The Effects of Daptomycin on Chemical Composition and Morphology of *Staphylococcus aureus*

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The effects of daptomycin on *Staphylococcus aureus* morphology and chemical composition were studied by comparative analyses of susceptible and daptomycin-resistant strains. The resistant strains were developed from wild-type (WT) susceptible cells by stepwise selection to increasingly higher levels of daptomycin. The minimal inhibitory concentrations of penicillin, streptomycin, tetracycline, and erythromycin were similar among resistant and susceptible strains. Daptomycin-resistant strains were concomitantly resistant to polymyxin B. The cellular percentages and phosphate content of cell walls, readily extractable lipids (REL), and peptidoglycan among the respective strains were similar except for a reduction in the cellular percentage of REL in resistant cells. The phosphate content of lipoteichoic acids that were extracted from resistant cells was drastically reduced. Both susceptible and resistant REL were fractionated by selective elution from silicic acid columns. The primary phospholipids of both strains were diphosphatidyl glycerol, phosphatidyl glycerol, and phosphatidyl serine. Only minor differences in fatty acids were noted. The growth of resistant cells in sublethal concentrations and susceptible cells in lethal concentrations of daptomycin resulted in unusual cell morphology characterized by various cell wall anomalies. (This study was presented in part at the 97th General Meeting of the American Society for Microbiology, Miami Beach, Florida, May 1997.)

INTRODUCTION

Daptomycin (LY146032) is an acidic lipopeptide with a high specificity for gram-positive bacteria (*1*). Early studies indicated that antimicrobial activity is initiated by the irreversible binding of daptomycin to cellular membranes in a calcium-dependent reaction (*2*) that leads to disruption of peptidoglycan synthesis and membrane integrity (*3,4*). However, no specific step in cell wall metabolism was identified as being sensitive to daptomycin. Although the exact mechanisms of action and target sites have not yet been determined, subsequent studies by Canepari and his colleagues (*5,6*) determined that daptomycin interferes with the synthesis of lipoteichoic acid. These same studies also noted that daptomycin caused an almost 50% reduction of [^{14}C]acetate into lipids, which suggests that daptomycin may affect other aspects of lipid metabolism as well.

The purpose of this study was to determine if the activity of daptomycin is specific for the metabolism of lipoteichoic acids or if it also includes interference with the metabolism of other cellular components, such as the lipid-containing compounds found in the readily extractable lipids (REL). This possibility was examined by developing daptomycin-resistant strains of *Staphylococcus aureus* from the daptomycin-susceptible wild-type (WT) parent strain and performing comparative analyses of selected cell wall/membrane components, antimicrobial susceptibilities, and morphology.

MATERIALS and METHODS

Bacterial strains and growth conditions: Wild type (WT) *S. aureus* strain FDA 209P (ATCC 6538P) was provided by Lilly Research Laboratories, Indianapolis, Indiana. One-liter batches of cells were cultivated at 37 °C in 2 L flasks with vigorous shaking. The growth media were either trypticase soy broth (TSB) or Mueller-Hinton broth that had been supplemented with 50 mg of Mg^{2+} and Ca^{2+} per liter

of media (CSMH) as recommended by Lilly Research Laboratories. Cation supplements were autoclaved and added to the Mueller-Hinton broth separately. Chemical analyses of the WT strain were performed with bacterial cells that had been grown to mid-to-late logarithmic growth phase, killed by 1% phenol, harvested by preparative centrifugation, washed with cold water, freeze-dried, and stored at -20 °C until used. Cells of the daptomycin-resistant strain were prepared by the same procedures as the WT strain except that the media contained 50 μg daptomycin/mL (CSMH-50) and the growth time was extended to 3 d due to slow growth.

Isolation of daptomycin resistant strains: Daptomycin-resistant strains were obtained from the daptomycin-susceptible *S. aureus* WT strain by stepwise selection of cells to increasingly higher concentrations of daptomycin. The selection process was initiated by transferring 0.20 mL of a culture growing at the minimal inhibitory concentration as determined during this study, 0.25 μg/mL, into 50 mL of fresh medium that contained 0.50 μg daptomycin/mL. Visible growth was observed at the higher concentration (0.5 μg daptomycin/mL) after 2 d. This selection process was repeated with the successive transfer of cells into medium containing progressively higher concentrations of antimicrobial agent (1, 2, 4, 8, 12, 16, 20, 25, 30, 35, 40, and 50 μg daptomycin/mL). The inoculation of cells into the next higher concentration of the antimicrobial agent resulted in an abundant, overnight growth until daptomycin levels reached 35 μg/mL, and higher. Visible growth was not observed in these higher concentrations until after 3-5 d incubation. Cells growing in concentrations of 50 μg daptomycin/mL were subcultured onto CSMH plates, and 20 isolates were selected for transfer to CSMH-50 plates. The isolate that produced the largest colonies on CSMH-50 upon repeated transfers between CSMH and CSMH-50 media was designated DAR2 and used as the prototype daptomycin-resistant strain throughout this study. The DAR2 strain was routinely maintained on CSMH-50 plates and transferred every 2 wk.

Determination of minimal inhibitory concentration (MIC): The MICs of erythromycin, penicillin G, polymyxin B, streptomycin and tetracycline for the WT and DAR2 strains in CSMH were determined by tube dilution techniques previously described by Conrad et al. (*7*). Possible synergistic/antagonistic interactions of daptomycin and other antimicrobials in DAR2 cells were evaluated by the dilution of the designated antimicrobial in CSMH media containing 12.5 μg/mL daptomycin. Aqueous stock solutions of all antimicrobials were filter sterilized and kept at -20°C until needed.

Electron microscopy: The morphology of daptomycin-susceptible (WT) and resistant (DAR2) *S. aureus* cells were compared by scanning and transmission electron microscopy. WT and DAR2 cells were grown to mid-logarithmic phase in CSMH and CSMH-50 media, respectively. Two mL of each culture were sedimented in a table-top centrifuge, and the pellets were washed twice with 2 mL of cold phosphate buffer saline (PBS). After the second wash, the cells were resuspended in 1 mL of PBS, fixed by the addition of 1 mL of 6% gluteraldehyde, and left overnight at 4 °C. The effects of bactericidal levels of daptomycin on cellular morphology were examined by adding 500 μg/mL of daptomycin to logarithmically growing WT cells and incubating for 3 h. The cells were harvested and prepared for examination by the previously described methods, and electron microscopy was performed using standard procedures.

Analytical methods: The RELs were extracted from freeze-dried cells by chloroform-methanol (2:1) using the Conrad et al. modification (*8*) of the method of Folch et al. (*9*). The cellular percentages of REL were measured gravimetrically by the evaporation of extract in tared, aluminum weigh pans. The RELs were fractionated by elution from a column of acid-treated silicic acid (volume ca 10 mL) by the successive application of 10 column volumes of chloroform, 40 column volumes of acetone, and 10 column volumes of methanol. The fractions were taken to dryness with a gentle stream of nitrogen and

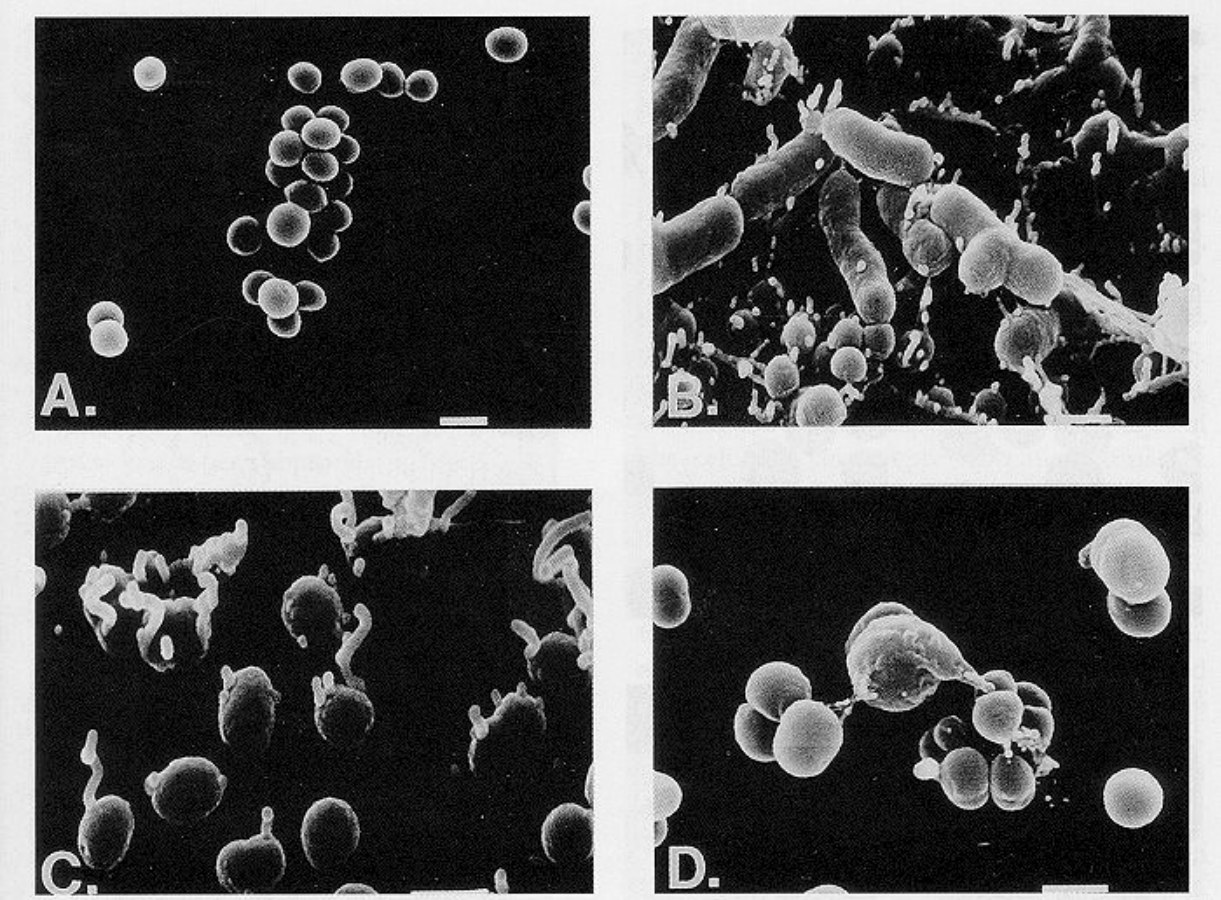

Figure 1. SEM demonstrating effects of daptomycin on *S. aureus* strains of varying susceptibilities. (A) Wild-type (susceptible) control with no daptomycin. (B) and (C) Wild-type exposed to lethal concentration of daptomycin (500 μg/mL, 3 h). (D) DAR2 strain (resistant) grown in sublethal concentration of daptomycin (50 μg/mL, 24 h). Bar = 1μm.

transferred with 2:1 chloroform-methanol. The chloroform, acetone, and methanol eluates were then analyzed by thin-layer chromatography (TLC) on silica gel plates using a multisolvent system as described by Dunnick and O'Leary (*10*). TLC plates were visualized by charring with dilute H_2SO_4 at 150 °C. The REL components were identified by comparative Rf values, cochromatography with authentic standards, and reactions with TLC spray reagents used as recommended by Applied Science Laboratories (Technical Bulletin No. 34). Fatty acids in the REL were transmethylated by BF_3 in methanol by the method of Morrison and Smith (*11*). Using standard procedures (Supelco, GC Bulletin 767), fatty acids in whole cells were first saponified (NaOH) and acidified (HCl) prior to transmethylation by the same procedures used for the REL fractions. Fatty acid methyl esters were identified by comparative relative retention times and cochromatography with authentic standards. Using the procedures of Peterson et al. (*12*), cell walls, peptidoglycan, and teichoic acids were isolated from cells that were previously defatted by REL extraction. Phosphate was measured by the method of Lowry and Tinsley (*13*).

RESULTS

Effects of daptomycin on morphology: Scanning electron microscopy (SEM) clearly demonstrated that daptomycin has profound effects on the morphology of susceptible and resistant strains of *S. aureus. S. aureus* WT cells (Fig. 1A) were drastically altered by exposure to lethal concentrations of daptomycin (Figs. 1B,C). Antimicrobial-induced modifications included filamentous growth, ghosts, antler-like protrusions, mini-cells, elongated

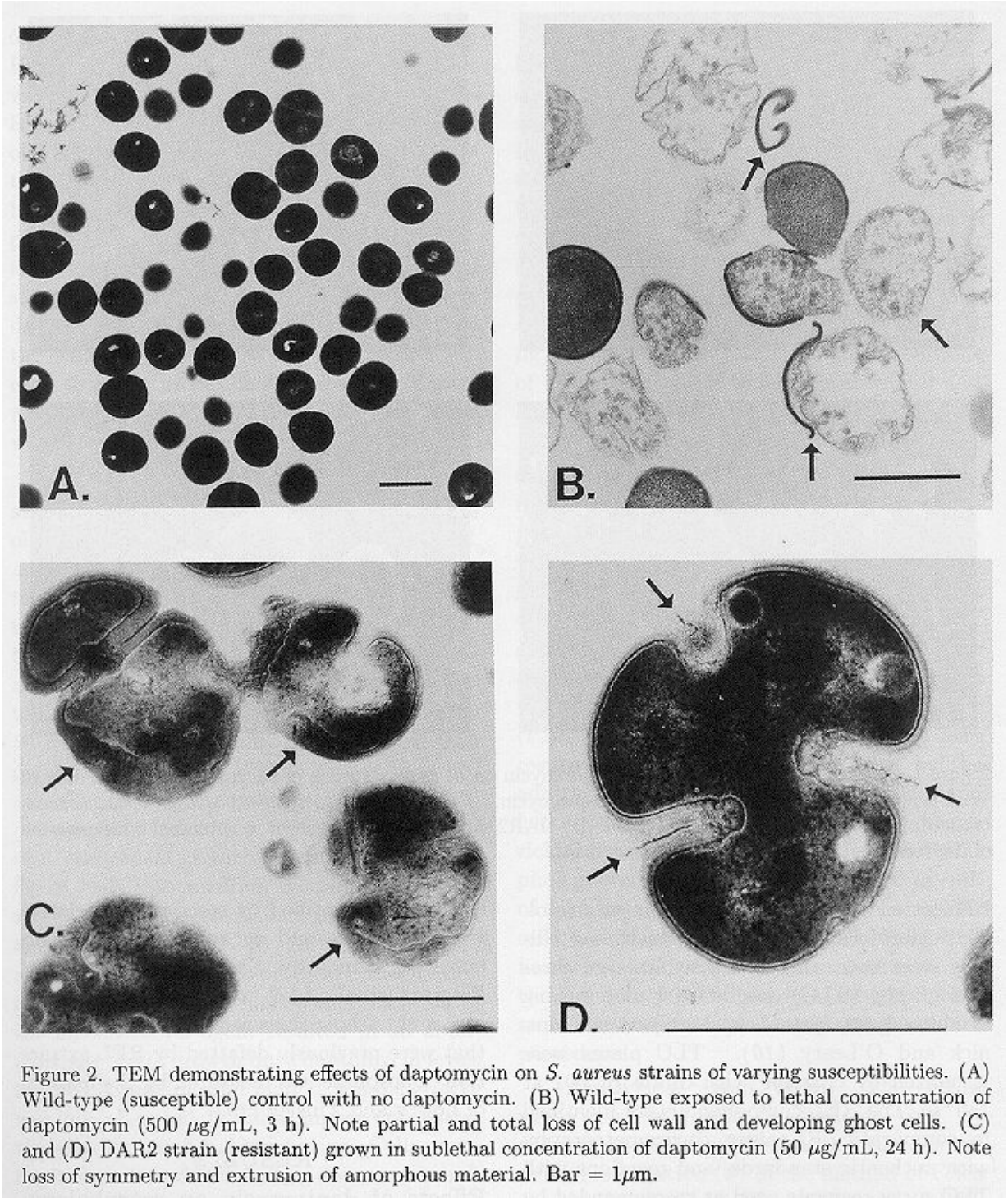

Figure 2. TEM demonstrating effects of daptomycin on *S. aureus* strains of varying susceptibilities. (A) Wild-type (susceptible) control with no daptomycin. (B) Wild-type exposed to lethal concentration of daptomycin (500 μg/mL, 3 h). Note partial and total loss of cell wall and developing ghost cells. (C) and (D) DAR2 strain (resistant) grown in sublethal concentration of daptomycin (50 μg/mL, 24 h). Note loss of symmetry and extrusion of amorphous material. Bar = 1μm.

linear cells, blebs, and deep web-like fissures. Although this aberrant morphology was most prominent in killed cells, DAR2-resistant cells grown in the presence of daptomycin also presented many of these same characteristics (Fig. 1D), which were consistent with results by Wale et al. (*14*). The extent of these cell wall perturbations was further illustrated by transmission electron microscopy (TEM) comparing normal WT cells (Fig. 2A) with killed cells (Fig. 2B), which have a total or partial loss of the cell wall. In addition, DAR2 cells presented protrusions of unidentified, capsular-like material (Figs. 2C,D) that were not observed in either killed or normal WT cells (Figs. 2A,B). This unknown, extruded material was noted in DAR2 cultures grown in either the presence or absence of daptomycin.

Effects of daptomycin adaptation on S. aureus cellular composition: Comparative analyses of WT and DAR2 strains indicated that selection for daptomycin resistance resulted in some quantitative, chemical changes of selected cellular components (Table 1). For example, the cellular percentage of the REL fraction of the resistant DAR2 strain, 1.3%, was considerably lower than that of the susceptible *S. aureus* WT strain, 3.1%. Otherwise, only minor quantitative differences were noted among the cellular percentages of the cell wall and peptidoglycan of resistant and susceptible cells. The relative percentage of phosphate in these respective fractions were similar, except for that of the lipoteichoic acids isolated from DAR2 cells. In that case, the phosphate content of DAR2 lipoteichoic acids was seven fold lower than that found in WT cells. This lack of major quantitative differences was somewhat surprising considering the striking morphological differences and daptomycin- induced cell leakage.

TABLE 1. Cell wall components in *Staphylococcus aureus* strains with varying susceptibilities to daptomycin.

	Strain			
	WT		DAR2	
Component	Cell.[a] %	PO_4 ppt[b]	Cell.[a] %	PO_4 ppt[b]
Cell Wall	28.0	68.8	21.0	65.2
Peptidoglycan	14.9	19.5	20.4	15.8
Lipo... acid[c]	<0.1	216.7	0.1	35.5
REL	3.1	13.1	1.3	10.6

[a] Cellular
[b] ppt = parts per thousand (μg/mg).
[c] Lipoteichoic acid.

Despite the quantitative differences in their respective REL, the fatty acids found in *S. aureus* WT and DAR2 strains were similar except for increased levels of a-C17 and decreased levels of C17 and C19 (Table 2) in DAR2. Moreover, these small differences were not even as pronounced as those noted when the WT strain was grown on another carbon source such as trypticase soy broth (TSB). For example, compared to CSMH-grown cells, WT cells grown on TSB presented higher concentrations of C18 and C20, a loss of C17, reduced levels of a-C17, and the occurrence of a-C21. Fatty acid methyl esters prepared by saponification and acidification of WT and DAR2 whole cells (data not shown) were indistinguishable from methyl esters prepared from their respective REL fractions.

TABLE 2. Fatty acid composition of REL fractions extracted from *Staphylococcus aureus* strains with varying susceptibilities to daptomycin.

	Area Percent[a]		
	Strain / Medium		
Fatty Acid	WT / CSMH[b]	WT / TSB[c]	DAR 2 / CSMH-50[d]
a-C15	41.93±1.02	39.28±2.75	45.33±1.43
i-C16	1.38±0.31	2.34±1.04	1.69±0.34
C16	1.98±0.21	2.40±1.25	2.17±0.41
a-C17	26.26±0.82	14.90±1.23	34.74±1.08
C17	2.57±0.28	0	0
i-C18	2.29±0.39	1.86±0.60	1.90±0.95
C18	3.27±0.18	11.43±0.85	2.41±1.20
a-C19	10.40±0.49	9.04±0.64	9.01±0.40
C19	6.82±0.39	1.79±0.67	0.59±0.41
C20	3.09±0.73	15.03±1.22	2.16±0.84
a-C21	0	2.30±0.87	0

[a] Values normalized to equal 100% by ignoring minor and unidentified peaks that ranged from 4 to 8% of the total in typical GLC chromotograms. Standard deviations calculated from 4 to 6 separate determinations.
[b] Calcium-supplemented Mueller-Hinton broth.
[c] Trypticase soy broth.
[d] Calcium-supplemented Mueller-Hinton broth with 50 μg/mL daptomycin.

WT and DAR2 REL were fractionated by column chromatography and examined by TLC. Only minor quantitative differences were noted between their REL components, which included pigments, glycolipids, and phospholipids (data not shown). The primary phospholipids of both strains were tentatively identified as diphosphatidyl glycerol, phosphatidyl glycerol, and osphatidyl serine.

MICs of selected antimicrobials in daptomycin-susceptible and -resistant strains of *S. aureus:* When evaluated in antimicrobial-free medium, the MICs of erythromycin, penicillin G, streptomycin, and tetracycline were similar for WT-susceptible and DAR2-resistant strains (Table 3). The MICs of these same antimicrobials were similar for DAR2 grown in CSMH media containing 12.5 μg/mL daptomycin. However, compared to WT, the MIC of polymyxin B for DAR2 was twenty-fold higher in antimicrobial-free medium, which increased to over sixty-four-fold when determined in medium containing sublethal concentrations of daptomycin.

TABLE 3. MICs of selected antimicrobials against strains of *Staphylococcus aureus* susceptible (WT[a]) and resistant (DAR2[b]) to daptomycin.

	MIC (μg/mL)[c]		
	Media		
	CSMH[d]		CSMH+[e]
Antimicrobial	WT	DAR2	DAR2
Erythromycin	0.4	0.2	0.4
Penicillin G	0.1	0.1	<0.1
Polymyxin B	50.0	1000.0	3200.0
Streptomycin	12.5	25.0	6.3
Tetracycline	0.8	0.8	0.8

[a] Daptomycin MIC 0.25 μg/mL.
[b] Daptomycin MIC 50 μg/mL.
[c] Average of 3–6 determinations.
[d] Cation-supplemented Mueller-Hinton (CSMH) broth. Read at 24 h.
[e] CSMH plus 12.5 μg/mL daptomycin. Read at 60 h due to slow growth.

Isolation of daptomycin-resistant strains: WT *S. aureus* FDA 209P strain, MIC of 0.25 μg/mL, was stepwise selected to a daptomycin resistance level of 50 μg/mL, Because cells did not revert to susceptibility even when repeatedly subcultured on CSMH media, daptomycin resistance in the prototype daptomycin-selected strain (DAR2) appeared to be stable. Likewise, spontaneous mutation to high levels of daptomycin resistance appears to be an infrequent event. This was demonstrated by the overnight incubation of WT-susceptible cells in CSMH media that contained concentrations of daptomycin ranging from 20 to 500 μg/mL, followed by subculturing onto CSMH and CSMH-50 media. Abundant growth on CSMH, but not on CSMH-50, indicated that surviving cells had neither adapted nor mutated to daptomycin resistance. The DAR2 strain retained typical staphylococcal characteristics, such as positive coagulase and catalase activities.

DISCUSSION

Electron microscopy in this study clearly demonstrates that lethal and sublethal concentrations of daptomycin disrupt the cellular integrity of *S. aureus* and form unusual and bizarre morphological forms. It is uncertain as to which specific, metabolic pathway was affected, but these abnormalities do not appear to be the consequences of major chemical changes in the lipids in the REL fraction. Preliminary analyses did, however, detect significant quantitative differences in the phosphates that are associated with the lipoteichoic acids isolated from resistant and susceptible cells. Disruptions in lipoteichoic acid metabolism are consistent with the current proposals that lipoteichoic acids are the primary target of daptomycin and underscores the vital role that these linear molecules play in maintaining the normal morphology of *S. aureus.* It also follows that, if daptomycin affects only the metabolism of lipoteichoic acids, collateral effects on REL and peptidoglycan metabolism in *S. aureus* are most likely the indirect effects of impaired membrane function rather than direct inhibition of a specific metabolic step.

The concomitant development of resistance to polymyxin B and daptomycin in DAR2 strain was unexpected and appears somewhat incongruous with our current understanding of these antimicrobials. For example, polymyxin B and daptomycin have completely different spectra of activity with each being highly specific for gram-negative and gram-positive bacteria, respectively (*1,15*). Assuming that daptomycin interferes with the synthesis of lipoteichoic acids, the general ineffectiveness of daptomycin for gram-negative bacteria is, therefore, attributable to the lack of appropriate target sites. Although the

mechanism of action of polymyxin B is still debatable, it is generally agreed that its activity is initiated by binding to the outer membrane, which leads to the disruption of the cytoplasmic membrane (*16*). Depending on the model system, susceptibility to polymyxin B has been linked with profound phospholipid alterations (*17*), and resistance has been associated with the presence or absence of 4-amino-arabinose esterified onto lipid A (*18*). It is unlikely that any of these relationships are pertinent to the polymyxin B resistance noted in DAR2 strain because daptomycin did not significantly affect the phospholipid content of *S. aureus,* which has neither outer membranes nor lipid A.

Although polymyxin B and daptomycin have very different spectra of activity, they nevertheless share some physical characteristics. Daptomycin is characterized chemically by a cyclic polypeptide anionic headgroup and amide-linked acyl side chain. The polymyxins are structurally and sterically similar, except that they have a polycationic headgroup (*19*). In the case of polymyxin B, the resultant amphipathic configuration is believed to facilitate an intercalation of antimicrobial into the bipolar cytoplasmic membrane. It is unknown whether the lipophilic and lipophobic regions of daptomycin act in an analogous fashion. Polymyxins are not considered appropriate chemotherapeutic agents for staphylococci, presumably due to their lack of binding sites and inability to traverse the normally impermeable protective cell wall. TEM in this study demonstrated that daptomycin leads to an obvious loss of cell wall material in killed cells and appears to have structurally compromised the cell wall of the DAR2 strain. Under these circumstances, one would anticipate that the weakened condition of the DAR2 cell wall would enhance the passage of polymyxin B to the otherwise vulnerable, cytoplasmic membrane. However, contrary to these expectations, adaptation to daptomycin resistance was accompanied by a simultaneous gain in resistance to polymyxin B. Polymyxin B resistance in the DAR2 strain was further enhanced when daptomycin was included in the test medium. This observation raises the possibility that daptomycin and polymyxin B are competing for the same target site. On the other hand, this seems implausible considering the differences in charge of these two antimicrobials and resultant intrinsic differences in electrostatic repulsion/attraction, which would appear to preclude any meaningful interaction at a common site.

In summary, we have found that daptomycin affects drastic morphological changes in *S. aureus.* These cellular changes were accompanied by alterations in lipoteichoic acids and minor changes in the REL fraction. Studies are currently underway to characterize chemically peptidoglycans and lipoteichoic acids isolated from susceptible and resistant cells. The intriguing possibility that resistance/susceptibility to daptomycin and polymyxin B are somehow linked will be further explored by examining the interactions between daptomycin and polymyxin B in gram-negative strains with varying degrees of susceptibility to polymyxin that results from mutation, stepwise selection, or naturally occurring resistance.

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