml, and cell growth resumed when the medium VAN concentration decreased below a critical level of about 1.0 to 1.5 μg/ml. The β-lactam antibiotics promoted the depletion of free VAN and therefore led to a shortened time of BIVR cell recovery from VAN-mediated growth inhibition.

Several mechanisms are conceivable for the VAN resistance of BIVR. First, the BIVR cells produce enzymes that replace D-Ala–D-Ala with D-Ala–D-lactate as reported previously for MRSA (5). Second, the BIVR cells had thickened peptidoglycan layers that trapped VAN from the medium into the cell wall layers and blocked the passage of VAN, as reported previously for Mu50 cells (7, 8). Third, BIVR cells deplete free VAN from the medium in the presence of β-lactam antibiotics, causing rapid growth restoration from VAN-mediated inhibition. We verified these possibilities. The first possibility was ruled out, since the gene *vanA* or *vanB*, encoding the enzyme involved in D-Ala–D-lactate synthesis, was undetectable in BIVR cells when examined by PCR, and VAN binding to D-Ala–D-lactate was much weaker than binding to D-Ala–D-Ala. The second possibility was tested by taking electron micrographs of the BIVR cells under VAN-induced growth inhibition and those that had recovered from VAN inhibition. We found that the cell surface of nongrowing BIVR cells was thickened and appeared irregular, as reported previously for Mu50 cells (11). The result was consistent with a recent observation that non-BIVR MRSA isolates showed a thickened and irregular cell wall structure when the cells were under the influence of VAN and nongrowth conditions (24). Therefore, the appearance of a thickened and irregular cell surface structure under the influence of VAN is a general phenomenon in *S. aureus* and is not unique to BIVR cells. Accordingly, β-lactam antibiotic is unrelated to the VAN-mediated cell wall thickening and irregularity. The third possibility, that the rapid depletion of VAN from the BIVR growth environment, which is promoted by β-lactam antibiotics, is the most likely mechanism for VAN resistance in BIVR cells as presented in this study. This type of growth restoration was much faster than that in the absence of β-lactam antibiotics. This finding is consistent with the fact that BIVR cells are resistant to VAN in the simultaneous presence of β-lactam antibiotics. The role of β-lactam antibiotics in the VAN resistance of BIVR is likely to be the promotion of cell wall metabolism by some means producing an elevated level of VAN binding sites. However, cell wall metabolism in gram-positive bacteria including *S. aureus* is poorly understood.

The mechanism by which β-lactam antibiotics promote the depletion of VAN remains to be elucidated. However, the following discussion may assist in an understanding of the mechanism of BIVR. Jacobs previously reported that β-lactam antibiotics damage the peptidoglycan network, generating a large amount of peptidoglycan fragments, which are actively transported into the cell for recycling (17). This cascade reaction induces β-lactamase in gram-negative bacteria. In an analogy to this episode, it is likely that peptidoglycan recycling and signal transduction systems operate in gram-positive bacteria, including *S. aureus*, which stimulates the synthesis of nascent peptidoglycan. Although large amounts of peptidoglycan precursors may be exported onto and accumulated on the external surface of the cytoplasmic membrane, these precursors cannot be cross-linked to preexisting peptidoglycans because the D-Ala–D-Ala terminals of the peptidoglycan network are already occupied by VAN, and consequently, they entrap free VAN in the environment. Eventually, the VAN concentration in the BIVR growth environment may decrease to below the threshold concentration, and the BIVR cells resume growth.

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