Serial Daptomycin Selection Generates Daptomycin-Nonsusceptible 
*Staphylococcus aureus* Strains with a Heterogeneous 
Vancomycin-Intermediate Phenotype

Ilana Lopes Baratella da Cunha Camargo,† Hui-Min Neoh,‡ Longzhu Cui, and Keiichi Hiramatsu*

Department of Bacteriology, Juntendo University, Hongo 2-1-1, Bunkyo-ku, Tokyo 113-8421, Japan

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In order to better understand the mechanism of daptomycin resistance, we generated a daptomycin-nonsusceptible derivative strain, strain 10Δ3d1 (MIC = 3.0 µg/ml), by in vitro exposure of methicillin-resistant *Staphylococcus aureus* strain N315ΔIP (MIC = 0.5 µg/ml) to daptomycin. We also obtained a daptomycin-susceptible phenotypic revertant strain, strain 10Δ3d1-10 (MIC = 1.0 µg/ml), by passing strain 10Δ3d1 in drug-free medium for 10 days. The resultant triple-isogenic strains were analyzed for their phenotypes and gene expression by microarray analysis. No significant differences in the membrane fluidities of 10Δ3d1 and 10Δ3d1-10 compared to the membrane fluidity of N315ΔIP were observed. Resistant strain 10Δ3d1 had the highest membrane potential, followed by strains 10Δ3d1-10 and N315ΔIP. The vancomycin and teicoplanin MICs also increased. Teichoic acid genes (*tagA*, *tagC*), *mprF* encoding lysyl-phosphatidylglycerol, and *cls* encoding cardioliopin synthase were downregulated in 10Δ3d1 and 10Δ3d1-10. The *vraF* and *vraG* genes, which encode ATP binding cassette transporter proteins, were upregulated in 10Δ3d1. The *vraSR* two-component regulatory system was upregulated, and electron microscopy revealed that the cell wall of 10Δ3d1 was significantly thicker than that of the parental strain. Taken together, daptomycin exposure selected a daptomycin-nonsusceptible strain with a phenotype similar to that of heterogeneous vancomycin-intermediate *S. aureus* and a transcription profile that partially overlapped that of heterogeneous vancomycin-intermediate *S. aureus*.

After the isolation of the first methicillin-resistant *Staphylococcus aureus* (MRSA) strains with reduced susceptibility to vancomycin in 1997, the need for a drug with a new target increased (20, 21). Due to the multiple-drug resistance profile of most *S. aureus* strains isolated nowadays, there are few therapeutic options available. Daptomycin is a calcium-dependent cyclic lipopeptide approved by the U.S. Food and Drug Administration in 2003 and, afterwards, by the European Agency for Evaluation of Medicinal Products for the treatment of complicated skin and soft tissue infections caused by susceptible bacteria. The U.S. Food and Drug Administration also licensed daptomycin for the treatment of *S. aureus* bacteremia and right-sided infective endocarditis (8, 15). Although it has already been approved, studies of daptomycin, which started 20 years ago, have not yet completely clarified its mechanism of action (1, 2).

The cytoplasmic membrane seems to be the main target of daptomycin. A model of action was proposed in which it was suggested that daptomycin first binds to calcium, reducing its charge from +3 to −1, before it binds to the membrane through its lipophilic tail (26, 48). It was demonstrated that daptomycin may aggregate in solution in the presence of at least an equimolar ratio of calcium to daptomycin, changing its conformation. However, to interact with the membrane, the aggregate formed may need to be dissociated (49) before it interacts with the phosphatidylglycerol (PG) head groups, which induces a positive curvature strain on the lipids (26). Daptomycin exhibits rapid bactericidal activity in vitro and causes membrane depolarization, which leads to potassium release and cell death (48). Despite the effectiveness of daptomycin that has been shown during clinical trials, strains nonsusceptible both in vitro and in vivo have already been isolated (19, 25, 53, 47, 50).

The exact mechanism of daptomycin resistance remains unknown, but some characteristics of the resistance phenotype have been described. The heterogeneous resistance profile was observed with daptomycin-nonsusceptible derivative strains, and they were found to have higher membrane potentials than their parental strains (27, 47). Recently, Jones et al. (23) also observed a heterogeneous pattern of resistance, but they did not observe any difference in membrane potential between nonsusceptible and susceptible strains during exponential and postexponential growth. However, the nonsusceptible strains were not depolarized by daptomycin.

Kaatz et al. (27) observed that smaller numbers of daptomycin molecules bind to the resistant strain than to its parental strain (27). They also demonstrated a loss of a membrane protein of 81 kDa in one of the nonsusceptible strains. Genetic changes in *mprF* (also called *fmtC*) and *yycG* (also called *vicK*) were correlated with the reduced susceptibility to daptomycin in both laboratory-derived and clinically isolated *S. aureus* strains (16). On the other hand, Julian et al. (25) analyzed...
more daptomycin-nonsusceptible clinical isolates and con-
cluded that some, but not all, nonsusceptible strains had a
mutation in mprF, but they did not detect a mutation in yycG.

Besides the membrane, a change in the cell wall may be
involved in daptomycin resistance. Cui et al. (11) described
a strong positive correlation between reduced daptomycin sus-
cceptibility and vancomycin resistance in vancomycin-interme-
diate S. aureus (VISA) strains, suggesting that the thickened
cell wall could be a physical barrier to daptomycin. Julian et al.
(25) investigated the muropeptide profile of a series of clinical
isogenic strains; the parent was susceptible to both daptomycin
and vancomycin, whereas its subsequent isolates were daptom-
ycin nonsusceptible and had a VISA phenotype. The strains
were serially isolated from a patient with endocarditis who
underwent therapy first with vancomycin and second with dap-
tomycin. The reduction of cross-linking of peptidoglycan and a
reduced degree of muramic acid O-acetylation were observed in
the daptomycin-nonsusceptible VISA strains, suggesting the
presence of a certain degree of modification of the cell wall.
Recently, Muthaiyan et al. (36) observed that daptomycin
induces the Staphylococcus aureus cell wall stress stimu-
lation and genes responsive to membrane depolarization. Therefore,
It seems that daptomycin may also influence cell wall synthesis.

In order to better understand the mechanism of daptomycin
resistance, we generated a laboratory-derived daptomycin-
nonsusceptible strain and analyzed its biological characteristics
in comparison with those of the daptomycin-susceptible parent
strain. This work reports that a part of the VISA phenotype,
as an increase in the level of yraSR expression and a
thickened cell wall, is cogenerated in the process of daptomy-
in resistance development.

MATERIALS AND METHODS

Bacterial strains. N315ΔIP is a MRSA strain that has been described previ-
ously (31). It is a derivative of pre-MRSA strain N315 whose mecI gene is intact
and in which methicillin resistance is not expressed (31). N315ΔIP represents the
genotype of hospital-acquired MRSA strains in Japan whose mecI gene is inac-
tivated by mutations. The penicillinase plasmid of N315 was further eliminated
from N315ΔIP to make the strain more pertinent as a model for the tracing of
the development of vancomycin-intermediate resistance in Japanese hospital-
adquired MRSA strains, since the representative heterogeneous VISA and VISA
strains Mu3 and Mu50, respectively, lacked the penicillinase plasmid. N315ΔIP
was classified as sequence type 5, staphylococcal chromosomal cassette mec type
IIa, and its agr type is II. Strain 10+3d1 is daptomycin nonsusceptible and a
 derivative of N315ΔIP obtained in this study. It was isolated after exposure to
daptomycin in two steps, as follows. A total of 10^7 CFU of N315
was spread on Mueller-Hinton agar (MHA) plates supplemented with 50 μg/ml calcium and
0.5 μg/ml daptomycin (Cubic Pharmaceuticals, Lexington, MA). Colonies were
recovered, and those with increased MICs were spread as described above on
plates containing 1 μg/ml daptomycin. Colonies were recovered, and the MICs
were determined. The colony with the highest MIC was subcultured for 6 con-
secutive days on plates containing 1 μg/ml daptomycin to stabilize the strain.
Strain 10+3d1 has an MIC of 3 μg/ml and is considered nonsusceptible by the
CLSI (9). Strain 10+3d1-10 is a daptomycin-susceptible derivative strain obtained
by serially cultivating 10+3d1 in 4 ml of drug-free Mueller-Hinton broth (MHB)
at 37°C. After 24 h, 4 μl was transferred to 4 ml of fresh medium. This procedure
was repeated for 10 consecutive days. All strains were checked for their isoge-
necy by pulsed-field gel electrophoresis (data not shown) and were stocked at
~80°C in 40% glycerol.

Antibiotic susceptibility tests. The daptomycin MICs were determined by the
broth dilution method with concentrations 0.125, 0.25, 0.50, 1.0, 2.0, 4.0,
and 6.0 μg/ml. The test medium was MHB supplemented with calcium, according
to the criteria of the CLSI (9). MICs were also determined by Etest (AB Biodisk,
Solna, Sweden) in MHA, as recommended by the manufacturer, since Etest
results have already been demonstrated to have a good correlation with those of
the CLSI method (24). In order not to miss a slowly growing resistant cell
subpopulation, the MICs were evaluated not only at an incubation time of 24 h
but also at an incubation time of 48 h (22). The MICs of vancomycin, teicoplanin,
linezolid, oxacinil, imipenem, bacitracin, fosfomycin, and colistin were deter-
mind by Etest in MHA; with fosfomycin, however, MHA was supplemented
with 25 μg/ml α-glucose-6-phosphate. The Etest with daptomycin was also per-
formed in the presence of gadolinium (III) chloride at 200 μM in MHA. Gad-
olinium chloride is a well-known mechanism-sensitive channel blocker (18) that can
decrease the membrane potential of the cell and that could give us new insight
into the importance of the high membrane potential of daptomycin-resistant
strains.

Population analysis. Analysis of the daptomycin-nonsusceptible popula-
tion of strain 10+3d1 was performed by comparing it to the population of
parental train N315ΔIP, as described previously (20). An appropriately diluted
overnight culture was spread onto MHA plates supplemented with 50 μg/ml
calium and daptomycin at various concentrations. The plates were theoreti-
bated at 37°C for 48 h to 72 h before the colonies were enumerated. The
population curve was drawn by calculating and plotting the number of resistant
cells theoretically contained in 50 μl of the undiluted culture.

Growth curve. Overnight cultures of the test strains were diluted 1/1,000 in 10
ml fresh MHB and were grown at 37°C with shaking at 25 rpm in a photore-
cording incubator (TN-2612; Advantec, Tokyo, Japan). The optical density (OD)
was automatically monitored every 2 min up to an OD at 600 nm (OD600) of 0.8.
For growth curve and doubling time determinations, the OD versus time was
plotted for each strain in the exponential growth phase. The doubling times were
then calculated as follows: [(t2 − t1) × log 2]/(log OD600 at t2 − log OD600 at t1),
where t1 is sampling time 1 and t2 is sampling time 2.

Membrane potential measurement. The membrane potential was determined
by flow cytometry by using a FACSAttix apparatus (BD, Biosciences, San Jose,
CA) with 1,3-diphenyl-1,3,5-hexatriane (DPH; Sigma) as a probe, as described by
Bayer et al. (48), and the membrane potential kit; Molecular Probes, Eugene, OR,
as described before (48), except for the following modifications. Strains were grown to exponential
phase (OD600 = 0.3) in MHB supplemented with 50 μg/ml calcium and diluted
1:100 in 1 ml of the component C provided by the manufacturer. Negative
controls consisted of cells treated with carbonyl cyanide 3-chlorophenylhydra-
zide (CCCP), which was provided with the kit, and gadolinium (III) chloride
(Sigma, St. Louis, MO), which was used at 200 μM (final concentration). In order to
distinguish depolarized cells from cells with damaged membranes, the imper-
meant DNA-binding dye TO-PRO-3 (concentration, 500 nM) was combined with
the cells, and an additional control consisting of N315ΔIP heat-killed cells
was included. A total of 30 μM DiOC2(3) was added to each sample, and the
tubes were incubated at room temperature for 4 min. Flow cytometry was
performed with 10,000 cells per run by using a 488-nm beam from an argon ion
laser to excite the DiOC2(3). The green and red fluorescence were detected as
1:1.48 and 1:7.48, respectively. The ratios of these values allowed us to compare the
membrane potentials of these strains. TO-PRO-3 was excited with a 633-nm beam
from a helium-neon laser, as recommended (48), and the detection of an elevated
red fluorescence with this dye (detected through a 695-nm long-pass filter) was
indicative of membrane damage (48). The membrane potential was measured as the fluorescence ratio (red/green) by using
the population mean fluorescence intensities.

Cell membrane labeling and fluorescence polarization measurement.
Membrane fluidity was determined by measuring fluorescence polarization with 1,6-
diphenyl-1,3,5-hexatriene (DPH; Sigma) as a probe, as described by Bayer et al.
(3). In brief, an overnight culture of S. aureus was washed two times with cooled
0.05 M Tris-HCl (pH 7.6) buffer by centrifugation (at 5,000 × g for 15 min), and
the culture was suspended in digestion buffer with a bacterial density of about 10^9
CFU/ml. The cell wall-free staphylococcal protoplasts were prepared as de-
scribed previously (10). For cell membrane labeling with DPH, a 2 mM solution
of DPH was prepared in tetrahydrofuran, and 400 μl of this solution was added
to 50 μl of 0.05 M Tris-HCl (pH 7.6). Excess tetrahydrofuran was removed by
flushing the solution with nitrogen. The protoplasts were then incubated in the
DPH solution at a final concentration of 2 μM for 60 min at 30°C. Fluorescence
polarization was measured after incubation of the labeled sample at 35°C for 30
min with a fluorescence spectrophotometer (F-4500-plus-FP; Hitachi, Tokyo,
Japan). The excitation and emission wavelengths were 360 and 426 nm, respec-
tively. The corrected fluorescence was determined by subtracting the emissions
of the unlabelled control protoplasts from those of the labeled protoplasts.
The degree of fluorescence polarization, or the polarization index (PI), was cal-
culated from the following formula, as described by Bayer et al. (3): \[ PI = \frac{(I_{Ex} - I_{Em})}{I_{Ex} + I_{Em}} \]
where \( I_{Ex} \) is the corrected fluorescence intensity and subscripts \( V \) and \( H \) indicate the values obtained with vertical or horizontal

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orientation of the analyzer, respectively. The lower that the PI value is, the more fluid the membrane is (3).

Electron microscopic cell wall thickness. The preparation of *S. aureus* cells for transmission electron microscopy and the examination of *S. aureus* cells by transmission electron microscopy were performed as described previously (12). Morphometric evaluation of the cell wall thickness was performed by using photometric images at a final magnification of ×30,000. Thirtynine cells of each strain with nearly equatorial cut surfaces were measured for the evaluation of cell wall thickness, and the results were expressed as the mean value ± standard deviation.

Autolysis assay. The autolysis assay was performed as described previously (39). Basically, overnight cultures of the test strains were diluted 1/1,000 in 10 ml fresh LB broth (pH 7.2) and were grown at 37°C with shaking at 25 rpm in the photorecording incubator indicated above. The OD was monitored automatically every 2 min, and the cells were grown to an OD600 of 0.7. Afterwards, the cells were pelleted by centrifugation at 7,500 rpm for 5 min at 4°C. The cells were then washed twice and resuspended in 0.01 M sodium phosphate buffer (pH 7.0). The cell suspension was then incubated at 37°C with continuous shaking at 25 rpm. The decrease in the OD was monitored every 2 min for 20 h with the same photorecording incubator described above.

DNA preparation and microarray analysis. The bacteria were grown in 10 ml MHB to exponential phase (OD600 = 0.3) onto MHA for daptomycin MIC determination by Etest. SA2179 was amplified with the primers 5′-TTC-3′ and 5′-AAATAGTCGACGGTACCGAAAGGAGAAAGGATTAGGTTCCA-3′, in which the underlined nucleotides in the two primers represent added BamHI and PstI sites, respectively. The integrities of the cloned genes were confirmed with 20 mM sodium acetate) was immediately added, followed by addition of 600 μl of 3 M sodium acetate. The samples were then frozen and thawed three times at −80°C and 65°C, respectively. Phenol-chloroform extraction and ethanol precipitation were then carried out. After that, the resulting RNA pellet was subjected to digestion with RNase-free DNase I (Roche, Mannheim, Germany) at 37°C for 30 min. The RNA samples were then purified again by phenol-chloroform extraction and ethanol precipitation. The pellets were then resuspended in 25 μl diethyl pyrocarbonate-treated water. The construction and analysis of the DNA microarray has been described previously (13). To confirm the reproducibility of the assay, RNA extraction and hybridization were performed in duplicate for each experiment. The results are given as the ratio of the average signal intensity of 100×3d1 to that of N315ΔIP to compare nonsusceptible and parental strains and the ratio of the average signal intensity of 100×3d1-10 to that of N315ΔIP to compare what changed and how similar the transcriptional profiles of the susceptible and the parental strain are.

Construction of overexpression plasmid and electroporation. Plasmid DNA was purified from Escherichia coli by using an SV Miniprep DNA purification system (Promega Corporation, Madison, WI), according to the manufacturer’s instructions. The isolation of DNA from *S. aureus* cells, restriction endonuclease digestion, ligation reactions, and DNA cloning were carried out as described previously (13). Electroporation of *S. aureus* was performed with a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA) with a pulse controller, as described previously (31).

Overexpression plasmids were constructed by cloning the PCR-amplified response regulator genes SA2179 and SA2151 from 100×3d1 into plasmid vector pRS and cloning pRS in N315ΔIP. SA2179 was amplified with the primers 5′-AAATAGATTCGCGAAAGTGATAGTGCGGGAAATGGTTTG-3′ and 5′-AAATAGTCGACGGTACCGAAAGGAGAAAGGATTAGGTTCCA-3′, in which the underlined nucleotides in the two primers represent added EcoRI and Sall sites, respectively. SA2151 was amplified with the primers 5′-ATAATGGATGGTCGATAGTGAGATGCTATAGTGAATAGAAGAAACGGAAGAACT-3′ and 5′-ATAATCTGGATGGTCCATCTTGTTAAGCTGGCAGGATTGATTTTCC-3′, in which the underlined nucleotides in the two primers represent added BamHI and PstI sites, respectively. The integrities of the cloned genes were ascertained by sequencing each overexpression plasmid. The resultant plasmids, p2179 and p2151, respectively, were then introduced into N315ΔIP, resulting in strains ΔIP(2179) and ΔIP(2151), respectively. Overexpression was carried out overnight in MHB plus 10 μg/ml chloramphenicol. Afterwards, the culture was spread at an OD600 of 0.3 onto MHA for daptomycin MIC determination by Etest.

Microarray data accession number. The profiles of strains 100×3d1 and 100×3d1-1 may be found under CIBEX accession no. CBX67.

RESULTS

Daptomycin-nonsusceptible and -susceptible strains derived from N315ΔIP. After two steps of daptomycin exposure, a daptomycin-nonsusceptible strain, strain 100×3d1, whose MICs were 2.0 μg/ml by the broth microdilution method and 3.0 μg/ml by Etest was isolated. Its phenotypic revertant (which was daptomycin susceptible), strain 100×3d1-10, was then obtained by serial passage of 100×3d1 for 10 days in drug-free MHB. Its daptomycin MIC was 1.0 μg/ml by both Etest (Table 1) and the broth microdilution method, and the strain was considered susceptible to daptomycin by the CLSI criteria (9).

Before the following experiments were started, the strains were confirmed to be isogenic by their identical pulsed-field gel electrophoresis patterns (data not shown). The MICs of several drugs for this isogenic set were determined by Etest (Table 1), and the values were read after 48 h at 37°C. It was noted that besides the increase in the daptomycin MIC from 0.5 to 3 μg/ml in strain 100×3d1, its vancomycin MIC increased from 0.75 to 3.0 μg/ml, its teicoplanin MIC increased from 0.5 to 6.0 μg/ml, and its imipenem MIC increased from 3.0 to >32 μg/ml (Table 1). The daptomycin MICs decreased in passage-derived susceptible strain 100×3d1-10, and the MICs of vancomycin and teicoplanin also decreased (Table 1). On the other hand, decreases in the MICs of beta-lactam antibiotics were not observed.

### Table 1. MICs of N315ΔIP and derivatives determined by Etest in MHA

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (μg/ml)</th>
<th>N315ΔIP</th>
<th>100×3d1</th>
<th>100×3d1-10</th>
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<tr>
<td>Daptomycin</td>
<td>0.5</td>
<td>3.0</td>
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<tr>
<td>Daptomycin + 200 μM GdCl₃</td>
<td>0.5</td>
<td>3.0</td>
<td>ND*</td>
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<td>Vancomycin</td>
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<td>&gt;256</td>
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<td>&gt;32</td>
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<tr>
<td>Fosfomycin²</td>
<td>12</td>
<td>0.5</td>
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<td>Colistin</td>
<td>256</td>
<td>192</td>
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</table>

*ND, not determined.  
²MIC of the subpopulation. 
Supplemented with 25 μg/ml of β-glucose-6-phosphate.

Daptomycin-nonsusceptible and -susceptible strains derived from N315ΔIP. After two steps of daptomycin exposure, a daptomycin-nonsusceptible strain, strain 100×3d1, whose MICs were 2.0 μg/ml by the broth microdilution method and 3.0 μg/ml by Etest was isolated. Its phenotypic revertant (which was daptomycin susceptible), strain 100×3d1-10, was then obtained by serial passage of 100×3d1 for 10 days in drug-free MHB. Its daptomycin MIC was 1.0 μg/ml by both Etest (Table 1) and the broth microdilution method, and the strain was considered susceptible to daptomycin by the CLSI criteria (9).

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*ND, not determined.  
²MIC of the subpopulation. 
Supplemented with 25 μg/ml of β-glucose-6-phosphate.
Overview of gene expression. We compared the microarray transcription profile of 10×3d1 with that of N315ΔIP in drug-free medium to figure out which genes are correlated with daptomycin resistance. Afterwards, we compared the microarray transcription profile of 10×3d1-10 with that of N315ΔIP to determine how similar gene expression in 10×3d1 and 10×3d1-1 (CIBEX accession no. CBX67) and see which genes have changed their expression levels after 10 days of serial passage in drug-free medium.

Transcription analysis showed upregulation of some enzymes of fatty acid metabolism and repression of phospholipid biosynthesis. Because the major target of daptomycin described so far is the membrane, we carefully observed the genes encoding the enzymes involved in fatty acid and phospholipid biosynthesis whose expression changed in strains 10×3d1 and 10×3d1-10 compared to their expression in strain N315ΔIP.

The expression of the enzymes involved in the metabolic process of fatty acid (SA0223 to SA0226) was enhanced in strain 10×3d1 (ratios > 2.0) but not in strain 10×3d1-10 (Table 2). On the other hand, vraA and vraB, which encode the long-chain fatty acid coenzyme A (CoA) ligase and acetyl-CoA c-acetyltransferase, respectively, along with vraC, which is included in this operon, were repressed only in 10×3d1-10. These data suggest that enhanced fatty acid metabolism may be correlated with enhanced resistance to daptomycin.

The expression of some genes involved in phospholipid biosynthesis in strains 10×3d1 and 10×3d1-10 also changed. The gene encoding cardiolipin synthase (cls) was repressed in the two strains (0.47 and 0.30, respectively), and mprF (or fmtC) was slightly repressed in both strains (0.61 and 0.51, respectively). mprF was sequenced, and there was no mutation in 10×3d1 (data not shown). These results show that the alterations in enzymes involved in the biosynthesis of phospholipids persisted even in the strain cultivated for 10 days in drug-free medium.

Besides fatty acid metabolism and phospholipid synthesis, the genes required for glycolipid synthesis, lipoteicoic acid anchoring (SA0875 [ypfP], SA0874 [laaA]), and teichoic acid synthesis (SA0592 [tagA]) were also repressed. Teichoic acid gene repression was accentuated in the susceptible strain (Table 2). The expression of the ltaS (SA0674) gene, which encodes polyglycerol phosphate synthase (LtaS), which is required for lipoteichoic acid synthesis, was not altered in strain 10×3d1 compared to that in N315ΔIP, but it was repressed in 10×3d1-10 (Table 2). The autolysin gene (atl), however, was upregulated in both strains.
<table>
<thead>
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<th>Gene action and ORF</th>
<th>Gene</th>
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<th>Transcription ratio</th>
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Regulators. Some two-component systems were expressed differently in strain 10\textsuperscript{3d1} and its susceptible derivative strain compared to their expression in strain N315\textsuperscript{ΔIP}. The vraS and vraR genes were upregulated only in the daptomycin-nonsusceptible strain (Table 2). The expression of the gra\textit{S} gene was slightly increased in the nonsusceptible strain, strain 10\textsuperscript{3d1} (Table 2), and the vra\textit{R} and gra\textit{S} genes did not show any transcriptional difference in susceptible strain 10\textsuperscript{3d1-10} compared to that in N315\textsuperscript{ΔIP}. The entire operon containing vra\textit{SR} and the gra\textit{RS} genes were sequenced, and no mutations were found.

The other two-component systems, SA2152 and SA2179, which have not yet been explored by our group, had only one of their components increased in strain 10\textsuperscript{3d1} but not in strain 10\textsuperscript{3d1-10} (Table 2). However, the introduction of the SA2179 and SA2151 genes on multicopy plasmids into N315\textsuperscript{ΔIP} did not raise the daptomycin MICs, which were between 0.38 and 0.50 μg/ml by Etest.

Genes related to the electron transport system. When an electron transport system is enhanced, it increases the membrane potential by pumping protons out of the cell. Several enzymes correlated with the electron transport system were upregulated in strains 10\textsuperscript{3d1} and 10\textsuperscript{3d1-10} compared to their expression in strain N315\textsuperscript{ΔIP}. NAD-dependent formate dehydrogenase, encoded by \textit{fdh} (SA0171), was the enzyme whose transcription level increased the most in 10\textsuperscript{3d1}, reaching a ratio of 82.28 when its transcription level was compared with that in N315\textsuperscript{ΔIP}. This gene was still upregulated in the susceptible strain to levels considered high (ratio = 3.18); however, its upregulation was much less so than that in the resistant strain (ratio = 82.28). Other sets of the genes involved in the electron transport system, such as succinate dehydrogenases and quinol oxidases, were upregulated in both strains (Table 2). Among these, the open reading frames (ORFs) SA0204, which encodes an NAD(P)H dehydrogenase homologue, and SA2311, which encodes a hypothetical protein similar to NAD(P)H flavin oxidoreductase, were upregulated only in strain 10\textsuperscript{3d1}.

MscL is a mechanosensitive channel with a large conductance that acts as an emergency valve in the bacterial cytoplasmic membrane by preventing excessive turgor pressure, which can cause the lysis of bacterial cells (32). This channel was already described as being involved in the calcium release induced by membrane depolarization in cytoncobacterial cells (38). Strains 10\textsuperscript{3d1} and 10\textsuperscript{3d1-10} presented increased levels of transcription of \textit{mscL} (3.36 and 4.58, respectively).

Transporters and cell wall-related genes. The expression of the following transporters was upregulated only in resistant strain 10\textsuperscript{3d1}. ORF SA0172, which encodes a membrane protein similar to LmrP, a multidrug transporter, was upregulated only in strain 10\textsuperscript{3d1} (ratio = 2.32), as were the vra\textit{FG} genes (Table 2).

Finally, we observed an increased level of transcription of the cell wall-related gene \textit{murI} only in strain 10\textsuperscript{3d1}. None of the penicillin-binding proteins was altered in the strains tested in this study (data not shown). Almost all genes in the capsule operon were found to be upregulated only in strain 10\textsuperscript{3d1-10} (Table 2).

Membrane potential and membrane fluidity. Strain 10\textsuperscript{3d1} presented a higher membrane potential than the parental strain (fluorescence ratios = 10.52 and 7.19, respectively). The membrane potential of strain 10\textsuperscript{3d1-10} decreased to 9.19 after it was cultured for 10 days in drug-free MHB, although it was still higher than that of strain N315\textsuperscript{ΔIP} (Fig. 3). It is already known that gadolinium (III) chloride can inhibit MscL (18), and since this channel was upregulated in the \textit{S. aureus} strains evaluated in our study, we inhibited MscL by adding 200 μM GdCl\textsubscript{3} solution to the culture. Studies of the viability of cells incubated in MHB with 200 μM GdCl\textsubscript{3} showed that this substance did not affect the total number of CFU per ml on MHA plates (data not shown). In the presence of 200 μM GdCl\textsubscript{3}, strains N315\textsuperscript{ΔIP} and 10\textsuperscript{3d1} presented lower membrane potentials (fluorescence ratios = 1.75 and 2.03, respectively), although they were still higher than those in the presence of the proton ionophore CCCP (Fig. 3). Thus, we determined the daptomycin MICs of 10\textsuperscript{3d1} and N315\textsuperscript{ΔIP} strains on MHA in the presence of GdCl\textsubscript{3}, but the daptomycin MICs did not change.

Jones et al. reported an increase in membrane fluidity in daptomycin-nonsusceptible strains (23). However, we did not observe any significant difference in membrane fluidity among strains 10\textsuperscript{3d1}, 10\textsuperscript{3d1-10}, and N315\textsuperscript{ΔIP}. The Pls from six independent measurements at 35°C were 0.296 ± 0.022, 0.296 ± 0.043, and 0.276 ± 0.019 for N315\textsuperscript{ΔIP}, 10\textsuperscript{3d1-10}, and 10\textsuperscript{3d1}, respectively.

Thickness of cell wall and autolysis assay. Although the autolysin gene was upregulated in strains 10\textsuperscript{3d1} and 10\textsuperscript{3d1-10}, in comparison to the autolytic activity in N315\textsuperscript{ΔIP}, these strains presented little increase in autolytic activity during 2 h of incubation (Fig. 4). We also measured the cell wall thicknesses of these strains in order to investigate if any change in the cell wall occurred by exposing the strain to daptomycin.

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**Table 2**—Continued

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Impressively, 10\textsuperscript{3}d1 had a very thick cell wall of 41.81 nm, while N315 presented a cell wall thickness of only 26.65 nm (Fig. 5). After 10 days of passage in drug-free medium, 10\textsuperscript{3}d1-10 showed a thinner (37.51 nm) cell wall than 10\textsuperscript{3}d1.

**DISCUSSION**

Several studies have been performed with daptomycin-resistant strains derived in vitro (16, 19, 33, 47, 50), yet the mech-
anism of daptomycin resistance remains a mystery. A decrease in daptomycin binding to the cell was observed in daptomycin-nonsusceptible MRSA strains (28). Jung et al. (26) showed that the daptomycin-calcium complex binds to the cell membrane via the negatively charged phospholipid head groups. Recently, Jones et al. (23) contributed immensely to the study of daptomycin-nonsusceptible methicillin-susceptible \textit{S. aureus} strains and observed many different aspects in the membrane, such as (i) enhanced membrane fluidity, (ii) increased translocation of the positively charged phospholipid lysophosphatidylglycerol (LPG) to the outer membrane leaflet, and (iii) an increased net positive surface charge. In addition, Jones et al. (23) described a reduced susceptibility to daptomycin-induced depolarization, permeabilization, and autolysis, as well as a significantly lowered surface binding of daptomycin and increased cross-resistance to the cationic antimicrobial host defensins hNP-1 and tPMP-1. Although the membrane has been regarded as the main target for daptomycin, our group observed a strong correlation between the presence of a thickened cell wall and daptomycin resistance. Thickening of cell wall peptidoglycan layers is the characteristic feature of VISA strains (11).

This study was conducted with MRSA strain N315ΔIP, a mecI-inactivated derivative strain of N315 from which penicillinase plasmid was eliminated, which represents the genotype of the most dominant hospital-acquired MRSA strains in Japan. VISA strain Mu50 also belongs to this group (30). Two steps of selection of N315ΔIP with daptomycin was enough to observe several significant alterations in the expression of the membrane- and cell wall-associated genes. The resistant strain obtained in our study presented a heterogeneous daptomycin-resistant population pattern compared to that presented by strain N315ΔIP. Besides reduced daptomycin susceptibility, strain 10*3d1 also had decreased susceptibility to other drugs, namely, vancomycin, teicoplanin, and imipenem. Thus, we obtained a strain with a heterogeneous VISA phenotype by exposing the parental strain only to daptomycin (Table 1). A transcriptional feature of VISA strain Mu50 was also observed in strain 10*3d1, that is, increased levels of transcription of \textit{vraSR} and \textit{vraFG} (39). Muthaiyan et al. (36) also reported that VraSR and the cell wall stress stimulon might be involved in the action of daptomycin.

Besides the feature in common with VISA strains, several alterations of transcription were specific to daptomycin resistance. Regarding the genes related to phospholipid biosynthesis, we noticed that the level of transcription of cardiolipin synthase gene (\textit{cls}) was repressed in both strains (strain 10*3d1 and 10*3d1-10) compared to that in N315ΔIP. Cardiolipin is a phospholipid that has a negatively charged (−2) head group and plays a role in the preservation of osmotic stability and the stabilization of spherical cytoplasmic membranes (37, 40). Cardiolipin has already been described as a proton reservoir, and its presence can decrease the membrane potential (28, 29, 51). Thus, the repression of the cardiolipin synthase gene observed in strain 10*3d1 may contribute to a high membrane potential and a low level of daptomycin binding to the membrane by reducing its negative charge.

Curiously, another gene related to phospholipid biosynthesis that was slightly repressed in strain 10*3d1 was \textit{mprF}. MprF is an enzyme responsible for the addition of an l-lysine to the negatively charged head group of PG, which forms LPG, which is a positively charged phospholipid (41). On the contrary to what has been described by another group (16), we did not find any mutation in the \textit{mprF} gene in the strains evaluated in our study. The apparent involvement of \textit{mprF} might corroborate the results of Julian et al. (25) and Pillai et al. (43), who showed that some, but not all, daptomycin-nonsusceptible strains have a mutation in \textit{mprF}. However, both strain 10*3d1 and strain 10*3d1-10 presented the same levels of repression of \textit{mprF} and \textit{cls} transcription compared to the level in strain N315ΔIP. Therefore, the repression of only cardiolipin synthase and/or \textit{mprF} is not enough to explain daptomycin resistance in this study.

Differences in phospholipid content may alter the membrane fluidity and the cell surface charge (41, 51). Alterations in membrane charge have been observed to repulse and prevent the attachment of some substances to the membrane (5). Recently, Jones et al. (23) studied daptomycin-nonsusceptible methicillin-susceptible \textit{S. aureus} strains and observed that the membrane was more fluid than that of the parental strain.

![Image](http://aac.asm.org/Downloadedfrom/4296 CAMARGO ET AL. ANTIMICROB. AGENTS CHEMOTHER.)
although there was no change in the fatty acid composition. They also found a less negatively charged membrane along with a higher proportion of LPG in the outer membrane leaflet of the nonsusceptible strains, but they found no statistically significant change in the phospholipid content (23). The findings of our study suggest a change in the amount of enzymes produced for phospholipid biosynthesis; but the PG, LPG, and cardiolipin contents should be judged by more extensive study of the composition and charge of the cell membrane. In our study, an increase of membrane fluidity was not observed for strain 10\textsuperscript{3d1}.

The membrane potential was one of the phenotypic differences found between strains 10\textsuperscript{3d1} and 10\textsuperscript{3d1-10}. It was already described that daptomycin-nonsusceptible strains obtained by serial passage in broth with increasing concentrations of daptomycin or during clinical treatment have higher membrane potentials than the wild type (27, 47). In our study, the membrane potential determined from the fluorescence intensity of \textit{DiOC\textsubscript{4}(3)} demonstrated that nonsusceptible strain 10\textsuperscript{3d1}, for which the daptomycin MIC was 3.0 \(\mu\)g/ml, presented a higher membrane potential than strain N315\textsuperscript{15\textsubscript{S}}. Susceptible strain 10\textsuperscript{3d1-10}, for which the daptomycin MIC was 1.0 \(\mu\)g/ml, had a lower membrane potential than the nonsusceptible strain, but it was still higher than that of N315\textsuperscript{15\textsubscript{S}}. The expression of some membrane proteins may be involved in the mechanism of the membrane potential in these strains. These proteins include MscL, cardiolipin, and proteins associated with the redox loop system, which involve succinate dehydrogenase, quinol oxidases, and dehydrogenases.

MscL has a major function in decreasing the intracellular pressure by releasing cytoplasmic osmotolites (35). It was described to be involved with calcium release in the cyanobacterium \textit{Synechocystis}. \textit{Synechocystis} MscL mutant strains, in which the mscL gene was interrupted by a kanamycin resistance gene, have more depolarized cells than the wild type in the resting state (38). We tested the MscL inhibitor GdCl\textsubscript{3} (18) in our strains and observed that although there was a decrease in the membrane potential, the daptomycin MIC did not change. This result corroborates the findings of another group that recently performed time-kill curve assays in the presence of MscL (34). Moreover, it is reported that a derivative strain of a methicillin-susceptible \textit{Staphylococcus aureus} strain was found to be daptomycin nonsusceptible, but there was no difference in the membrane potential between it and its parental strain (23). Therefore, it is not clear whether a high membrane potential contributes to the mechanism of daptomycin resistance or is only a consequence of changes in the membrane.

Several membrane transporters were altered in strain 10\textsuperscript{3d1} compared to those in the parental strain. SA0172 encodes a hypothetical protein similar to integral membrane protein LmrP that was upregulated only in the nonsusceptible strain. The multidrug transporter LmrP of \textit{Lactococcus lactis} was described as a proton-motive-force-driven hydrophobic “vacuum cleaner” that senses the hydrophobic substrates (such as cationic peptides) bound to the membrane and removes them to maintain the integrity of the membrane (4, 17). The increased activity of this protein might require a high membrane potential, as observed in strain 10\textsuperscript{3d1}.

The other genes encoding ATP binding cassette (ABC) transporter proteins, \textit{vraF} and \textit{vraG}, were also upregulated in strain 10\textsuperscript{3d1}. These transporter proteins may contribute to the vancomycin resistance associated with activated cell wall peptidoglycan synthesis (35). Matsuo and Hiramatsu recently observed that a \textit{vraFG}-deletant strain of Mu50 had a decrease in daptomycin resistance (M. Matsuo and K. Hiramatsu, unpublished observations). Interestingly, the \textit{mure} gene, which was already cited as being involved in cell wall synthesis, was upregulated only in 10\textsuperscript{3d1}. Finally, \textit{vraSR} were found to be upregulated only in 10\textsuperscript{3d1}. \textit{vraSR} is a two-component system that positively modulates the regulation of cell wall biosynthesis (30). The thickened cell wall and the increased vancomycin resistance of 10\textsuperscript{3d1} coincided with the increased level of \textit{vraSR} transcription. In VISA strain Mu50, two sets of mutated two-component systems, \textit{vraS}+\textit{R} and \textit{graR}+\textit{S} (the asterisks designate mutated genes) are responsible for vancomycin resistance (39). The overexpression of \textit{vraSR} in Mu50 is caused by a mutation in the \textit{vraS} gene (39; Y. Katayama, unpublished data). The expression of the mutated gene \textit{graR} but not that of wild-type \textit{graR} raises the daptomycin MICs of daptomycin-nonsusceptible \textit{S. aureus} strains (39). Although \textit{graR} is not mutated in 10\textsuperscript{3d1} nor is its transcription appreciably increased in 10\textsuperscript{3d1}, the ABC transporter genes \textit{vraFG}, which are positively regulated by \textit{graR} in Mu50, were upregulated in 10\textsuperscript{3d1}. If \textit{vraFG}, together with \textit{vraSR}, were direct contributors to the VISA phenotype, it would be reasonable that 10\textsuperscript{3d1} shares the VISA phenotype. Thus, although the regulator alteration was not common among the strains, a different combination of regulator alterations may yield similar physiological conditions required for such a phenotype as thickened cell wall or heterogeneous vancomycin resistance.

The thickening of the cell wall observed in VISA strains has been associated with the peptidoglycan-clogging mechanism, which prevents the passage of vancomycin through a thickened peptidoglycan layer (12). However, unlike vancomycin, daptomycin does not have binding sites in the peptidoglycan, so the clogging effect may not apply to daptomycin resistance. However, if daptomycin molecules aggregate themselves with calcium ions before they pass through the peptidoglycan layers, the peptidoglycan mesh may serve as an obstacle for daptomycin access to the membrane. Alternatively, the thickened cell wall of strain 10\textsuperscript{3d1} may be protecting the cells from rupture due to the increased membrane fluidity caused by the action of daptomycin. Jones et al. (23) observed that the membrane of a daptomycin-nonsusceptible strain was more fluid than that of the parental strain. Fluid membranes have less resistance to turgor pressure and need a stronger cell wall to protect the cell. In our study, the membrane of the daptomycin-nonsusceptible strain was not more fluid than that of the parental strain. Therefore, it remains to be tested whether cell wall thickening alone directly contributes to daptomycin resistance.

Muthaiyan et al. (36) studied the daptomycin response of a \textit{VraSR} mutant strain, and they indicated that the inhibition of peptidoglycan biosynthesis (directly or indirectly) is a part of the mode of action of daptomycin. In this regard, the activation of cell wall synthesis driven by the \textit{vraSR} upregulator would be important for the survival of cells exposed to daptomycin.

Finally, the transcription of teichoic acid synthesis genes was repressed in both strain 10\textsuperscript{3d1} and strain 10\textsuperscript{3d1-10}. All these
results suggest that the cell wall composition was substantially modified during the development of daptomycin resistance. We have tried to analyze the genetic basis for daptomycin resistance. We noticed that both membrane and cell wall synthesis pathways are affected, and the latter seemed to be similar to the pathway that affects vancomycin resistance in VISA strains. A few mutations exquisitely placed in the extremely intertwined S. aureus regulatory network seem to generate a range of types of physiological status protective against both vancomycin and daptomycin.

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REFERENCES


44. Reference deleted.

45. Reference deleted.

46. Reference deleted.


