

Studies on the Mechanism of Telavancin Decreased Susceptibility in a Laboratory-Derived Mutant

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Telavancin is a novel semisynthetic lipoglycopeptide derivative of vancomycin with a dual mode of action. This study sought to understand the mechanisms of decreased telavancin susceptibility in a laboratory-derived *Staphylococcus aureus* mutant Tlv^{DS}MED1952. There were extensive changes in the transcriptome of Tlv^{DS}MED1952 compared to the susceptible parent strain MED1951. Genes upregulated included cofactor biosynthesis genes, cell wall-related genes, fatty acid biosynthesis genes, and stress genes. Downregulated genes included lysine operon biosynthesis genes and *lrgB*, which are induced by telavancin in susceptible strains, *agr* and *kdpDE* genes, various cell surface protein genes, phenol-soluble modulins, several protease genes, and genes involved in anaerobic metabolism. The decreased susceptibility mutant had somewhat thicker cell walls and a decreased autolytic activity that may be related to decreased proteolytic peptidoglycan hydrolase processing. Membrane fatty acid changes correlated with increased membrane fluidity were observed. It seems likely that there are multiple genetic changes associated with the development of decreased telavancin susceptibility. The Tlv^{DS} mutant showed some similar features to vancomycin-intermediate *S. aureus* and decreased daptomycin susceptibility strains, but also exhibited its own unique features.

Introduction

THE DEPLOYMENT OF penicillin in the nineteen forties and the discovery of penicillinase-producing, penicillin-resistant strains of *Staphylococcus aureus* shortly thereafter have been followed by epidemic waves of antibiotic-resistant *S. aureus*.² In the present day, we have community-associated and hospital-associated methicillin-resistant *S. aureus* (MRSA), vancomycin-intermediate *S. aureus* (VISA), and vancomycin-resistant *S. aureus* (VRSA).^{12,15,29} This situation has led to attempts to develop novel antimicrobial agents for therapy of infections with these antibiotic-resistant strains.

One of these novel agents is telavancin, a lipoglycopeptide developed from the parent molecule vancomycin.¹⁹ This agent exhibits potent activity against methicillin-sensitive *S. aureus* (MSSA), MRSA, VISA, and VRSA both *in vitro* and *in vivo*.^{5,10} Telavancin, marketed as ATIVIVTM, has been approved by the Food and Drug Administration for the treatment of complicated skin and skin structure infections, and in Europe for treatment of nosocomial pneumonia known or suspected to be caused by MRSA. This molecule has a dual mode of action on *S. aureus*

causing the inhibition of peptidoglycan biosynthesis and membrane depolarization.^{11,23,39}

Although we are unaware of the development of telavancin-resistance or decreased susceptibility in clinical strains, the potential fate of a novel antimicrobial agent that is introduced into use is the development of resistance or decreased susceptibility to varying degrees and over varying timescales. For example, while frank resistance may be rare, modest increases in minimum inhibitory concentrations (MICs) have occurred to vancomycin and daptomycin resulting in vancomycin and daptomycin decreased susceptibility isolates.^{12,25} It is desirable then to have knowledge of the mechanisms involved in resistance or decreased susceptibility to each antimicrobial agent, especially an agent with a dual mode of action.

In a recent study, Kosowska-Shick *et al.*¹⁷ reported on their attempts to select telavancin-resistant strains in MRSA. One stable mutant was obtained after 43 days in multistep resistance selection studies from one of the ten MRSA strains tested, which was also a vancomycin-susceptible strain (VSSA). In this article, we describe a variety of approaches we have taken to try to understand, for the first time, the mechanisms involved in decreased telavancin susceptibility.

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Materials and Methods

Strains studied and growth conditions

The strains studied were the telavancin-susceptible parent strain MED1951 and telavancin decreased susceptibility (Tlv^{DS}) mutant MED1952 kindly provided by Dr. Peter Applebaum, which were designated as parental SA248 and passage day 43 isolate, respectively, in the original publication describing these strains.¹⁷ The strains were grown in Mueller-Hinton broth supplemented with 25 mg of Ca²⁺ liter⁻¹ and 12.5 mg of Mg²⁺ liter⁻¹. The growth rates of the strains were assessed by determining the mean generation times of cultures in the exponential phase of growth. Antibiotic MICs were determined as described by Kosowska-Shick *et al.*¹⁷ or by the E-test.

Transcriptional profiling

The strains were grown to an OD₆₀₀ of 0.4 (50 ml of culture in a 250-ml Erlenmeyer flask) with shaking (210 rpm) at 37°C. Transcriptional profiling was carried out using *S. aureus* genome microarrays version 8.0 provided by the Pathogen Functional Genomics Resource Center (PFGRC) of the National Institutes of Allergy and Infectious Diseases (NIAID) as described in previous publications from this laboratory.^{26,39} The full genome array consists of 70-mer oligonucleotides representing 4589 ORFs from the *S. aureus* strain COL, Mu50, MW2, N315, MRSA252, MSSA476, USA300-FPR3757, and pLW043. Each ORF is printed three times on the array. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo/) and are accessible through the GEO Series accession number GSE40697.

Microarray validation by real-time reverse transcription polymerase chain reaction

This was carried out as described by Song *et al.*³⁹ The primers used are shown in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/mdr).

Autolysis, preparation of cell walls, and peptidoglycan hydrolase profiles

Triton X-100 stimulated autolysis of whole cells grown in Ca²⁺- and Mg²⁺-supplemented MHB was determined as described by Gustafson *et al.*⁸ Crude cell walls retaining autolytic activity (CCW) and purified cell walls (PCW), which have been boiled and digested with trypsin and do not retain autolytic activity, were prepared as described previously.^{16,30} An autolysin extract was prepared by suspending washed exponential phase cells in a 0.01 M K₂PO₄-K₂HPO₄ buffer (pH 7.2) and subjecting them to three freeze-thaw cycles (−80°C for 1 hr at 37°C for 10 min).^{16,30} The suspension was centrifuged (13,380 g, 10 min) and the supernatant was used as the freeze-thaw autolysin extract for digestion of PCW. The peptidoglycan hydrolase profiles of these extracts were determined by zymography by renaturation following sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a gel containing 0.2% (wt/vol) *Micrococcus luteus* cells (Sigma) as previously described.^{16,20}

Fatty acid composition

Washed exponential phase cells were saponified and methylated and fatty acid methyl esters were analyzed on an Agilent 5890 dual-tower gas chromatograph. Fatty acids were identified using the MIDI microbial identification system (Sherlock 4.5 Microbial identification system) at Microbial ID.⁵⁰

Total carotenoid determination

This was determined by measuring the OD₄₆₅ of cells extracted with methanol at 55°C for 3 min.¹⁸

Measurement of membrane fluidity

This was determined as described previously.³⁸ In brief, midexponential phase cells (OD₆₀₀ 0.6) were washed twice with 0.85% NaCl. Then, the cells were resuspended in 0.85% NaCl containing 2 μM 1,6-diphenyl-1,3,5-hexatriene (DPH; Sigma) to an OD₆₀₀ of 0.3 and incubated at 30°C for 1 hr. A 1 mM DPH solution was prepared in tetrahydrofuran, and 200 μl was added to 50 ml of 0.85% NaCl. DPH fluoresces in the hydrophobic regions of the lipid bilayer, but does not fluoresce in an aqueous environment.¹ Excess tetrahydrofuran was removed by flushing with nitrogen. Fluorescence polarization was measured using a QuantaMasterTM40 spectrofluorometer (Photon Technology International, Inc.). The excitation and emission wavelength for DPH were 360 and 426 nm, respectively. The higher the value is, the lower the membrane fluidity. The experiment was performed twice, and the mean polarization values were compared for significant differences by using the *t* test.

Transmission electron microscopy

Preparation and examination of *S. aureus* cells by transmission electron microscopy were performed as described previously.⁴⁵ Thin sections stained with uranyl acetate and lead citrate were examined in a Zeiss 10-C transmission electron microscope operating at 60 kV. The cell wall thickness was determined using photographic images at 50,000× final magnification. Thirty five cells of each strain with nearly equatorial cut surfaces were measured, and results are expressed as mean values ± standard deviation.

δ-Hemolysis assay for Agr dysfunction

δ-hemolysin production was measured by streaking the strains adjacent to a β-hemolysin disk (Remel) on a Tryptic Soy Agar plate with 5% sheep blood.³⁴ Results were observed after overnight incubation at 37°C. Agr dysfunction was evaluated by the absence of δ-hemolysis within the β-hemolysis zone due to lack of synergistic hemolysis.

Results and Discussion

Characteristics of the strains

The telavancin MICs of the strains were MED1951 0.12 μg ml⁻¹ and Tlv^{DS}MED1952 1 μg ml⁻¹, an eightfold increase in MIC. Tlv^{DS}MED1952 was derived through multistep resistance selection with a subinhibitory concentration of telavancin at day 43. The elevated MIC of the mutant was stable in that it remained the same after ten passages in the absence

of an antibiotic. There was also a fourfold increase in vancomycin MIC going from 0.5 to 2.0 $\mu\text{g ml}^{-1}$; a twofold increase in daptomycin MIC going from 1.0 to 2.0 $\mu\text{g ml}^{-1}$; a 2.6-fold increase in oxacillin MIC going from 12 to 32 $\mu\text{g ml}^{-1}$ in strains MED1951 and Tlv^{DS}MED1952, respectively. The seesaw effect of decreasing oxacillin susceptibility seen in daptomycin decreased susceptibility isolates⁴⁸ was not observed here. Tlv^{DS}MED1952 did not demonstrate heteroresistance to telavancin or vancomycin in the population analysis profile. The mutant grew at a somewhat slower rate, which was about 67% of that of the parent strain. This is in contrast to what can be found in some VISA and daptomycin-resistant strains, where growth is often markedly slower than their susceptible parent strains.^{12,37}

Changes in gene expression in Tlv^{DS}MED1952 compared to telavancin-susceptible parent strain MED1951

Compared to the strain MED1951, 330 genes were increased in expression and 311 genes were decreased in expression using a twofold cut off in the strain Tlv^{DS}MED1952. Changes in gene expression of selected genes are shown in Table 1 and the full transcriptome is shown in Supplementary Tables S2 and S3 in the Supplementary material. Considerable number of genes in the categories energy metabolism, cellular processes, cell envelope, regulatory functions, and transport and binding proteins were upregulated (Supplementary Table S2 Supplementary material), and the distribution between these different categories is given in Supplementary Table S4.

Upregulated genes

Some of the most highly upregulated genes were as follows: SAV2569 *isaA* 5.3-fold; SAV2299 *ssaA* 9.7-fold; SACOL2291 staphyloxanthin biosynthesis protein 6.3-fold; SACOL2280 *ureA* 33.8-fold plus other *ure* operon member genes; SAV2530 putative L-serine dehydratase 15.1-fold and SAV2531 putative beta-subunit of L-serine dehydratase 12-fold; SAV0017 *purA* 14.3-fold; SAV2095 similar to SceD precursor 25.1-fold.

In the cell envelope category, there were 2–2.3-fold increases in the expression of *murF*, *murC*, *pbp2*, and *lrm* (*tarO*), the first enzyme in teichoic acid biosynthesis, and *drp35*, a gene encoding a protein with the Ca^{2+} -dependent lactonase activity that is upregulated by cell wall antibiotics.^{41,46} Two genes involved in metabolism of the peptidoglycan interpeptide bridge were upregulated: *femB*, which is involved in adding a glycine residue to the bridge, and *lytM*, a glycylglycine endopeptidase. *lytM*, *isaA*, and *ssaA*, which were also upregulated, are members of the WalKR regulon. The WalKR (YycGF) two-component system is essential in *S. aureus*, and plays a significant role in the control of the expression of several peptidoglycan hydrolase genes.⁶ *LytM* is a glycyl-glycyl endopeptidase. *SsaA* and *IsaA* are CHAP-domain (Cysteine, Histidine-dependent Aminohydrolases/Peptidases) peptidoglycan hydrolases. Delaune *et al.*⁴ have suggested that these enzymes play a role in relaxation of crosslinking in the peptidoglycan cross bridge, especially in the absence of WalKR. The *sceD* precursor gene was upregulated 25.1-fold. *SceD* is a peptidoglycan lytic transglycosylase.⁴⁰ However, despite upregulation of these

peptidoglycan hydrolase genes, overall autolysis of Tlv^{DS}MED1952 was lower compared with MED1952, see below.

bacA, undecaprenyl pyrophosphate phosphatase; *mvaK1*, mevalonate kinase; *mvaS*, isoprenoid synthesis, *ppS* (undecaprenyl diphosphate synthase) were upregulated. These genes are involved in the biosynthesis of undecaprenyl phosphate, which is a critical component of lipid-I and lipid-II peptidoglycan intermediates, and lipid intermediates involved in teichoic acid biosynthesis. This may be a correlate of enhanced or altered cell wall synthesis in Tlv^{DS}MED1952. Similar changes in expression of isoprenoid biosynthesis genes have also been noted in a mutant showing decreased susceptibility to the membrane-active household disinfectant pine oil,¹⁸ which, like the lipoglycopeptide telavancin, is membrane active.

Genes involved in the biosynthesis of several cofactors, prosthetic groups, and carriers, including pantothenate and coenzyme A, thiamine, heme, porphyrin, cobalamin, biotin, menaquinone and ubiquinone, pyridoxine, and glutathione, were upregulated. This suggests that there is a significant change in metabolism in this strain with possibly a greater dependence on the biosynthesis of these molecules.

betA and *gbsA*, encoding choline and glycine betaine aldehyde dehydrogenase, respectively, were upregulated. These enzymes metabolize choline to glycine betaine,¹⁴ a compatible solute involved in osmoregulation that has beneficial effects on the protein structure.³¹ In addition, there was upregulation of the heat shock protein genes *clpP*, *htrA*, and *groES* (about 1.9-fold). With the upregulation of genes involved in accumulation of protective solutes and proteins to deal with stress, Tlv^{DS}MED1952 may be primed to deal with the challenge posed by exposure to telavancin.

Several genes involved in the fatty acid metabolism were upregulated—acetyl-CoA acetyl transferase homolog, *fabG1*, *fabH*, *accB*, acetyl CoA carboxylase, and *fabD*. Tlv^{DS} MED 1952 shows an altered fatty acid composition from MED1951, see below.

A variety of intermediary metabolism genes were upregulated. *ure* member genes of the urease operon were highly upregulated. Urease degrades urea to NH_3 and CO_2 . One possible role of NH_3 production may be to neutralize any excess acidity that may be produced through changes in the metabolism of the mutant. Also, a putative L-serine dehydratase, which breaks serine down to pyruvate and ammonia, was upregulated 15.1-fold.

arg, encoding arginase, and *rocA*, Δ' -pyrroline-5-carboxylate dehydrogenase, showed an increased expression and are part of the arginase pathway whereby arginine can be utilized as a source of carbon and energy resulting in the production of glutamate.⁴²

Several genes of the purine biosynthesis operon were overexpressed. This is consistent with decreased expression of *purR*, the negative repressor of the *pur* operon.

A variety of genes involved in protein synthesis, including ribosomal protein genes, tRNA and rRNA base modification genes, and tRNA aminoacylation genes, were upregulated suggesting some changes in protein synthesis in the mutant.

Various regulatory genes were upregulated, including *sarA*, *rot*, *treR* (1.9), *tcaR* (1.7), and *ccpA* (1.6). *SarA* is involved in the control of expression of *agr*,³² a quorum-sensing locus

TABLE 1. EXPRESSION OF SELECTED GENES OF TLV^{DS} MED1952 COMPARED TO MED1951

Locus ID	Gene symbol	Gene-protein name	Subcategory	Fold
Cell envelope				
SAV2082	<i>murF</i>	UDP-N-acetylmuramoylalanyl-D-glutamyl-2, 6-diaminopimelate-D-alanyl-D-alanyl ligase	Biosynthesis and degradation of murein sacculus and peptidoglycan	2.2
SACOL1790	<i>murC</i>	UDP-N-acetylmuramate—L-alanine ligase	Biosynthesis and degradation of murein sacculus and peptidoglycan	2
SACOL1490	<i>pbp2</i>	penicillin-binding protein 2	Biosynthesis and degradation of murein sacculus and peptidoglycan	2.2
SACOL1411	NA	femB protein	Biosynthesis and degradation of murein sacculus and peptidoglycan	2.4
SAV0276	<i>lytM</i>	peptidoglycan hydrolase	Biosynthesis and degradation of murein sacculus and peptidoglycan	4.6
SAV2569	<i>isaA</i>	immunodominant antigen A	Other	5.3
SAV0747	<i>llm</i>	lipophilic protein affecting bacterial lysis rate and methicillin resistance level	Role category not yet assigned	2.9
SACOL0033	<i>mecA</i>	penicillin-binding protein 2'	Biosynthesis and degradation of murein sacculus and peptidoglycan	−2.3
SAR0260	<i>lrgB</i>	antiholin-like protein LrgB	Other	−2.3
SAR0136	<i>sasD</i>	putative surface anchored protein	Other	−17.8
SAV2515	NA	probable transmembrane protein smpB	Other	−28.1
Fatty acid and phospholipid metabolism				
SAV0354	NA	acetyl-CoA C-acetyltransferase homolog	Biosynthesis	3.7
SAV1456	NA	similar to biotin ligase	Biosynthesis	2.4
SACOL1245	<i>fabG1</i>	3-oxoacyl-(acyl-carrier-protein) reductase	Biosynthesis	2.4
SACOL0987	<i>fabH</i>	3-oxoacyl-(acyl carrier protein) synthase	Biosynthesis	2.3
SACOL1280	<i>cdsA</i>	phosphatidate cytidyltransferase	Biosynthesis	2.2
SACOL1572	<i>accB</i>	acetyl-CoA carboxylase, biotin carboxyl carrier protein	Biosynthesis	2.1
SACOL1244	<i>fabD</i>	malonyl CoA-acyl carrier protein transacylase	Biosynthesis	2
Agr regulon				
SACOL2026	<i>agrA</i>	accessory gene regulator protein A	Pathogenesis	−6.1
SAV2036	<i>agrB</i>	accessory gene regulator B	Nitrogen metabolism	−8.8
SAV2038	<i>agrC</i>	accessory gene regulator C	Role category not yet assigned	−9.9
SAV2037	<i>agrD</i>	AgrD protein	Role category not yet assigned	−6.1
SAS1940a	NA	delta-hemolysin precursor	Toxin production and resistance	−30.5
SAV0111	<i>spa</i>	Immunoglobulin G-binding protein A precursor	Role category not yet assigned	−26.1
Protein fate				
SAV1048	<i>sspA</i>	serine protease	Role category not yet assigned	−8
SACOL1970	<i>sspB2</i>	cysteine protease precursor SspB	Pathogenesis	−4.5
SAV2637	<i>aur</i>	zinc metalloproteinase aureolysin	Role category not yet assigned	−20.4
SAV1046	<i>sspC</i>	cysteine protease	Role category not yet assigned	−2.2
Regulatory functions				
SAR0071	<i>kdpB</i>	potassium-transporting ATPase B chain	Cations and iron carrying compounds	−27.7
SAR0070	<i>kdpA</i>	potassium-transporting ATPase subunit A	Cations and iron carrying compounds	−15.9
SAV2078	<i>kdpD</i>	sensor protein	Other	−7.6
SAR0068	<i>kdpE</i>	response regulator protein	DNA interactions	−2.4
Amino acid biosynthesis				
SAV1329	<i>thrC</i>	threonine synthase	Aspartate family	−4.1
SACOL1364	<i>thrB</i>	homoserine kinase	Aspartate family	−3.5
SAV1328	<i>dhoM</i>	homoserine dehydrogenase	Aspartate family	−3.3
SAV1396	<i>dapB</i>	dihydrodipicolinate reductase	Aspartate family	−3.1
SAV1330	<i>thrB</i>	homoserine kinase homolog	Aspartate family	−3
SAV1397	<i>dapD</i>	tetrahydrodipicolinate acetyltransferase	Aspartate family	−2.9
SAV0012	NA	putative homoserine-o-acetyltransferase	Aspartate family	−2.9

in *S. aureus*. Rot is a global regulator with both positive and negative effects on *S. aureus* gene expression,³³ and some members of the Rot regulon were changed in expression.

A range of transporter genes were upregulated. Also, genes of various hypothetical proteins were upregulated.

Downregulated genes

The distribution of downregulated genes in various functional categories is given in Supplementary Table S4. Considerable numbers of cell envelope, cellular processes, energy metabolism, regulatory function, and transport and binding proteins were downregulated.

Some of the most highly downregulated genes were as follows: SAV215 probable transmembrane protein SmpB –28.1-fold; SAR0136 *sasD* –17.8-fold; SAV0158 *capJ* –13.6-fold and other cap operon genes; SAS1940a delta-hemolysin precursor –30.5-fold; *agrB* –8.8-fold; SAV0990 similar to peptide-binding protein OppA –16.7-fold; SAS0202 putative pyruvate formate-lyase activating enzyme –13-fold; *pflB* –12.2-fold; *hutU* –10.3-fold; SAV2514 probable transport protein –28.9-fold; SAR0071 potassium-transporting ATPase B chain –27.7-fold; SAR0070 potassium-transporting ATPase submit A; *oppC* –10.3-fold; SACOL1187 phenol soluble modulins –40-fold; SACOL1186 phenol soluble modulins –29.1-fold; *spa* immunoglobulin G binding protein A precursor –26.1-fold; *aur* zinc metalloproteinase aureolysin –20.4-fold.

In the category of cell envelope genes, various genes of the capsular polysaccharide biosynthetic operon (*cap*) were strongly downregulated.

Genes of the lysine biosynthetic operon were downregulated, as was *lrgB*, an anti-holin protein (Table 1). Interestingly, these genes are overexpressed in response to the membrane-depolarizing and membrane-active effects of telavancin, daptomycin, bacitracin, and various other antimicrobial peptides.^{21,26,39,44} Although upregulation of these genes by the cell is viewed as a response to combat telavancin challenge,³⁹ perhaps their downregulation makes the mutant less responsive to telavancin in some way.

The delta hemolysin precursor gene was downexpressed 30.5-fold. Expression of delta hemolysin is a marker of the

agr system, which is a quorum-sensing system that controls the expression of virulence and other genes.^{13,28} *agrA*, *agrB*, *agrC*, and *agrD* were also markedly downregulated. This suggested that Tlv^{DS}MED1952 was likely to be *agr*[–] and indeed this was confirmed by the blood agar plate assay by lack of synergistic hemolysis within the β -hemolysin zone,³⁴ in contrast to MED1951, which was *agr*⁺ (Fig. 1). There is also an association between the VISA phenotype and defects in *agr*.⁴³

Several protease genes were downregulated: *sspB2*, cysteine protease precursor; *sspA*, cysteine protease; *sspC*, cysteine protease; and *aur*, zinc metalloproteinase aureolysin. Peptidoglycan hydrolase profiles of Tlv^{DS}MED1952 autolysin extract showed decreased proteolytic processing of peptidoglycan hydrolases in this strain (see below), which may be related to decreased transcription of protease genes. A *sspC* *S. aureus* mutant showed a diminished autolytic activity.³⁵

Various cell surface-associated protein genes were under expressed: *sasD*, *clfB*, *sdrE*, *isaB*, and *spa*, protein A precursor. It would seem that Tlv^{DS}MED1952 would present a significantly different cell surface than its parent strain. Downregulation of *spa* gene expression is a common characteristic of VISA strains.¹² This may be related to possible alterations in the peptidoglycan structure in Tlv^{DS}MED1952, particularly involving the interpeptide bridge in that *lytM* and *femB* are increased in expression.

Several genes involved in anaerobic metabolism were downregulated, including *nar* genes involved in nitrate and nitrite metabolism, *pflB*, formate acetyltransferase; formate lyase-activating enzyme; and *ldh1*, lactate dehydrogenase. Possibly, Tlv^{DS}MED1952 has a greater dependence on aerobic metabolism than its parent strain.

kdpD and *kdpE*, a potassium two-component sensor and response regulator were downregulated. Xue *et al.*⁴⁷ have provided evidence that the main function of KdpDE is not in K⁺ transport via the KdpFABC system, but is in the regulation of the virulence factor expression. Genes SAR0071 and SAR0070 encoding proteins of the *kdpFABC* gene cluster are controlled by *kdpDE*. Genes regulated include *spa*, *cap*, *hla*, *aur*, and *hlgB*. Also, *agr*/RNAIII strongly activated the transcription of *kdpDE*.⁴⁷ Clearly, downregulation of the *agr*

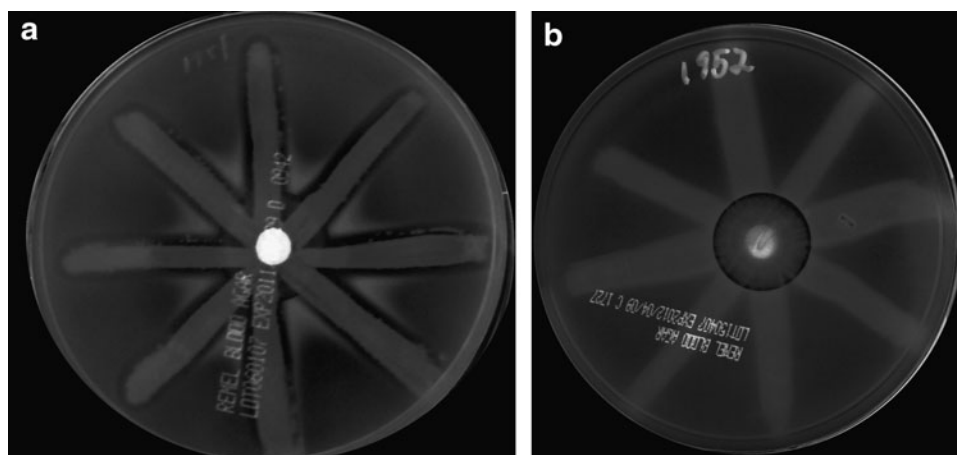


FIG. 1. Visualization of *agr* dysfunction by δ -hemolysin assay. (a) MED1951, (b) Tlv^{DS}MED1952.

system may well be involved in downregulation of *kdpDE*, and thereby, further genes affected by it. Gardete *et al.*⁷ have noted downregulation of major virulence determinants in a clinical VISA.

oppC and *oppF* were strongly downregulated, as well as a gene similar to *oppA*. Transport of peptides may be impaired in Tlv^{DS}MED1952. Various other transport genes were also downregulated.

SACOL1187 and SACOL1186, encoding phenol soluble modulins, were highly downregulated. Phenol-soluble modulins are small peptides of about twenty amino acids that destroy neutrophils at low concentrations,³ with a significant role in *S. aureus* pathogenesis.

smpB was significantly downregulated. SmpB, small protein B, is a probable transmembrane protein that binds to transfer-messenger RNA (tmRNA).^{9,36} Decreased transcription of *smpB* may lead to decreased cellular levels of SmpB and may result in altered regulation of gene expression.^{9,36}

Verification of transcriptional profiling results by real-time reverse transcription polymerase chain reaction

The fold changes in gene expression of selected up- and downregulated genes as indicated by microarray and real-time reverse transcription polymerase chain reaction (RT-PCR) results are shown in Supplementary Table S5. RT-PCR confirmed the up- or downregulation of the selected genes, with some quantitative differences in the magnitude of gene expression in some cases.

Ultrastructure

The cell walls of Tlv^{DS}MED1952 were about 30% thicker than those of MED1951 (32.4 ± 0.75 nm versus 25.0 ± 3.0 nm, Fig. 2). Markedly thicker cell walls are often a characteristic of VISA and daptomycin-resistant strains.^{29,48} This may be related to the upregulation of some cell wall biosynthesis genes noted above. However, otherwise, the cells appeared normal, with normal division septa.

Autolysis

It is a common characteristic of VISA and daptomycin-resistant strains that they show a decreased autolytic activity compared to their susceptible parent strains.^{12,16,37} Tlv^{DS}MED1952 showed a somewhat decreased Triton X-100-

stimulated autolysis (Fig. 3a), although some VISA and daptomycin-resistant strains show a more severe reduction in the autolytic activity.^{12,16,37} In an effort to investigate decreased autolysis in Tlv^{DS}MED1952 further, we determined the autolytic activity of isolated CCW that retain autolytic activity. The CCW from the strain Tlv^{DS}MED1952 also had a lower autolytic activity than those from the strain MED1951 (Fig. 3b). This indicates that the decreased autolysis is due to either alterations in the structure of the peptidoglycan, which is the autolysin substrate, or in the autolysin amount or activity. We next examined the susceptibility of PCW, which have no autolytic activity due to inactivation of autolysins by boiling, to freeze-thaw autolysin extract from the strains. The PCW of Tlv^{DS}MED1952 were digested slowly by its own autolysin extract (Fig. 3c), but significantly more rapidly by the autolysin extract from the strain MED1951. The autolysin extract from this strain digested its own PCW rapidly (Fig. 3c). This indicates that major changes in the peptidoglycan structure in Tlv^{DS}MED1952 sufficient to explain the decreased autolytic activity in this strain are unlikely. In support of this there was little difference in the lysostaphin digestion of PCW from the two strains (data not shown).

We further characterized the freeze-thaw autolysin extract by studying the peptidoglycan hydrolase profile by zymography. When the peptidoglycan hydrolase profile of the autolysin freeze-thaw extracts of the strains were compared at equal protein concentrations, the Tlv^{DS}MED1952 extract showed different characteristics than the MED1951 extract (Fig. 4). The bands of the Tlv^{DS}MED1952 extract were less intense; it was devoid of lower molecular weight bands and had higher molecular weight bands, showing deficiency in autolysin processing.^{16,20} This is consistent with downregulation of protease genes (Table 1 and Supplementary Table S3 Supplementary material) that may be involved in peptidoglycan hydrolase processing.

Fatty acid composition, carotenoid content, and membrane fluidity

The total fatty acid compositions of the strains were determined and are shown in Supplementary Table S6. The content of straight-chain fatty acids was decreased to 9.4% in Tlv^{DS}MED1952 from 17.5% in MED1951, and the sum of branched-chain fatty acids increased to 90.6% from 82.5% in Tlv^{DS}MED1952 compared to MED1951. The anteiso:iso

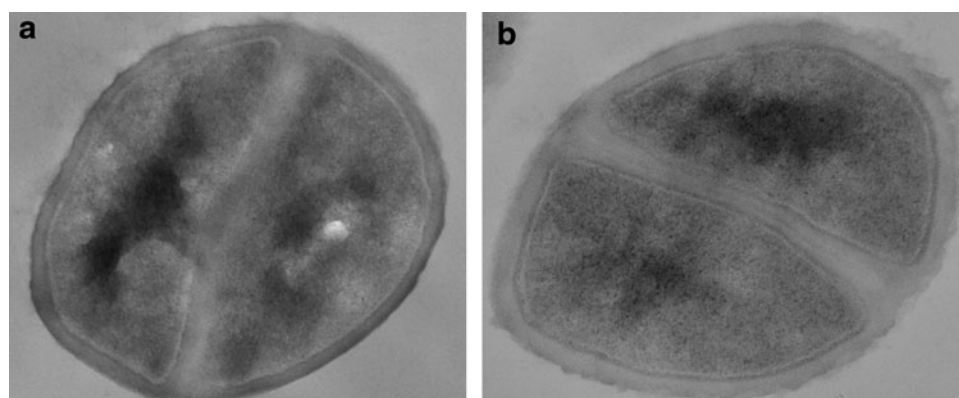


FIG. 2. Ultrastructure of the strains. (a) MED1951 (b) Tlv^{DS}MED1952. Magnification 50,000 \times .

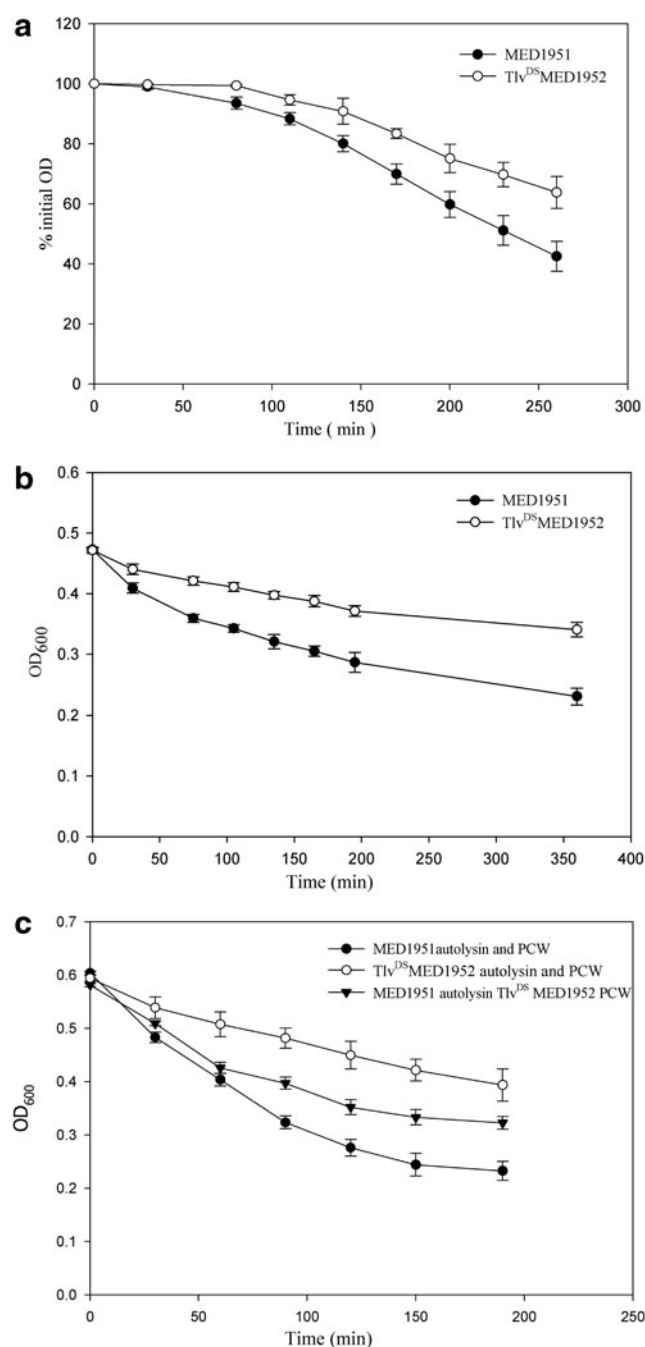


FIG. 3. The autolysis activity of MED1951 and Tlv^{DS}MED1952. **(a)** Triton X-100 stimulated whole-cell autolysis; **(b)** autolysis of CCW; **(c)** activity of freeze-thaw autolysin extracts on PCW; MED1951 PCW with MED1951 autolysin extract; Tlv^{DS}MED1952 PCW with Tlv^{DS}MED1952 autolysin extract; Tlv^{DS}MED1952 PCW with MED1951 autolysin extract.

ratio was increased to 6.8 in Tlv^{DS}MED1952 compared to 3.4 in MED1951. These changes are indicative of increased membrane fluidity in strain Tlv^{DS}MED1952.⁴⁹ Perhaps, counter intuitively, increased membrane fluidity has been associated with decreased susceptibility to membrane-active molecules in *S. aureus*, including organic solvents and daptomycin.^{1,24,27,38}

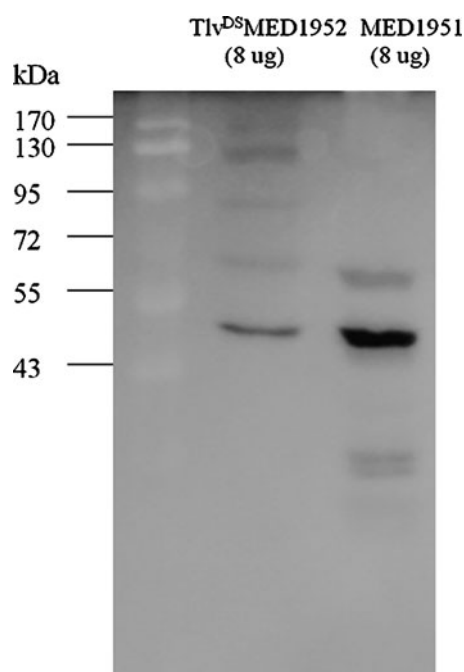


FIG. 4. Peptidoglycan hydrolase profiles of freeze-thaw autolysin extracts. Eight micrograms of protein were loaded on each lane.

The OD₄₆₅ value of the warm methanol extracts of Tlv^{DS}MED1952 were 73% of those of MED1951. This indicates no major changes in the carotenoid content, which can impact membrane fluidity.^{22,24}

A statistically significant difference ($p < 0.05$) in the polarization value (0.5 ± 0.019) was detected for MED1951 compared to the polarization value of Tlv^{DS}MED1952 (0.46 ± 0.016). The lower polarization value indicates a more fluid membrane in Tlv^{DS}MED1952.^{1,38}

In conclusion, there were multiple changes in the transcriptome of the decreased susceptibility mutant that may be involved in its decreased telavancin susceptibility. Also, expression of various virulence factors was decreased. There were significant cell envelope changes in the mutant involving thicker cell walls, decreased autolysis, and membrane fatty acid composition.

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Disclosure Statement

No competing financial interests exist.

References

- Bayer, A.S., R. Prasad, J. Chandra, A. Koul, M. Smriti, A. Varma, R.A. Skurray, N. Firth, M.H. Brown, S.P. Koo, and M.R. Yeaman. 2000. *In vitro* resistance of *Staphylococcus aureus* to thrombin-induced platelet microbicidal protein is

- associated with alterations in cytoplasmic membrane fluidity. *Infect. Immun.* **68**:3548–3553.
2. Chambers, H.F., and F.R. Deleo. 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nat. Rev. Microbiol.* **7**:629–641.
 3. Clarke, S.R. 2010. Phenol-soluble modulins of *Staphylococcus aureus* lure neutrophils into battle. *Cell Host Microbe*. **7**: 423–424.
 4. Delaune, A., O. Poupel, A. Mallet, Y.-M. Coic, T. Msadek, and S. Dubrac. 2011. Peptidoglycan crosslinking relaxation plays an important role in *Staphylococcus aureus* WalKR-dependent cell viability. *Plos One* **6**:e17054.
 5. Draghi, D.C., B.M. Benton, K.M. Krause, C. Thornsberry, C. Pillar, and D.F. Sahm. 2008. Comparative surveillance study of telavancin activity against recently collected gram-positive clinical isolates from across the United States. *Antimicrob. Agents Chemother.* **52**:2383–2388.
 6. Dubrac, S., I.G. Boneca, O. Poupel, and T. Msadek. 2007. New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in *Staphylococcus aureus*. *J. Bacteriol.* **189**:8257–8269.
 7. Gardete, S., C. Kim, B.M. Hartmann, M. Mwangi, C.M. Roux, P.M. Dunman, H.F. Chambers, and A. Tomasz. 2012. Genetic pathway in acquisition and loss of vancomycin resistance in a methicillin-resistant *Staphylococcus aureus* (MRSA) strain of clonal type USA300. *Plos Pathogens* **8**:e1002505.
 8. Gustafson, J.E., B. Berger-Bächi, A. Strässle, and B.J. Wilkinson. 1992. Autolysis of methicillin-resistant and-susceptible *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **36**:566–572.
 9. Hanawa-Suetsugu, K., M. Takagi, H. Inokuchi, H. Hime-no, and A. Muto. 2002. SmpB functions in various steps of trans-translation. *Nucleic Acids Res.* **30**:1620–1629.
 10. Hegde, S.S., N. Reyes, R. Skinner, and S. Difuntorum. 2008. Efficacy of telavancin in a murine model of pneumonia induced by methicillin-susceptible *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **61**:169–172.
 11. Higgins, D.L., R. Chang, D.V. Debarov, J. Leung, T. Wu, K.M. Krause, E. Sandvik, J.M. Hubbard, K. Kaniga, D.E. Schmidt, Jr., Q. Gao, R.T. Cass, D.E. Karr, B.M. Benton, and P.P. Humphrey. 2005. Telavancin, a multifunctional lipoglycopeptide, disrupts both cell wall synthesis and cell membrane integrity in methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **49**:1127–1134.
 12. Howden, B.P., J.K. Davies, P.D. Johnson, T.P. Stinear, and M.L. Grayson. 2010. Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanism, laboratory detection, and clinical implications. *Clin. Microbiol. Rev.* **23**:99–139.
 13. Johnson, J.K., A.D. Harris, M.D. Shardell, J.C. McGregor, K.A. Thom, and E.N. Perencevich. 2011. Increased mortality with accessory gene regulator (*agr*) dysfunction in *Staphylococcus aureus* among bacteremic patients. *Antimicrob. Agents Chemother.* **55**:1082–1087.
 14. Kaenjak, A., J.E. Graham, and B.J. Wilkinson. 1993. Choline transport activity in *Staphylococcus aureus* induced by osmotic stress and low phosphate concentrations. *J. Bacteriol.* **175**:2400–2406.
 15. Klevens, R.M., M.A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L.H. Harrison, R. Lynfield, G. Dumyati, J.M. Townes, A.S. Craig, E.R. Zell, G.E. Fosheim, L.K. McDougal, R.B. Carey, and S.K. Fridkin. 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* **298**:1763–1771.
 16. Koehl, J.L., A. Muthaiyan, R.K. Jayaswal, K. Ehlert, H. Labischinski, and B.J. Wilkinson. 2004. Cell wall composition and decreased autolytic activity and lysostaphin susceptibility of glycopeptide-intermediate *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **48**:3749–3757.
 17. Kosowska-Shick, K., C. Clark, G.A. Pankuch, P. McGhee, B. Dewasse, L. Beachel, and P.C. Appelbaum. 2009. Activity of telavancin against staphylococci and enterococci determined by MIC and resistance selection studies. *Antimicrob. Agents Chemother.* **53**:4217–4224.
 18. Lamichhane-Khadka, R., J.T. Riordan, A. Delgado, A. Muthaiyan, T.D. Reynolds, B.J. Wilkinson, and J.E. Gustafson. 2008. Genetic changes that correlate with the pine-oil disinfectant-reduced susceptibility mechanism of *Staphylococcus aureus*. *J. Appl. Microbiol.* **105**:1973–1981.
 19. Leadbetter, M.R., S.M. Adams, B. Bazzini, P.R. Fatherree, D.E. Karr, K.M. Krause, B.M. Lam, M.S. Linsell, M.B. Nodwell, J.L. Pace, K. Quast, J.P. Shaw, E. Soriano, S.G. Trapp, J.D. Villena, T.X. Wu, B.G. Christensen, and J.K. Judice. 2004. Hydrophobic vancomycin derivatives with improved ADME properties: discovery of telavancin (TD-6424). *J. Antibiot. (Tokyo)* **57**:326–336.
 20. Ledala, N., B. J. Wilkinson, and R. K. Jayaswal. 2006. Effect of oxacillin and tetracycline on autolysis, autolysis processing and *atl* transcription in *Staphylococcus aureus*. *Int. J. Antimicrob. Agents* **27**:518–524.
 21. Li, M., D.J. Cha, Y. Lai, A.E. Villaruz, D.E. Sturdevant, and M. Otto. 2007. The antimicrobial peptide-sensing system *aps* of *Staphylococcus aureus*. *Mol. Microbiol.* **66**: 1136–1147.
 22. Liu, G.Y., A. Essex, J.T. Buchanan, V. Datta, H.M. Hoffman, J.F. Bastian, J. Fierer, and V. Nizet. 2005. *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *J. Exp. Med.* **202**:209–215.
 23. Lunde, C.S., S.R. Hartouni, J.W. Janc, M. Mammen, P.P. Humphrey, and B.M. Benton. 2009. Telavancin disrupts the functional integrity of the bacterial membrane through targeted interaction with the cell wall precursor lipid II. *Antimicrob. Agents Chemother.* **53**:3375–3383.
 24. Mishra, N.N., G.Y. Liu, M.R. Yeaman, C.C. Nast, R.A. Proctor, J. McKinnell, and A.S. Bayer. 2011. Carotenoid-related alteration of cell membrane fluidity impacts *Staphylococcus aureus* susceptibility to host defense peptides. *Antimicrob. Agents Chemother.* **55**:526–531.
 25. Mishra, N.N., J. McKinnell, M.R. Yeaman, A. Rubio, C.C. Nast, L. Chen, B.N. Kreiswirth, and A.S. Bayer. 2011. *In vitro* cross-resistance of daptomycin and host defense cationic antimicrobial peptides in clinical methicillin-resistant *Staphylococcus aureus* (MRSA) isolates. *Antimicrob. Agents Chemother.* **55**:4012–4018.
 26. Muthaiyan, A., J.A. Silverman, R.K. Jayaswal, and B.J. Wilkinson. 2008. Transcriptional profiling reveals that daptomycin induces the *Staphylococcus aureus* cell wall stress stimulon and genes responsive to membrane depolarization. *Antimicrob. Agents Chemother.* **52**:980–990.
 27. Nielsen, L.E., D.R. Kadavy, S. Rajagopal, R. Drijber, and K.W. Nickerson. 2005. Survey of extreme solvent tolerance in gram-positive cocci: membrane fatty acid changes in *Staphylococcus haemolyticus* grown in toluene. *Appl. Environ. Microbiol.* **71**:5171–5176.

28. Novick, R.P., and E. Geisinger. 2008. Quorum sensing in staphylococci. *Annu. Rev. Genet.* **42**:541–564.
29. Pfeldt, R.F., and B.J. Wilkinson. 2004. The escalating challenge of vancomycin resistance in *Staphylococcus aureus*. *Curr. Drug Targets Infect. Disord.* **4**:273–294.
30. Qoronfleh, M.W., and B.J. Wilkinson. 1986. Effects of growth of methicillin-resistant and -susceptible *Staphylococcus aureus* in the presence of β -lactams on peptidoglycan structure and susceptibility to lytic enzymes. *Antimicrob. Agents Chemother.* **29**:250–257.
31. Record, M.T. Jr., E.S. Courtenay, D.S. Cayley, and H.J. Guttman. 1998. Responses of *E. coli* to osmotic stress: large changes in amounts of cytoplasmic solutes and water. *Trends Biochem. Sci.* **23**:143–148.
32. Reyes, D., D.O. Andrey, A. Monod, W.L. Kelley, G. Zhang and A.L. Cheung. 2011. Coordinated regulation by AgrA, SarA and SarB and SarR to control *agr* expression in *Staphylococcus aureus*. *J. Bacteriol.* **193**:6020–6031.
33. Saïd-Salim, B., P.M. Dunman, F.M. McAleese, D. Macapagal, E. Murphy, P.J. McNamara, S. Arvidson, T.J. Foster, S.J. Projan, and B.N. Kreiswirth. 2003. Global regulation of *Staphylococcus aureus* genes by *rot*. *J. Bacteriol.* **185**:2610–2619.
34. Schweizer, M.L., J.P. Furuno, G. Sakoulas, J.K. Johnson, A.D. Harris, M.D. Shardell, J.C. McGregor, K.A. Thom, and E.N. Perencevich. 2011. Increased mortality with accessory gene regulator (*agr*) dysfunction in *Staphylococcus aureus* among bacteremic patients. *Antimicrob. Agents Chemother.* **55**:1082–1087.
35. Shaw, L.N., E. Golonka, G. Szmyd, S.J. Foster, J. Travis, and J. Potempa. 2005. Cytoplasmic control of premature activation of a secreted protease zymogen: deletion of staphostatin B (*SspC*) in *Staphylococcus aureus* 8325–4 yields a profound pleiotropic phenotype. *J. Bacteriol.* **187**:1751–1762.
36. Shimizu, Y., and T. Ueda. 2002. The role of SmpB protein in trans-translation. *FEBS Lett.* **514**:74–77.
37. Shoji, M., L. Cui, R. Iizuka, A. Komoto, H.M. Neoh, Y. Watanabe, T. Hishinuma, and K. Hiramatsu. 2011. The *walK* and *clpP* mutations confer reduced vancomycin susceptibility on *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **55**:3870–3881.
38. Singh, V.K., D.S. Hattangady, E.S. Giotis, A.K. Singh, N.R. Chamberlain, M.K. Stuart, and B.J. Wilkinson. 2008. Insertional inactivation of branched chain α -keto acid dehydrogenase in *Staphylococcus aureus* leads to decreased branched-chain membrane fatty acid content and increased susceptibility to certain stresses. *Appl. Environ. Microbiol.* **74**:5882–5890.
39. Song, Y., C.S. Lunde, B.M. Benton, and B.J. Wilkinson. 2012. Further insights into the mode of action of the lipoglycopeptide telavancin through global gene expression studies. *Antimicrob. Agents Chemother.* **56**:3157–3164.
40. Stapleton, M.R., M.J. Horsburgh, E.J. Hayhurst, L. Wright, I.-M. Jonsson, A. Tarkowski, J.F. Kokai-Kun, J.J. Mond, and S.J. Foster. 2007. Characterization of IsaA and SceD, two putative lytic transglycosylases of *Staphylococcus aureus*. *J. Bacteriol.* **189**:7316–7325.
41. Tannake, Y., K. Morikawa, Y. Ohki, M. Yao, K. Tsumot, N. Watanabe, T. Ohta, and I. Tanaka. 2007. Structural and mutational analyses of Drp35 from *Staphylococcus aureus*. *J. Biol. Chem.* **282**:5770–5780.
42. Townsend, D.E., A. Kaenjak, R.K. Jayaswal, and B.J. Wilkinson. 2006. Proline is biosynthesized from arginine in *Staphylococcus aureus*. *Microbiology.* **142**:1491–1497.
43. Tsuji, B.T., M.J. Rybak, K.L. Lau, and G. Sakoulas. 2007. Evaluation of accessory gene regulator (*agr*) group and function in the proclivity towards vancomycin intermediate resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **51**:1089–1091.
44. Utaida, S., P.M. Dunman, D. Macapagal, E. Murphy, S.J. Projan, V.K. Singh, R.K. Jayaswal, and B.J. Wilkinson. 2003. Genome-wide transcriptional profiling of the response of *Staphylococcus aureus* to cell-wall-active antibiotics reveals a cell-wall-stress stimulon. *Microbiology* **149**:2719–2732.
45. Vijaranakul, U., M.J. Nadakavukaren, B.L. de Jonge, B.J. Wilkinson, and R.K. Jayaswal. 1995. Increased cell size and shortened peptidoglycan interpeptide bridge of NaCl-stressed *Staphylococcus aureus* and their reversal by glycine betaine. *J. Bacteriol.* **177**:5116–5121.
46. Wilkinson, B.J., A. Muthaiyan, and R.K. Jayaswal. 2005. The cell wall stress stimulon of *Staphylococcus aureus* and other gram-positive bacteria. *Curr. Med. Chem. Anti-Infective Agents.* **4**:259–276.
47. Xue, T., Y. You, D. Hong, H. Sun, and B. Sun. 2011. The *Staphylococcus aureus* KdpDE two-component system couples extracellular K^+ sensing and Agr signaling to infection programming. *Infect. Immun.* **79**:2154–2167.
48. Yang, S.J., Y.Q. Xiong, S. Boyle-Vavra, R. Daum, T. Jones, and A.S. Bayer. 2010. Daptomycin-oxacillin combinations in treatment of experimental endocarditis caused by daptomycin-nonsusceptible strains of methicillin-resistant *Staphylococcus aureus* with evolving oxacillin susceptibility (the “seesaw effect”). *Antimicrob. Agents Chemother.* **54**:3161–3169.
49. Zhang, Y.M., and C.O. Rock. 2008. Membrane lipid homeostasis in bacteria. *Nat. Rev. Microbiol.* **6**:222–233.
50. Zhu, K., D.O. Bayles, A. Xiong, R.K. Jayaswal, and B.J. Wilkinson. 2005. Precursor and temperature modulation of fatty acid composition and growth of *Listeria monocytogenes* cold-sensitive mutants with transposon-interrupted branched-chain α -keto acid dehydrogenase. *Microbiology* **151**:615–623.

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