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Phenotypic and genetic changes associated with the seesaw effect in MRSA strain N315 in a bioreactor model

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Abstract

Objectives: Over the past decade, daptomycin treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections has led to the emergence of daptomycin nonsusceptible (DAP-NS) MRSA strains and a subsequent interest in combinatorial antibiotic therapies. We investigated the phenotypic and genetic changes associated with the seesaw effect, which describes the correlation between daptomycin resistance and increased β-lactam susceptibility in DAP-NS MRSA and the reverse phenomenon of DAP-NS strains acquiring renewed susceptibility to daptomycin after β-lactam exposure.

Methods: A continuous bioreactor model was used to study the effects of incremental doses of daptomycin followed by oxacillin on MRSA strain N315. Minimum inhibitory concentrations for daptomycin and oxacillin were determined for the bioreactor-derived samples. Transmission electron microscopy and cytochrome C binding assays were used to measure cell wall thickness and cell membrane charge, respectively, in the bioreactor-derived samples. Whole-genome sequencing was used to identify mutations associated with the seesaw effect.

Results: Although daptomycin resistance conferred enhanced susceptibility to oxacillin, oxacillin treatment of DAP-NS strains was accompanied by a lowered minimum inhibitory concentration for daptomycin. Additionally, there was a reduction in relative positive cell surface charge and cell wall thickness. However, the mutations acquired in our DAP-NS populations were not accompanied by additional genomic changes after treatment with oxacillin, implicating alternative mechanisms for the seesaw effect.

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Conclusion: In this study, we successfully produced and characterized the seesaw effect in MRSA strain N315 in a unique bioreactor model.

Keywords

MRSA; Daptomycin nonsusceptibility; Seesaw effect; Cell wall thickness; Cell surface charge

1. Introduction

Staphylococcus aureus is the causative agent for a wide range of infections that are not responsive to many forms of clinical treatment [1]. Over the years, *S. aureus* has had a detrimental economic and societal impact on communities worldwide. The evolution of methicillin-resistant *S. aureus* (MRSA) strains has contributed significantly to increases in mortality from this pathogen. The treatment of *S. aureus* infections is further hindered by the emergence of strains that are nonsusceptible to multiple antibiotics, including vancomycin [1]. Recent use of daptomycin (DAP) as an alternative to vancomycin for treatment of complicated *S. aureus* infections has led to treatment failures owing to DAP nonsusceptible (DAP-NS) or resistant *S. aureus* strains [2].

DAP is a cyclic lipopeptide that binds to the cytoplasmic membrane of *S. aureus* in a calcium-dependent manner, ultimately leading to membrane depolarisation and cell death [3]. DAP nonsusceptibility is most frequently associated with mutations in the multipeptide resistance factor gene (*mprF*) [4–6]. The *mprF* gene encodes for lysyl-phosphatidylglycerol synthetase, responsible for lysinilating phosphatidylglycerol and translocating it to the outer membrane [7,8]. Mutations in *mprF* are thought to mediate DAP resistance by causing an increase in the lysyl-phosphatidylglycerol content of the cell membrane, which increases the net positive charge of the membrane and repels DAP [5]. Mutations in the cardiolipin synthase gene (*cls2*) have also been observed in DAP-NS strains and might further alter membrane composition by increasing the ratio of cardiolipin to phosphatidylglycerol, thereby leaving fewer target sites for DAP [9]. Other phenotypic changes that often appear in DAP-NS strains include increased cell wall thickening and changes in membrane fluidity that might prevent the antibiotic from accessing its target site [10,11].

Combination therapies of antistaphylococcal β-lactams with DAP have recently been explored to overcome the problem of generating DAP-NS strains [12]. Treatment with DAP and β-lactams leads to a phenomenon known as the “seesaw effect,” in which MRSA strains fail to develop DAP-NS, possibly by preventing *mprF* mutations from emerging [13]. Alternatively, the seesaw effect can describe the resensitisation of a DAP-NS MRSA strain to a β-lactam antibiotic by mechanisms that also involve mutations at the *mprF* locus [14]. Although multiple studies have documented the role of *mprF* in the seesaw effect, detailed genetic and phenotypic changes that accompany the seesaw effect are not yet fully understood.

In our previous study, we recapitulated an in vivo *S. aureus*– induced endocarditis model in a bioreactor and validated it as an effective method to monitor evolutionary changes associated with antibiotic treatment [4]. Among other mutations, we identified single nucleotide polymorphisms (SNPs) in *mprF* and *cls2,* which encode proteins involved in

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altering cell membrane charge and composition and are implicated in DAP nonsusceptibility [4]. Here, we show that the bioreactor can produce both phenomena of the seesaw effect: (1) a decrease in oxacillin minimum inhibitory concentrations (MICs) after DAP exposure and (2) a decrease in DAP MIC in DAP-NS strains after oxacillin treatment. Treatment with oxacillin was able to reverse some of the phenotypes that accompanied DAP nonsusceptibility, such as increased positive cell surface charge and increased cell wall thickness. Whole-genome sequencing revealed that mutations at *mprF* and other loci emerged after treatment with DAP. However, no additional mutations were observed after recovery from oxacillin treatment, suggesting that an alternative mechanism produces the seesaw effect.

2. Materials and methods

2.1. Bacterial strain, media, and antimicrobials

The DAP-susceptible *S. aureus* strain N315 (American Type Culture Collection - 29213), obtained from BEI Resources (catalog no. NR-45898), served as the parental strain for all experiments. The genome sequence of the parental isolate was obtained previously by Lasek-Nesselquist et al. [4]. The isolates obtained in this study derive from the parental N315 strain after exposure to incremental doses of DAP and oxacillin in a bioreactor. The bacteria from frozen stock were streaked onto blood agar plates (BD Biosciences) and incubated at 37°C with 5% CO_2 overnight for every experiment. For broth cultures, bacteria were grown in Mueller-Hinton broth (MHB) supplemented with 50 mg/L $CaCl_2$, 12.5 mg/L $MgCl_2$. Cation-adjusted MHB (CAMHB) cultures were additionally supplemented with NaCl 2 %w/v (0.34 mol/L) in accordance with the Clinical Laboratory Standards Institute standards to help determine oxacillin heteroresistant *S. aureus* [15].

Injectable DAP (Cubicin) was purchased from Albany Medical Center Outpatient Pharmacy (Albany, NY). United States Pharmacopeia reference standard oxacillin was purchased from Millipore Sigma. Oxacillin for injection (Wockhardt) was purchased from Cardinal Health.

2.2. Bioreactor culture

The bioreactor was set up as described previously [4]. A single colony of *S. aureus* strain N315 was used to inoculate 30 mL of CAMHB. The broth culture was grown overnight in a shaking incubator at 37°C, at a speed of 150 rpm, and was diluted the next day to an OD_{600} of 0.4 to yield 40 mL of CAMHB (starting culture). A final volume of 400 mL was set to revolve at 100 rpm at 37°C in the bioreactor. The bacteria were allowed to acclimate to the bioreactor environment for 24 h before treatment. DAP was added at concentrations of 6 μg/mL and 10 μg/mL, followed by the addition of 8 μg/mL of oxacillin. Samples were collected at different time points during the bioreactor run to determine the OD_{600} and colony-forming unit (CFU) counts. From the samples collected, frozen stock was made of population-based isolates and colony-based isolates.

2.3. Antimicrobial susceptibility studies

The MIC of DAP was determined by the Epsilometer test (E test) and broth microdilution, as described previously [4]. All susceptibility studies were performed using samples

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in duplicate, and each experiment was done at least thrice. Broth microdilution to determine the MIC value for oxacillin followed a similar protocol. In accordance with the Clinical Laboratory Standards Institute guidelines, CAMHB media was used. The starting concentration of 512 μg/mL of oxacillin (reference standard) was serially diluted to achieve final concentrations of 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0 μg/mL.

2.4. Disc diffusion assays

Bioreactor-derived bacterial isolates were tested for their susceptibility to different concentrations of oxacillin. Uniform lawns were created by streaking bacterial cultures with an OD_{600} of 0.2 (10^8 CFU/mL) in MHB with sterile cotton swabs premoistened with phosphate-buffered saline (PBS). Sterile discs with 10 μL of the desired drug concentration (16, 12, 8, 7, 6, 5, and 4 μg/mL of oxacillin) were placed on each plate using a sterile tweezer. Plates were incubated overnight at 37°C and 5% CO_2. Zones of inhibition around the discs were measured using a Vernier caliper.

2.5. Cytochrome C binding assay

This assay was performed to compare the relative positive charge of the cell membrane of the DAP-NS bioreactor-derived isolates before and after treatment with oxacillin. Bacterial isolates were grown overnight in CAMHB and then washed twice with 20 mM MOPS (3-(N-morpholino) propanesulfonic acid) buffer (pH 7.0) and brought to an OD_{600} of 0.15. The bacterial suspension was incubated with 0.5 mg/mL cytochrome C for 10 min at room temperature. The amount of cytochrome C that remained unbound in the supernatant was determined by measuring the OD_{530}.

2.6. Transmission electron microscopy

Transmission electron microscopy was performed using a previously published protocol [9] with minor modifications. Ultrathin sections were examined using a Tecnai F20 electron microscope operating at 200 KeV. Electron micrographs were recorded on a 4K × 4K charge coupled device (CCD) (TVIPS TemCam-F416) for analysis. Cell wall thickness was measured for the bioreactor-derived isolates using ImageJ. Cell wall thickness was calculated by measuring the thickness in each of the four quadrants of a cell and taking the average. A minimum of 27 cells was measured for each isolate.

2.7. DNA isolation, whole-genome sequencing, and analysis of DNA-Seq libraries

Three independent bioreactor experiments were conducted to examine the genomic effects of combination therapy on *S. aureus*. Genomic DNA was isolated from the bioreactor samples with the PureLink Genomic DNA Mini Kit (Invitrogen) as described previously [4].

Whole-genome sequencing was conducted by the Sequencing Core at the Wadsworth Center, New York State Department of Health (Albany, NY). Paired-end 250 × 250 bp libraries were prepared using the Nextra DNA library preparation kit (Illumina) and sequenced using the standard 500 cycle V2 protocol on the Illumina MiSeq. Analysis of DNA-seq libraries was performed as described previously [4].

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Raw reads generated from this study have been deposited in the SRA database at NCBI under the BioProject PRJNA770908 (http://www.ncbi.nlm.nih.gov/bioproject/770908).

3. Results and discussion

3.1. β-lactam and DAP-mediated seesaw effect in MRSA

In this study, we leveraged our previously established bioreactor model to investigate the acquisition of β-lactam susceptibility after the evolution of DAP-NS and the resensitisation of these strains to DAP after oxacillin treatment. After initial bacterial acclimatisation to the bioreactor, the bacteria were treated with incremental doses of 6 and 10 μg/mL of DAP, followed by 8 μg/mL oxacillin. The addition of 6 μg/mL of DAP resulted in a sudden drop in bacterial numbers and viability as measured by OD_{600} values and the number of CFUs per millitlitre (Fig. 1A,B). DAP-NS bacteria were allowed to recover to their starting concentrations before the addition of 10 μg/mL of DAP, which resulted in a 10-fold reduction in bacterial viability. The addition of 8 μg/mL oxacillin resulted in a moderate threefold reduction in bacterial viability (Fig. 1B). A similar trend was observed in the OD_{600} values (Fig. 1A). Although oxacillin treatment did not cause a dramatic reduction in bacterial viability in the bioreactor, disc diffusion assays indicated that bioreactor-derived N315 strains resistant to 6 and 10 μg/mL DAP were susceptible to 8 μg/mL of oxacillin, supporting the seesaw effect (Fig. 1C). Additionally, E-tests revealed that DAP MIC values dropped from 12 to 3 μg/mL after treatment of DAP-NS strains with oxacillin (Fig. 1D). The DAP-susceptible parental N315 strain, as well as the bioreactor-derived oxacillin-resistant strains (after recovery from 8 μg/mL oxacillin), were resistant to 16 μg/mL of oxacillin and did not show any zone of inhibition (Fig. 1C).

Several clinical studies have shown the effectiveness of DAP (or vancomycin) and β-lactam combinatorial therapy over DAP or vancomycin monotherapy [12]. However, a recent clinical trial showed no significant difference in the clinical outcome with the addition of β-lactam to DAP or vancomycin [16]. With conflicting data available from various clinical studies, our results indicate that sequential treatment of DAP followed by oxacillin could offer another line of treatment for MRSA infections.

3.2. Oxacillin treatment decreases cell surface charge of DAP-NS strains

Alterations in cell membrane morphology and electrostatic repulsion due to an increase in the relative positive charge of the cell membrane have been associated with DAP nonsusceptibility [17]. We determined the changes in cell surface charge of DAP-NS strains before and after treatment with oxacillin by using a cytochrome C binding assay. A greater amount of unbound cytochrome C indicates a more positive cell-surface charge. A significantly higher percentage of unbound cytochrome was observed in samples collected after recovery from 6 μg/mL DAP treatment and 3 h posttreatment with 8 μg/mL oxacillin as compared with no drug treatment (Fig. 2A). DAP-NS strains that recovered after oxacillin treatment exhibited lower cytochrome C binding, indicating a decrease in overall positive cell surface charge (Fig. 2A). Our results show that the evolution of DAP nonsusceptibility was accompanied by an increase in positive membrane charge, which was then reversed

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upon oxacillin treatment, which suggests that decreased positive charge of the cell surface after β-lactam treatment could lead to increased DAP binding.

3.3. Oxacillin treatment reverses the increased cell wall thickness phenotype of DAP-NS strains

Studies have shown a correlation between an increase in cell wall thickness and DAP nonsusceptibility [18], possibly due to increased production of cell wall teichoic acids and D-alanylation [11], which prevents DAP from accessing the membrane. However, cell wall thickening is not a universal phenomenon associated with DAP nonsusceptibility [19]. Here, we measured the cell wall thickness of DAP-NS strains before and after oxacillin treatment using transmission electron microscopy. There was a significant increase in the cell wall thickness in samples collected after recovery from 6 μg/mL DAP treatment compared with the control (30 vs. 46.7 nm, respectively) (Fig. 2B). The increase in cell wall thickness was reversed in samples collected after recovery from 8 μg/mL oxacillin treatment (31.2 nm) (Fig. 2B). Again, these results support our previous analyses, which showed the reversal of phenotypic changes associated with DAP nonsusceptibility after β-lactam treatment.

3.4. Oxacillin treatment reduces the frequency of DAP-NS mutants in a population

The three bioreactor experiments produced *S. aureus* DAP-NS populations characterised by mutations at the *mprF, cls2, clpP*, and *clpX* loci (Table 1). After recovery from 6 μg/mL DAP treatment, all populations were dominated by cells with changes to the *mprF* locus with other co-occurring SNPs appearing at varying frequencies (Table 1). In the third replicate, mutations in *mprF* and *clpP* that appeared after 6 μg/mL DAP exposure were replaced. After exposure to 8 μg/mL oxacillin, each population demonstrated a substantial decrease in the frequency of SNPs that had appeared after DAP treatment—in some cases by up to almost 90% (Table 1)—with no new mutations recorded.

Recent analyses of DAP-NS mutants that reverted to susceptibility either by passaging under nonselective conditions or treatment with β-lactams frequently displayed a reduction in cell wall thickness and positive membrane charge without loss of mutations at the *mprF* locus [13,20]. It appeared that additional mutations at the *mprF* locus or in other genes were required to induce susceptibility. Although phenotypic reversions also occurred in our DAP-NS bioreactor populations after oxacillin treatment, they reacquired DAP susceptibility without additional mutations. Thus, the decreased frequency of the *mprF, cls2,* and *clpX* mutations after oxacillin treatment suggests that selection against DAP-NS cells in a mixed population was responsible for the phenotypic and genetic reversions observed. In our model, reduced cell surface charge and decreased cell wall thickness were not consequences of cellular plasticity or changes in the mutational landscape but rather selection for wild-type forms that already existed.

In conclusion, the bioreactor model is an effective method to study the evolution of antibiotic nonsusceptibility. Our characterisation of MRSA DAP-NS populations generated in a bioreactor contributes to our understanding of the phenotypic and genomic changes associated with the seesaw effect and the reverse phenomenon. Our results suggest that the interplay between DAP and β-lactams can be exploited on both ends: to resensitise DAP-NS

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MRSA to β-lactams after DAP treatment or to decrease DAP resistance with continuous exposure to β-lactams.

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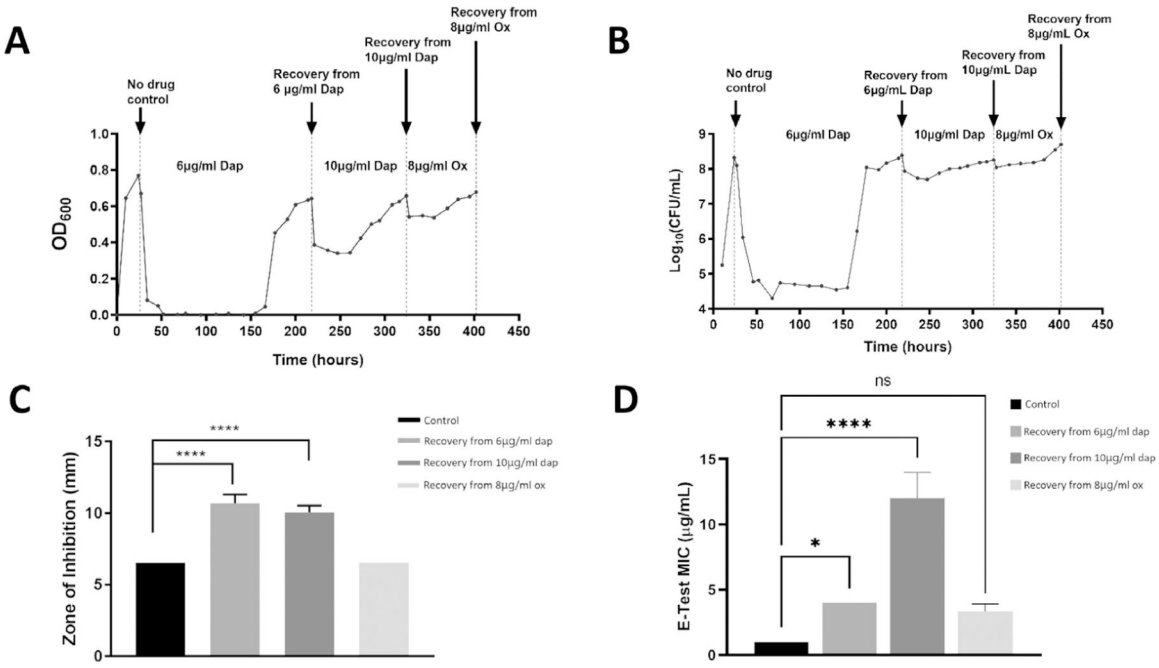

Fig. 1.

Oxacillin- and daptomycin (DAP)-mediated seesaw effect in DAP nonsusceptible (DAP-NS) methicillin-resistant *Staphylococcus aureus* (MRSA) strain using a bioreactor model. (A, B) Bacteria were allowed to acclimate to the bioreactor environment for 24 h, after which incremental doses of 6 and 10 μg/mL of DAP were added, followed by 8 μg/mL oxacillin. Samples were collected at frequent intervals to measure optical density at 600 nm (A) and determine viability by colony-forming unit (CFU) counting (B). The data are representative of three independent experiments. (C) Acquisition of oxacillin susceptibility in DAP-NS *S. aureus*. Disc diffusion assay was conducted on the collected samples to determine the susceptibility of DAP-NS strains to 8 μg/mL oxacillin. The data are an average of three independent experiments. Statistical analysis was carried out using one-way analysis of variance (ANOVA), and a P value of ≤0.05 was considered significant. **** $P < 0.001$. (D) DAP susceptibility studies for DAP-oxacillin combination therapy. The minimum inhibitory concentrations (MICs) of the bacterial aliquots collected at the indicated time points were determined by DAP Etest strip. The data are an average of three independent experiments

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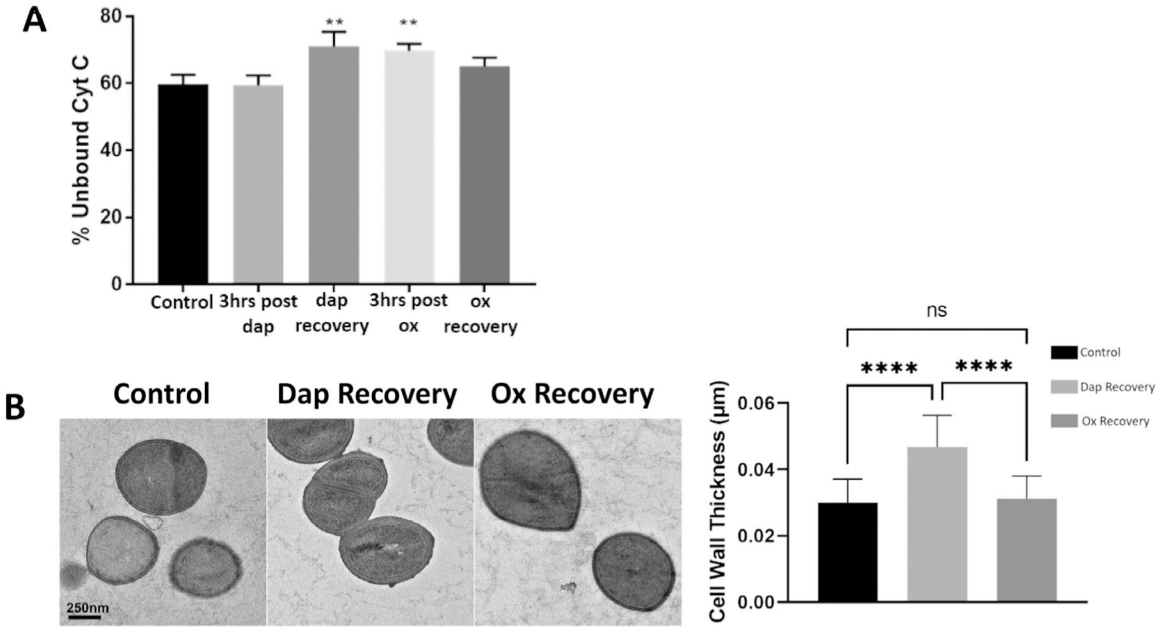

Fig. 2.

Changes in cell surface charge in daptomycin nonsusceptible (DAP-NS) *Staphylococcus aureus* after oxacillin treatment. (A) Bacterial samples obtained from the bioreactor at no drug treatment (control), 3 h posttreatment with 6 μg/mL DAP, recovery from 6 μg/mL DAP, 3 h posttreatment with 8 μg/mL oxacillin, recovery from 8 μg/mL oxacillin were incubated with 0.5 mg/mL cytochrome C, and the amount of unbound cytochrome C was calculated to determine the cell surface charge of DAP-susceptible (DAP-S), DAP-NS before and after oxacillin treatment. The data are an average of three independent experiments. Statistical analysis was carried out using one-way analysis of variance (ANOVA), and a P value of ≤0.05 compared to 24 h was considered significant. $^{**}P < 0.01$. (B) Bacterial samples obtained from the bioreactor at no drug treatment (control), recovery from 6 μg/mL DAP, and recovery from 8 μg/mL oxacillin were prepared for transmission electron microscopy (TEM) imaging. Scale bar: 250 nm. Cell wall thickness was measured using ImageJ with a minimum n value of 27. The data are an average of all the cells measured. Statistical analysis was carried out using one-way ANOVA, and a P value of ≤0.05 compared to 24 h was considered significant. $^{****}P < 0.001$.

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Table 1

Mutations acquired by N315 *Staphylococcus aureus* populations in three bioreactor experiments after exposure to increasing doses of daptomycin followed by a treatment with oxacillin

	Position	Locus tag	Gene abbreviation	Substitution	Amino acid change	No antibiotic	6 μg/mL daptomycin	10 μg/mL daptomycin	8 μg/mL oxacillin
Experiment 1	915302	SA0811	Subunit C of antiporter	C → T	M1I	0	47	NA	25
	1071295	SA0943–1	*pdhA*	C → A	Q152K	0	56	NA	29
	1364621	SA1193	*mprF*	C → T	S337L	0	95	NA	51
Experiment 2	1364663	SA1193	*mprF*	T → A	V248E	0	92	NA	64
	1706449	SA1498	*clpX*	T → A	I319F	0	68	NA	22
Experiment 3	827873	SA0723	*clpP*	C → T	Q82*	0	93	0	0
	1364621	SA1193	*mprF*	C → T	S337L	0	100	0	0
	1366087	SA1193	*mprF*	C → T	L826F	0	0	99	12
	1707292	SA1498	*clpX*	A → G	C38R	0	0	100	12
	2143187	SA1891	*cls2*	T → C	L26S	0	0	91	11
	2143994	SA1891	*cls2*	G → T	R295L	0	0	83	0.5

The percentage of the population with a given mutation is recorded for each antibiotic exposure and was determined by the number of reads that support the nonsynonymous nucleotide substitution. All positions were covered by a minimum depth of 20 reads, with the exception of the S337L MprF mutation in experiment 3, which was supported by 16 reads but at a frequency of 1.0 at 6 μg/mL daptomycin exposure and the Cls L26S mutation in experiment 3 at position 2143187, which was covered by 19 reads at 10 μg/mL daptomycin exposure. NA indicates a timepoint/antibiotic exposure not sampled. The Bioreactor model was used to recapitulate seesaw effect. Daptomycin nonsusceptible (DAP-NS) showed increased cell membrane positive charge and cell wall thickness. Oxacillin treatment reversed DAP-NS phenotypes. The frequency of DAP-NS–associated mutations decreased with oxacillin exposure.