Altering the Proclivity towards Daptomycin Resistance in Methicillin-Resistant
Staphylococcus aureus Using Combination with Other Antibiotics

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Running title: Antibiotic combinations alter daptomycin resistance

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Abstract

Daptomycin (DAP) is increasingly used as a part of combination therapy, particularly in complex MRSA infections. While multiple studies have reported the potential for synergy between DAP and adjunctive anti-infectives, few have examined the influence of adjunctive therapy on the emergence of DAP resistance. This study examined eight adjunctive antimicrobial combinations with DAP in vitro and the emergence of DAP resistance over time (up to 4 weeks) using clinical isolates of DAP susceptible MRSA (MIC 0.5 µg/ml) in which DAP resistance subsequently developed during patient therapy (MIC 3 µg/ml). In addition to DAP susceptibility testing, selected strains were examined for phenotypic changes associated with DAP resistance, including changes to cell wall thickness and cell membrane alterations.

The addition of either oxacillin or clarithromycin in media containing DAP significantly inhibited the development of DAP resistance through the entirety of the four-week exposure (10-32-fold MIC reduction vs DAP alone). Combinations with rifampin or fosfomycin were effective in delaying the emergence of DAP resistance through the end of week one only (week one MIC: 0.5 µg/ml; week four MIC: 24 µg/ml). Cell wall thickening was observed for all antibiotic combinations regardless of their effect on the DAP MIC (14-70% increase in CWT), while changes in cell membrane fluidity were variable and treatment dependent. DAP showed reduced activity against strains with DAP MICs of 1-12 µg/ml, but cell membrane integrity was still disrupted at concentrations achieved with doses greater than 10 mg/kg.

The emergence of DAP resistance in MRSA is strongly influenced by the presence of subinhibitory concentrations of adjunctive antimicrobials. These data suggest that combining...
DAP with oxacillin or clarithromycin may delay the development of DAP resistance in cases requiring prolonged antibiotic therapy.

**INTRODUCTION**

Since its approval in 2003, daptomycin (DAP) has been increasingly used in the treatment of various types of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA). However, by 2005 the first cases of DAP nonsusceptible MRSA (DAP MIC >1 µg/ml) were observed (7, 21, 43). Although the incidence remains low, DAP resistance is thought to emerge via the accumulation of multiple mutations, each contributing a slight increase to the DAP MIC (26, 49) with significant strain-to-strain variability in the affected pathways (13, 50).

When treating complicated infections, low free (f) DAP concentrations at the site of infection may encourage the selection of DAP resistant subpopulations and subsequent loss of susceptibility, particularly when bacterial inoculum is high and with prolonged exposure (21). The recommended administration of DAP every 24 hours results in a large difference in the serum peak and trough concentrations (at 6 mg/kg: $fC_{\text{max}} = 8$ µg/ml, $fC_{\text{min}} = 1$ µg/ml), potentially exposing the bacteria to repeated cycles of inhibitory-subinhibitory unbound antibiotic concentrations in strains with MICs > 0.5 µg/ml (11). The infection itself may also play a role, as it can be sequestered in privileged locations such as in biofilms, bone or cardiac vegetations where antibiotic effectiveness may be reduced (29, 39, 45). There is also growing evidence that prior exposure to other antimicrobials, particularly vancomycin (VAN) and host-derived cationic peptides, can encourage the development of DAP resistance in DAP-naïve
patients (9, 25). Therefore, even when following current best practices, conditions favoring the emergence of DAP resistant strains may occur.

In order to improve clinical success rates and minimize the emergence of DAP resistance, DAP is increasingly used in combination with adjunct antibiotics, although clinical studies supporting this practice are lacking (18). Several *in vitro* and animal infection models have shown additive or synergistic effects with DAP combination therapy (29, 42, 44). Previous work has examined the effect of combination exposures on the emergence of DAP resistance, although these studies are often limited by a number of factors including short exposure duration, use of static antibiotic concentrations, testing of reference strains rather than clinical isolates, and the few number of combinations examined (4, 20, 35, 38). Of note, Entenza and colleagues demonstrated that beta-lactam antibiotics significantly decrease the emergence of DAP resistance during a week-long subinhibitory exposure in Gram-positive isolates from a laboratory collection. Rifampicin and gentamicin were shown to have comparatively little effect, delaying the onset of resistance by no more than one day (12).

This study examined the effect of *in vitro* antibiotic combination exposures over 28 days on the emergence of DAP resistance in clinical isolates of MRSA with a known propensity for developing DAP resistance during patient treatment. Eight antimicrobials that have been used therapeutically in combination with DAP were selected (29, 42, 44). In addition to measuring changes in the DAP MIC, strains selected under these antimicrobial combinations were examined for phenotypic changes associated with DAP resistance, including changes to cell wall thickness and cell membrane physiology.
MATERIALS and METHODS

Bacterial strains. Three pairs of MRSA bloodstream isolates from 3 different patients in which DAP nonsusceptibility emerged were examined in this study. D592, J01 and O325 are the initial pre-treatment, DAP-susceptible MRSA strains from the three individual patients, and D712, J03 and O510 are the respective DAP nonsusceptible strains that emerged on therapy. D592/D712 and O325/O510 have been described in previous work (10). Clonal relationship between sequential paired isolates was confirmed by whole genome sequence analysis (data not shown).

Antimicrobials and Media. Clarithromycin (CLR), fosfomycin (FOF), gentamicin (GEN), oxacillin (OXA), rifampicin (RIF) and trimethoprim-sulfamethoxazole (SXT) were purchased from Sigma-Aldrich (Sigma, St Louis, MO, USA). DAP was provided by Cubist (Cubist Pharmaceuticals, Lexington, MA, USA). Linezolid (LZD) was obtained from Pfizer (Pfizer Inc, New York, NY, USA). Moxifloxacin (MXF) was obtained from Bayer (Bayer Corp, West Haven, CT, USA). Mueller-Hinton Broth II (BD, Sparks, MD, USA) supplemented with 50 µg/ml calcium (as CaCl₂) and 12.5 µg/ml magnesium (as MgCl₂) (MHB50) was used to grow S. aureus in liquid culture.

Susceptibility Testing. MICs of study bacteria to DAP, OXA, SXT, and VAN were determined by E-test as suggested by the manufacturer (bioMérieux, Marcy-l’Etoile, France). MICs to other antibiotics were determined by broth microdilution as per the Clinical and Laboratory Standards Institute (CLSI) guidelines (7). All samples were incubated at 35°C for 24 h. Reference strain ATCC 29213 was included for MIC determination testing as an internal control. All values obtained were within the acceptable CLSI range for this organism. DAP resistant strains that
emerged under antimicrobial *in vitro* selection were passaged twice in media without antibiotics prior to MIC determination.

**Strain Characterization.** All strains obtained or developed in this study were confirmed as *S. aureus* by microscopy and either lysostaphin testing (Remel, Lenexa, KS, USA) or icaA amplification (2). The presence of *S. aureus mecA* element was identified for individual strains according to the method of Zhang *et al* (52).

**DAP Exposure Conditions.** As a preliminary experiment, all three DAP-susceptible strains were grown in triplicate and exposed to increasing concentrations of DAP in the presence or absence of combination antibiotics for 14 days as outlined below. Changes to DAP MICs following both DAP exposure and combination exposures were similar among the three strains (data not shown). The strain selected for a more robust 28-day study was J01, a DAP-susceptible clinical isolate from a patient with left-sided endocarditis and multiple septic-embolic complications, including splenic and spinal epidural abscesses. Strain J01 was collected while the patient was receiving VAN monotherapy. Over the next 24 days the patient received a complex antibiotic regimen consisting sequentially of VAN alone, VAN+GEN, VAN+GEN+RIF, DAP alone (8 mg/kg) and DAP+GEN. Strain J03 was collected after the 24 day period while the patient was receiving DAP+GEN therapy. The patient ultimately required valve replacement and splenectomy and completed therapy with LZD followed by minocycline. Strain J01 was selected for further study because the DAP MIC of 0.5 µg/ml in this strain is comparable to MIC₉₀ values reported from surveillance studies (36). Isolated colonies of J01 were collected from MH50 agar + 0.125 µg/ml DAP, corresponding to ¼ MIC. Bacteria were resuspended in MHB50 to an OD₆₀₀ of 0.5 and diluted 1:100 in fresh
MHB50 containing 0.125 µg/ml DAP. The cultures were shaken overnight at 37ºC and 160rpm. Any cultures that showed visual growth (≥ OD₆₀₀ = 0.2) were collected, diluted 1:100 into MHB50 containing an elevated concentration of DAP and returned to the shaker. In this fashion, samples that showed visual growth after 24-hours’ incubation were used to inoculate each next day’s passage, and the concentration of DAP was increased stepwise. As permitted by growth, DAP concentrations were incremented by 0.25 µg/ml until overnight growth was observed at 1.0 µg/ml. The stepwise daily increment was increased to 0.5 µg/ml and maintained until overnight growth was observed at 5 µg/ml. The increment was increased further to 1 µg/ml until overnight growth was observed at 15 µg/ml. The increment was then increased to 5 µg/ml and maintained until end of study.

Five distinct passages started from isolated colonies were analyzed. Serial passages were performed for a total of 28 consecutive days and DAP MICs were determined on days 7, 14, 21 and 28, as described above.

**Combination Exposure Conditions.** Isolated colonies of the J01 parent strain were passaged for 28 days, as outlined above, except that the media contained a consistent concentration of a secondary antibiotic. In this fashion, the concentration of DAP was increased stepwise while the concentration of the secondary agent was held constant. Secondary antibiotics were added at the following concentrations, corresponding to ½ the initial MIC value established for strain J01: FOF (3.0 µg/mL in MHB50 also containing 25 µg/mL glucose-6-phosphate, as recommended (7)) GEN (0.5 µg/mL), LZD (0.062 µg/mL), MXF (1.0 µg/mL), OXA (16 µg/mL), RIF (0.002 µg/mL), SXT (0.3 µg/ml / 6 µg/ml, respectively). CLR was added at 50 µg/ml, corresponding to 20% of the initial MIC value established for strain J01, due to culture sterilization following sequential selection at ½ the initial MIC value.
**Cell Wall Thickness.** Cell wall thickness (CWT) was determined by transmission electron microscopy (TEM) as described previously (34). Samples were collected from 28-day exposure cultures after a minimum of 2 serial passages in media lacking antibiotic selection. Images were analyzed and measured using ImageJ 1.39t software. CWT measurements were made on a minimum of 15 cells per treatment using four separate quadrants of each cell.

**Membrane Fluidity Assay.** Membrane fluidity was determined spectrofluorometrically using 1,6-diphenyl-1,3,5-hexatriene (DPH) as previously described (3, 5) Overnight bacteria cultures were inoculated into fresh MHB50 medium and grown to an OD\textsubscript{600} of 0.2-0.5. Bacteria were collected via centrifugation, washed with normal saline (0.85% NaCl) and resuspended to an OD\textsubscript{600} of 0.3 in normal saline + 2 µM DPH. The DPH cell suspension was incubated for an hour at 37°C. Aliquots were transferred to preheated cuvettes and fluorescence was measured in an ISS Koala spectrofluorometer with a temperature-controlled cuvette holder maintained at 37°C. The probe was excited with vertically polarized light (λ\textsubscript{ex} = 360nm) and emission intensity was detected in both parallel and perpendicular planes (λ\textsubscript{em} = 426nm). Results were corrected by subtracting data from an unlabelled control reaction. The polarization index (p) was determined using the formula: 
\[ p = \frac{[I_V - I_H (I_{HV}/I_{HH})]}{[I_V + I_H (I_{HV}/I_{HH})]} \]
where (I_{HV}/I_{HH}) is the sensitivity ratio (G-factor) of the instrument for detecting DPH emission. Data were collected on 3 separate days with 9 readings from every sample recorded each day. Data are presented as the average ± standard deviation of the 27 total readings for each treatment. A lower fluorescence polarization value indicates a higher degree of membrane fluidity.

**Membrane Potential Dissipation Assay.** Membrane potential dissipation due to DAP was assayed via spectrofluorometry, as described elsewhere (30, 41). Briefly, overnight bacteria cultures were inoculated into fresh MHB50 medium and grown to an OD\textsubscript{600} of between 0.15 and
Cells were collected, washed with Dissipation Buffer, adjusted to an OD₆₀₀ of 0.25 in Dissipation Buffer + 2µM DiSC3(5) and incubated for 15 minutes at room temperature. Aliquots were then exposed for another 60 minutes at room temperature to one of the following DAP treatments corresponding to \( f_{C_{\text{max}}} \) values for these the indicated regimen: 6 mg/kg DAP (7 µg/ml, final m/v), 10 mg/kg DAP (11.5 µg/ml, final m/v), 12 mg/kg DAP (14 µg/ml DAP, final m/v). Ethanol (20%) and Dissipation Buffer were used for positive and negative controls, respectively. Samples were transferred to a cuvette, placed in an AMINCO-Bowman spectrofluorometer heated to 37ºC (\( \lambda_{\text{ex}} = 622\text{nm} ; \lambda_{\text{em}} = 670\text{nm} \)) and data were collected for a minimum of 30 seconds. Percentage depolarization activity is calculated as \( \frac{(F_D - F_C)}{(F_E - F_C)} \). Results are reported as the mean ± standard deviation of a minimum of three independent experiments.

Membrane Integrity. Staphylococcal membrane permeability after exposure to DAP was assayed with the LIVE/DEAD BacLight kit (Invitrogen, Madison, WI). Bacteria grown to an OD₆₀₀ of between 0.2 and 0.4 in MHB50 were resuspended to an OD₆₀₀ of 0.25 in 5 mM HEPES buffer + 50 µg/ml Ca²⁺. Each suspension was divided into 4 aliquots of 160 µl each, transferred to 96-well plates and each well was mixed with 40 µl of 5x antibiotic concentrate resulting in the following DAP exposures: 6 mg/kg DAP (7 µg/ml, final m/v), 10 mg/kg DAP (11.5 µg/ml, final m/v), 12 mg/kg DAP (14 µg/ml DAP, final m/v) or HEPES Buffer control. Suspensions were incubated for 3 hours at room temperature, brought to 5 µM SYTO-9 and 30 µM propridium iodide and incubated for an additional 15 minutes at room temperature in the dark. Fluorescence was measured in a Molecular Devices SpectraMax M2e spectrofluorometer (\( \lambda_{\text{ex}} = 485\text{nm} \)). Emission spectra were monitored at 530nm [SYTO-9] and 645nm [propridium iodide]. A live-dead fluorescence reading (\( F \)) was defined as the ratio of the SYTO-9 fluorescence to the...
propridium iodide fluorescence. Membrane integrity was reported as $F_D / F_C$. A lower value indicates more extensive membrane damage. Results are reported as the mean of a minimum of three independent experiments.

**Statistical Analysis.** All replicate measurements from each individual strain were considered to be random samples and comparisons were not made between strains obtained from different patients. Comparisons of MIC values were conducted with Wilcoxon rank sum tests. Comparisons of all other measurements were conducted with Student’s t-tests. Reported p-values are Holm adjusted to account for the multiple comparisons made within each measurement (16).

**RESULTS**

*Combination exposure can alter the development of DAP resistance.*

As demonstrated in **Figure 1**, inclusion of different antimicrobial agents during growth *in vitro* had significant differential effects on the rate at which DAP resistance developed. These effects appeared to be dependent on both duration of exposure and on the secondary antibiotic. Some combinations (DAP + FOF, DAP + RIF) were effective in delaying the emergence of DAP resistance through the end of week one but were ineffective in maintaining that suppression over the four-week course of study. More importantly, including either CLR or OXA in growth medium significantly and consistently reduced the development of DAP resistance through the entirety of the four-week period. While strains grown in a combination of DAP plus CLR did possess lower DAP MIC values at the end of the experiment than strains grown in DAP alone, those strains were still DAP-resistant (median 4-week DAP MIC=3 µg/ml; range: 1.5 µg/ml – 8 µg/ml). DAP plus OXA was the only combination exposure that largely prevented the
emergence of DAP resistance over the 4-week period (median 4-week DAP MIC=1 µg/ml; range: 0.02 µg/ml – 3 µg/ml). Conversely, by week two, bacteria grown in a combination of DAP and LZD were significantly more resistant to DAP than bacteria grown in DAP alone.

In order to assess the efficacy of different DAP treatment doses against DAP-susceptible MRSA and its \textit{in vivo}- or \textit{in vitro}-derived DAP-resistant relatives, we examined both cell membrane integrity and membrane potential dissipation activity of DAP (Tables 1 and 2). As anticipated, all strains show a dose-dependent decrease in membrane integrity. While the trends are suggestive, none of the \textit{in vivo}-derived strains showed any statistically significant differences in either membrane integrity or potential dissipation at any given dosage (Table 1). In contrast, all of the \textit{in vitro}-derived strains with the exception of those grown in DAP + OXA showed significantly higher preservation of membrane integrity in the presence of DAP relative to the DAP-susceptible J01 strain at all dosages ($p < 0.001$). The strains derived in the DAP + OXA selective environment showed significantly greater membrane disruption by DAP in a dose-dependent manner. Further, several of the \textit{in vitro}-derived strains exposed to combinations of antibiotics during growth demonstrated significant differences in membrane integrity at the 6 mg/kg dosage when compared to \textit{in vitro}-derived strains exposed to DAP alone. At higher dosages, only strains exposed to DAP + CLR or DAP + OXA had significantly reduced membrane integrity compared to strains exposed to DAP alone (Table 2).

\textit{Combination therapy alters the emergence of changes to the cell envelope in S. aureus.}

Cell envelope alterations associated with DAP resistance, including cell wall thickening and membrane fluidity restructuring were examined (Tables 1 and 2). No significant changes in cell
wall thickness were observed between the DAP-susceptible strains obtained from patients and their DAP-resistant pair (Table 1, Figure 2). However, strains exposed in vitro to DAP alone resulting in a median MIC of 32 µg/ml possessed significantly thicker cell walls than the J01 parent strain. Likewise, strains exposed to combination antibiotics in vitro contained significantly thicker cell walls in all combinations tested regardless of whether they delayed or accelerated the development of DAP resistance (p < 0.05 for DAP + GEN; p < 0.001 for all other combinations; Table 2, Figure 2).

All in vivo derived DAP resistant mutants tended to have increases in membrane fluidity compared to their DAP susceptible parent strains, but a statistically significant increase in membrane fluidity was observed only for the D series strain pair (Table 1). The membrane fluidity of the in vitro-derived MRSA strains was less predictable (Table 2). Combinations of DAP + GEN and DAP + OXA generated strains that contained significantly more fluid membranes relative to J01. However, other combinations produced strains with significantly less fluid membranes (DAP alone, DAP + FOF, DAP + LZD, DAP + MXF, DAP + SXT) or statistically indistinguishable membranes (DAP + CLR, DAP + RIF).

**DISCUSSION**

MRSA strain J01 was obtained as the original DAP-susceptible clinical isolate from a patient upon presentation with bacteremia that was ultimately determined to be the result of pacemaker-associated, left-sided endocarditis. Several metastatic foci of infection were identified, including spinal epidural abscess and splenic abscess. The MRSA lost susceptibility to DAP on DAP therapy. Several antimicrobial therapies were used alone and in combination, but were limited by
resistance, relapsing or persisting MRSA bacteremia, or adverse events to medication. The patient was ultimately cured by pacemaker removal, valve replacement, and splenectomy and finished 12 weeks of therapy with LZD and minocycline. J01 is therefore well-suited for studying the impact of antibiotic combination therapy on the emergence of resistance, as it is predisposed to develop resistance in vivo.

This study showed that growth in media containing different antibiotics in combination with DAP can significantly alter the propensity towards the emergence of DAP resistance in MRSA (Figure 1). Bacteria cultured in combinations of DAP + FOF or DAP + RIF displayed significantly lower DAP resistance at week one compared to culturing in DAP alone, but those distinctions were lost by week two. Of particular interest, combinations of DAP + OXA and DAP + CLR appear to significantly reduce the emergence of DAP resistance over 28 days of treatment, which is often a minimum recommended therapy duration for complicated infections, as was the case in the patient from whom the test strain was isolated. Published reports on the combination of DAP and FOF are few and have not addressed the effect of FOF on DAP resistance development (6, 31, 33). More data are available on the addition of RIF, which has been shown previously to delay the emergence of DAP resistance (12, 20) and can significantly enhance clearance of complex MRSA infection when given in combination with DAP (1, 38), possibly by suppressing the emergence of resistance to both agents (15). Combination with DAP + CLR has been shown to be superior to either antibiotic alone in the eradication of biofilms (29) but there have been no reports of macrolide antibiotics suppressing the emergence of DAP resistance.

The combination of DAP with beta-lactam antibiotics has been shown to be synergistic against S. aureus (32). Intriguingly, there also appears to be a seesaw effect wherein...
development of DAP resistance in strains that are initially beta-lactam resistant leads to partial
resensitization to the beta-lactam antibiotic (51). The combination of DAP with
antistaphylococcal beta-lactams has been used successfully to treat the most recalcitrant cases of
MRSA bacteremia (10). Having evolved several highly DAP-resistant strains, we were interested
to see if the OXA sensitivity in these strains had changed. We tested J01-S03, a strain grown in
both DAP and SXT that had an increase in the DAP MIC from 0.5 μg/ml on day one to 32 μg/ml
on day 28. The OXA MIC of this strain was determined by E-test as 0.38 μg/ml, greatly reduced
from an OXA MIC of 32 μg/ml in the J01 parent strain and less than the 4 μg/ml threshold
definitive of MRSA. This beta-lactam resensitization phenomenon was also observable among
the in vitro-derived DAP-resistant strains cultured in other antibiotics or in DAP alone (data not
shown).

MRSA strains commonly develop high-level methicillin resistance via acquisition of the
mecA gene driving production of a beta lactam-insensitive transpeptidase (46). We confirmed via
PCR amplification that the parent J01 strain and J01-S03 both contain the mecA gene normally
responsible for the MRSA phenotype. The development of methicillin-sensitivity in S. aureus
strains that maintain a mecA element is not novel and has been described elsewhere in
association with DAP resistance (51) and vancomycin resistance (28). The mechanisms
underlying this phenomenon are poorly understood and likely involve at least some changes that
are mecA-independent (47).

Given the similar trends of DAP + CLR and DAP + OXA for the suppression of DAP
resistance in our study, we decided to test if DAP resistant in vitro-derived MRSA demonstrated
a seesaw effect to CLR analogous to that seen for OXA. Indeed, the MIC to CLR of strain J01-
S03 was determined by broth microdilution as 16 μg/ml. While this strain is still considered
CLR-resistant, the CLR MIC is significantly reduced from 256 μg/ml in the J01 parent strain. As with the OXA seesaw effect, the CLR resensitization phenomenon was also observable among other in vitro-derived DAP-resistant strains cultured in other antibiotics or in DAP alone (median CLR MIC: 16 μg/ml). We highlight this because CLR was so efficient at suppressing bacterial growth that our experimental design was amended to include CLR at a lower percentage of the measured MIC (20%) compared to other antibiotics tested (50%).

To our knowledge, this is the first report of a seesaw effect observed between DAP and CLR. One potential factor known to contribute to DAP resistance is a mutation in the $yycFG$ two-component regulator system involved in cell membrane function (14). Interestingly, an early publication characterizing $yycFG$ in $S. aureus$ noted that a conditional-lethal mutant in this system rendered the bacteria hypersusceptible to macrolides (22). Upon MIC testing of our in vitro-derived DAP-resistant strains, we did find strains that were susceptible to clarithromycin (MIC ≤ 2 mg/L). It is of further interest whether mutations in $yycFG$ as a result of prolonged daptomycin exposure contribute to this phenomenon.

Bacterial populations often have heterogeneous susceptibility/non-susceptibility profiles and an antibiotic may still reduce the overall microbial burden when used at concentrations lower than the static MIC measured for the population (8). This heterogeneous resistance phenomenon has been characterized in $S. aureus$ with vancomycin (17, 23) and has also been observed with DAP (19, 27, 37, 40). These MIC distributions are difficult to measure with standard susceptibility methods (48). This is relevant to our study because several of the in vitro generated strains produced in this study have MICs greater than the serum free drug concentrations normally attainable under standard and high dose (> 6 mg/kg) DAP regimens.
The in vitro-derived MRSA in our study contain higher MIC values than the J01 progenitor strain for all treatments except the DAP + OXA combination. Therefore, we would predict that the in vitro-derived MRSA would maintain superior membrane integrity when exposed to DAP. Indeed, when compared to the membrane integrity of J01, the integrity of in vitro-derived MRSA was significantly elevated at all DAP dosages and for all treatments \( (p < 0.001) \) with the exception of the DAP + OXA combination treatment. This assay can also be utilized to provide a more direct indication of DAP activity against what may be a heterologous population. Membrane integrity was not reduced at higher dosage exposure concentrations for strains where the measured MIC is higher than the physiologically-relevant concentrations tested in the assay (Table 2). This suggests that high dose DAP therapy would likely not provide any antimicrobial contribution when administered against highly resistant strains with DAP MICs \( \geq 12 \) µg/ml but may have some membrane effects, albeit relatively minor, against resistant strains with MICs between 1.5 and 12 µg/ml. All reported clinical S. aureus strains with DAP resistance fall below a MIC of 12 µg/ml.

Cell wall thickening is commonly, but not necessarily, associated with decreases in DAP susceptibility (50). While no significant changes in cell wall thickness were observed between the DAP-susceptible and DAP-resistant strains obtained in vivo from clinical patients, significant cell wall thickening was observed for in vitro-derived DAP resistant bacteria isolated under pressure from DAP alone or with any secondary antibiotic (Table 2, Figure 2). These differences between patient samples and lab-derived strains could be due to the greater stresses caused by stepwise increases in DAP concentration in vitro or to a lack of in vivo pressures that could normally suppress the production of thickened cell walls. In these experiments, we were limited by available resources and therefore analyzed only the strain with the median DAP MIC from
each treatment arm. Therefore, statistical analyses are only valid between the individual strains and not necessarily for the comparison of antibiotic treatments as a whole.

In general, DAP resistant isolates obtained from patients show increased membrane fluidity as compared to their DAP susceptible progenitor. These differences, while an observable trend, are not usually statistically significant until multiple pairs are compared (25). Similarly in our study, the membrane fluidity of the DAP resistant bacteria obtained from patients was increased compared to their DAP susceptible partners, but only significantly for one pair (Table 1). In contrast, Mishra et al. associate strains that develop DAP resistance in vitro and strains that overproduce carotenoids with both less fluid membranes and increases in DAP resistance (24, 26). Thus, either increases or decreases in membrane fluidity in vitro could lead to DAP resistance. The membrane fluidity of the in vitro-derived DAP resistant bacteria in our study was inconsistent with some strains increasing in fluidity, some decreasing and others remaining statistically the same (Table 2). Therefore, membrane fluidity appears to be a poor predictor of DAP resistance in strains with high MICs generated in vitro. The difference in these fluidity trends may be due to the high level of DAP resistance produced in vitro compared to the relatively low-level resistance found in the in vivo DAP resistant strains. Alternatively, host factors such as production of cationic peptides may only select in vivo for strains that have increased membrane fluidity. Overall, our data suggests that changes in membrane fluidity may be influenced by which antibiotics are used in combination.

In conclusion, this study clearly demonstrated a significant impact of antibiotics coadministered with DAP on the proclivity towards the loss of DAP susceptibility in MRSA. OXA, and to a lesser extent CLR, effectively suppressed the emergence of DAP resistance when coadministered with DAP. In contrast, LZD appeared to hasten the development of DAP
resistance. It should be noted that in this study, exposure to adjunctive antibiotics was maintained at 0.2x - 0.5x MIC. Therefore, active concentrations achieved in vivo may lead to different effects. While the preliminary data with CLR are encouraging, further study is needed to evaluate if adjunctive CLR therapy may provide a treatment benefit to DAP monotherapy. The data with DAP+OXA provide additional evidence favoring the concomitant use of beta-lactam adjunctive therapy alongside DAP in the treatment of serious MRSA infections, not only for the benefit of synergy that has been previously demonstrated (10), but also for preventing the loss of DAP susceptibility.

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Figure legends

Figure 1. DAP MIC development of J01 following *in vitro* antibiotic exposure. Solid line, DAP alone; Dashed line, DAP + secondary antimicrobial agent. (A) DAP + CLR (B) DAP + FOF (C) DAP + GEN (D) DAP + LZD (E) DAP + MXF (F) DAP + OXA (G) DAP + RIF (H) DAP + SXT. Data points are displayed as the median MIC from five independent replicates of each treatment. Data points with an asterisk (*) denote statistically significant differences between DAP alone and DAP + secondary antimicrobial agent on comparative days ($p \leq 0.05$).

Figure 2. Cell wall thickness modifications of J01 following *in vitro* antibiotic exposure. (A) J01 control (B) J03 control (C) DAP (D) DAP + FOF (E) DAP + GEN (F) DAP + OXA. An asterisk (*) denotes statistically significant differences between the indicated image and the J01 control ($p < 0.001$). Cell wall thickness measurements provided below each image represent the average of 60 measurements across 15 cells per treatment.
Table 1. Strain characteristics of DAP susceptible parent MRSA strains and their in vivo-derived DAP-resistant mutants. The potential dissipation and membrane integrity assays utilized DAP concentrations corresponding to the $C_{\text{max}}$ at the doses listed. Lower polarization index ($p$) values indicate a greater degree of membrane fluidity. Values marked with an asterisk (*) denote statistically significant differences between the parent strain and its in vivo-derived partner (**, $p < 0.01$). Comparisons of membrane integrity between J01 and J03 were statistically significant at all three dosages, however significance was lost after statistical adjustment for multiple comparisons.

<table>
<thead>
<tr>
<th>Strain</th>
<th>DAP MIC (µg/ml)</th>
<th>VAN MIC (µg/ml)</th>
<th>6 mg/kg (7 µg/ml)</th>
<th>10 mg/kg (11.5 µg/ml)</th>
<th>12 mg/kg (14 µg/ml)</th>
<th>6 mg/kg (7 µg/ml)</th>
<th>10 mg/kg (11.5 µg/ml)</th>
<th>12 mg/kg (14 µg/ml)</th>
<th>Cell Wall Thickness (nm)</th>
<th>Fluidity ($p$)</th>
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</thead>
<tbody>
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<td>J01</td>
<td>0.5</td>
<td>2</td>
<td>20 ± 5 %</td>
<td>26 ± 6 %</td>
<td>27 ± 7 %</td>
<td>48 ± 3 %</td>
<td>28 ± 3 %</td>
<td>27 ± 2 %</td>
<td>32.5 ± 3.9</td>
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<td>J03</td>
<td>3</td>
<td>2</td>
<td>12 ± 3 %</td>
<td>19 ± 2 %</td>
<td>20 ± 3 %</td>
<td>79 ± 10 %</td>
<td>43 ± 6 %</td>
<td>34 ± 4 %</td>
<td>31.9 ± 3.5</td>
<td>0.299 ± 0.047</td>
</tr>
<tr>
<td>O325</td>
<td>0.09</td>
<td>1</td>
<td>27 ± 6 %</td>
<td>36 ± 10 %</td>
<td>58 ± 32 %</td>
<td>39 ± 2 %</td>
<td>19 ± 2 %</td>
<td>18 ± 1 %</td>
<td>31.8 ± 5.3</td>
<td>0.294 ± 0.016</td>
</tr>
<tr>
<td>O510</td>
<td>2</td>
<td>1</td>
<td>15 ± 7 %</td>
<td>26 ± 5 %</td>
<td>42 ± 19 %</td>
<td>55 ± 30 %</td>
<td>25 ± 10 %</td>
<td>27 ± 9 %</td>
<td>29.8 ± 6.3</td>
<td>0.291 ± 0.022</td>
</tr>
<tr>
<td>D592</td>
<td>0.75</td>
<td>2-3</td>
<td>6 ± 8 %</td>
<td>7 ± 6 %</td>
<td>11 ± 3 %</td>
<td>23 ± 10 %</td>
<td>25 ± 12 %</td>
<td>21 ± 13 %</td>
<td>44.7 ± 8.6</td>
<td>0.304 ± 0.020</td>
</tr>
<tr>
<td>D712</td>
<td>4</td>
<td>3</td>
<td>0 ± 2 %</td>
<td>2 ± 4 %</td>
<td>7 ± 5 %</td>
<td>27 ± 1 %</td>
<td>26 ± 3 %</td>
<td>24 ± 7 %</td>
<td>43.8 ± 6.6</td>
<td>0.289 ±0.026**</td>
</tr>
</tbody>
</table>

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Table 2. Strain characteristics of *in vitro*-derived DAP-resistant MRSA. Each strain was evolved from J01 and passaged for 28 days in the antibiotics indicated, as described in Materials and Methods. Cell wall thickness and fluidity data are reported for the strain with the median DAP MIC value of the five isolates derived per antibiotic treatment regimen. Membrane integrity data are reported as the average of all five isolates derived per antibiotic treatment regimen. Values marked with a dagger (†) denote statistically significant differences between the treatment arm indicated and the DAP-only treatment arm (single dagger, *p* ≤ 0.05; double dagger, *p* ≤ 0.01). Values marked with an asterisk (*) denote statistically significant differences between the treatment arm indicated and parent strain J01 (*, *p* ≤ 0.05; **, *p* ≤ 0.01). Lower polarization index (ρ) values indicate a greater degree of membrane fluidity.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median MIC (µg/ml)</th>
<th>MIC Range (µg/ml)</th>
<th>Membrane Integrity</th>
<th>Cell Wall Thickness (nm)</th>
<th>Fluidity (p)</th>
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</thead>
<tbody>
<tr>
<td>Parent strain</td>
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<tr>
<td>J01</td>
<td>0.5</td>
<td>0.5</td>
<td>48 ± 3 %</td>
<td>28 ± 3 %</td>
<td>27 ± 2 %</td>
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<td></td>
<td></td>
<td>32.5 ± 3.9</td>
<td>0.305 ± 0.020</td>
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<tr>
<td>Single agent exposure</td>
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<tr>
<td>DAP</td>
<td>32</td>
<td>8 – 32</td>
<td>95 ± 4 %</td>
<td>92 ± 6 %</td>
<td>89 ± 6 %</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>36.9 ± 6.1 *</td>
<td>0.330 ± 0.027 **</td>
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<tr>
<td>Combination exposures</td>
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<tr>
<td>DAP + OXA</td>
<td>1</td>
<td>0.02 – 3</td>
<td>62 ± 16 %† ††</td>
<td>46 ± 26 %† ††</td>
<td>39 ± 23 %† ††</td>
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<td>43.6 ± 7.6 † † †</td>
<td>0.290 ± 0.033</td>
</tr>
<tr>
<td>DAP + CLR</td>
<td>3</td>
<td>1.5 – 8</td>
<td>84 ± 16 %†</td>
<td>58 ± 14 %† ††</td>
<td>45 ± 10 %† ††</td>
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<tr>
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<td>49.8 ± 8.6 † † †</td>
<td>0.304 ± 0.028</td>
</tr>
<tr>
<td>DAP + FOF</td>
<td>12</td>
<td>3 – 64</td>
<td>83 ± 14 %†</td>
<td>79 ± 20 %</td>
<td>71 ± 25 %</td>
</tr>
<tr>
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<td>55.4 ± 13.6 † † †</td>
<td>0.329 ± 0.033 *</td>
</tr>
<tr>
<td>DAP + RIF</td>
<td>24</td>
<td>16 – 48</td>
<td>95 ± 8 %</td>
<td>96 ± 12 %</td>
<td>96 ± 13 %</td>
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<tr>
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<td>44.4 ± 10.1 † † †</td>
<td>0.323 ± 0.046</td>
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<tr>
<td>DAP + SXT</td>
<td>32</td>
<td>12 – 96</td>
<td>94 ± 5 %</td>
<td>95 ± 7 %</td>
<td>86 ± 7 %</td>
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<td>43.0 ± 6.7 † † †</td>
<td>0.338 ± 0.039 **</td>
</tr>
<tr>
<td>DAP + GEN</td>
<td>32</td>
<td>8 – 128</td>
<td>94 ± 10 %</td>
<td>90 ± 13 %</td>
<td>88 ± 20 %</td>
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<td>38.8 ± 6.8 † † †</td>
<td>0.282 ± 0.027 **</td>
</tr>
<tr>
<td>DAP + LZD</td>
<td>64</td>
<td>32 – 192</td>
<td>100 ± 6 %†</td>
<td>100 ± 9 %</td>
<td>96 ± 9 %</td>
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<tr>
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<td>45.6 ± 7.1 † † †</td>
<td>0.338 ± 0.040 **</td>
</tr>
<tr>
<td>DAP + MXF</td>
<td>96</td>
<td>4 – 128</td>
<td>85 ± 11 %†</td>
<td>83 ± 15 %</td>
<td>74 ± 21 %</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>ND</td>
<td>0.328 ± 0.029 *</td>
</tr>
</tbody>
</table>