ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2007, p. 3627–3633 Vol. 51, No. 10
0066-4804/07/$08.00+0 doi:10.1128/AAC.00431-07

Fluorescence Ratio Imaging Microscopy Shows Decreased Access of Vancomycin to Cell Wall Synthetic Sites in Vancomycin-Resistant *Staphylococcus aureus*[▽]

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Received 28 March 2007/Returned for modification 10 June 2007/Accepted 17 July 2007

A new method of fluorescence ratio imaging microscopy was used to compare the in vivo binding capacity and the access of a fluorescent derivative of vancomycin to the cell wall synthetic sites in isogenic pairs of vancomycin-susceptible and -resistant laboratory mutants and vancomycin-intermediate and -susceptible clinical isolates of *Staphylococcus aureus*. Live cells of resistant strains were found to bind approximately 1.5 times more antibiotic, but there was no correlation between the increased binding capacity and the MICs of the strains. In both susceptible and resistant bacteria, the subcellular sites of wall synthesis were localized to the division septa, but the rate of diffusion of drug molecules to these sites was reduced in resistant cells. The findings allow a reinterpretation of the mechanism of vancomycin resistance in which the path of vancomycin to its lethal target (lipid II) is considered to be through the division septum and therefore is dependent on the stage of the staphylococcal cell cycle.

Vancomycin is one of the few antibiotics that have remained effective against methicillin-resistant *Staphylococcus aureus* and its multidrug-resistant clones (19). Methicillin and other beta-lactam antibiotics inhibit cell wall synthesis by binding to and inactivating the penicillin-binding proteins (PBPs), enzymes catalyzing the last stages of peptidoglycan biosynthesis. Vancomycin inhibits cell wall synthesis through a completely different mechanism, by forming stable complexes with the C-terminal D-Ala-D-Ala residues present in the lipid II-linked disaccharide pentapeptide cell wall precursors and in the nascent, un-cross-linked peptidoglycan. This blocks access of PBPs to their substrates, resulting in the inhibition of cell wall biosynthesis (18).

Two different mechanisms of resistance to vancomycin have been identified among clinical isolates of *S. aureus*. In the few highly vancomycin-resistant *S. aureus* strains recently identified in the United States (vancomycin MICs $\geq$ 32 μg/ml) (2, 3), the mechanism of resistance appears to involve the acquisition of the Tn*1546*-linked *vanA* gene complex (29), presumably from a vancomycin-resistant *Enterococcus faecalis*. The *vanA*-associated mechanism of resistance in vancomycin-resistant *E. faecalis* was shown to involve changes in the normal pathway of peptidoglycan synthesis: the production of cell wall precursor molecules in which the C-terminal D-Ala-D-Ala residues are replaced with D-Ala-D-Lac, a depsipeptide which is not recognized by the vancomycin molecule but can be utilized as a substrate for the PBPs (1, 28). Recent studies demonstrated that the gene complex identified in vancomycin-resistant *S. aureus* strains carries the same resistance mechanism (20).

A completely different type of staphylococcal vancomycin resistance was first identified in 1997 in Japan (13) and has since then been described in many different countries (8). These vancomycin-intermediate *Staphylococcus aureus* (VISA) isolates show only a modest decline in vancomycin susceptibility (MIC increases up to 8 to 16 μg/ml) which nevertheless is sufficient to cause complications in therapy, including treatment failure (12, 23).

Although almost a decade has passed since the first report of VISA strains, their mechanism of resistance is still not well understood. Virtually all clinical VISA strains studied have a thickened cell wall (5) which, in some isolates, is associated with an increase in peptidoglycan synthesis (11), while in others it seems to be related to a decrease in cell wall turnover and/or autolysis (23, 24). VISA strains retain cell wall precursors with the normal D-Ala-D-Ala C termini, and the mechanism of reduced susceptibility does not seem to involve acquired genetic determinants but is more reminiscent of the complex resistance mechanisms identified in some vancomycin-resistant laboratory mutants (24, 25). In these mutants, and in the best-studied VISA isolates, decreased susceptibility to vancomycin is associated with various degrees of reduction in the cross-linking of peptidoglycan strands, which leads to an increase in the number of free D-Ala-D-Ala residues in the cell wall (12, 22, 24). In *S. aureus*, the D-Ala-D-Ala residues are maintained in the mature peptidoglycan due to the low carboxypeptidase activity and, therefore, they constitute potential nonlethal binding sites for vancomycin. An increased capacity of cell walls from vancomycin-resistant laboratory mutants to bind vancomycin has been demonstrated (25), and models of vancomycin resistance have been proposed in which the accumulation of such "decoy" targets in the cell wall of resistant bacteria would obstruct the pathway of the antibiotic molecules through the mesh-like cell wall to their lethal target,

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▽ Published ahead of print on 23 July 2007.

located at the sites of cell wall synthesis on the plasma membrane (22, 25). Binding of the large vancomycin molecules to the nonlethal targets would further hinder the progress of antibiotic molecules to their lethal target, the lipid-linked peptidoglycan precursor (12, 22). This "drug capture" (22) or "clogging" phenomenon (12) was proposed to represent an essential feature of the mechanism of decreased vancomycin susceptibility in VISA isolates and laboratory mutants as well (12, 21, 24).

Linking of the numerous altered morphological, physiological, and biochemical changes observed in clinical VISA isolates with the proposed resistance mechanism was hampered by the lack of available isogenic susceptible isolates which could be used for valid comparisons. The recent identification of isogenic vancomycin-susceptible and -resistant (VISA) isolates has provided such pairs of comparable clinical isolates with which differences between the properties of the susceptible and the matching VISA isolates can be explored (14, 21, 24).

In this paper we describe the use of a new method, fluorescence ratio imaging microscopy, to compare the in vivo vancomycin binding capacity, the localization of the subcellular sites of cell wall biosynthesis, and the access of vancomycin to its lethal target at the cell membrane, in live cells of vancomycin-susceptible and -resistant *S. aureus*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The following bacterial strains were used in this study: *S. aureus* reference strain NCTC8325-4 (R. Novick), vancomycin susceptible (vancomycin MIC, 1 μg/ml); two isogenic pairs of vancomycin-resistant and -susceptible strains, laboratory mutant VM50 (MIC, 100 μg/ml) and its parental strain, COL (MIC, 3 μg/ml) (25); clinical isolate JH9 (MIC, 8 μg/ml) and its parental strain, JH1 (MIC, 1 μg/ml) (21). All *S. aureus* strains were grown in tryptic soy broth (TSB; Difco), at 37°C with aeration. Growth was followed by monitoring the optical density at 600 nm (OD_{600}).

Analysis of cell wall composition. *S. aureus* NCTC8325-4 cells were grown for 16 h in TSB. Cultures were diluted to an OD_{600} of 0.0025 and allowed to grow for approximately eight generations in fresh TSB supplemented with different concentrations of D-serine (0, 0.003, 0.006, 0.009, 0.0125, 0.03, 0.06, and 0.125 M). Cells were harvested, and peptidoglycan was purified as previously described (6). The muropeptide composition of peptidoglycan was analyzed by high-performance liquid chromatography (HPLC).

Quantification of vancomycin binding by fluorescence microscopy. *S. aureus* NCTC8325-4 cells were grown for 16 h in TSB containing different concentrations of D-serine (0, 0.003, 0.006, 0.009, 0.0125, 0.03, 0.06, and 0.125 M), diluted in the same medium and grown to mid-exponential phase (OD_{600}, 0.7). Cultures were labeled with a 1:1 mixture of vancomycin (Sigma) and the fluorescent BODIPY FL conjugate of vancomycin (VanFL; Molecular Probes), at a final concentration of 1 μg/ml, for 5 min at room temperature with agitation. Sample cells corresponding to each D-Ser concentration were mixed with an internal control, which consisted of NCTC8325-4 cells grown in TSB supplemented with 0.06 M of D-serine and labeled with the same mixture of vancomycin as well as with the DNA dye Hoechst 33342 (3 μg/ml; Molecular Probes) for 5 min at room temperature with agitation. DNA labeling allows the control cells to be distinguished from the sample cells, on the same slide. Sample cultures mixed with the internal control were then examined by fluorescence microscopy using a Leica DRM2 microscope and an exposure time of 100 ms, which was confirmed not to lead to signal saturation under these experimental conditions.

Fluorescence of bound vancomycin was quantified at three points in each sample cell (at least 100 cells per assay), all localized in the peripheral cell wall and not at the septa, using the software Leica FW4000. The average fluorescence of each cell was corrected by subtracting the signal of the background. The same quantification procedure was done for internal control cells. For each photograph, the ratio between the average fluorescence of sample cells and the average fluorescence of internal control cells was determined. Lastly, values from different photographs of the same sample were averaged.

The same methodology was used to compare COL, VM50, JH1, and JH9 cells (grown in TSB only) with internal control NCTC8325-4 cells grown in TSB with 0.06 M of D-serine.

Localization of new cell wall synthesis. The localization of new cell wall synthesis in strains JH1/JH9 and COL/VM50 was done as previously described (16). Briefly, cells were grown for 16 h in TSB containing 0.125 M of D-serine, to promote the incorporation of this amino acid into the cell wall. The culture was diluted 1/250 into the same medium, grown to an OD_{600} of 0.6, washed, and resuspended in the same volume of TSB without D-serine. The cells were then incubated for 10 min (JH1 and JH9) or 15 min (COL and VM50) at room temperature, to allow the incorporation of D-alanine into the cell wall, labeled with a 1:1 mixture of vancomycin and VanFL (final concentration of 0.5 μg/ml) for 5 min at room temperature, and analyzed by fluorescence microscopy.

Microscopy analysis of vancomycin diffusion into *S. aureus* cells. To determine if vancomycin molecules could reach the division septum of vancomycin-labeled *S. aureus* cells, JH1/JH9 and COL/VM50 cells were first grown to mid-exponential phase (OD_{600} of 0.7). Nonfluorescent vancomycin was added at a final concentration of 30 μg/ml for JH1/JH9 and 100 μg/ml for COL/VM50, cells were incubated for 1 hour at room temperature with agitation, washed one time with TSB, and then labeled with a 1:1 mixture of vancomycin and VanFL (final concentration of 1 μg/ml), DNA dye Hoechst 33342 (3 μg/ml), and the membrane dye FM5-95 (6 μg/ml; Molecular Probes) for 5 min at room temperature with agitation. Cells were then analyzed by fluorescence microscopy using a Leica DRM2 microscope.

To decrease the vancomycin diffusion rate in vancomycin-labeled *S. aureus* cells, the experiment described above was repeated but cells were incubated for 5 min at 4°C before labeling with a 1:1 mixture of vancomycin and VanFL (final concentration of 1 μg/ml) for 5 min (JH1/JH9 pair) and 10 min (COL/VM50) at 4°C with agitation.

In both experiments, fluorescence of bound vancomycin was determined exclusively in cells with completely formed septa, inferred from the presence of a complete septal membrane and of two segregated chromosomes. Fluorescence was measured, in each cell, across a line perpendicular to the division septum. This line scan quantifies vancomycin fluorescence at each lateral wall and at the septum. Cells were assigned to one of three classes based on the ratio between the septal/lateral wall fluorescence being less than 1 (class I), approximately equal to 1 (±10%) (class II), or greater than 1 (class III). Over 100 cells were quantified for each strain.

RESULTS AND DISCUSSION

Modulation of the amounts of D-alanyl-D-alanine units in the mature cell wall of *S. aureus*. In order to develop and validate a fluorescence microscopy-based method for quantification of the in vivo vancomycin binding capacity of *S. aureus*, a set of samples with different vancomycin binding capacities was required. For this purpose, the number of vancomycin targets (D-Ala-D-Ala residues) in the peptidoglycan of reference strain NCTC8325-4 was modified by growing the bacteria in different concentrations of D-serine. The presence of D-serine in the growth medium leads to the incorporation of this amino acid as the C-terminal residue of muropeptides, replacing the usual D-alanine (7). Vancomycin does not efficiently bind D-Ala-D-Ser residues (10, 27) and, therefore, the in vivo vancomycin binding capacity of the bacteria should decrease in parallel with the increasing concentration of D-Ser in the growth medium.

S. aureus NCTC8325-4 was grown to mid-exponential phase in TSB containing increasing concentrations of D-serine, ranging from 0 to 0.06 M. Further increasing the D-Ser concentration to 0.125 M did not result in a higher incorporation of this amino acid in the cell wall (data not shown). The peptidoglycan was purified and its muropeptide composition analyzed by HPLC (Fig. 1A). As expected, increasing the concentration of D-Ser in the growth medium resulted in a gradual change in the composition of peptidoglycan, from a state containing normal muropeptides, terminating in D-Ala, to a state containing mu-

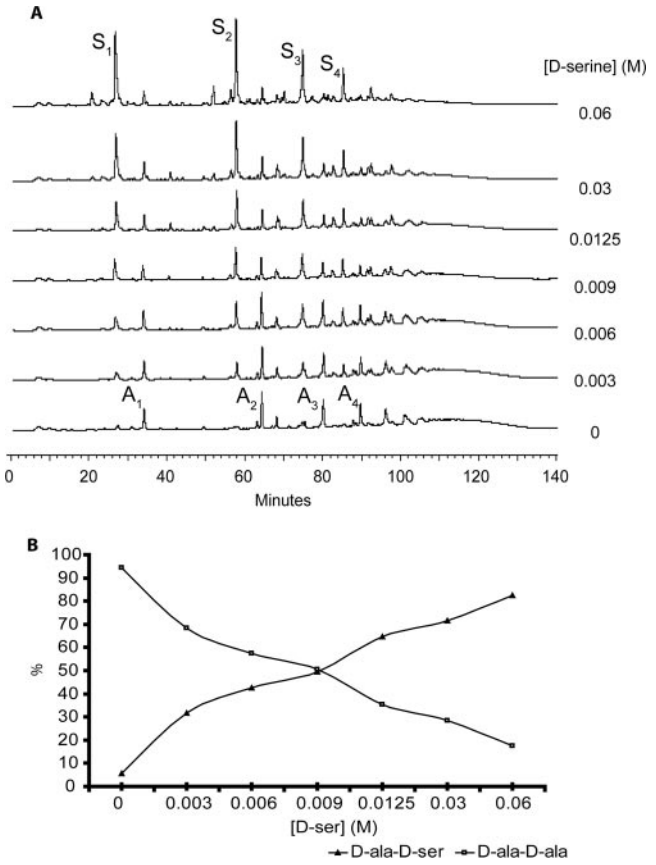

FIG. 1. Modulation of the cell wall composition of *S. aureus* NCTC8325-4 by growth in different concentrations of D-serine. (A) HPLC analysis of the muropeptide composition of the peptidoglycan of NCTC8325-4 grown in TSB and TSB containing increasing concentrations of D-serine. As the D-serine concentration in the growth medium increases, the normal muropeptides ending in D-Ala (A_1 to A_4) are replaced by D-Ser-ending muropeptides (S_1 to S_4). Monomers, dimers, trimers, and tetramers are labeled 1, 2, 3, and 4, respectively. (B) Values obtained by quantifying the peaks corresponding to D-Ala- and D-Ser-ending muropeptides in the profiles from panel A, represented as a percentage of the total amount of peptidoglycan (note that only monomers to tetramers were included in the calculations and that the *x*-axis scale is not linear).

ropeptides terminating in D-Ser. As vancomycin has a low affinity for these D-Ser-terminating muropeptides, the number of high-affinity vancomycin targets is reduced, implying an effective modulation of the number of D-alanyl-D-alanine residues capable of binding vancomycin molecules to the mature cell wall (Fig. 1B).

Correlation between the total number of vancomycin targets in the cell wall and the in vivo vancomycin binding capacity of *S. aureus*. Previous methods to quantify vancomycin binding to staphylococcal cells were either indirect, such as the measurement of the disappearance of vancomycin from the growth medium or determination of the time required for regrowth after vancomycin exposure (4), or in vitro and quite laborious methods, such as the quantification of vancomycin bound to purified cell walls (25) or to purified cell membranes (4).

To directly quantify the total amount of vancomycin bound by live cells, NCTC8325-4 cells were grown in the presence of the same concentrations of D-serine as used for the HPLC analysis of the peptidoglycan composition. A 1:1 mixture of vancomycin and an active fluorescent derivative of the same antibiotic (VanFL) was added to each sample, at a concentration of 1 μg/ml. Doubling the vancomycin concentration did not significantly alter the results, indicating that the vancomycin concentration used was not limiting (data not shown). The samples were then submitted to fluorescence microscopy analysis. To enable an accurate comparison of the quantification of cellular bound vancomycin in different samples (and therefore different microscopy images), an internal control was mixed with each sample and analyzed in the same microscopy slide (an example can be seen in Fig. 2A). The internal control used was NCTC8325-4 grown in the presence of 0.06 M of D-Ser and labeled with the DNA dye Hoechst 33342, so that the cells from the internal control could be distinguished from the sample cells. Hoechst 33342 labeling does not interfere with VanFL fluorescence (data not shown). For each microscopy image, fluorescence was quantified in sample cells and in internal control cells, and results are presented as the ratio between the average fluorescence of sample cells and the average fluorescence of control cells. This ratio was then plotted against the percentage of D-Ala-D-Ala residues of each sample (Fig. 2B).

The linear correlation obtained for the total amount of vancomycin bound to live staphylococcal cells, as determined by fluorescence microscopy, and the total number of vancomycin targets, as determined by HPLC analysis of the peptidoglycan, validates the use of fluorescence ratio imaging microscopy for in vivo quantification of vancomycin binding.

Comparison of the vancomycin binding capacity of isogenic pairs of vancomycin-susceptible and -resistant *S. aureus* strains. We have used the fluorescence ratio imaging microscopy method to study two isogenic pairs of resistant and susceptible *S. aureus* strains. Strain JH9 (vancomycin MIC, 8 μg/ml) is a VISA strain isolated from a patient undergoing vancomycin therapy (21). JH1 (MIC, 1 μg/ml) is the "parental" susceptible strain, recovered from the same patient before the onset of vancomycin therapy (21). The second pair of *S. aureus* strains was the laboratory strain VM50 (MIC, 100 μg/ml), obtained by step selection with vancomycin from the parental strain, COL (MIC, 3 μg/ml) (25).

The vancomycin binding capacities of the four strains were studied as described above, using *S. aureus* NCTC8325-4 grown in the presence of 0.06 M of D-serine as an internal control for every microscopy image. In both pairs of strains, vancomycin-resistant cells had an increased in vivo vancomycin binding capacity (Fig. 2C). However, there was no correlation between the quantitative increase in vancomycin binding capacity and the increase in the vancomycin MIC. This finding is in agreement with the report in which the in vitro vancomycin binding capacity of cell walls purified from strain COL or VM50 were compared, and the maximum binding capacity difference observed was about 2.5-fold (25).

The fact that fluorescence imaging techniques allow determination of in vivo binding of vancomycin to individual staphylococcal cells allowed us to further explore the resistance mechanism in the isogenic pairs of VISA and vancomycin-resistant laboratory mutant strains.

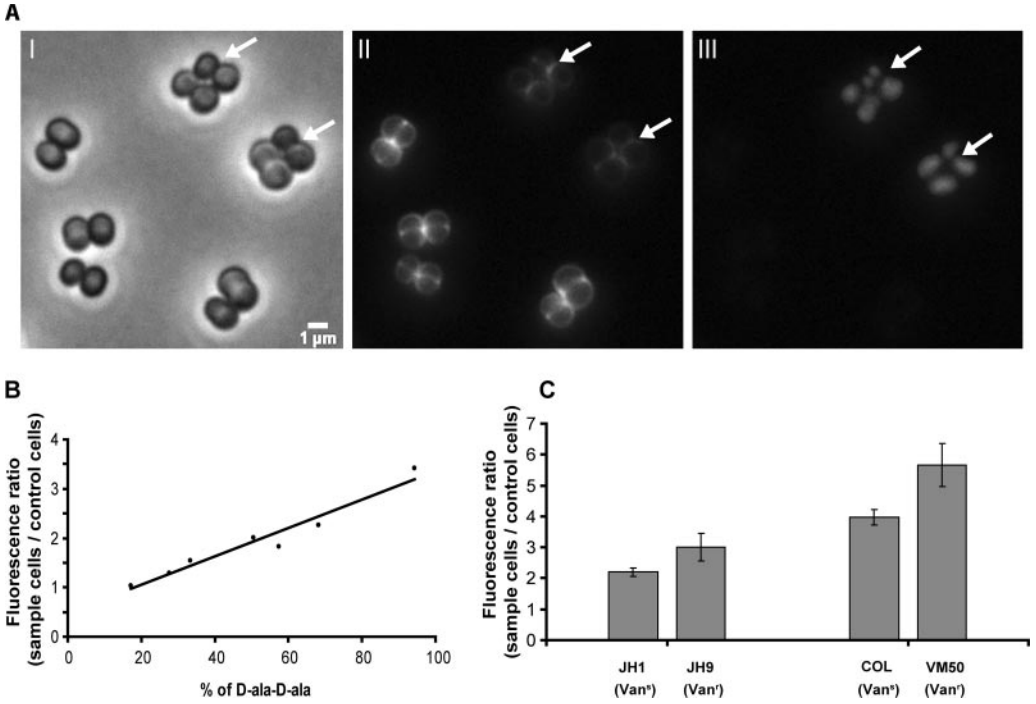

FIG. 2. In vivo fluorescence ratio imaging microscopy for quantification of vancomycin binding to *S. aureus* cells. (A) A mixture of sample cells (in this example NCTC8325-4 grown in TSB without D-Ser and labeled with Van/VanFL) and internal control cells (NCTC8325-4 grown in TSB containing 0.06 M of D-serine and labeled with Van/VanFL and DNA dye Hoechst 33342) was analyzed by fluorescence microscopy. A phase-contrast image (I), VanFL fluorescence (II), and DNA labeling with Hoechst 33342 (III) are shown. The presence of the DNA dye in control cells allows differentiation between sample cells and control cells (indicated by a white arrow) in the same microscopy image. (B) VanFL fluorescence was quantified (see Materials and Methods), and the ratio of VanFL fluorescence in sample cells versus control cells was calculated. The value of this ratio for sample cells grown in the presence of different D-serine concentrations is plotted, showing a linear correlation obtained between the vancomycin bound to live staphylococcal cells and the number of vancomycin targets ($R^2 = 0.948$). (C) VanFL fluorescence of the two isogenic pairs of vancomycin-susceptible and -resistant strains, JH1/JH9 and COL/VM50, normalized against the fluorescence of control cells (same as in panel A) in the same microscopy image. This value is directly correlated to the vancomycin binding capacity of each strain.

Localization of the subcellular sites of cell wall synthesis in vancomycin-susceptible and -resistant strains of *S. aureus*. Recent fine-structure studies indicated that vancomycin has to cross approximately 25 nm (26), corresponding to 20 to 40 layers of peptidoglycan (12), before it reaches the plasma membrane. However, the lethal target of vancomycin should be located where cell wall synthesis is taking place. In the vancomycin-susceptible RN4220 *S. aureus* strain, the main location of cell wall synthesis was shown to be at the division septum and not over the entire plasma membrane surface (16). Major enzymatic components of the cell wall synthetic machinery, such as PBP1 and PBP2, were also localized to the same septal site (15–17). Were this also the case for vancomycin-resistant strains, then vancomycin molecules would have to reach the tip of the division septum (and not merely the cytoplasmic membrane at any point of the cell surface) in order to inhibit cell wall synthesis. In this case, the access of vancomycin to its lethal target could be studied by comparing the VanFL fluorescence at the division septum versus the lateral wall, under a variety of experimental conditions.

In order to compare the main location of cell wall synthesis in the vancomycin-resistant strains VM50 and JH9 and their susceptible controls, we have used a previously described method (16) in which cells are grown in the presence of an excess of D-serine, leading to incorporation of this amino acid

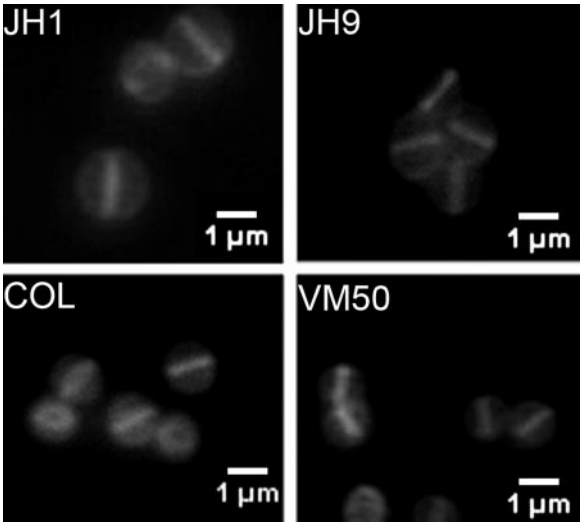

FIG. 3. Localization of cell wall synthesis in isogenic pairs of vancomycin-susceptible and -resistant strains. Images show VanFL labeling of JH1/JH9 and COL/VM50 cells after growth with an excess of D-serine and transient incubation with D-alanine, which results in specific labeling of new peptidoglycan. In all cases, the main location for cell wall synthesis is at the division septum.

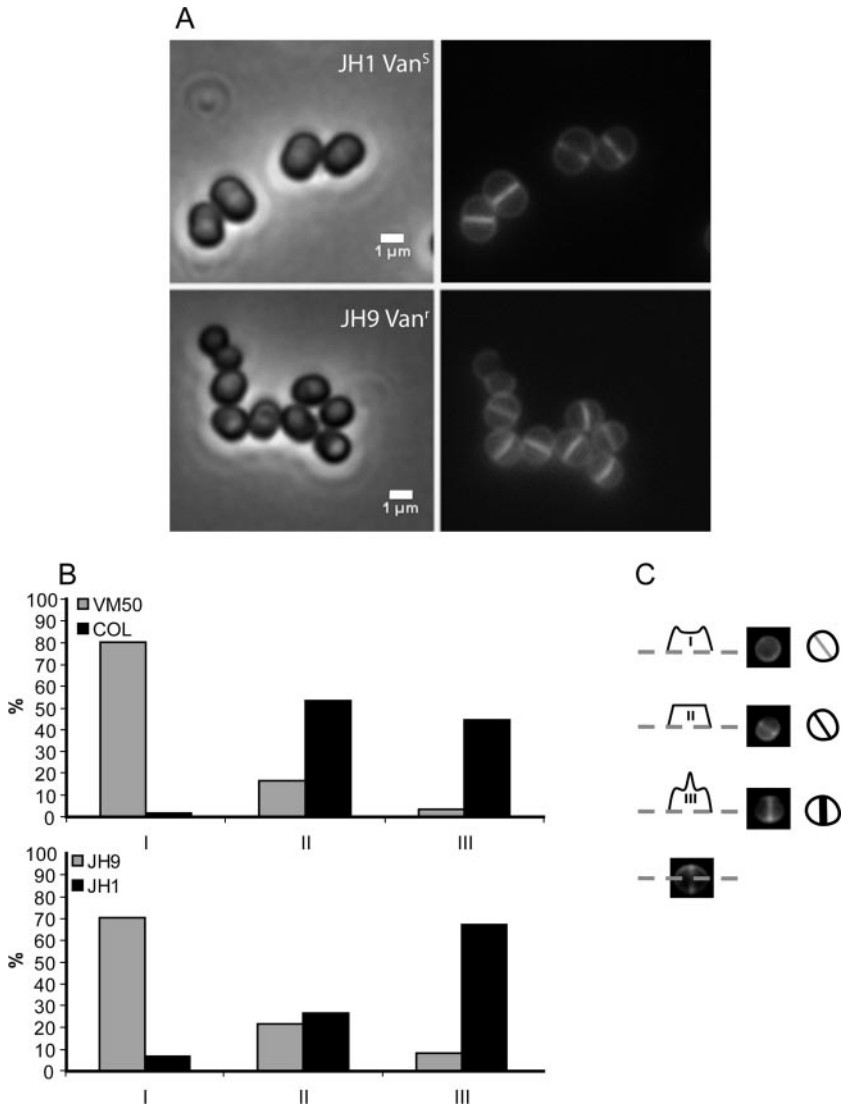

FIG. 4. Diffusion of a fluorescent derivative of vancomycin (VanFL) through the cell wall of *S. aureus* cells prelabeled with nonfluorescent vancomycin. (A) Fluorescent vancomycin was able to reach the division septum in both vancomycin-susceptible strain JH1 and resistant strain JH9. (B) Vancomycin diffusion, at 4°C, was qualitatively evaluated by determining the ratio of vancomycin fluorescence at the septum and at the lateral wall. Cells were assigned to one of three classes based on the ratio between the septal/lateral wall fluorescence being less than 1 (class I), approximately equal to 1 (class II), or greater than 1 (class III). (C) Profile of the line scan used to measure fluorescence (left), a fluorescence image (middle), and a schematic view (right) of a cell corresponding to each class.

as the C-terminal residue of the peptidoglycan muropeptides. This results in a cell wall for which vancomycin has very low affinity. Subsequent growth in the presence of D-Ala leads to the incorporation of this amino acid primarily into lipid II and newly synthesized and un-cross-linked chains of the peptidoglycan on the outer surface of the plasma membrane. Cells were then labeled with VanFL, which binds only the D-Ala-D-Ala-containing peptidoglycan, allowing visualization of the place where new cell wall synthesis is occurring. Figure 3 shows that the main location for cell wall synthesis in parental strains COL and JH1 was the division septum and that this main location was maintained in the vancomycin-resistant strains VM50 and JH9.

Access of vancomycin to sites of cell wall synthesis in resistant and susceptible staphylococci. A key common feature of models proposed for the mechanism of vancomycin resistance in VISA strains and in laboratory mutants is that in the resistant bacteria the increased cell wall thickness and/or the increased proportion of "false" binding sites in the mature cell wall forms a physical barrier that impedes and/or slows down penetration of the antibiotic molecules to their lethal target (12, 22). "Capture" of vancomycin molecules by these false targets should further impede penetration of the cell wall barrier.

To test this hypothesis in live bacteria, both the susceptible and resistant isolates of each isogenic pair were pretreated with nonlabeled vancomycin for 1 hour followed by the addition of VanFL and visualization of fluorescence intensity at the septum. Figure 4A indicates that the reagent was able to reach the sites of cell division in each of the types of bacteria, indi-

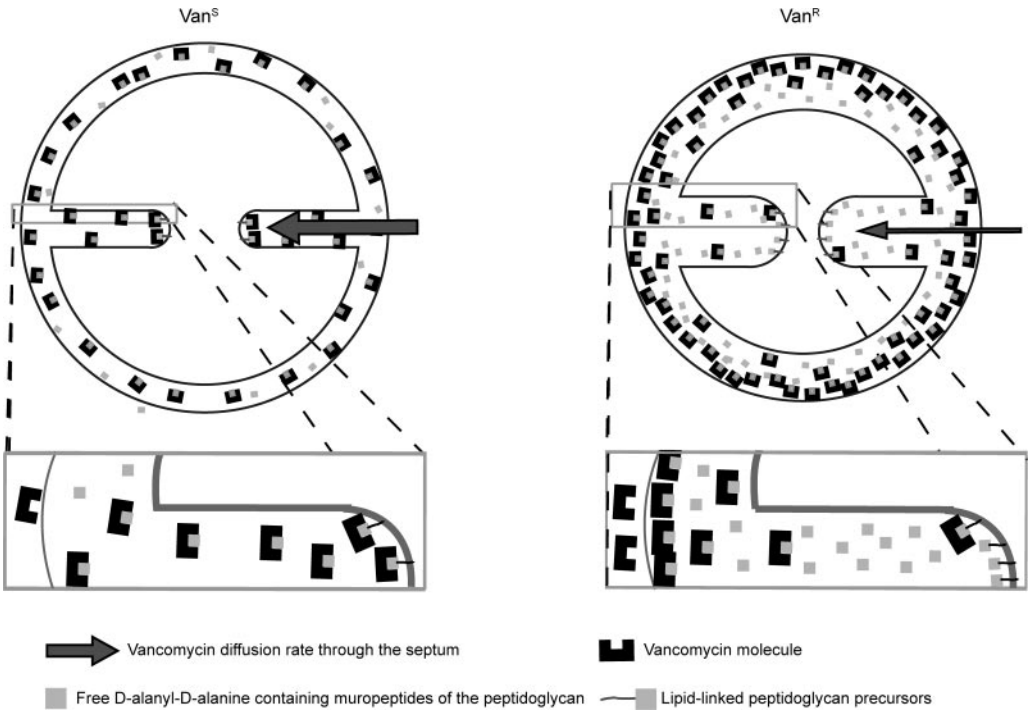

FIG. 5. Model for vancomycin resistance in VISA strains. The path of vancomycin to its lethal target (lipid II) should be through the division septum. In resistant cells (Van^r), the diffusion rate of vancomycin molecules to the septal tip is decreased, lowering the effective concentration of antibiotic that reaches the lipid-linked peptidoglycan precursor (lipid II) at the site of cell wall synthesis, per unit time, and therefore tilting the balance in favor of continued cell wall synthesis. This model implies that vancomycin efficiency varies during the cell cycle, as the path from the outside of the cell to the lethal targets is shorter when the septum starts to be formed and longer when septum synthesis approaches completion.

cating the absence of a physical barrier to the passage of antibiotic molecules.

Next, we proceeded to determine if there was a difference between the in vivo diffusion rate of vancomycin through the vancomycin-saturated cell walls of the isogenic pairs of susceptible and resistant strains.

The experiment described above was repeated, but the temperature was reduced to 4°C before and during VanFL addition, in order to slow down the diffusion of the VanFL through the bacterial surface. The ratio of VanFL fluorescence at the division septum to the VanFL fluorescence at the lateral wall was then measured. The slower the diffusion rate, the lower this ratio is expected to be, as less vancomycin reaches the septum in the time frame of the experiment. Cells were assigned to one of three classes based on the ratio between the septal/lateral wall fluorescence being less than 1 (class I), approximately equal to 1 (class II), or greater than 1 (class III). In susceptible cells, this ratio can be higher than 1, i.e., the VanFL fluorescence can be higher at the septum than at the lateral wall, because the septal cell wall (which will be later split into two lateral walls) is thicker and the concentration of lipid-linked peptidoglycan precursor is higher in this region. Figure 4B shows that, in contrast to susceptible JH1 and COL cells, the majority of JH9 and VM50 cells had less fluorescence at the septum than at the lateral wall, indicating that vancomycin access to its target at the septum was delayed in the resistant strains.

These results strongly suggest that the compositional alterations and/or increased thickness in the cell wall of the resistant bacteria can slow down the "arrival" of the vancomycin molecule to the cell wall synthetic sites. This finding is in general agreement with the key points of models proposed for the vancomycin-resistant phenotype of laboratory mutants (22) and also with a recent report that described, in quantitative terms, the lower diffusion rate of vancomycin through the thicker cell walls of VISA-type isolates (4).

However, if the path of vancomycin to its lethal target is through the division septum, as strongly suggested in Fig. 3 and 4, and a complete septum is synthesized before separation of the two daughter cells is initiated (9), then the thickness of the lateral cell wall represents only a minor fraction of this path, except in early stages of septum synthesis (Fig. 5). Therefore, the general increase in cell wall thickness and/or compositional changes that occur in the peripheral cell wall of the vancomycin-resistant bacteria may only be indirectly related to the mechanism of vancomycin resistance. Only cell wall compositional changes (including an increase in the number of "false" binding sites) that occur in the septum would contribute to the mechanism of decreased susceptibility to vancomycin. In resistant bacteria, the slower diffusion of vancomycin molecules to the septal tip would decrease the effective concentration of the antibiotic that reaches the lipid II at the site of cell wall synthesis per unit time, thus allowing a sufficient proportion of lipid II to escape vancomycin, i.e., allowing cell wall synthesis to outpace the inhibitory action of vancomycin (Fig. 5).

The fact that vancomycin has to diffuse across different distances depending on the stage of septum synthesis indicates that it may be interesting to determine the effect of vancomycin

in single cells, over the bacterial cell cycle. Studies of antibiotic resistance should gain new insights from integrating the information that has become available in recent years about the cell cycle of bacteria and the subcellular localization of the various essential processes that take place in bacterial cells.

ACKNOWLEDGMENTS

This work was supported by grant POCI/BIA-BCM/56493/2004 from Fundação para a Ciência e Tecnologia, Portugal, awarded to Mariana G. Pinho, and by grants RO1 AI37275 and RO1 AI45738 from the National Institutes of Health, U.S. Public Health Service, awarded to Alexander Tomasz.

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