Unprotonated Short-Chain Alkylamines Inhibit Staphylolytic Activity of Lysostaphin in a Wall Teichoic Acid-Dependent Manner

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ABSTRACT Lysostaphin (Lst) is a potent bacteriolytic enzyme that kills *Staphylococcus aureus*, a common bacterial pathogen of humans and animals. With high activity against both planktonic cells and biofilms, Lst has the potential to be used in industrial products, such as commercial cleansers, for decontamination. However, Lst is inhibited in the presence of monoethanolamine (MEA), a chemical widely used in cleaning solutions and pharmaceuticals, and the underlying mechanism of inhibition remains unknown. In this study, we examined the cell binding and killing capabilities of Lst against *S. aureus* ATCC 6538 in buffered salt solution with MEA at different pH values (7.5 to 10.5) and discovered that only the unprotonated form of MEA inhibited Lst binding to the cell surface, leading to low Lst activity, despite retention of its secondary structure. This reduced enzyme activity could be largely recovered via a reduction in wall teichoic acid (WTA) biosynthesis through tunicamycin treatment, indicating that the suppression of Lst activity was dependent on the presence and amount of WTA. We propose that the decreased cell binding and killing capabilities of Lst are associated with the influence of uncharged MEA on the conformation of WTA. A similar effect was confirmed with other short-chain alkylamines. This study offers new insight into the impact of short-chain alkylamines on both Lst and WTA structure and function and provides guidance for the application of Lst in harsh environments.

IMPORTANCE Lysostaphin (Lst) effectively and selectively kills *Staphylococcus aureus*, the bacterial culprit of many hospital- and community-acquired skin and respiratory infections and food poisoning. Lst has been investigated in animal models and clinical trials, industrial formulations, and environmental settings. Here, we studied the mechanistic basis of the inhibitory effect of alkylamines, such as monoethanolamine (MEA), a widely used chemical in commercial detergents, on Lst activity, for the potential incorporation of Lst in disinfectant solutions. We have found that protonated MEA has little influence on Lst activity, while unprotonated MEA prevents Lst from binding to *S. aureus* cells and hence dramatically decreases the enzyme's bacteriolytic efficacy. Following partial removal of the wall teichoic acid, an important component of the bacterial cell envelope, the inhibitory effect of unprotonated MEA on Lst is reduced. This phenomenon can be extended to other short-chain alkylamines. This mechanistic report of the impact of alkylamines on Lst functionality will help guide future applications of Lst in disinfection and decontamination of health-related commercial products.

KEYWORDS *Staphylococcus aureus*, lysostaphin, peptidoglycan, tunicamycin, unprotonated amine, wall teichoic acids

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Antibiotics are the gold standard for treating bacterial infections in both humans and animals for over 70 years. Nevertheless, life-saving antibiotics have triggered, through their widespread and sometimes improper use, prevalent antibiotic resistance among bacterial pathogens. An alternative to antibiotics is the naturally present bacteriolytic enzymes, including endolysins, autolysins, virion-associated lysins, and bacteriolysins, hydrolases that selectively cleave certain structures of bacterial cell wall peptidoglycan (1, 2). With high efficiency, low incidence of eliciting bacterial resistance, high biocompatibility, and environmental friendliness, these antimicrobial enzymes have been explored for their applications in wound healing, antifouling, food packaging, etc. (1, 3–6). For nonpharmaceutical applications, the incorporation of bacteriolytic enzymes into commercial products for decontamination and disinfection has attracted industrial attention, owing to bacterial contamination in industrial and communal settings and the need to minimize pervasive use of antibiotics. For example, the broad-spectrum peptidoglycan hydrolase lysozyme has been successfully formulated and encompassed into animal feed and pet care products to mitigate bacterial infections (7–10). Similarly, lysostaphin (Lst) has been used in commercial paint formulations to kill *Staphylococcus aureus* on contact, including patient-isolated methicillin-resistant *S. aureus* (MRSA) strains (11).

Lst is a bacteriolysin secreted from *Staphylococcus simulans* biovar *staphylolyticus* for the eradication of the competing bacteria *S. aureus* and closely related species, functioning as an endopeptidase that specifically digests the pentaglycine cross-bridge between peptidoglycan strands in the target cell wall (12). Lst (246 amino acids) consists of an N-terminal catalytic domain (amino acids 1 to 137) and a C-terminal substrate-binding domain (amino acids 154 to 246) joined by a flexible linker (amino acids 138 to 153) (13, 14). Each domain is functional on its own and can be fused with other protein domains to generate novel chimeric enzymes (15–18). Attributed to its superior activity and specificity, and in addition to its formulation into an antibacterial paint (11), Lst has been exploited for integration into end products in health-related industries, such as in personal/pet care products, either as a direct treatment of *S. aureus* infections or for preservation of the products from bacterial contamination over long-term storage and use. These end products are usually composed of surfactants, emulsifiers, stabilizers, etc., many of which are harsh chemicals that can impose significant challenges to Lst efficacy and stability. Because Lst, like all bacteriolytic enzymes, must reach the cell wall peptidoglycan to act, knowledge of how the solution environment affects Lst binding and subsequent activity would lead to viable solutions to developing highly effective antistaphylococcal disinfectants. Furthermore, with bacteriolytic enzymes that target other pathogens, such information may enhance the ability of such enzymes to be formulated into other disinfectant solutions.

One commonly used industrial emulsifier is monoethanolamine (MEA), which exists ubiquitously in household cleansers and laundry detergents for the neutralization of fatty acids, in personal and pet care products as a surfactant, and in cosmetics and pharmaceuticals for pH adjustment and preparation of emulsions, etc. In addition, MEA can be used by some bacteria as a sole source of carbon and/or nitrogen (19) or for integration into cell wall components (20, 21). We have discovered here that MEA, while not interfering with Lst functionality below pH 9.5 (the pK_a of MEA), significantly inhibits the staphylolytic activity of Lst above pH 9.5 (e.g., when MEA is unprotonated), even though the enzyme retains near-full catalytic activity against live *S. aureus* cells. Because many disinfectants are formulated into alkaline environments, we were motivated to understand the underlying mechanism of cell susceptibility to Lst in the presence of MEA and other alkylamines.

One structural feature used by Gram-positive bacteria for survival in the presence of antimicrobials is wall teichoic acids (WTAs) (22–26), the negatively charged glycopolymers covalently anchored within the inner leaflet of peptidoglycan and extending out from the cell (27, 28). In *S. aureus*, *Listeria monocytogenes*, *Clostridium difficile*, and *Bacillus anthracis*, WTAs have been shown to regulate the binding of autolysins and endolysins to the cell surface (29–32), and cells without WTAs tend to be more sensitive

to lytic enzymes, anionic dyes, and antibiotics (29, 33–35). However, the influence of WTAs in tuning Lst activity in the presence of alkylamines is unexplored and thus should be addressed to develop Lst-based disinfectants or cleaning solutions with selective and effective staphylolytic activity. For this reason, in the present work, we have studied how MEA and related alkylamines inhibit Lst activity and gained an understanding of how WTAs influence such inhibition. Such mechanistic insight may be exploited to ensure that Lst can be combined with various disinfectant solutions that contain MEA or other alkylamines for application as a highly effective anti-staphylococcal formulation.

RESULTS

Activity of Lst in the presence of MEA and its structural analogs. To understand the effect of MEA on Lst function, the enzyme activity was determined using a standard plating assay against exponentially growing *S. aureus* ATCC 6538 cells as a function of pH and MEA concentration. In the absence of MEA, cell viability decreased marginally with increasing pH, and Lst (0.75 μ M) on its own was less active at higher pH, reducing cell viability by ~ 4.5 log units at pH 10.5 compared with >6 log units at pH 7.5 (Fig. 1a). To rule out the possibility that cells were weakened, and thus more sensitive to Lst at high pH, thereby leading to high apparent enzyme activity, a cell wall-based turbidity assay was performed. At pH 10.5, 0.75 μ M Lst degraded isolated cell wall fragments at approximately half the rate of that at lower pHs (Fig. 1b), demonstrating that Lst remains partially functional at the high pH. The addition of MEA did not significantly impact cell viability at all pHs tested (Fig. 1c). In addition, at and below pH 9.5 (pK_a of MEA), there was no noticeable effect of MEA at concentrations up to 125 mM on Lst activity against *S. aureus* cells. However, at pH 10.5, MEA strongly deactivated Lst in a concentration-dependent manner (Fig. 1d).

To investigate whether the pH dependence of Lst activity is related to the protonation state of MEA, several structural analogs of MEA were examined (Fig. 1e; see also Fig. S1 in the supplemental material). When the amino group in MEA was replaced with a hydroxyl or carboxyl group, which is either neutral or negatively charged in the tested pH range, Lst activity was largely unaffected at a concentration of up to 125 mM each compound (Fig. S1). However, when the hydroxyl group of glycolic acid was substituted with an amino group (glycine with $pK_a = 9.8$), Lst inactivation was observed in a dose-dependent manner at pH 10.5 but not at pH 7.5 (Fig. 1e), similar to that observed with MEA. These results suggest that the unprotonated amino group of MEA and glycine negatively impacts the staphylolytic activity of Lst.

Inactivation of Lst in unprotonated MEA is associated with reduced cell binding. The low activity of Lst against *S. aureus* cells at high pH in the presence of MEA may be due to denaturation of the enzyme by MEA, disruption of the interaction between Lst and the peptidoglycan binding sites, or a combination of the two. To assess the denaturation possibility, we examined the effect of MEA as a function of pH on Lst secondary structure, as quantified by circular dichroism (CD) spectroscopy. As shown in Fig. 2a, the secondary structure of Lst in the presence or absence of 100 mM MEA, as reflected in the CD spectra, was almost identical across the pH range of 7.5 to 10.5. These results suggest that the reduced activity of Lst in unprotonated MEA at pH 10.5 was most likely not due to enzyme denaturation. However, we cannot rule out some subtle changes in the enzyme's tertiary structure, such as the exposure of hydrophobic amino acids, which led to a less efficient, albeit still functional, enzyme-substrate interaction and catalysis.

With respect to the hypothesis that unprotonated MEA disrupts the interaction of Lst with peptidoglycan, we constructed an enhanced green fluorescent protein (EGFP)-Lst binding domain (LBD) fusion protein. This allowed us to monitor the binding of LBD on *S. aureus* cell surface through flow cytometry. LBD bound strongly to cells both with and without 100 mM MEA at pH 7.5, as indicated by the shift of the signal compared to cells alone (Fig. 2b). However, at pH 10.5, while moderately strong LBD binding was detected in buffer, minimal LBD binding was observed in the presence of 100 mM MEA

FIG 1 Impact of pH and MEA concentration on Lst activity. (a) Viability of exponentially growing *S. aureus* ATCC 6538 cells treated without or with Lst (0.75 μ M) as a function of pH in 20 mM sodium phosphate buffer (also containing 137 mM NaCl and 3 mM KCl). (b) Rates of turbidity decrease of isolated *S. aureus* ATCC 6538 cell wall fragments in the absence or presence of Lst as a function of pH. (c) Viability of exponentially growing *S. aureus* ATCC 6538 cells in sodium phosphate buffer containing increasing concentrations of MEA. (d) Effect of MEA concentration on cell lytic activity of Lst at various pHs. (e) Activity of Lst as a function of glycine concentration at pH 7.5 and 10.5. The staphylolytic activity of Lst is represented as the difference in cell viability between no Lst treatment (negative control) and Lst treatment at the same pH and chemical concentration. Data represent the mean $\pm$ standard deviation of triplicate measurements.

(Fig. 2b), suggesting that unprotonated MEA prevented LBD from binding to the *S. aureus* cell surface. This result is consistent with our finding that Lst has lower cell lytic activity at higher pH in buffer (Fig. 1a) and is almost inactive in unprotonated MEA (Fig. 1d).

Partial removal of WTA promotes Lst binding and catalytic activity in unprotonated MEA. *S. aureus* peptidoglycan, the direct target of Lst, is buried within a thick layer of WTA, which acts as a barrier to many antimicrobial reagents (36). To determine whether WTA mediates the loss of cell binding and bactericidal activity of Lst in unprotonated MEA, we treated the cells with tunicamycin (TUN), an antibiotic that inhibits WTA biosynthesis. Cells grown in tryptic soy broth (TSB) supplemented with 0.1% (vol/vol) dimethyl sulfoxide (DMSO) were used as a control, since TUN was dissolved in this solvent and diluted 1,000-fold into TSB. Cell growth was slightly slower in 0.05 μ g/ml TUN, and growth inhibition was more apparent at higher TUN concentrations (Fig. 3a); specifically, at 2 μ g/ml tunicamycin, cell growth was reduced by 21% (the maximum specific growth rate was reduced by 22%).

Following WTA removal, the amounts of WTA that remained on the cells were analyzed by polyacrylamide gel electrophoresis. As shown in Fig. 3b, cells grown in TSB

FIG 2 Influence of MEA on Lst structure and substrate-binding capacity. (a) Secondary structure of Lst in 20 mM sodium phosphate buffer (containing 137 mM NaCl and 3 mM KCl) or 100 mM MEA (dissolved in the same buffer) at various pHs measured by circular dichroism spectroscopy. (b) Binding capability of EGFP-LBD to *S. aureus* ATCC 6538 cells in buffer or 100 mM MEA at pH 7.5 and 10.5 measured by flow cytometry. FITC, fluorescein isothiocyanate.

or in the presence of DMSO appeared to have a similar level of WTA, whereas cells treated with as low as 0.05 $\mu\text{g/ml}$ TUN in DMSO had a lower level of WTA. As the TUN concentration increased, there was a clear drop in the amount of WTA generated by the cells. At 2 $\mu\text{g/ml}$ TUN, WTA biosynthesis was dramatically reduced versus the DMSO

FIG 3 Influence of TUN treatment on *S. aureus* ATCC 6538 growth and WTA biosynthesis. (a) Cell growth and maximum specific growth rate (inset) in TSB medium containing 0.1% DMSO or different concentrations (micrograms per milliliter) of TUN (dissolved in DMSO). (b) Polyacrylamide gel (20%) electrophoresis of WTA extracted from cells grown in TSB or TSB supplemented with DMSO or TUN. The dots at the lower portion of the gel are bromophenol blue as a tracking dye.

FIG 4 Flow cytometry analysis of EGFP-LBD binding to log-phase *S. aureus* ATCC 6538 cells in 20 mM sodium phosphate buffer (also containing 137 mM NaCl and 3 mM KCl) or 100 mM MEA (dissolved in the same buffer) at different pHs. Cells were harvested from TSB medium plus 0.1% DMSO (a to d, control) or TSB plus 0.25 $\mu\text{g/ml}$ TUN (e to h).

control, although as described above, cell growth was approximately one-fifth slower than in the absence of TUN. The isolated WTA did not show a ladder-form pattern on the gel as typically observed with other *S. aureus* strains and other bacteria (37–39); instead, a large spot with some smear was observed. This was probably due to a lack of WTA glycosylation in this *S. aureus* strain (40). To prepare cells with low enough WTA content but sufficient growth rate indicative of healthier cells, we used 0.25 $\mu\text{g/ml}$ TUN in subsequent studies.

Cells grown in the presence of 0.1% DMSO bound EGFP-LBD (Fig. 4a to d) in a pattern similar to that with untreated cells (Fig. 2b). In accordance with the lack of LBD binding to wild-type cells in MEA at pH 10.5, DMSO treatment did not result in LBD affinity to cells in MEA at this pH. Upon partial removal of WTA from *S. aureus*, the binding affinity of EGFP-LBD to cells was significantly enhanced either in buffer or in MEA below pH 9.5, as indicated by the shift in peaks and the increase in peak heights (Fig. 4e and f compared to Fig. 4a and b). At pH 10.5, cell-LBD binding was weaker than that at lower pHs, which is in agreement with the binding pattern of cells grown in the absence of TUN. Nevertheless, LBD was able to bind TUN-treated cells in MEA at pH 9.5 and 10.5 with strong and moderate affinity, respectively, although the binding was stronger in the absence of MEA under these conditions.

Based on these results, the loss of Lst activity in unprotonated MEA (at pH 10.5) appears to stem from a lack of enzyme-substrate binding as a result of interference by WTA. It may be reasoned, then, that with sufficient access to cell wall peptidoglycan, Lst staphylolytic activity could be at least partially restored. To test this hypothesis, the susceptibility of TUN-treated cells to Lst in MEA was assessed. Consistent with the strong binding observed for cells treated with 0.25 $\mu\text{g/ml}$ TUN (Fig. 4), these cells were slightly more susceptible to Lst at every concentration of MEA at pH ≤ 9.5 than with untreated or DMSO-treated cells (Fig. 5a to c). At pH 10.5, cell viability in the buffer control (no Lst) did not change noticeably with increasing MEA content (Fig. S2a). As

FIG 5 Activity of Lst at pH 7.5 (a), pH 8.5 (b), pH 9.5 (c), and pH 10.5 (d) against *S. aureus* ATCC 6538 cells grown to mid-log phase in TSB medium supplemented with 0.25 $\mu\text{g/ml}$ TUN (TUN). Lst activity against cells grown in TSB or TSB plus 0.1% DMSO (DMSO) are shown as controls. (e) Activity of Lst as a function of pH in the presence of 100 mM MEA against exponentially growing *S. aureus* ATCC 33807 cells harvested from TSB medium, TSB plus 0.1% DMSO, or TSB plus 0.25 $\mu\text{g/ml}$ TUN. (f) Lst activity against isolated cell wall peptidoglycan with (+) or without (–) WTA at pH 10.5 in the presence (+) or absence (–) of 100 mM MEA. The staphylolytic activity of Lst is represented as a log reduction in cell viability from triplicate measurements, and in all cases, the negative control (no Lst) was subtracted from the log kill results at the corresponding pH and MEA concentration for cells with the same treatment.

expected, TUN treatment led to a >3 -log reduction in cell viability even in the presence of 125 mM MEA, while cells harvested from TSB or TSB plus DMSO without TUN were recalcitrant to Lst in MEA at a concentration as low as 50 mM (Fig. 5d and S2b). It is worth noting that not all the TUN-treated cells were sensitized to Lst in the presence of unprotonated MEA, and this is because the amount of TUN applied was not sufficient to completely shut down WTA biosynthesis (Fig. 3b and 4h). To investigate whether the effect of MEA protonation state on Lst activity was strain specific, we tested Lst on the unrelated *S. aureus* strain ATCC 33807 and observed similar results, in which Lst-induced cell lysis was inhibited by unprotonated MEA, and cell vulnerability to Lst was markedly enhanced after TUN treatment (Fig. 5e).

Unlike in intact cells where peptidoglycan is shielded by WTA, isolated peptidoglycan is readily accessible to Lst from the side adjacent to the cell, as opposed to the outer side of the peptidoglycan that contains WTA. Thus, removing WTA during isolation of the peptidoglycan would open up additional binding sites for Lst on the peptidoglycan surface, which should be reflected in higher degradation rates. To test this hypothesis and to further support the notion that WTA plays a role in regulating peptidoglycan accessibility to Lst, we removed WTA from isolated cell wall fragments by trichloroacetic acid treatment and compared Lst-induced solubilization (degrada-

FIG 6 Activity of Lst in the presence of different short-chain alkylamines (a) and at (b) 100 mM concentration as a function of pH - pK_{a} . Activity was measured against exponentially growing *S. aureus* ATCC 6538 cells harvested from TSB medium. Data are presented as the mean $\pm$ standard deviation of triplicate measurements, and the negative control (no Lst) was subtracted from the log kill results at the corresponding pH for each amine.

tion) of cell wall materials with and without bound WTA at pH 10.5 in the turbidity assay (Fig. 5f). In the absence of MEA, the rate of peptidoglycan hydrolysis was approximately one-third faster following WTA removal. In the presence of 100 mM unprotonated MEA (pH 10.5), the rate of peptidoglycan hydrolysis was lower than in the absence of MEA; however, Lst-catalyzed hydrolysis of WTA-removed peptidoglycan was nearly 2.6-fold faster than on peptidoglycan containing WTA. This higher reactivity supports the aforementioned hypothesis that the removal of WTA enables Lst to gain access to its peptidoglycan binding sites and that unprotonated MEA interacts with WTA to block Lst's access to its peptidoglycan binding sites, and this reduced enzyme binding results in lower cell lytic activity.

Inactivation of Lst by unprotonated amino groups is also observed with other short-chain alkylamines. The charge status of MEA and glycine is obviously linked to their similar pK_{a} values, and therefore, it is not possible to separate the effects of pH and protonation state with these two amines. To investigate whether the unprotonated state of MEA and, similarly, glycine, was influenced by the combination of high pH and the presence of the unprotonated amines, we examined Lst activity in the presence of other small-molecule alkylamines with a pK_{a} of <10 (Fig. 6a) at pH 7.5 to 10.5. When the pH was lower than their respective pK_{a} values, the amines did not significantly reduce Lst activity, and the small drop in enzyme activity was mainly due to a pH effect (consistent with that shown in Fig. 1). At pH at or above the pK_{a} , however, Lst activity was significantly diminished in all of the amines tested (Fig. 6b). It is interesting to note that ethylenediamine had only a minor impact on Lst activity when only one amino group was protonated ($\text{pH} - \text{pK}_{\text{a}1} < 0$), while it totally blocked Lst activity when both amino groups were unprotonated ($\text{pH} - \text{pK}_{\text{a}1} > 0$). Furthermore, when the ethanolamines were compared, it appeared that the unprotonated primary amine (MEA) had the strongest effect on Lst activity, while the unprotonated tertiary amine (triethanolamine) had the lowest inhibition effect.

To further test whether the unprotonated amine-driven deactivation of Lst could be reversed by WTA removal, we measured the Lst sensitivities of cells treated with TUN. As shown in Fig. 7, cells with lower levels of WTA had at least 1.5-log units higher susceptibility to Lst in the presence of unprotonated amines, while these cells were susceptible at comparable levels to Lst with protonated amines compared to cells treated with DMSO alone.

FIG 7 Effect of TUN treatment on cell sensitivity to Lst in the presence of various short-chain alkylamines. The activity was measured against exponentially growing *S. aureus* ATCC 6538 cells harvested from TSB plus 0.1% DMSO (DMSO) or TSB plus 0.25 $\mu\text{g/ml}$ TUN (TUN) in the presence of 25 mM (+25) or 100 mM (+100) ethylenediamine (a), diethanolamine (b), and triethanolamine (c). The staphylocytic activity of Lst is represented as the difference in cell viability between no Lst treatment (negative control) and Lst treatment at the same pH and chemical concentration against the same type of cells. Data are presented as the mean $\pm$ standard deviation of three replicates.

DISCUSSION

Lst is a potent enzyme against both planktonic cells and biofilms of *S. aureus* in either simple saline solutions or rich growth media (12, 41). In the current study, we discovered that Lst presented differential staphylocytic activity in the presence of MEA, i.e., high activity at pH less than the pK_a and low to no activity at pH greater than the pK_a , as a function of MEA concentration. However, Lst was highly functional in a wide alkaline pH range (pH 7.5 to 10.5) in the absence of MEA and with a near-native secondary structure, and the enzyme remained active against isolated cell wall materials at all pHs tested, even in the presence of MEA. The effect of MEA structural analogs on Lst activity allowed us to identify the unprotonated amino group as the structural feature involved in Lst inhibition against intact *S. aureus* cells. Cell binding studies suggested that MEA at pH 10.5 eliminated LBD-cell interactions. The same inhibitory effect was observed when other alkylamines were assayed for Lst activity. Interestingly, when cellular WTA biosynthesis was inhibited with TUN, Lst bound efficiently to *S. aureus* cells, which were also highly susceptible to Lst in 100 mM alkylamines at pH greater than the pK_a . These results suggest that the unprotonated amino group does not directly inhibit Lst; rather, it interacts with the *S. aureus* WTA to modulate the accessibility of Lst binding sites on the cell peptidoglycan, thus tuning cell susceptibility to Lst, which in turn indirectly affects Lst activity.

Some bacteria can incorporate MEA from growth media to WTAs and lipoteichoic acids; however, MEA-containing WTAs cannot be recognized by lytic enzymes, and cells incorporating MEA cannot undergo autolysis at the end of stationary phase (20, 21, 42). In our study, cells were tested in a buffer that does not sustain cell growth, and the addition of MEA did not noticeably increase cell viability at pH 7.5 to 9.5 but instead slightly decreased cell viability at pH 10.5 (Fig. 1c). As a result, it is expected that MEA could not be utilized by *S. aureus* for growth or metabolism, and very little, if any, MEA could be incorporated into the cell wall when the cells were metabolically inactive.

Moreover, the Lst recognition and binding epitope on the *S. aureus* peptidoglycan are cross-linked short murein peptides instead of the WTA (43). Consequently, the loss of Lst activity in MEA is not likely to be caused by MEA substitution on the WTA, but rather a lack of Lst binding sites on the cell wall. Once WTA biosynthesis was partially reduced by TUN, the negative effect of MEA at pH 10.5 was mostly removed.

TUN is a dual-acting inhibitor of cell wall synthesis in *S. aureus*. At low concentrations, it blocks the function of TarO and thus stops the initial step in WTA biosynthesis. At high concentrations, TUN inhibits Mray, an indispensable enzyme in peptidoglycan assembly, hence negatively impacting cell growth (37, 44); however, TUN has a higher selectivity for TarO than for Mray (37). *S. aureus* cells without WTA, achieved either through high-dose treatment with TUN or through knockout of the *tarO* gene, have altered peptidoglycan morphology and defective cell division when visualized by electron microscopy, so a WTA-deficient strain would not be ideal for the study of amine-WTA-Lst interactions in our system (37, 45). In the current study, 0.25 $\mu\text{g/ml}$ TUN was used, as it is a sufficient concentration to remove a substantial amount of WTA (Fig. 3b) but low enough to ensure specificity against TarO, yet not affecting Mray, thereby minimally affecting peptidoglycan structure. The near-normal cell growth rate in 0.25 $\mu\text{g/ml}$ TUN further suggested minor, if any, damage to the peptidoglycan. In addition, it appears that *S. aureus* strain ATCC 6538 has a strong requirement of WTA for basic survival and metabolism. This hypothesis is supported by 2 $\mu\text{g/ml}$ TUN, which slowed cell growth by 21% (Fig. 3a), whereas cells with this treatment still had a detectable amount of extracted WTA. In comparison, WTA biosynthesis in the Newman strain is completely abolished by even 0.1 $\mu\text{g/ml}$ TUN, while cell growth is reduced by $\sim 30\%$ in 2.5 $\mu\text{g/ml}$ TUN (37), and MRSA strain COL is entirely depleted of WTA at a sub-MIC dose of 4 $\mu\text{g/ml}$ TUN (46).

Based on the results of the present study, we hypothesize that reduced Lst cell binding is at least partly attributed to a conformational change in the WTA. Indeed, WTA conformation is believed to govern the exposure of peptidoglycan to proteins with peptidoglycan binding affinity and specificity (29, 47). In addition, teichoic acids, either WTAs or lipoteichoic acids, undergo conformational transitions upon environmental changes (20, 47–51). In a previous study, we suggested that inactivation of the lytic enzyme CD11 in rich growth media, but not in phosphate-buffered saline (PBS), against *Clostridium difficile* is associated with a WTA conformational change in rich media versus in the buffer (29).

Supplementation of unprotonated versus protonated amines may have a totally different influence on WTA conformation through specific intermolecular interactions. Unprotonated MEA interacts strongly with water molecules due to the formation of hydrogen bonds at both the hydroxyl and amino groups with water, thus exhibiting kosmotropic properties that stabilize WTA's rigid structure, which is critical for the protection of peptidoglycan from lytic enzymes and leads to cell resistance to Lst. Protonated MEA, with only the hydroxyl group involved in hydrogen bonding, interacts relatively weakly with water and forms strong ion-pairs through its NH_3^+ group with the negatively charged phosphate groups of the WTA. Hence, protonated MEA behaves less like a kosmotrope and more like a weak chaotrope. Owing to their polyelectrolyte nature (50, 52), WTAs tend to have collapsed structure and undergo dehydration in the presence of chaotropic ions (53), providing access of peptidoglycan to lytic enzymes and leading to cell lysis. Finally, the addition of salts has also been reported to convert WTAs from a rigid conformation into random coils (50). Nonetheless, in the current study, the inhibitory effect of unprotonated amines is less likely to be associated with the high salt concentration (160 mM total salts), since the same trend was observed in a low-salt solution (40 mM total salts), i.e., only the unprotonated MEA reduced Lst activity, which was greatly reversed by TUN treatment (Fig. S3).

S. aureus ATCC 6538 produces enterotoxin C and is commonly used for testing the antimicrobial activities of disinfectants, sanitizers, and antimicrobial preservatives. Since different strains have various cell wall compositions and structures, and hence diverse susceptibility to lytic enzymes (54, 55), a second *S. aureus* strain, ATCC 33807, which

does not produce enterotoxin C and is not closely related to ATCC 6538, was assessed combinatorially as a function of pH, MEA concentration, and the presence of WTA on Lst-initiated cell lysis, and similar results were obtained. This suggests that the inhibitory effect of unprotonated MEA and the role played by WTA are not strain specific.

It has been proposed that there is a proton gradient on the external side of the cell peptidoglycan, generated by cell respiration and retained by WTAs, that leads to a local pH change, which regulates the pH-sensitive activity of bacteriolytic enzymes in a WTA-dependent manner (56, 57). Once this gradient is dissipated by the respiratory inhibitor sodium azide, *S. aureus* cells become highly sensitive to the autolysin AtlA (56). This report, although elucidating the role of the WTA in maintaining an external proton gradient, is less informative in explaining the correlation of the proton gradient with autolysin activity. Specifically, cells treated with azide, which also stops ATP generation and thus turns off many cellular activities, may have a weakened cell wall and become more vulnerable to lytic enzymes. In our study, we found that at physiological pH, the addition of small molecules with different charge properties (positively charged, neutral, or negatively charged) did not noticeably affect Lst activity. This suggests that at least for *S. aureus* strain ATCC 6538, the proton gradient, if present, does not influence Lst activity.

In summary, we have shown that unprotonated alkylamines reduce Lst activity against *S. aureus*, and this inhibitory effect is mediated by the WTA present on the cell surface. This knowledge is important in designing Lst-containing disinfectant formulations and may open up new avenues to fine-tuning the activities of bacteriolytic enzymes in specific environments.

MATERIALS AND METHODS

Plasmid construction. The codon-optimized gene encoding mature lysostaphin (UniProt entry P10547 for the preproprotein) was purchased from GenScript (NJ, USA) and PCR amplified with the forward primer 5'-GGAATTCCATATGGCCGCAACGCACGAA-3' and the reverse primer 5'-CCGCTCGAGTTT GATGGTGCCCCACA-3' (the underlined sequences are the restriction sites, and the italicized sequences include nucleotides added to facilitate digestion). The purified PCR product and the empty vector pGS21a (GenScript) were digested with restriction enzymes NdeI (NEB) and XhoI (NEB), ligated by T4 DNA ligase (NEB), and transformed into *Escherichia coli* DH5 α competent cells. Positive transformants were validated by colony PCR and plasmid sequencing (Genewiz). The plasmid with the correct sequence was named pGS21a-Lst. The gene sequence encoding the binding domain of Lst was PCR amplified and fused with EGFP into plasmid pCDFDuet-1 using the In-Fusion cloning kit (Clontech), giving rise to the recombinant plasmid pEGFP-LBD.

Protein expression and purification. Plasmid pGS21a-Lst was transformed into *E. coli* BL21 Star (DE3) competent cells (Invitrogen) and plated on Luria-Bertani (LB; MP Biomedicals) agar (Sigma) plates containing 100 μ g/ml ampicillin (Gold Biotechnology). A single colony was picked to inoculate 50 ml of LB medium supplemented with 100 μ g/ml ampicillin. After 15 h of cultivation at 37°C and 220 rpm, 5 ml of culture was transferred to 250 ml of fresh LB medium containing 100 μ g/ml ampicillin and incubated at 37°C and 220 rpm. When the optical density at 600 nm (OD₆₀₀) reached 0.6 (~3.5 h), isopropyl- β -D-thiogalactopyranoside (IPTG) was added to a 0.5 mM final concentration. After another 5 h of incubation at room temperature and 180 rpm, cells were centrifuged at 4°C and 4,000 rpm for 20 min, resuspended in 20 ml of native purification buffer (50 mM sodium phosphate [pH 8], 500 mM NaCl), aliquoted into two tubes, and frozen at -80°C. Plasmid pEGFP-LBD was transformed into *E. coli* BL21 Star (DE3) cells and expressed in a way similar to that described above, except that the antibiotic used was 50 μ g/ml streptomycin (Gold Biotechnology) and expression was conducted at room temperature for 20 h.

For protein purification, one tube of frozen cells was thawed at room temperature and supplemented with 0.5 mM phenylmethanesulfonyl fluoride (Sigma) and 1 mg of DNase I from bovine pancreas (Sigma). After sonication (Sonics Vibra-Cell) at 60% power and 3-s pulses for 5 min on ice, the cell lysate was centrifuged at 4,000 rpm and 4°C for 20 min. The supernatant was incubated in a 20-ml purification column (Bio-Rad) with 1 ml of wet high-density nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Gold Biotechnology) prewashed with deionized (DI) water and equilibrated with native purification buffer. Following 2.5 h of incubation at 4°C and 125 rpm, the column was washed with 60 resin volumes of native wash buffer (50 mM sodium phosphate [pH 8], 500 mM NaCl, 20 mM imidazole) and eluted with 15 ml native elution buffer (50 mM sodium phosphate [pH 8], 500 mM NaCl, 250 mM imidazole). The eluted protein was dialyzed in 8- to 10-kDa dialysis tubes with cellulose ester membrane (Spectrum Labs) against PBS (pH 7.4), with a dilution factor of $>5 \times 10^6$. The dialyzed protein solution was sterilized with a 0.22- μ m-pore-size polyvinylidene difluoride (PVDF) filter (EMD Millipore) and stored at 4°C.

Measurement of enzyme activity by plating. The activity of Lst against live *S. aureus* ATCC 6538 or ATCC 33807 cells was measured with the plating assay. An overnight culture of *S. aureus* (30 μ l) was transferred to 3 ml fresh TSB medium (MP Biomedicals) or to TSB supplemented with 3 μ l DMSO (Sigma) or 3 μ l of 0.25 mg/ml TUN in DMSO (prepared from a 10 mg/ml stock solution in DMSO purchased from

Alfa Aesar) in a 15-ml culture tube and shaken at 220 rpm and 37°C until it reached an OD₆₀₀ of 0.6 (~2.5 h). Cells were spun at 10,000 rpm and room temperature for 2 min, washed with 2 ml of sterile PBS, and suspended in PBS to a final OD₆₀₀ of 1. In a sterile 96-well plate, 20 μ l of cells and 160 μ l of 20 mM sodium phosphate (also containing 137 mM NaCl and 3 mM KCl) at pH 7.6, 8.6, 9.7, or 10.7 were mixed with 20 μ l PBS (control) or 20 μ l Lst (0.2 mg/ml for a final working concentration of 0.75 μ M) (after mixing, the final pH was 7.5, 8.5, 9.5, or 10.5). The plate (with lid) was wrapped in foil to reduce evaporation and incubated at room temperature and 120 rpm for 3 h. Cells were then serially diluted, spread on TSB agar plates, and grown at 37°C for 16 h. Colonies were counted for the calculation of cell viability.

The effects of different compounds on Lst activity was assayed similarly. Cells were washed with and resuspended in PBS. Then, 140 μ l of sodium phosphate buffer at a specific pH and 20 μ l of cells were mixed with 20 μ l of amine at that specific pH (prepared as a 10 $\times$ stock solution dissolved in sodium phosphate buffer and adjusted to the respective pH) and 20 μ l PBS (control) or Lst (0.2 μ g/ml) (after mixing, the final pH was 7.5, 8.5, 9.5, or 10.5). Cells were incubated at room temperature and 120 rpm for 3 h, serially diluted, and spread on TSB agar plates. After overnight growth at 37°C, colonies were counted.

Turbidity assay. Cell wall fragments were prepared following a previously published protocol (29). For WTA removal, 1 ml isolated cell wall fragments (at an OD₆₀₀ of 6) was centrifuged and suspended in 4 ml of 5% trichloroacetic acid (50). After 24 h of incubation at 4°C, the mixture was centrifuged at 12,000 rpm for 10 min, washed 10 times, and resuspended in DI water. To test the effect of pH on Lst activity on isolated cell wall materials, 20 μ l of cell wall materials (with or without WTA, diluted to an initial OD₆₀₀ of 5) were mixed in a 96-well plate with 160 μ l of sodium phosphate buffer at different pHs (or 140 μ l buffer plus 20 μ l of MEA at the corresponding pH for the effect of the amine) and 20 μ l of PBS (control) or Lst (0.2 mg/ml). The OD₆₀₀ was tracked for 2 h with 15-s intervals.

Circular dichroism spectroscopic measurements. CD spectra were recorded in a 1-mm quartz cuvette at room temperature using a Jasco 815 spectrometer. The measurement was performed in a wavelength range of 260 to 200 nm with a scanning speed of 100 nm/min and a bandwidth of 1 nm. The baseline was established with sodium phosphate buffer. The Lst concentration was 0.9 mg/ml. Every sample (300 μ l in volume) was measured five times and averaged automatically.

Flow cytometry analysis. *S. aureus* overnight culture was seeded into 3 ml fresh TSB medium supplemented with DMSO or TUN and grown at 37°C and 220 rpm until reaching an OD₆₀₀ of 0.6. Cells were washed with PBS and suspended in PBS to a final OD₆₀₀ of 2. In a microcentrifuge tube, 100 μ l cells were mixed with 800 μ l sodium phosphate buffer (at a specific pH) plus 100 μ l PBS (negative control), 800 μ l sodium phosphate buffer plus 100 μ l EGFP-LBD (0.1 mg/ml; positive control), or 700 μ l sodium phosphate buffer plus 100 μ l amine (1 M at the corresponding pH in sodium phosphate buffer) plus 100 μ l EGFP-LBD. Cells were incubated at room temperature for 5 min, spun at 12,000 rpm for 3 min, washed twice with PBS, and resuspended in 500 μ l PBS. The GFP signal was measured on a LSRII flow cytometer (BD Sciences).

Extraction and gel analysis of WTA. An overnight culture of *S. aureus* cells (0.5 ml) was subcultured into 50 ml fresh TSB, TSB plus 50 μ l DMSO, or TSB plus 50 μ l TUN at 0.05, 0.1, 0.25, 0.5, 1.0, or 2.0 mg/ml (diluted in DMSO from the 10 mg/ml stock solution in DMSO). After further cultivation at 37°C and 220 rpm, cells were harvested by centrifugation (4,000 rpm and 4°C for 20 min) when the OD₆₀₀ reached 0.6. WTA was extracted using a modified method based on a previously published protocol (58). Briefly, the cell pellet was washed with 50 mM Tris-HCl (pH 8) and resuspended in 4% sodium dodecyl sulfate in 50 mM Tris-HCl (pH 8) to a final OD₆₀₀ of 1. The cell suspension was boiled at 95°C for 1 h, pelleted at 14,000 $\times g$ for 10 min, and washed once with 2% (wt/vol) NaCl in 50 mM Tris-HCl (pH 8) and five times with 50 mM Tris-HCl (pH 8). Cell sacculus was further digested with 3 ml of 0.1 mg/ml proteinase K (Sigma) in the presence of 20 mM Tris-HCl (pH 8) and 0.5% sodium dodecyl sulfate at 50°C for 4 h, washed 10 times with DI water, and incubated in 300 μ l of 0.1 M NaOH at room temperature and 120 rpm for 16 h. The dissolved WTA solution was collected through centrifugation at 14,000 $\times g$ for 10 min and stored at 4°C. The extracted WTA was analyzed by polyacrylamide gel electrophoresis, as previously described (29).

Growth study. An overnight culture of *S. aureus* cells (30 μ l) was subcultured into 3 ml fresh TSB or TSB supplemented with 3 μ l of DMSO or 3 μ l of different concentrations of TUN. Cell growth was maintained at 37°C with 220 rpm shaking. Every 30 min, 100 μ l of culture was withdrawn and diluted with 100 μ l PBS in a 96-well plate for OD₆₀₀ measurement. Fresh medium mixed 1:1 with PBS was used as a blank control.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00693-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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