# Morphological and Biochemical Alterations in *Staphylococcus epidermidis* Stepwise Adapted to Vancomycin Resistance

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**A vancomycin-susceptible laboratory strain of *Staphylococcus epidermidis* was stepwise adapted to grow in increasing concentrations of vancomycin ultimately reaching a maximum of 30µg/mL. The resultant vancomycin-resistant strain (VRSE) was stable and did not revert to susceptibility on repeated sub-culturing. Analysis of VRSE by electron microscopy indicated prominent morphological alterations including thickened and defective cell walls, abnormal septation and intracellular morula-like structures. These morphological alterations were found in resistant cells, which had grown in either the presence or absence of vancomycin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses indicated that resistant cells grown in the presence of vancomycin are missing a protein band (ca. 48000 daltons) found in resistant cells grown in the absence of vancomycin. Vancomycin-resistant cells are more resistant to the lytic effects of lysostaphin than are susceptible wild-type cells.** 

## INTRODUCTION

The incidence of disease attributed to coagulase-negative staphylococci such as *Staphylococcus epidermidis* has drastically increased in recent years. This is especially relevant to patients with prosthetic joints and other implanted biomaterials (*1,2*). Successful chemotherapy of this group of organisms has been further complicated by the global spread of methicillin-resistant *S. epidermidis* (MRSE) strains (3). In recent years the primary methods of treating MRSE and methicillin-resistant *S. aureus* (MRSA) strains have been glycopeptides such as vancomycin, which interferes with cell wall biosynthesis by binding to the D-ala-D-ala terminus of muramic acid pentapeptide (*3,4*). The recent emergence of vancomycin- resistance among these groups is not surprising considering the metabolic versatility of the staphylococci and the well-documented overuse of antimicrobials. Although vancomycin resistance is relatively uncommon at the present, any level of resistance is troublesome because of the scarcity of alternative chemotherapies. The exact mechanism of action of vancomycin resistance is unknown but resistant clinical isolates have been reported with morphological and physiological changes when compared to susceptible isolates (*5,6*).

The purpose of this study was to characterize morphological and biochemical alterations occurring in a *S. epidermidis* wild-type (SEWT) strain, which had been stepwise adapted to a relatively high level of vancomycin resistance.

## MATERIALS and METHODS

**Bacterial Strains and Culture Conditions:** The MRSA strain was a generous gift from the Microbiology Laboratory of St. John's Hospital ( Tulsa, OK). *S. aureus* ATCC 12598 (methicillin-susceptible) was obtained from Carolina Supply (Burlington, NC) and was designated as *S. aureus* wild-type (SAWT). SEWT was a lab strain obtained from the teaching program at OSU-COM (Tulsa, OK). A vancomycin-resistant *S. epidermidis* strain (VRSE) was derived from SEWT strain by the stepwise adaptation procedure described below. Identifications of SEWT and vancomycin-resistant strains were confirmed by

16S rRNA analyses performed by Midilabs (Newark, DE). Working cultures of all strains were routinely maintained on blood agar plates. Growth of cells used in experiments was initiated by inoculating isolated colonies from blood agar plates into flasks containing 50 mL Mueller-Hinton (MH) broth. All cells used in this study were grown in Mueller-Hinton broth (CSMH) supplemented with $Mg^{2+}$ (12.5 mg/L) and $Ca^{2+}$ (25 mg/L) and were aerated with vigorous shaking at 37ºC. Susceptibility to lysostaphin (Sigma, St. Louis, MO) was measured by adding 1 unit of enzyme activity/mL (1ng/mL) to exponentially growing cells in 50 mL of CSMH. Cultures were incubated at 37ºC and absorbance at 620 nm was measured every 10 min.

**Isolation of Vancomycin Resistant Strains**: The VRSE strain was derived from the SEWT strain by a modification of the stepwise adaptation procedure described by Conrad et al. (*7*). However, in contrast to these previous studies in which staphylococcal resistance to daptomycin developed in large increments, stepwise adaptation to vancomycin resistance in this study proceeded in very small increments of 1-2 µg/mL per step. Stepwise adaptation continued until a maximal resistance level of 30 µg/mL vancomycin was achieved. Attempts to obtain higher levels of resistance by this procedure were deemed not feasible because of very slow and minimal growth.

**Antimicrobial Susceptibilities:** Antimicrobial susceptibilities and the development of resistance were determined by various methods using standard protocols and manufacturer's recommendations. These techniques included minimum inhibitory concentration (MIC) by microtiter plates, disk diffusion, and the Etest method (*8-10*). All antimicrobials were obtained from Sigma (St. Louis, MO). Stock solutions of antimicrobials were filter-sterilized and frozen at –20ºC until used. Overnight broth cultures of each strain were adjusted to an optical density of 0.20 at 620 nm. Depending on the type of assay, the cultures were either swabbed onto CSMH agar plates prior to adding antimicrobial disks or strips, or dispensed in 5 µl aliquots into microtiter wells containing appropriate dilutions of the antimicrobials.

**Transmission Electron Microscopy:** VRSE, SAWT, and MRSA cultures were grown in CSMH media as indicated (± 30 µg/mL vancomycin). Two milliliters of liquid culture from each strain were sedimented by centrifugation at 6000 rpm. The supernatant fluids were removed and discarded and the pellets were washed twice in phosphate buffered saline (PBS). The washed cells were then fixed in 1 mL of 6% glutaraldehyde and 100 mM sodium cacodylate buffer and then refrigerated overnight at 4º C. For transmission electron microscopy (TEM), the cells were washed in 100 mM sodium cacodylate buffer, pH 7.3, and fixed in 2% osmium tetroxide. Samples were then dehydrated through a graded series of acetone/resin dilutions. The final solution was 100 % resin, which was maintained overnight at 64º C. The resultant resin blocks were cut by Ultracut E microtome into thick (0.5 µm) and thin (55 nm) sections. Thick sections were placed on glass slides and stained with Mallory's stain. Thin sections were placed on copper posts and stained with uranyl acetate and Reynolds's lead stain. All sections were examined by TEM by using standard procedures (*11*).

**Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Membrane Proteins:** Cells to be analyzed were grown to stationary phase in 1 L of CSMH (± 30µg/mL vancomycin as indicated) and harvested by centrifugation at 6000 rpm for five min. The supernatant fluid was discarded and the pellets were washed twice with PBS and gently stirred overnight at room temperature in 50 mL of lysis medium containing 10 mM Tris-HCl buffer (pH 8.0), 1 mg lysostaphin and 10 mg lysozyme. The next day, the treated cells were homogenized with a Janke and Kunkel tissue homogenizer and centrifuged at 15,300x g for 30 min. The pellets were discarded and the supernatant fluids were centrifuged at 170,000 x g for 45 min (*12*). These pellets were reconstituted in the same buffer and protein concentrations were

determined by the method of bicinchoninic acid (BCA) (*13*). Samples of membrane proteins were transferred into 10 % SDS buffer and heated for 3 min at 95ºC. The lanes of 4% stacking gels and 10 % separating gels were loaded with 100 µg protein/lane and subjected to the electrophoresis procedure of Laemmeli (*14)* for 6-8 h. The electropherograms were developed with Comassie blue and destained with acetic acid/methanol by standard procedures. Molecular weights of the respective protein bands were estimated by comparison with commercial molecular weight standards (Bio-Rad, Richmond, CA**).**

## RESULTS and DISCUSSION

Electron microscopy demonstrated that the stepwise adaptation of vancomycin susceptible *S. epidermidis* to vancomycin resistance (VRSE) resulted in prominent morphological alterations (Fig. 1). Some of the most notable of these anatomical changes were located in the cell wall, which is also the active site of vancomycin. These perturbations in VRSE morphology were noted in either the absence (Fig. 1C) or the presence (Fig. 1D) of vancomycin. Specific changes included; abnormal septation**,** incomplete and defective cell walls, and in many cells thickened cell walls which were two to three times normal size (single arrows). However, the clusters of intracellular morula-like bodies (double arrows) found in VRSE cells indicated that morphological alterations related to vancomycin resistance were not strictly limited to cell walls. Similar types of cellular anomalies have been previously noted in clinical isolates of vancomycin-resistant *S. epidermidis* (*5*). Morphological effects of vancomycin on susceptible staphylococci were examined by growing the susceptible SAWT and MRSA strains in the presence (30 µg/mL) and absence of vancomycin. Cells grown in the absence of vancomycin had typical staphylococcal morphology (Figs. 1A and 1B) whereas overnight exposure of these susceptible strains to vancomycin resulted in the almost complete lysis of intact bacteria to cellular debris (data not shown).

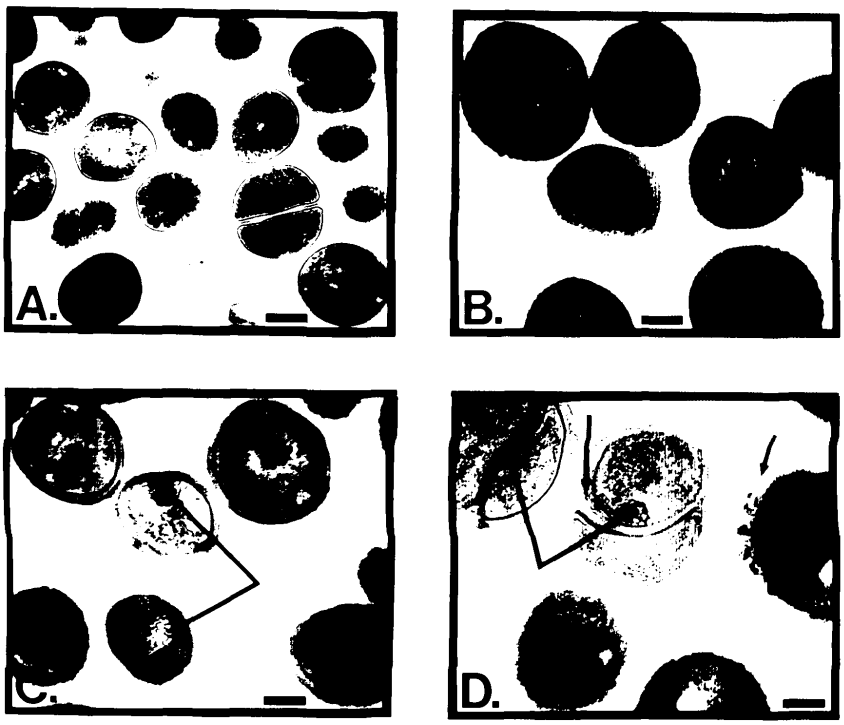


Figure 1. TEM demonstrating the effects of stepwise adaptation to vancomycin on *S. epidermidis.* Wild-type susceptible staphylococcal controls (A) SAWT and (B) MRSA with no vancomycin. VRSE grown in absence (C) and presence (D) of vancomycin. Note morula-like structures (double arrows) and thick walls and defective walls (single arrows). Bar = 1µm.

Determination of antimicrobial sensitivities (MIC's) demonstrated that SEWT is susceptible to penicillin G, oxacillin and vancomycin at concentrations less than 1 µg/mL. VRSE remained susceptible to penicillin G and oxacillin at the same concentrations but was resistant to vancomycin at 32 µg/mL.

The VRSE strain remained vancomycin resistant even after 17 passages on antimicrobial-free blood agar plates indicating that its resistance is likely the result of a stable mutation or possibly even several stable mutations. Although the molecular basis of this resistance remains unresolved, preliminary experiments with lysostaphin suggested that the peptidoglycans of resistant cells have been either biochemically or architecturally altered. Lysostaphin is an endopeptidase that cleaves the peptide bonds associated with the pentaglycine structure common to the peptidoglycan of staphylococcal species (*15*). When lysostaphin was added to exponentially growing staphylococcal cultures, the intensity of its lytic effects differed depending upon the strain's susceptibly or resistance to vancomycin (Fig. 2). For example absorbance of the susceptible SEWT strain declined 75% in 60 min, while under similar conditions the absorbance of the resistant VRSE strain declined only 23%. The effects of lysostaphin were even more pronounced in cultures of SAWT, which declined 93%. Controls for this experiment were samples of these same cultures, which were subdivided into fresh medium containing 10% lysis buffer solution minus lysostaphin. Cells in the lysostaphin-free medium continued in the exponential growth phase (data not shown).

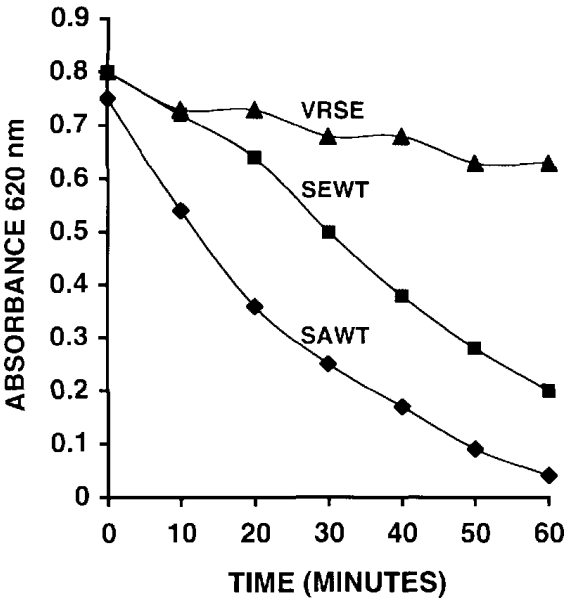


Figure 2. Effects of lysostaphin on staphylococcal strains with varying susceptibilities to vancomycin. Lysostaphin was added to each culture at a concentration of 1 ng/mL of culture. VRSE is resistant to vancomycin (>30µg/mL). SEWT and SAWT are susceptible to vancomycin (< 2 µg/mL.).

There are at least three possible explanations for vancomycin resistance in VRSE. It is possible that the antimicrobial was either destroyed or inactivated by the bacteria but this theory was not addressed during this study. Another possibility is that the antimicrobial target site (peptidoglycan) has been altered in such a manner that it no longer recognizes and binds the antimicrobial to initiate its lethal effects. This hypothesis would be consistent with the previously observed altered responses to lysostaphin. The third possibility is that resistance is the result of alterations to protein(s) that are essential for either facilitating the uptake of the antimicrobial or for transporting it to its active site. To this end we performed SDS-PAGE comparative analyses of membrane proteins extracted from VRSE growing in the presence and absence of vancomycin (Fig. 3). A prominent protein band was found in cells growing in the absence of vancomycin but was absent (top arrow) in VRSE cells grown in the presence of vancomycin. However, this protein band appeared to be present in the susceptible SAWT and MRSA strains. Interestingly the apparent molecular weight (ca. 48000 daltons) of this band approximates that of penicillin binding protein 4 (PBP4), a protein closely associated with staphylococcal resistance/susceptibility to β-lactam antimicrobials. PBP4 functions as a carboxypeptidase and transpeptidase (*16*) as well as affecting cross-linking of the peptidoglycan (*17*). The significance of the absence of this protein from cultures grown in the presence of vancomycin is unclear, but may be related to the lysostaphin

resistance noted for VRSE. Incomplete cross-linking of the cell wall resulting from the lack of this enzyme could conceivably result in a defective peptidoglycan incapable of interacting with vancomycin to interfere with cell wall synthesis. β-lactam uptake and binding experiments are underway to determine if this specific protein band functions as either PBP4 or a similar protein. This possibility is consistent with a previous report that PBP4 is missing from glycopeptide-resistant strains of *S. aureus* (*18*). It was of interest that staphylococcal species such as *S. aureus* and *S. epidermidis* have numerous protein bands of common molecular weights such as the bands noted at 23,000 (bottom arrow).

In summary, vancomycin resistance in *S. epidermidis* is accompanied by significant morphological and biochemical changes. Comparative biochemical analyses of resistant and susceptible strains are in progress to determine if resistance to vancomycin and other antimicrobials can be attributed to specific alterations in chemical composition or architecture of peptidoglycans. These analyses will also include investigations of the proteins, which affect the uptake, accessibility or transport of vancomycin to its active site.

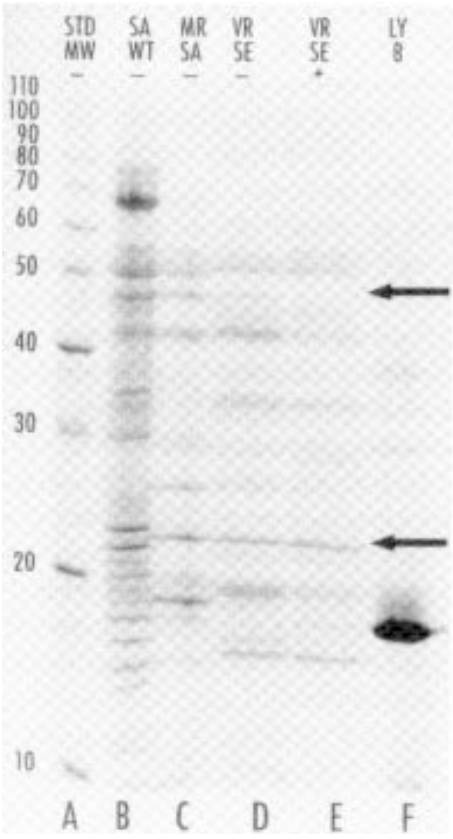


Figure 3. SDS-PAGE of membrane proteins from VRSE strain grown in presence (E) and absence (D) of vancomycin. Susceptible SAWT (B) and MRSA (C) strains shown for comparison. LYB is the lysis buffer containing lysostaphin and lysozyme.

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