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Dual Targeting of Cell Wall Precursors by Teixobactin Leads to Cell Lysis

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Teixobactin represents the first member of a newly discovered class of antibiotics that act through inhibition of cell wall synthesis. Teixobactin binds multiple bactoprenol-coupled cell wall precursors, inhibiting both peptidoglycan and teichoic acid synthesis. Here, we show that the impressive bactericidal activity of teixobactin is due to the synergistic inhibition of both targets, resulting in cell wall damage, delocalization of autolysins, and subsequent cell lysis. We also find that teixobactin does not bind mature peptidoglycan, further increasing its activity at high cell densities and against vancomycin-intermediate *Staphylococcus aureus* (VISA) isolates with thickened peptidoglycan layers. These findings add to the attractiveness of teixobactin as a potential therapeutic agent for the treatment of infection caused by antibiotic-resistant Gram-positive pathogens.

Antibiotic resistance development is a major threat to human health. Constant development of novel antibiotics is required to keep pace with the emergence and spread of antibiotic resistance in bacterial pathogens (1). The majority of antibiotics in use today are derivatives of molecules discovered in the early to mid-twentieth century. The lack of novel compounds, coupled with the emergence and spread of antibiotic resistance, has resulted in an increasingly dangerous situation (2–4).

One approach to discover novel antibiotics is to improve our ability to cultivate microorganisms that produce them. Traditional culturing methods allow access to an estimated 1% of the biodiversity in soil. A novel cultivation technique, using an isolation chip or iChip, provides access to an untapped reservoir of natural product antibiotics, produced by bacteria that had previously eluded cultivation efforts. One such organism, *Eleftheria terrae*, a previously uncultivated Gram-negative betaproteobacterium, was found to produce a novel depsipeptide antibiotic, called teixobactin. Teixobactin inhibits cell wall biosynthesis and represents a new class of antibiotics (5).

The bacterial cell wall contains layers of peptidoglycan, a cross-linked matrix of linear glycan chains (6). Peptidoglycan cross-linking is the target of β-lactam and glycopeptide antibiotics. In Gram-positive bacteria, teichoic acid (TA) is also a major component of the cell wall. TA includes wall teichoic acid (WTA), connected to peptidoglycan, or lipoteichoic acid (LTA), anchored in the cytoplasmic membrane. TA plays important roles in bacterial physiology (7, 8), and teichoic acid biosynthesis is an important target for antibiotic development (9). Recently, it was shown that inhibition of teichoic acid biosynthesis can restore susceptibility to methicillin in methicillin-resistant *Staphylococcus aureus* (MRSA) (10).

In a previous study, we found that teixobactin binds lipid II, a precursor of peptidoglycan biosynthesis, and lipid III, a precursor of teichoic acid biosynthesis (5). Interestingly, teixobactin was capable of superior bactericidal and bacteriolytic activity compared to other cell wall-acting antibiotics, the β-lactam oxacillin, or the glycopeptide vancomycin. Also, resistance to teixobactin was not detected in a number of *in vitro* studies. Here, we show that the ability of teixobactin to simultaneously inhibit peptidoglycan and teichoic acid biosynthesis triggers synergistic effects, resulting in increased cell wall damage, delocalization of autolysins, and subsequent lysis and cell death. Furthermore, we demonstrate that teixobactin does not bind mature peptidoglycan and hence is capable of effectively targeting vancomycin-intermediate *S. aureus* (VISA) strains that have increased cell wall density.

MATERIALS AND METHODS

Antimicrobial agents, bacterial strains, and primers. Teixobactin was purified according to the procedure described previously (5). Vancomycin and tunicamycin were purchased from Sigma-Aldrich. The bacterial strains and primers are listed in Table S2 in the supplemental material. JE2, HG003, and SA113 were used throughout the study. HG003 and SA113 are closely related laboratory methicillin-susceptible *S. aureus* (MSSA) strains. JE2 is a MRSA USA300 isolate. All strains displayed the same MIC to teixobactin. Strains were selected for specific assays due to preexisting characterized mutants in those strain backgrounds.

MIC. The MIC was determined by the broth microdilution method according to CLSI guidelines. Muller-Hinton broth (MHB) was supplemented with 0.1% Tween 80 to prevent the absorption of compounds to plastic surfaces. Cell concentration was adjusted to about 5×10^5 CFU/ml, and cells were incubated for 20 h at 37°C. The MIC was defined as the lowest concentration of antimicrobial agents that resulted in no visible

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growth. The MICs of teixobactin and vancomycin in the presence of purified cell wall components were measured with the method described above. Purified peptidoglycan suspension was added to MHB to an optical density at 600 nm (OD_{600}) of 0.1. The medium containing antibiotics and peptidoglycan was preincubated at 37°C for 1 h before adding bacterial cells.

Scanning electron microscopy. Overnight cultures of JE2 in MHB were diluted 1:100 in 100 ml TSB and incubated at 37°C and 225 rpm to an OD_{600} of 0.4 to 0.6. Antibiotics were added at 10× MIC, and the cells were cultivated for an additional 4 h. Cultures were filtered through a cellulose membrane. The cells on the membrane were fixed with 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h and treated in a graded series of 1% OsO_4 with 0.1 M sodium cacodylate buffer. Cells were then dehydrated in ethanol and critical-point dried using CO_2. The samples were sputter-coated with a 5-nm platinum coating and examined at a 3.0-kV accelerating voltage with a Hitachi S-4800 field emission scanning electron microscope.

Time-dependent killing. An overnight culture of *S. aureus* was diluted in MHB and incubated at 37°C and 225 rpm for 3 h. Antibiotics were added at 10× MIC, and cultures were incubated at 37°C and 225 rpm. At intervals, 100-μl aliquots were removed and centrifuged at 10,000 × *g* for 1 min and resuspended in 100 μl of sterile phosphate-buffered saline (PBS). Tenfold serially diluted suspensions (10 μl) were plated on Muller-Hinton agar plates. The plates were incubated at 37°C overnight.

Bacteriolytic assay. The bacteriolytic activity in supernatant of cell cultures was determined by the change of turbidity of the substrate, heat-killed RN4220 cells (11). The cells were incubated at 37°C until reaching mid-exponential phase (OD_{600} of 0.4 to 0.6). Antibiotics were added at 10× MIC, and cultures were incubated at 37°C and 225 rpm for 4 h. Filter-sterilized culture supernatant was mixed with heat-killed cells adjusted to an OD_{600} of 0.5 and incubated at 37°C for 6 h. The turbidity was measured every 30 min. The remaining quantity was expressed as the ratio to initial values.

Zymographic analysis. Murein hydrolase profiles were analyzed by zymogram (12, 13). Antimicrobial agents at 10× MIC were added to mid-exponential-phase JE2 cells in 15 ml Trypticase soy broth (TSB), and the cells were cultivated for an additional 4 h. Cultures were centrifuged and supernatants and cells were collected separately. The supernatants were concentrated using an Ultra-4 3000 molecular-weight-cutoff filter (Amicon) to 500 μl. The concentration of protein was determined by the Bio-Rad protein assay method by following the manufacturer's instructions. The cells were washed with 0.1 M Tris-HCl (pH 6.8) once and treated with sample buffer (2% SDS with β-mercaptoethanol) and incubated at 65°C for 5 min. After centrifugation, the supernatants were stored at −80°C until analysis could be performed. The samples were electrophoresed in an 8% SDS-PAGE gel containing heat-killed RN4220 cells as a substrate (final OD_{600}, 10). After electrophoresis, the gel was washed with Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, and 3 mM KCl, pH 7.5) buffer containing 1% Hanks buffer (Sigma) and 2.5% Triton-X and incubated in TBS buffer containing 1% Hanks buffer and 10 mM $CaCl_2$ at 37°C overnight. The clear zone, which indicated a protein with autolysis activity, appeared as a dark band in Fig. 2B (see also Fig. S1A in the supplemental material).

Quantitative real-time PCR. RNA purification was performed as previously reported (14). Antimicrobial agents at 10× MIC were added to mid-exponential-phase USA300 cells, and the cells were incubated for 1 h. The cells were collected and treated with RNAprotect bacterial reagent (Qiagen) to ensure RNA integrity. Cells were pelleted, suspended in 50 mM EDTA with 0.6 mg/ml lysostaphin (Sigma), and incubated at 37°C for 2 min to lyse the cell. Total RNA was isolated using an RNeasy minikit (Qiagen) according to the manufacturer's instructions. To remove DNA, a Turbo DNA-free kit (Ambion) was used. The integrity of purified RNA was confirmed by an RNA 600 Nano kit and bioanalyzer (Agilent). RNA was reverse transcribed into cDNA using high-capacity cDNA reverse transcription kits (Applied Bioscience). Quantitative real-time PCR was conducted with using Power SYBR green master mix (Thermo Fisher) and the ABI Prism 7000 sequence detection system (Applied Bioscience). The *16S* gene was used as the internal standard.

Western blotting. For Western blotting, the samples were run in SDS-PAGE gel (NuPAGE with 4 to 12% Bis-Tris gel; Novex). The proteins were transferred onto an Immobilon-P polyvinylidene difluoride (PVDF) membrane (0.45-μm pore size; Millipore) with the XCell II blot module (Invitrogen). Once transferred, immunoblotting was carried out using a WesternBreeze chemiluminescent Western blot immunodetection kit according to the manufacturer's instructions (Invitrogen). The membrane was washed, blocked with nonspecific antibody, and treated with anti-Atl antibody (12) for 1 h. Finally, the membrane was washed and incubated with anti-rabbit IgG secondary antibody. After incubation of the membrane with substrate, the blot was analyzed by a gel imaging system (Bio-Rad).

Isolation of peptidoglycan. Isolation of peptidoglycan for competition assay was described previously (15). Exponential-phase HG003 cells were harvested by centrifugation, washed with ice-cold saline, and resuspended in 2 ml of saline. The cells were boiled for 20 min. After centrifugation, the cells were resuspended with saline, mixed with glass beads, and lysed by bead beater (2 rounds, with 20 s at the highest speed and cooling on ice for 5 min). After harvesting the suspensions, the cells were collected by centrifugation, suspended in 1 ml 2% SDS, and boiled for 30 min. After cooling to room temperature, the cells were washed with distilled water at least 5 times. The pellets were dissolved in 0.1 M Tris-HCl (pH 6.8) with trypsin (0.5 mg/ml; Sigma) and incubated at 37°C overnight. Finally, the pellets were washed with distilled water 3 times. This suspension was stored at −80°C until used for competition assay.

Isolation and detection of cell wall teichoic acid. Isolation of teichoic acid was conducted as follows (16). Antimicrobial agents (teixobactin and vancomycin [10× MIC] as well as tunicamycin [0.025× MIC]) were added to mid-exponential-phase HG003 cells in MHB, and the cells were cultivated for an additional 4 h. Cell pellets were collected by centrifuge, washed with 50 mM 2-(N-morpholino)ethanesulfonic acid (MES; pH 6.5), resuspended in 0.5 ml 50 mM MES (pH 6.5) with 4% (wt/vol) SDS, and boiled for 1 h. The cells then were collected by centrifugation and washed with 50 mM MES (pH 6.5) with 4% SDS twice, washed with 50 mM MES (pH 6.5) with 2% (wt/vol) NaCl, and finally washed with 50 mM MES (pH 6.5). The cells were resuspended with 20 mM Tris-HCl (pH 8.0) with trypsin (0.5 mg/ml) and incubated at 37°C overnight. Following this, the pellets were washed with distilled water at least 3 times. Pellets were then suspended with 0.1 ml of 0.1 M NaOH and incubated at room temperature overnight. Supernatants were used for competition assay or detection of WTA by SDS-PAGE. Samples were run in SDS-PAGE gel (16% Tris-Tricine gel; Novex), and the gel was stained by a Pierce silver stain kit (Thermo Fisher) by following the manufacturer's procedure.

Purification of Atl R_{1-3} and conjugation with Cy3 dye. The three-Atl-repeat domain, R_{1-3} (17, 18), was expressed with a N-terminal His_6 tag in *Escherichia coli* M15 using the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible plasmid pEQ30Atl R_{1-3} (19, 20). Cells were cultivated in 2× yeast extract-tryptone broth (YT) to an OD of 0.5 at 37°C and then induced with 0.5 mM IPTG for 4 h at 20°C. The cells were harvested by centrifugation and washed twice in PBS containing complete protease inhibitor cocktail (Roche); the cell pellet was lysed using a French press. The crude extract was centrifuged (5,000 × *g*), and R_{1-3}–His was affinity purified with nickel-nitrilotriacetic acid (Ni-NTA) superflow (Qiagen) as described by the manufacturer. The imidazole elution buffer was then exchanged by PBS using a Vivaspin 2 column with a 10-kDa cutoff (Sartorius). For protein conjugation with Cy3, the Amersham Cy3 antibody labeling kit (GE) was used. Conjugation of $His6R_{1-3}$ and the separation of protein from free dye were performed as described by the manufacturer.

Epifluorescence microscopy. Localization of Atl on the cell surface exposed to antibiotics was determined similarly to a previous study (21). Antimicrobial agents at 10× MIC were added to mid-exponential-phase HG003 cells in MHB, and the cells were cultivated for an additional 30

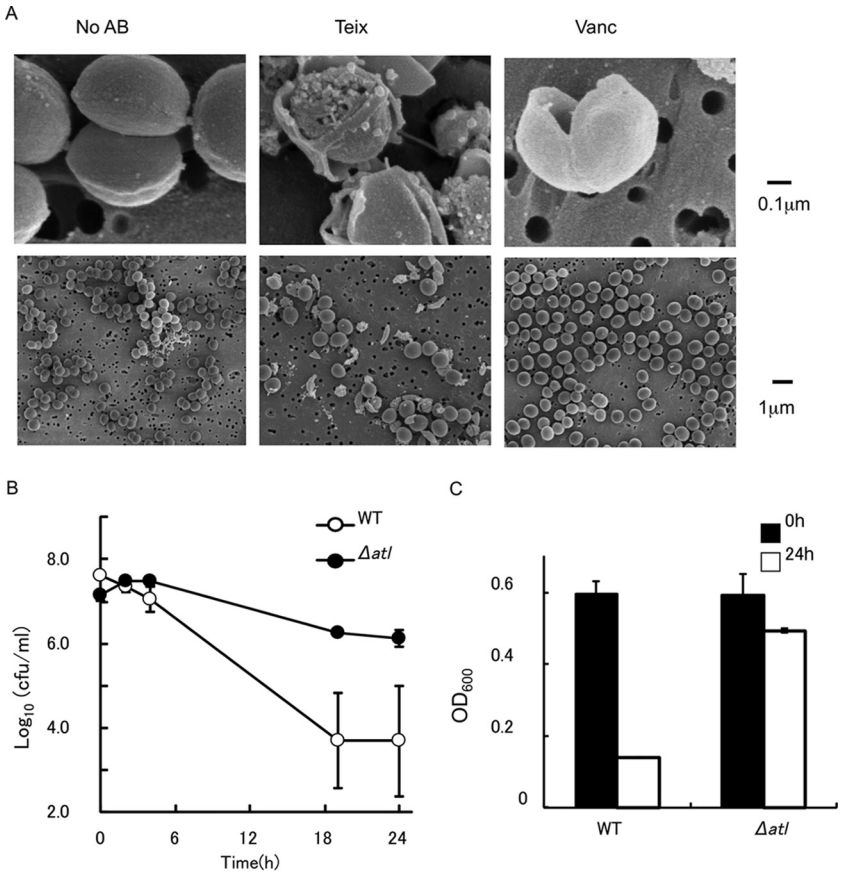

FIG 1 Antibacterial activity of teixobactin is dependent on autolysin. (A) Scanning electron microscopy of *S. aureus* JE2 revealed cell wall damage in response to teixobactin (10× MIC). The scale bars for upper and lower pictures indicate 0.1 μm and 1 μm, respectively. AB, antibody; Teix, teixobactin; Vanc, vancomycin. (B) Time-dependent killing by teixobactin (10× MIC) against JE2 and its isogenic Δ*atl* mutant. (C) The turbidity of JE2 and the Δ*atl* mutant before and after exposure of teixobactin (10× MIC) for 24 h (right). The OD_{600} was measured. These data represent the means and standard deviations (SD) from 3 independent experiments.

min. The cells were washed twice with PBS and incubated with Cy3-$R_{1–3}$ for 5 min at room temperature. After washing twice with PBS, a 2-μl cell suspension was applied to glass slides and distribution of fluorescence was confirmed with a Leica fluorescence microscopy system.

RESULTS

Teixobactin-induced lysis is dependent on the Atl autolysin. Teixobactin has been shown to have excellent bactericidal activity against *S. aureus* (5). We examined the effect on cell morphology of teixobactin and vancomycin after 4 h of exposure at 10× MIC by electron microscopy (Fig. 1A). Exposure to teixobactin results in collapse of the cell wall, while vancomycin damage to the cell wall was less severe.

β-Lactam-induced lysis is known to be mediated by Atl, the major cell wall autolysin of *S. aureus* (17, 22). To investigate the contribution of Atl to the activity of teixobactin, the antibacterial activity against an Δ*atl* mutant from the Nebraska transposon insertion library (available through BEI [https://www.beiresources.org/]) was examined and compared to wild-type strain JE2. Although the MIC of teixobactin was not affected by mutation of *atl* (see Table S1 in the supplemental material), the bactericidal activity was markedly reduced. Teixobactin did not significantly reduce the viable cell count of the Δ*atl* mutant after 24 h of exposure, compared to a 3-log reduction in CFU per milliliter in the wild-type strain (Fig. 1B). Also, 24 h of teixobactin exposure did not cause lysis in an *atl* mutant, confirming the lysis phenotype is Atl dependent (Fig. 1C).

Teixobactin causes a decrease in *atl* expression. Teixobactin's bactericidal activity is dependent on Atl; hence, we decided to examine the lytic capacity of the supernatant of a culture treated with teixobactin. Atl undergoes maturation by proteolytic processing, resulting in generation of two extracellular lytic enzymes, an amidase and a glucosaminidase (12, 14, 23). If teixobactin treatment resulted in increased lytic enzyme production, we would expect the supernatant to have increased lysis capacity. To test this, we performed bacteriolytic assays with supernatants of treated and untreated cultures (Fig. 2A). The untreated control supernatant had marked lytic activity and strongly reduced the turbidity of a cell suspension over time. On the other hand, the supernatant of a teixobactin-treated culture did not have any visible lytic capacity. To further investigate this, we examined the lysis profile of the extracellular and cell wall-anchored Atl with zymography (Fig. 2B). Both supernatant and cell wall-associated protein isolated from cells treated with 10× MIC of teixobactin for 4 h showed reduced murein hydrolase activity compared to those from untreated samples (see Fig. S1A in the supplemental material). Furthermore, real-time PCR revealed that expression of

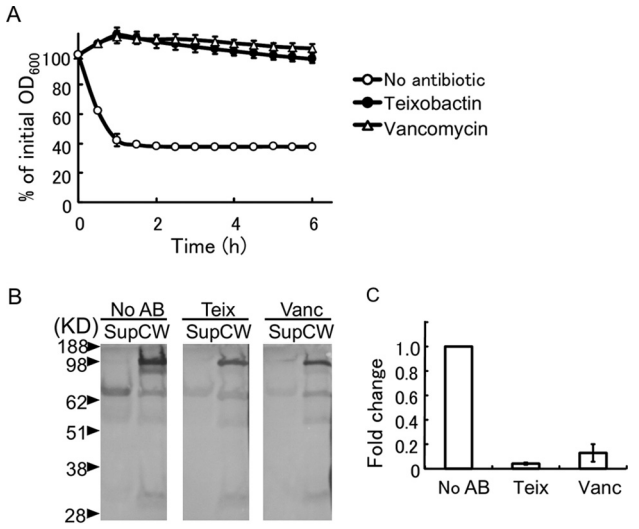

FIG 2 Teixobactin causes downregulation of *atl* expression. (A) Bacteriolytic assay using supernatant from JE2 incubated with teixobactin (10× MIC) or vancomycin (10× MIC) for 4 h. (B) Zymography of teixobactin-treated samples on SDS-PAGE gel containing heat-killed *S. aureus* RN4220 as a substrate. The dark bands indicate the clear zone in the SDS-PAGE gel, which is caused by the lysis of substrate. Left and right lanes were supernatant (Sup) and cell wall-associated samples (CW), respectively. (C) Comparison of *atl* transcription by quantitative real-time PCR. The bars indicate the relative values compared to the no-antibiotic-treatment sample. These data represents the means and SD from 3 independent experiments.

atl in teixobactin-treated cells was about 25-fold lower than that in untreated control cells (Fig. 2C). Finally, the reduced Atl levels were confirmed by Western blot analysis (see Fig. S1B). Vancomycin also reduced the murein hydrolase activity (Fig. 2A to C), which has been reported previously (24, 25). These results suggest that lipid II inhibition results in the activation of a signal transduction pathway that results in reduced autolysin expression, presumably to reduce cell death in the presence of this extreme cell wall stress. Importantly, the reduction of autolysins in the supernatant in response to antibiotic occurs after 2 to 4 h of exposure (see Fig. S1A).

Teixobactin causes Atl-dependent cell lysis but paradoxically causes inhibition of autolysin expression. Hence, the teixobactin-treated cell wall must be more susceptible to autolysins present at the time of addition of the antibiotic.

Teixobactin-induced lysis is enabled by inhibition of cell wall teichoic acid biosynthesis. The localization of Atl at the septum to mediate cell division has been proposed to occur via exclusion from the rest of the cell wall by the presence of teichoic acids (21). In the absence of WTA, Atl binding on the cell surface is delocalized, causing lysis (21). Furthermore, WTA are required for β-lactam resistance in methicillin-resistant *S. aureus*, and the cells which lack WTA are sensitized to β-lactam-induced cell lysis (26–28). The genes involved in teichoic acid biosynthesis are called the *tar* genes (for teichoic acid ribitol). TarO (previously referred to as TagO), an *N*-acetylglucosamine-1-phosphate transferase, catalyzes the first step in this biosynthetic pathway, and mutation of *tarO* results in a teichoic acid-deficient strain (29). We examined cell lysis of wild-type and *tarO* mutant strains in the presence of vancomycin and teixobactin. As previously reported, teixobactin causes increased lysis of the wild-type strain compared to vancomycin. Both antibiotics caused lysis of the *tarO* mutant, demonstrating that lipid II inhibition results in cell lysis, but only in a teichoic acid null background (Fig. 3A and B). As teixobactin blocks lipid II and lipid III, this suggests that the lytic activity of teixobactin is due to the combined inhibition of both targets. To

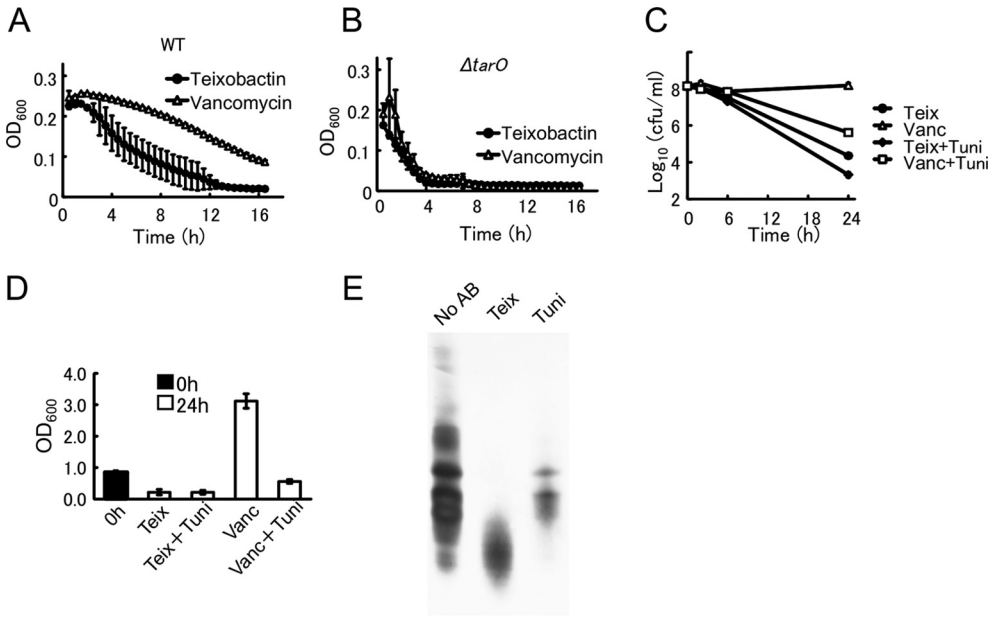

FIG 3 Inhibition of WTA biosynthesis is responsible for teixobactin-mediated lysis. The lysis of SA113 (A) and the SA113 Δ*tarO* mutant (B) was induced by teixobactin (10× MIC) or vancomycin (10× MIC). The OD_{600} was measured every 30 min for 16 h. Time-dependent killing (C) and the change of turbidity (D) of HG003 by combination of tunicamycin (Tuni; 0. 4 μg/ml; 0.025× MIC) and teixobactin (10× MIC) or vancomycin (10× MIC). These data represent the means and SD from 3 independent experiments. (E) The detection of WTA purified from HG003 exposed to antibiotics in SDS-PAGE gel. The concentrations of teixobactin and tunicamycin were 5 μg/ml (10× MIC) and 0.4 μg/ml (0.025× MIC), respectively. The gel was stained by a silver stain kit.

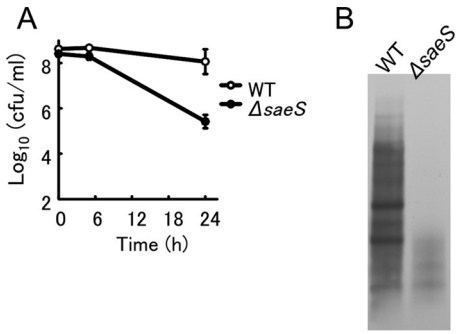

FIG 4 Mutation of *saeS* results in sensitization to vancomycin due to decreased WTA. (A) Time-dependent killing of JE2 Δ*saeS* mutant by vancomycin (10× MIC). These data represent the means and SD from 3 independent experiments. (B) The detection of WTA purified from JE2 Δ*saeS* mutant in stationary phase. The gel was stained by a silver stain kit.

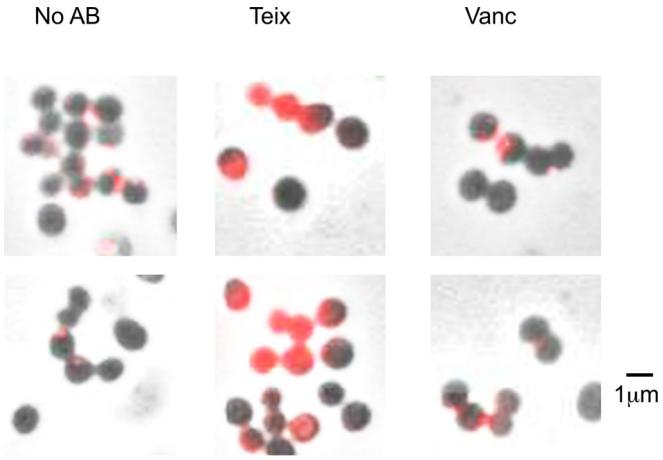

FIG 5 Teixobactin treatment causes Atl delocalization. *In vitro* binding of amidase repeats (Cy3-$R_{1–3}$) with the cell treated with teixobactin. *S. aureus* HG003 exposed to teixobactin (10× MIC) or vancomycin (10× MIC) was incubated with Cy3-$R_{1–3}$ and visualized with fluorescence microscopy. The phase contrast and fluorescent images (red color) were merged. The scale bar represents 1 μm.

further examine a potential synergistic relationship between inhibition of peptidoglycan and teichoic acid biosynthesis, we examined the bactericidal and lytic activity of vancomycin with tunicamycin, a known inhibitor of WTA synthesis against cultures of the *S. aureus* laboratory strain HG003 (27) (Fig. 3C and D). We found that indeed, the combination of tunicamycin and vancomycin results in increased killing relative to vancomycin alone, and cell lysis was increased (Fig. 3C and D). We next examined the effect of teixobactin on teichoic acid levels. Teichoic acids were isolated from the cell wall before and after 4 h of teixobactin treatment. Results clearly showed that teixobactin treatment results in markedly reduced quantities of teichoic acid in the cell wall (Fig. 3E).

In an attempt to identify the signal transduction pathway leading to *atl* downregulation, we examined the MIC activity of teixobactin, oxacillin, and vancomycin against 12 mutants of two-component regulatory systems (see Table S3 in the supplemental material). These mutants were from the JE2 ordered mutant library. The Δ*saeS* mutation resulted in a dramatic 16-fold reduction in MIC to teixobactin but only a modest 2-fold decreased MIC to vancomycin. We then performed killing experiments and found that vancomycin displayed bactericidal killing against a Δ*saeS* mutant but not the JE2 parental strain (Fig. 4A). Surprisingly, preliminary analysis showed an apparent decreased abundance of WTA in the Δ*saeS* mutant (Fig. 4B). SaeRS is known to regulate virulence determinants, including surface proteins, toxins, and capsule biosynthesis components (30–32), and the *saeR* mutant shows high susceptibility to β-lactams in *Staphylococcus epidermidis* (33). This increased susceptibility to cell wall-acting antibiotics may be due to decreased teichoic acids in the cell wall. This would further corroborate the role of teichoic acids in prevention of lysis mediated by lipid II inhibition. Again, teixobactin's ability to inhibit both peptidoglycan and teichoic acid biosynthesis explains its ability to kill and lyse *S. aureus* so efficiently. More work is required to more precisely measure the impact of *saeS* mutation on teichoic acid content of the cell wall and elucidate how the SaeRS two-component system could influence WTA levels in *S. aureus*.

Teixobactin treatment results in delocalization of autolysins. It has been proposed that teichoic acids control autolysin binding to peptidoglycan by an exclusion principle, whereby cell wall teichoic acid interaction with peptidoglycan inhibits autolysin binding (21, 34). Consequently, an absence of teichoic acids at the septum facilitates appropriate localization of autolysins during cell division. It was also shown that mutation of *tarO* results in delocalization of amidase (21). Atl possesses three repeat sequences, each about 150 amino acids long ($R_{1–3}$), which bind to peptidoglycan (19, 35). We examined the localization of Atl using a fluorescently labeled $R_{1–3}$ repeat domain (Cy3-$R_{1–3}$) (21). We found that Cy3-$R_{1–3}$ delocalization occurs in cells treated with teixobactin for 30 min, similar to that previously seen in a *tarO* mutant. These experiments were performed against the model laboratory strain HG003 (Fig. 5; see also Fig. S2 in the supplemental material). This suggested that teixobactin treatment resulted in delocalization of Atl due to inhibition of WTA biosynthesis of the cell. This delocalization explains the intense lytic capacity of teixobactin. Vancomycin did not cause delocalization of the amidase over a similar time period. A longer 4-h treatment with vancomycin did result in delocalization of the fluorescent amidase. Delocalization by teixobactin is rapid and does not allow the cell time to reduce autolysin production and limit Atl-mediated damage. Collectively, these results suggest that coinhibition of lipid II and lipid III by teixobactin causes a pronounced weakening of the cell wall compared to lipid II inhibition alone. This results in increased delocalization of autolysins, leading to cell lysis and death.

Teixobactin does not bind cell wall peptidoglycan. We have previously shown that teixobactin has killing activity superior to that of vancomycin against dense populations of *S. aureus* (5). This is due in part to teixobactin's ability to inhibit teichoic acid production. However, a further important limitation of vancomycin activity, particularly at high cell densities, is the binding of vancomycin to mature peptidoglycan. Vancomycin has no antibacterial activity when bound to mature peptidoglycan. This explains the reduced bactericidal activity of vancomycin against dense populations of cells. Furthermore, this circumstance can lead to vancomycin-intermediate resistance. Many VISA strains have thicker cell walls with altered cross-linking (36). This leads to increased binding of vancomycin to mature peptidoglycan at the D-Ala-D-Ala pentapeptide and sequestration of the antibiotic. Teixobactin does not bind the pentapeptide; hence, it may not

TABLE 1 MIC values in the presence of PG[a]

Turbidity of PG (OD_{600})	MIC (μg/ml)	
	Teixobactin	Vancomycin
0	0.25	1
0.004	0.5	2
0.02	0.5	4
0.1	0.5	16

[a] PG, peptidoglycan.

bind the mature cell wall peptidoglycan. To test this, we purified peptidoglycan from *S. aureus* HG003 and performed MIC testing after a preincubation of vancomycin or teixobactin with purified peptidoglycan (Table 1). Interestingly, the MIC to vancomycin increased 16-fold after this preincubation due to binding with peptidoglycan decreasing the concentration of active antibiotic in the medium. Incubation with teixobactin, on the other hand, resulted in only a modest 2-fold increase in MIC, and this did not increase with higher concentrations of peptidoglycan (note that the intrinsic variability in MIC determination is also 2-fold). This showed that teixobactin does not bind mature peptidoglycan, which likely contributes to its activity against dense populations of *S. aureus* and its activity against all VISA strains examined.

DISCUSSION

Teixobactin represents a recently discovered class of antibiotics, and it exhibits a number of unique and desirable characteristics. These include an apparent absence of resistance development and an improved lytic capacity compared to the cell wall-acting antibiotics oxacillin and vancomycin. In this study, we sought to further explore the mechanism of action of teixobactin and how it yields the intense lytic and bactericidal activity against *S. aureus*, including VISA isolates. We find that teixobactin exhibits excellent bacterial killing, likely due to the synergistic inhibition of both peptidoglycan and WTA biosynthesis. In spite of downregulation of Atl, inhibition of teichoic acid biosynthesis and peptidoglycan results in significant Atl-mediated lysis and cell death. We also find that teixobactin does not bind to and hence is not antagonized by mature peptidoglycan, explaining activity against VISA isolates. We find that sub-MIC levels of tunicamycin enhanced the bactericidal activity of vancomycin without affecting the MIC. At this concentration (0.4 μg/ml), tunicamycin inhibits the biosynthesis of WTA without affecting cell growth. This showed that lack of WTA enhanced the killing by lipid II inhibition. Bactericidal activity of teixobactin plus tunicamycin was stronger than that of vancomycin plus tunicamycin. This may be due to the different stages of teichoic acid biosynthesis that are inhibited by tunicamycin and teixobactin. In the WTA biosynthesis pathway, the first two enzymes, TarO and TarA, are not essential under laboratory conditions, while most of the downstream factors are essential (37). The mechanism of this lethality may be due to accumulation of toxic intermediates or to depletion of cellular pools of cell wall precursors (37). Teixobactin is thought to inhibit the later steps of WTA biosynthesis by binding to lipid III outside the cell membrane (5). Interestingly, the teichoic acid profile from cells treated with tunicamycin or teixobactin is markedly different, presumably due to the different stages of biosynthesis inhibited by each compound.

Teixobactin causes the downregulation of *atl* gene expression. It has already been reported that inhibition of peptidoglycan biosynthesis elicits various secondary responses and downregulation of *atl* expression (24, 38). In prokaryotes, sensing and signal transduction is primarily conducted by two-component systems (TCSs), consisting of a histidine kinase sensor and a cognate response regulator. There are 16 pairs of TCSs in *S. aureus*, and some of them are involved in autolysis control, biofilm formation, cell wall synthesis, or drug resistance (39). Among these systems, WalKR is essential for viability and is involved in cell wall metabolism. A strain expressing a constitutively active form of WalR displays upregulated *atl* transcription (40). GraRS is a well-studied system which regulates the resistance to cationic antimicrobial peptides. A Δ*graRS* mutant displays reduced expression of *atl* (41). These findings suggest that *atl* transcription is inactivated via WalKR and/or GraRS systems under cell wall stress conditions. Here, we find that mutation of *saeS* of the SaeRS two-component system resulted in increased sensitivity to killing by vancomycin independent of an effect on *atl* expression. Mutation of *saeS* results in loss of teichoic acid in the cell wall, resulting in sensitivity to lysis by lipid II inhibition. With this in mind, inhibitors of two-component systems, particularly WalKR, GraRS, and SaeRS, have the potential to further sensitize cells to cell wall-acting antibiotics and may result in improved bactericidal activity.

Intriguingly, teixobactin exhibits potent activity against *M. tuberculosis* (5). Mycobacterium has a unique cell wall structure composed of arabinogalactan, peptidoglycan, and mycolic acid (42). The mechanism of ethambutol, an antituberculosis agents, is inhibition of arabinogalactan biosynthesis (43). Recently, it was reported that the inhibitor of WecA, which is the ortholog of TarO and is involved in arabinogalactan biosynthesis, has good antitubercular activity (44). It will be interesting to examine teixobactin's proposed ability to inhibit arabinogalactan and how the dual inhibition of peptidoglycan and arabinogalactan synthesis may result in enhanced bactericidal activity against this important human pathogen.

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REFERENCES

1. **Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Bartlett J.** 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis **48:**1–12. http://dx.doi.org/10.1086/595011.
2. **Bragginton EC, Piddock LJ.** 2014. UK and European Union public and

charitable funding from 2008 to 2013 for bacteriology and antibiotic research in the UK: an observational study. Lancet Infect Dis **14:**857–868. http://dx.doi.org/10.1016/S1473-3099(14)70825-4.

3. **Chambers HF, Bartlett JG, Bonomo RA, Chiou C, Cosgrove SE, Cross HR, Daum RS, Downing M, Evans SR, Knisely J, Kreiswirth BN, Lautenbach E, Mickley BS, Patel R, Pettigrew MM, Rodvold KA, Spellberg B, Fowler VG, Jr.** 2014. Antibacterial resistance leadership group: open for business. Clin Infect Dis **58:**1571–1576. http://dx.doi.org/10.1093/cid/ciu132.
4. **Schaberle TF, Hack IM.** 2014. Overcoming the current deadlock in antibiotic research. Trends Microbiol **22:**165–167. http://dx.doi.org/10.1016/j.tim.2013.12.007.
5. **Ling LL, Schneider T, Peoples AJ, Spoering AL, Engels I, Conlon BP, Mueller A, Schaberle TF, Hughes DE, Epstein S, Jones M, Lazarides L, Steadman VA, Cohen DR, Felix CR, Fetterman KA, Millett WP, Nitti AG, Zullo AM, Chen C, Lewis K.** 2015. A new antibiotic kills pathogens without detectable resistance. Nature **517:**455–459. http://dx.doi.org/10.1038/nature14098.
6. **Vollmer W, Blanot D, de Pedro MA.** 2008. Peptidoglycan structure and architecture. FEMS Microbiol Rev **32:**149–167. http://dx.doi.org/10.1111/j.1574-6976.2007.00094.x.
7. **Neuhaus FC, Baddiley J.** 2003. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. Microbiol Mol Biol Rev **67:**686–723. http://dx.doi.org/10.1128/MMBR.67.4.686-723.2003.
8. **Xia G, Kohler T, Peschel A.** 2010. The wall teichoic acid and lipoteichoic acid polymers of *Staphylococcus aureus*. Int J Med Microbiol **300:**148–154. http://dx.doi.org/10.1016/j.ijmm.2009.10.001.
9. **Pasquina LW, Santa Maria JP, Walker S.** 2013. Teichoic acid biosynthesis as an antibiotic target. Curr Opin Microbiol **16:**531–537. http://dx.doi.org/10.1016/j.mib.2013.06.014.
10. **Lee SH, Wang H, Labroli M, Koseoglu S, Zuck P, Mayhood T, Gill C, Mann P, Sher X, Ha S, Yang SW, Mandal M, Yang C, Liang L, Tan Z, Tawa P, Hou Y, Kuvelkar R, DeVito K, Wen X, Xiao J, Batchlett M, Balibar CJ, Liu J, Xiao J, Murgolo N, Garlisi CG, Sheth PR, Flattery A, Su J, Tan C, Roemer T.** 2016. TarO-specific inhibitors of wall teichoic acid biosynthesis restore beta-lactam efficacy against methicillin-resistant staphylococci. Sci Transl Med **8:**329ra32. http://dx.doi.org/10.1126/scitranslmed.aad7364.
11. **Mani N, Tobin P, Jayaswal RK.** 1993. Isolation and characterization of autolysis-defective mutants of *Staphylococcus aureus* created by Tn917-lacZ mutagenesis. J Bacteriol **175:**1493–1499.
12. **Bose JL, Lehman MK, Fey PD, Bayles KW.** 2012. Contribution of the *Staphylococcus aureus* Atl AM and GL murein hydrolase activities in cell division, autolysis, and biofilm formation. PLoS One **7:**e42244. http://dx.doi.org/10.1371/journal.pone.0042244.
13. **Saising J, Dube L, Ziebandt AK, Voravuthikunchai SP, Nega M, Götz F.** 2012. Activity of gallidermin on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. Antimicrob Agents Chemother **56:**5804–5810. http://dx.doi.org/10.1128/AAC.01296-12.
14. **Houston P, Rowe SE, Pozzi C, Waters EM, O'Gara JP.** 2011. Essential role for the major autolysin in the fibronectin-binding protein-mediated *Staphylococcus aureus* biofilm phenotype. Infect Immun **79:**1153–1165. http://dx.doi.org/10.1128/IAI.00364-10.
15. **Bertsche U, Weidenmaier C, Kuehner D, Yang SJ, Baur S, Wanner S, Francois P, Schrenzel J, Yeaman MR, Bayer AS.** 2011. Correlation of daptomycin resistance in a clinical *Staphylococcus aureus* strain with increased cell wall teichoic acid production and D-alanylation. Antimicrob Agents Chemother **55:**3922–3928. http://dx.doi.org/10.1128/AAC.01226-10.
16. **Meredith TC, Swoboda JG, Walker S.** 2008. Late-stage polyribitol phosphate wall teichoic acid biosynthesis in *Staphylococcus aureus*. J Bacteriol **190:**3046–3056. http://dx.doi.org/10.1128/JB.01880-07.
17. **Götz F, Heilmann C, Stehle T.** 2014. Functional and structural analysis of the major amidase (Atl) in *Staphylococcus*. Int J Med Microbiol **304:**156–163. http://dx.doi.org/10.1016/j.ijmm.2013.11.006.
18. **Zoll S, Schlag M, Shkumatov AV, Rautenberg M, Svergun DI, Götz F, Stehle T.** 2012. Ligand-binding properties and conformational dynamics of autolysin repeat domains in staphylococcal cell wall recognition. J Bacteriol **194:**3789–3802. http://dx.doi.org/10.1128/JB.00331-12.
19. **Biswas R, Voggu L, Simon UK, Hentschel P, Thumm G, Götz F.** 2006. Activity of the major staphylococcal autolysin Atl. FEMS Microbiol Lett **259:**260–268. http://dx.doi.org/10.1111/j.1574-6968.2006.00281.x.
20. **Hirschhausen N, Schlesier T, Schmidt MA, Götz F, Peters G, Heilmann C.** 2010. A novel staphylococcal internalization mechanism involves the major autolysin Atl and heat shock cognate protein Hsc70 as host cell receptor. Cell Microbiol **12:**1746–1764. http://dx.doi.org/10.1111/j.1462-5822.2010.01506.x.
21. **Schlag M, Biswas R, Krismer B, Kohler T, Zoll S, Yu W, Schwarz H, Peschel A, Gotz F.** 2010. Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl. Mol Microbiol **75:**864–873. http://dx.doi.org/10.1111/j.1365-2958.2009.07007.x.
22. **Sugai M, Yamada S, Nakashima S, Komatsuzawa H, Matsumoto A, Oshida T, Suginaka H.** 1997. Localized perforation of the cell wall by a major autolysin: *atl* gene products and the onset of penicillin-induced lysis of *Staphylococcus aureus*. J Bacteriol **179:**2958–2962.
23. **Heilmann C, Hussain M, Peters G, Götz F.** 1997. Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. Mol Microbiol **24:**1013–1024. http://dx.doi.org/10.1046/j.1365-2958.1997.4101774.x.
24. **Kuroda M, Kuroda H, Oshima T, Takeuchi F, Mori H, Hiramatsu K.** 2003. Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. Mol Microbiol **49:**807–821.
25. **Sieradzki K, Tomasz A.** 2006. Inhibition of the autolytic system by vancomycin causes mimicry of vancomycin-intermediate *Staphylococcus aureus*-type resistance, cell concentration dependence of the MIC, and antibiotic tolerance in vancomycin-susceptible *S. aureus*. Antimicrob Agents Chemother **50:**527–533. http://dx.doi.org/10.1128/AAC.50.2.527-533.2006.
26. **Brown S, Xia G, Luhachack LG, Campbell J, Meredith TC, Chen C, Winstel V, Gekeler C, Irazoqui JE, Peschel A, Walker S.** 2012. Methicillin resistance in *Staphylococcus aureus* requires glycosylated wall teichoic acids. Proc Natl Acad Sci U S A **109:**18909–18914. http://dx.doi.org/10.1073/pnas.1209126109.
27. **Campbell J, Singh AK, Santa Maria JP, Jr, Kim Y, Brown S, Swoboda JG, Mylonakis E, Wilkinson BJ, Walker S.** 2011. Synthetic lethal compound combinations reveal a fundamental connection between wall teichoic acid and peptidoglycan biosyntheses in *Staphylococcus aureus*. ACS Chem Biol **6:**106–116. http://dx.doi.org/10.1021/cb100269f.
28. **Maki H, Yamaguchi T, Murakami K.** 1994. Cloning and characterization of a gene affecting the methicillin resistance level and the autolysis rate in *Staphylococcus aureus*. J Bacteriol **176:**4993–5000.
29. **Weidenmaier C, Kokai-Kun JF, Kristian SA, Chanturiya T, Kalbacher H, Gross M, Nicholson G, Neumeister B, Mond JJ, Peschel A.** 2004. Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections. Nat Med **10:**243–245. http://dx.doi.org/10.1038/nm991.
30. **Voyich JM, Vuong C, DeWald M, Nygaard TK, Kocianova S, Griffith S, Jones J, Iverson C, Sturdevant DE, Braughton KR, Whitney AR, Otto M, DeLeo FR.** 2009. The SaeR/S gene regulatory system is essential for innate immune evasion by *Staphylococcus aureus*. J Infect Dis **199:**1698–1706. http://dx.doi.org/10.1086/598967.
31. **Mrak LN, Zielinska AK, Beenken KE, Mrak IN, Atwood DN, Griffin LM, Lee CY, Smeltzer MS.** 2012. *saeRS* and *sarA* act synergistically to repress protease production and promote biofilm formation in *Staphylococcus aureus*. PLoS One **7:**e38453. http://dx.doi.org/10.1371/journal.pone.0038453.
32. **Luong TT, Sau K, Roux C, Sau S, Dunman PM, Lee CY.** 2011. *Staphylococcus aureus* ClpC divergently regulates capsule via *sae* and *codY* in strain Newman but activates capsule via *codY* in strain UAMS-1 and in strain Newman with repaired *saeS*. J Bacteriol **193:**686–694. http://dx.doi.org/10.1128/JB.00987-10.
33. **Lou Q, Ma Y, Qu D.** 2016. Two-component signal transduction system SaeRS is involved in competence and penicillin susceptibility in *Staphylococcus epidermidis*. J Basic Microbiol **56:**358–368. http://dx.doi.org/10.1002/jobm.201500488.
34. **Frankel MB, Schneewind O.** 2012. Determinants of murein hydrolase targeting to cross-wall of *Staphylococcus aureus* peptidoglycan. J Biol Chem **287:**10460–10471. http://dx.doi.org/10.1074/jbc.M111.336404.
35. **Oshida T, Sugai M, Komatsuzawa H, Hong YM, Suginaka H, Tomasz A.** 1995. A *Staphylococcus aureus* autolysin that has an N-acetylmuramoyl-L-alanine amidase domain and an endo-beta-N-acetylglucosaminidase domain: cloning, sequence analysis, and characterization. Proc Natl Acad Sci U S A **92:**285–289. http://dx.doi.org/10.1073/pnas.92.1.285.
36. **Hiramatsu K.** 2001. Vancomycin-resistant *Staphylococcus aureus*: a new

model of antibiotic resistance. Lancet Infect Dis **1:**147–155. http://dx.doi.org/10.1016/S1473-3099(01)00091-3.
37. **Brown S, Santa Maria JP, Jr, Walker S.** 2013. Wall teichoic acids of gram-positive bacteria. Annu Rev Microbiol **67:**313–336. http://dx.doi.org/10.1146/annurev-micro-092412-155620.
38. **Utaida S, Dunman PM, Macapagal D, Murphy E, Projan SJ, Singh VK, Jayaswal RK, Wilkinson BJ.** 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. http://dx.doi.org/10.1099/mic.0.26426-0.
39. **Kuroda M, Ohta T, Uchiyama I, Baba T, Yuzawa H, Kobayashi I, Cui L, Oguchi A, Aoki K, Nagai Y, Lian J, Ito T, Kanamori M, Matsumaru H, Maruyama A, Murakami H, Hosoyama A, Mizutani-Ui Y, Takahashi NK, Sawano T, Inoue R, Kaito C, Sekimizu K, Hirakawa H, Kuhara S, Goto S, Yabuzaki J, Kanehisa M, Yamashita A, Oshima K, Furuya K, Yoshino C, Shiba T, Hattori M, Ogasawara N, Hayashi H, Hiramatsu K.** 2001. Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. Lancet **357:**1225–1240. http://dx.doi.org/10.1016/S0140-6736(00)04403-2.
40. **Delaune A, Dubrac S, Blanchet C, Poupel O, Mader U, Hiron A, Leduc A, Fitting C, Nicolas P, Cavaillon JM, Adib-Conquy M, Msadek T.** 2012. The WalKR system controls major staphylococcal virulence genes and is involved in triggering the host inflammatory response. Infect Immun **80:**3438–3453. http://dx.doi.org/10.1128/IAI.00195-12.
41. **Herbert S, Bera A, Nerz C, Kraus D, Peschel A, Goerke C, Meehl M, Cheung A, Götz F.** 2007. Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci. PLoS Pathog **3:**e102. http://dx.doi.org/10.1371/journal.ppat.0030102.
42. **Brennan PJ, Nikaido H.** 1995. The envelope of mycobacteria. Annu Rev Biochem **64:**29–63. http://dx.doi.org/10.1146/annurev.bi.64.070195.000333.
43. **Takayama K, Kilburn JO.** 1989. Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*. Antimicrob Agents Chemother **33:**1493–1499. http://dx.doi.org/10.1128/AAC.33.9.1493.
44. **Ishizaki Y, Hayashi C, Inoue K, Igarashi M, Takahashi Y, Pujari V, Crick DC, Brennan PJ, Nomoto A.** 2013. Inhibition of the first step in synthesis of the mycobacterial cell wall core, catalyzed by the GlcNAc-1-phosphate transferase WecA, by the novel caprazamycin derivative CPZEN-45. J Biol Chem **288:**30309–30319. http://dx.doi.org/10.1074/jbc.M113.492173.