Electrostatic-Mediated Affinity Tuning of Lysostaphin Accelerates Bacterial Lysis Kinetics and Enhances *In Vivo* Efficacy

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ABSTRACT Drug-resistant bacterial pathogens are a serious threat to global health, and antibacterial lysins are at the forefront of innovative treatments for these life-threatening infections. While lysins' general mechanism of action is well understood, the design principles that might enable engineering of performance-enhanced variants are still being formulated. Here, we report a detailed analysis of molecular determinants underlying the *in vivo* efficacy of lysostaphin, a canonical anti-MRSA (methicillin-resistant *Staphylococcus aureus*) lysin. Systematic analysis of bacterial binding, growth inhibition, lysis kinetics, and *in vivo* therapeutic efficacy revealed that binding affinity, and not inherent catalytic firepower, is the dominant driver of lysostaphin efficacy. This insight enabled electrostatic affinity tuning of lysostaphin to produce a single point mutant that manifested dramatically enhanced processivity and lysis kinetics and trended toward improved *in vivo* efficacy. More generally, these studies provide important insights into the complex relationships between lysin electrostatics, bacterial targeting, cell lysis efficiency, and *in vivo* efficacy. The lessons learned may enable engineering of other high-performance antibacterial biocatalysts.

KEYWORDS MIC, MRSA, affinity tuning, antibacterial lysin, electrostatic interaction, enzyme kinetics, *in vivo* efficacy, lysostaphin, minimal inhibitory concentration, protein engineering

Staphylococcus aureus is a prolific and dangerous bacterial pathogen that causes a wide variety of infections ranging from relatively mild skin infections to life-threatening endocarditis and pneumonia (1). According to a recent analysis by the U.S. Centers for Disease Control and Prevention, *S. aureus* bloodstream infections alone resulted in 119,247 hospitalizations and 19,832 deaths in 2017 (2). Treatment of *S. aureus* infections is a growing challenge due to the bacterium's capacity to rapidly develop resistance toward antibacterial chemotherapies (3). Methicillin-resistant *Staphylococcus aureus* (MRSA) is broadly resistant to β -lactam drugs, and it is now widespread in both community and hospital settings (4). Of even greater concern is the growing incidence of strains that are also resistant to the last-line therapies vancomycin, linezolid, and daptomycin (5–10). These trends have led to a crisis point for modern medicine (11–13), creating an imperative for discovery and development of new antibacterial agents possessing novel mechanisms of action.

Bacteriolytic enzymes, or lysins, are at the forefront of innovative strategies to treat staphylococcal infections, including those caused by multidrug-resistant isolates (14–17). Although most lysins are of bacteriophage origin, lysostaphin (LST) is a highly potent and selective staphylocidal lysin whose native source is the bacterium *Staphylococcus simulans* (18), an environmental competitor of *S. aureus*. LST, a type IIIa bacteriocin, has a canonical

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lysin structure comprising an N-terminal catalytic domain (CAT; residues 1 to 137), a C-terminal cell wall binding domain (CBD; residues 154 to 246), and a flexible linker tethering these two domains (residues 138 to 153) (19). The glycyl-glycine zinc endopeptidase CAT is responsible for catalytic cleavage of cross-linking pentaglycine bridges in *S. aureus* peptidoglycan, while the CBD targets the enzyme to the bacterial cell surface (20).

Since its discovery in the mid-1960s, LST has undergone extensive preclinical development and has been evaluated in numerous clinical trials (18). In one striking example, a highly drug-resistant, multifocal MRSA infection was cleared by a single intravenous administration of LST (21). This wealth of successful preclinical and clinical experience has, however, shown that LST's microbial origins prompt potent antidrug immune responses in mammals, including human patients (18, 22). Concern that immunogenicity could undermine efficacy and cause safety issues represents a long-standing barrier to successful translation of LST therapies to the marketplace (18).

Employing state-of-the-art computational protein design and directed evolution strategies, we recently developed LST variant F12, a deimmunized lysostaphin bearing 14 amino acid substitutions designed to silence immunogenic T cell epitopes while maintaining high-level antibacterial activity. In our initial report of F12, we demonstrated that the enzyme evades human immune cell surveillance, dampens both cellular and antibody immune responses in human HLA transgenic mice, and enables safe and highly efficacious repeated dosing to treat recurrent MRSA infections (23). Interestingly, compared to LST, F12 was found to have a 10- to 20-fold loss of *in vitro* potency, as measured by standardized MIC assays. At the same time, F12 exhibited enhanced *in vivo* efficacy relative to LST, even when tested in an acute infection model using naive mice such that antidrug immunity is immaterial. F12's enhanced *in vivo* efficacy was subsequently shown to correlate with unexpected serum synergy (24), but this serum effect could not fully explain the discrepancy between F12's reduced potency *in vitro* and its enhanced efficacy *in vivo*.

Here, we describe a rigorous analysis of the relative staphylocidal activities of F12 versus a native-like LST control enzyme, where both enzymes bear an S126P mutation to facilitate expression in *Pichia pastoris* (25). As functional readouts, we report the results of MIC assays, kinetic analysis of *S. aureus* lysis, quantitative cell wall binding experiments, and efficacy in an *in vivo* murine bacteremia model. Through a series of systematic reverse engineering experiments, we traced key mutations that drive F12's enhanced *in vivo* activity and reveal the underlying mechanism: finely tuned cell wall binding affinity. Using this information as a guide, we show that a single charge-reversing mutation in the LST progenitor molecule can reproduce or even surpass the enhanced performance of F12. Thus, in addition to providing a mechanistic explanation for the previously unknown origins of F12's enhanced efficacy, we suggest a more generalized strategy for engineering antibacterial lysins with optimized clinical performance.

RESULTS

F12 and LST *in vitro* and *in vivo* activities are decoupled. We had previously shown that, compared to that of LST, F12's lower *in vitro* MIC potency did not correlate with its enhanced *in vivo* efficacy in a human HLA transgenic mouse model (23). To ensure that this phenomenon was not an artifact of the transgenic mouse strain, we repeated the efficacy studies in this investigation using standard C57BL/6J mice. Consistent with our prior results, F12 exhibited an approximate 20-fold reduction in MIC potency (Fig. 1A; see also Table S1 in the supplemental material) but was significantly more efficacious in the C57BL/6J bacteremia model (Fig. 1B; $P = 0.0299$).

To more fully understand F12's *in vitro* activity profile, we analyzed its bacterial lysis kinetics using turbidimetric light scattering assays. Compared to LST, F12 manifested 60 to 70% faster kinetics (Fig. 1C), as quantified by both specific rate and time to 50% reduction in the optical density of the bacterial suspension (TOD_{50}) (Table S1). Interestingly, time-kill assays using NCCLS methods (26) showed that F12 was less active than LST (Fig. S2). This result was consistent with F12's reduced MIC potency but contrasted with turbidimetric assays and *in vivo* efficacy, suggesting a possible growth medium effect. Regardless, the

FIG 1 Performance parameters for wild-type (WT) lysostaphin (LST) and a deimmunized variant (F12). (A) MRSA growth inhibition activities for enzymes. MIC 50% and MIC 90% values are provided in Table S1. (B) *In vivo* efficacy of enzymes in a murine model of MRSA bacteremia (dose, 200 μg per mouse; $n = 10$ per group except PBS [$n = 5$]). F12 is more efficacious than the WT ($P = 0.029$, Gehan-Breslow-Wilcoxon test). (C) Lysis kinetics for full-length enzymes. Lytic rate and time to 50% OD₆₅₀ drop are provided in Table S1. (D) Lysis kinetics for isolated CAT domains from each enzyme; quantitative metrics are provided in Table S1. (E) *S. aureus* binding of isolated CBDs from each enzyme fused to mCherry fluorescent reporter. Areas under the curves (RFU·μg/ml) are 28,525 for LST and 6,762 for F12.

turbidimetric results prompted a closer examination of F12's improved light scattering kinetics. It seemed unlikely that the seven deimmunizing mutations in F12's catalytic domain imparted a fortuitous increase in catalytic efficiency, but to test this hypothesis, we quantified the lysis kinetics for the isolated F12 and LST CAT domains. Not surprisingly, the F12 CAT was found to have slower kinetics than the LST CAT (Fig. 1D), indicating that the enhanced kinetics of the full-length variant were dominated by the CBD. We therefore conducted a quantitative analysis of relative binding for the F12 and LST CBDs, and we observed that the F12 CBD bound *S. aureus* cells 4-fold less tightly than its LST counterpart (Fig. 1E). In summary, compared to LST, F12 exhibits lower inherent CAT activity, weaker CBD binding to bacteria, and reduced *in vitro* potency, but at the same time, the full-length variant manifests faster bacterial lysis kinetics and significantly enhanced *in vivo* efficacy compared to those of the wild type.

Reverse engineering localizes dominant F12 mutations. The above results revealed that F12's enhanced *in vivo* efficacy corresponded with faster *in vitro* lysis kinetics, and they further suggested that the reduced binding affinity of the F12 CBD contributed to these effects. To confirm this hypothesis, we generated two chimeric lysin constructs: Chi1 consisted of the wild-type CAT fused to the F12 CBD, and Chi2 was the complementary construct with the F12 CAT and wild-type CBD (Fig. 2A). Chi1 and Chi2 exhibited similar MIC potency, which was improved relative to F12 but less than that of LST (Fig. 2B). Of greater interest, Chi1, bearing the F12 CBD, possessed lysis kinetics similar to those of F12, whereas Chi2, bearing the LST CBD, exhibited slower kinetics similar to those of LST (Fig. 2C). Most importantly, the *in vivo* efficacy of Chi1 and Chi2 tracked with their *in vitro* lysis kinetics. Chi1 was significantly more efficacious than Chi2 ($P = 0.0134$), where Chi1 efficacy was similar to that of F12 and Chi2 efficacy was similar to that of LST (Fig. 2D). These results provided additional evidence that lysis kinetics are more predictive of *in vivo* efficacy than MIC potency, and they definitively localized F12's performance-enhancing mutations to the CBD.

FIG 2 Deconvolving mutational effects in F12. (A) Schematic representation of the domain-level and subdomain-level chimeric lysins. (B) Growth inhibition activity of enzymes, reported as MIC 90%. See also Table S1. (C) Lytic rates of full-length enzymes, normalized to LST. See also Table S1. (D) *In vivo* efficacy of enzymes in a murine model of MRSA bacteremia (dose, 200 μ g per mouse; $n = 11$ per group). Chi1 is significantly more efficacious than Chi2 ($P = 0.0126$, Gehan-Breslow-Wilcoxon test). (E) Lytic rates of full-length enzymes, normalized to LST. See also Table S1. Bar graphs represent means and standard deviations from duplicate experiments.

We next contemplated the possibility that a subset of the six F12 CBD mutations was the dominant source of its improved kinetics and efficacy. This prompted construction of variants Chi3, Chi4, and Chi5, in which we engrafted three groups of collocated F12 mutations into the LST backbone (Fig. 2A and Fig. S1). Kinetic analysis of these variants revealed that Chi4 and Chi5 appeared to have slightly improved lysis kinetics compared to those of LST, whereas Chi3 kinetics appeared marginally slower (Fig. 2E). The observed differences in Chi3, Chi4, and Chi5 were not as pronounced as those seen with Chi1 and 2, indicating that F12's improved kinetics were an aggregate function of many CBD mutations. At the same time, the slightly higher lysis rates of Chi4 versus Chi3 and Chi5 suggested that the single R186T mutation in Chi4 (Fig. S1) was a substantial contributor to F12's enhanced kinetics.

Electrostatic control of lysin binding affinity and antibacterial activity. Reverse engineering of the F12 CBD pointed to a single charge neutralizing mutation (R186T) as a key factor in F12's improved antibacterial activity, which suggested electrostatics as a possible driving force. We considered whether a charge-reversing mutation might impart additional performance benefits, and we next constructed two additional point mutants: LST(R186E) and F12(T186E). As hypothesized, the R186E charge-reversing muta-

FIG 3 Performance analysis of glutamic acid 186 mutation in LST and F12 proteins. (A) Lysis kinetics for full-length enzymes. Lytic rate and time to 50% OD₆₅₀ drop are provided in Table S1. (B) *S. aureus* binding of isolated CBDs from each enzyme fused to mCherry fluorescent reporter. Areas under the curve (RFU $\cdot \mu$ g/ml) are 28,525 for LST, 12,264 for LST(R186E), 6,762 for F12, and 2,338 for F12(T186E). (C) *In vivo* efficacy of enzymes in a murine model of MRSA bacteremia (dose, 200 μ g per mouse; $n = 6$ for charge variants and $n = 10$ for LST and F12). All groups have significantly higher efficacy than F12(T186E); LST and LST(R186E) are not significantly different. *, $P < 0.05$, and **, $P < 0.01$ (Gehan-Breslow-Wilcoxon test).

FIG 4 Solvent ionic strength effects on lysis kinetics and bacterial binding. Low-salt buffer is shown in blue, and high-salt buffer is shown in red. (A) *S. aureus* binding by isolated CBDs from each enzyme fused to mCherry fluorescent reporter. Binding signal at 100 μ g/ml of CBD in low- and high-salt buffer is shown. (B) Lytic rates of full-length enzymes in low- and high-salt buffer, normalized to LST. Bar graphs represent means and standard deviations from duplicate experiments.

tion rendered LST lysis kinetics equal to those of F12, and the F12(T186E) point mutant was likewise as fast as or perhaps faster than F12 (Fig. 3A and Table S1). Additionally, the LST(R186E)-CBD and F12(T186E)-CBD exhibited weaker binding to *S. aureus* cells than did their respective parental templates (Fig. 3B). Lastly, the *in vivo* efficacies of the two point mutants were compared. Consistent with its weaker cell binding and faster lysis kinetics, LST(R186E) manifested efficacy similar to that of F12 (Fig. 3C). In contrast, the F12(T186E) charge variant suffered a loss in efficacy, rendering it significantly less active than either parent enzyme or the LST(R186E) point mutant. This was an unexpected result, given that the F12(T186E) variant had exhibited reduced cell binding and best-in-study lysis kinetics.

The cumulative results of the above-described studies suggested a model wherein (i) CBD electrostatics are a key determinant of lysostaphin-*S. aureus* binding strength, (ii) reduced bacterial binding relative to that of wild-type LST results in faster lysis kinetics, and (iii) *in vitro* lysis kinetics are generally predictive of *in vivo* efficacy. However, the F12(T186E) charge variant broke this trend. To interrogate the seemingly contradictory results from F12(T186E), we tested *S. aureus* binding and lysis kinetics for different constructs in the presence of 200 mM NaCl. For LST, which possesses detrimentally high binding affinity, high salt reduced CBD binding 2-fold (Fig. 4A) and increased lytic activity 2-fold (Fig. 4B and Table S1). For variants with more biologically favorable binding strength, i.e., F12 and LST(R186E), the addition of salt manifested a less pronounced (0.3-fold) reduction in CBD binding strength and had a similarly small impact on lysis kinetics. Lastly, for the F12(R186E) variant, which had the lowest inherent CBD binding strength (Fig. 3B), the addition of salt had no impact on CBD binding affinity (Fig. 4A), but it reduced lysis kinetics approximately 2-fold (Fig. 4B). Importantly, this dampening of F12(R186E) lytic activity in high-salt buffer correlated with the variant's loss of efficacy *in vivo* (Fig. 3C).

A modeling analysis of the electrostatic potential fields generated by each enzyme further illuminated this charge-driven phenomenon. Constructs bearing the high-affinity wild-type LST CBD exhibit a pronounced positive field proximal to their CBDs (Fig. 5A and D). Constructs bearing the intermediate affinity F12 or LST(R186E) CBD show a notable contraction of the electrostatic field around their CBDs, though the fields maintained a pronounced net positive character (Fig. 5B, C, and F). The F12(T186E) construct, having the lowest cellular binding capacity, suffered a nearly complete loss of the net positive field proximal to its CBD (Fig. 5E). The models therefore suggest that placing an anionic glutamate in position 186 of the F12 CBD breaches a tipping point with respect to required electrostatic attraction to bacterial cell targets. This hypothesis is consistent with the observed effects of salt on CBD binding strength and enzyme lysis kinetics (Fig. 4).

FIG 5 Electrostatic potential fields for homology models of various lysostaphin constructs. Positive potential is in blue, and negative is in red. Potentials are both mapped to the protein surface and contoured as 1.8 kT/e fields. (A) LST; (B) F12; (C) Chi1; (D) Chi2; (E) F12(T186E); (F) LST(R186E). Constructs exhibiting higher *in vivo* efficacy than wild-type LST are boxed. The N terminus of the LST CAT domain is located on the distal surface of the image as rendered and is marked as a cyan "[N]" on panel A. The C terminus of the LST CBD is directly visible on the image as rendered and is marked with a cyan "C" on panel A.

DISCUSSION

The increasing incidence of drug-resistant bacteria combined with declining antibiotic development efforts have led to a global health crisis, putting at risk a cornerstone of modern medicine: the capacity to prevent and treat bacterial infections (11–13). The threat posed by drug-resistant pathogens has spurred a search for new antimicrobial agents acting by different modalities (27). Antibacterial lysins kill pathogens via unique mechanisms of action, and these bactericidal biocatalysts are among the vanguard of innovative antimicrobial strategies under development (14–17). To date, lysin biotherapies have been derived mostly from native sequences and chimeragenesis of CATs and CBDs from natural sources (14, 28). However, future research and development efforts are likely to include precise mutagenic tuning of lysin activity, stability (29), strain selectivity, immunogenicity (23, 30, 31), and other clinically relevant properties (29). Understanding how to assess outcomes from future molecular engineering efforts will be important for capitalizing on the full potential of engineered lysin biotherapies (32).

The antibacterial activity of lysins can be tested by various *in vitro* assays (33). Two common approaches are MIC assays and turbidimetric light scattering assays. The first quantifies the ability of lysins to inhibit the growth of bacteria and provide a means of comparing potencies to those of conventional chemotherapies. The second quantifies cell lysis kinetics and provide a direct measure of lysin catalytic activity against target bacteria. Although MIC potency and lysis kinetics are both derived from cell wall degradation, these two activity measures are not necessarily correlated. For example, correlations between native lysostaphin MIC potency and lysis kinetics were found to deviate across different *S. aureus* strains (33). Separately, when tested against a single MRSA target strain, the MICs and lysis kinetics of several distinct bacteriophage lysins

showed no uniform trend (34). Furthermore, the correlation between lysins' *in vitro* activities and *in vivo* efficacies is similarly muddled (32). Thus, there remains a gap in fundamental knowledge pertaining to engineering lysins for improved clinical potential.

To help address this knowledge gap, we have evaluated the relationship between *in vitro* and *in vivo* performance parameters using a panel of lysin variants built on a common molecular scaffold, i.e., lysostaphin. Our results show that lysis kinetics, as opposed to MIC potency, more reliably predicted *in vivo* efficacy among the panel of variants. In the one case where lysis kinetics were decoupled from *in vivo* efficacy, the ionic strength of the assay buffer helped explain the deviation. The effects of synthetic growth medium may contribute to the broad decoupling of LST's MIC potency and kinetics/*in vivo* efficacy, but the underlying molecular mechanism remains unknown. Additionally, it is not a given that these trends will manifest with other bacteriolytic enzymes, though, as noted above, the literature contains many similarly confounding examples of discrepancies between MIC potency and *in vivo* efficacy. Regardless, the results presented here suggest that analysis of lysis kinetics may be an important metric in assessing outcomes for lysin engineering programs.

Canonical lysin structures, including lysostaphin, consist of a CAT and CBD that are responsible for cell wall cleavage and targeting, respectively (20). Although it has long been recognized that CBDs are indispensable for the high activity of most lysins (35), the relationship between CBD affinity and lysin activity is not fully understood. In general, a wide range of affinities has been reported for different lysins. In a study of the *Listeria*-specific lysins Ply118 and Ply500, it was shown that the affinities of their CBDs for the target bacterium were in the nanomolar range (36). The authors hypothesized that the high affinity of lysin CBDs evolved to localize lysins to their cognate host cell, thereby limiting translysis and premature release of phage from other nearby host cells. In contrast, the isolated CBD of phage lysin PlySs2, a highly active antistaphylococcal lysin now in clinical trials, showed no obvious binding to target staphylococci (37). In general, there is no consensus design basis to guide engineering of optimal CBD targeting moieties.

Using our panel of lysostaphin variants, we have dissected the relationship between lysin binding affinity and antibacterial activity *in vitro* and *in vivo*, and our results point to a "Goldilocks" paradigm. When binding affinity to bacterial cells is too low, there is insufficient targeting to the cell surface and poor activity. When binding affinity is too high, enzymes may experience excessive local sequestration on the cell surface, which also lowers activity. Between these extremes, variants with intermediate binding affinity efficiently home to the bacterial surface while manifesting optimal binding dynamics, enhanced processivity, and higher antibacterial activity. This conclusion is consistent with a recent nuclear magnetic resonance (NMR) and X-ray structural analysis of the LST CBD (38). In this elegant study, the authors concluded that the domain's two independent binding sites, which have different ligand selectivity but uniformly low affinity, allow the enzyme to processively "walk" across the cell surface, enhancing CAT-mediated hydrolysis of pentaglycine cross-links. Here, we expand upon those insights with the surprising observation that wild-type LST has evolved excessive and therefore suboptimal binding affinity, at least in the context of systemic administration to combat infections in animals. Indeed, we disclose here three unique variants of LST [F12, Chi1, LST(R186E)] whose CBDs impart reduced cellular affinity yet greater therapeutic efficacy. Given the observation that the highly efficacious, clinical-stage lysin PlySs2 (39) also has low CBD binding affinity (37), we propose that optimization of lysin binding strength could represent a general strategy for enhancing the therapeutic potential of these next-generation antibiotics.

As a corollary to the concept of affinity optimization, the results presented here demonstrate electrostatic tuning as a strategy for achieving optimal cell wall binding. It is known that the surface of Gram-positive bacteria is negatively charged, largely due to anionic polymers such as wall teichoic acids (WTA) (40). However, the contribution

of cell wall anionicity to lysin susceptibility has been controversial. Studies have shown that increasing the positive charge on lysin CATs can improve CBD-independent activity, suggesting a positive effect of charge-charge interactions (41). However, other studies have demonstrated that WTA can sequester and limit lysostaphin access to the cell wall, leading to lysostaphin resistance (42). In this study, through reverse engineering, we traced a substantial portion of F12's enhanced antibacterial activity to a single charge-neutralizing mutation, R186T. We used this knowledge to show that a similar adjustment to LST affinity could be imparted by a single charge reversal mutation, R186E, generating a point mutant that replicated F12's enhanced *in vitro* lysis kinetics as well as its potent *in vivo* efficacy. Thus, we demonstrate that precise mutagenic modulation of lysostaphin electrostatics can reduce excessive cell-binding affinity and improve therapeutic performance. Notably, in a previous series of studies we reported analogous electrostatic binding optimization of human lysozyme (43–45), a broad-spectrum bacteriolytic enzyme that lacks a CBD. Others have also shown that electrostatic tuning can improve lysin activity in other contexts (46), and together, these results suggest that electrostatic tuning is a general strategy by which to optimize lysins' bacterial affinity, processivity, and overall activity.

To further probe the generality of electrostatic-driven enhancements in lysostaphin activity, we evaluated *in vitro* lysis kinetics in both low- and high-ionic-strength buffers. It has long been recognized that the addition of sodium chloride can increase the *in vitro* lytic activity of wild-type lysostaphin; however, the underlying mechanism has not been fully elucidated (34, 47). By independently measuring the lytic and binding activities of LST in both low- and high-salt media, we determined that the enzyme's enhanced lysis kinetics in salt correlated with a reduction in CBD binding affinity in that environment. More generally, the ionic-strength studies revealed that lysin activity enhancement by salt was critically dependent on the inherent binding affinity of the given construct. Constructs with detrimentally high affinity, such as LST, benefitted from electrostatic shielding in high-salt buffer, exhibiting a concomitant reduction in CBD binding strength and enhancement of lytic activity. Conversely, constructs with detrimentally low binding strength, such as F12(R186E), manifested no change in their inherently low CBD binding affinity but a considerable loss of lytic activity in high- versus low-salt buffer. Lastly, constructs with optimal affinity, such as F12 and LST(R186E), experienced intermediate reductions in binding affinity but no change in lytic activity upon the addition of salt. Importantly, the *in vitro* salt effects among this set of variants tracked with their *in vivo* efficacies. While there remains additional work to fully understand the functional significance of lysin electrostatics, these results provide a tantalizing example of how lysis kinetics, measured under varied ionic strengths, might be used to benchmark charge engineering efforts and perhaps even predict impacts on *in vivo* efficacy.

In summary, the systematic analyses described here provide mechanistic insights into the origins of F12's enhanced antibacterial potency relative to that of wild-type LST. These insights point to a general model by which molecular engineering of lysin affinity might be used to develop improved antibacterial biotherapies, and they more specifically illuminate the complex interplay between lysostaphin electrostatics, affinity for *S. aureus* cells, microbial lysis kinetics, and *in vivo* efficacy. We speculate that the lessons learned in this study might guide future development of other high-performance antibacterial lysin therapies.

MATERIALS AND METHODS

Genes, plasmids, and strains. Genes encoding LST and F12 were synthesized as described previously (25). The gene encoding the mCherry fluorescent reporter was from our lab. The chimeric lysins and mCherry-cell wall binding domain fusion proteins were constructed by overlapping PCR with the primers listed in Table S2. Primers were ordered with standard desalting from IDT Technologies (Coralville, IA). Restriction enzymes and Phusion DNA polymerase for molecular cloning were purchased from New England BioLabs (Ipswich, MA). All other reagents and supplies were from VWR Scientific (Philadelphia, PA), unless specifically noted.

Pichia pastoris expression vector pPIC9 and *P. pastoris* strain GS115(his4) were purchased from

Invitrogen (Grand Island, NY). *Escherichia coli* DH5 α [F^- Φ 80*lacZ* Δ M15 Δ (*lacZ*YA-*argF*)U169 *recA1 endA1 hsdR17*(r_K^- , m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*] and MRSA strain USA400 were from the American Type Culture Collection (Manassas, VA).

Protein expression and purification. LST, F12, and their derivative chimeric lysins were secreted from *P. pastoris* and purified as previously described (25). Importantly, the “wild-type” LST enzyme and all variants discussed here encoded an S126P mutation relative to native lysostaphin, where this substitution facilitates expression in yeast. Endotoxin was removed from the protein preparation by Triton X-114 extraction (48), and endotoxin levels in all samples were less than 0.1 EU/mg of protein. mCherry-cell wall binding domain fusion proteins (RFP-CBD) were also secreted from *P. pastoris* and purified with HisPur nickel-nitrilotriacetic acid (Ni-NTA) resin (Thermo Fisher Scientific) according to the manufacturer’s protocol.

MIC assay. The MICs of LST, F12, and their derivative chimeric lysins were determined by the microplate method (33). Using 96-well polystyrene plates, 100- μ l aliquots of purified lysin were serially diluted in phosphate-buffered saline (PBS; 2.7 mM KCl, 1.5 mM KH_2PO_4 , 8.9 mM Na_2HPO_4 , 136.9 mM NaCl [pH 7.4]) supplemented with 0.1% bovine serum albumin. Each well was then inoculated with 100 μ l of $\sim 10^6$ -CFU/ml MRSA strain USA400 in tryptic soy broth (TSB), yielding a total volume of 200 μ l per well. Microplates were then incubated at 37°C for 24 h. The inhibitory activity of purified LST was determined by measuring light scattering at 650 nm in a microplate reader. MIC 50% is the concentration of enzyme required to reduce the optical density of an overnight bacterial culture by 50% compared to an untreated control. MIC 90% is the concentration of enzyme required to reduce the optical density of an overnight bacterial culture by 90% compared to an untreated control. Relative results between MIC 50% outcomes and MIC 90% outcomes were generally consistent. All assays were performed in duplicate.

Kinetic turbidity reduction assay. MRSA strain USA400 was cultured overnight in TSB, pelleted by centrifugation, and washed once with 20 mM phosphate buffer (pH 7.5). The cells were then resuspended in same buffer, and this stock bacterial suspension was aliquoted into replicate wells of a 96-well flat-bottom polystyrene plate (Nunc; 269620) (100 μ l; A_{650} of about 1.0 in the plate). Lysis reactions were initiated by adding purified full-length lysins to a final concentration of 1 μ g/ml or 50 μ g/ml for catalytic domains. The kinetics of bacterial lysis were followed by measuring the light scattering at 650 nm every 5 min for a total of 60 min. The activity of the various lysostaphin constructs was quantified as the rate, calculated from the steepest linear portion of the curve, or as the time to reach half the starting optical density of the initial bacterial suspension (TOD_{50}). All assays were performed in triplicate.

Time-kill assay. Time-kill assays were performed using the method described by the NCCLS (26). In brief, 10^6 CFU/ml of MRSA USA400 was inoculated into 25 ml of cation-adjusted Mueller Hinton II broth (caMHIIb) medium containing F12 (100 ng/ml) or LST (16 ng/ml). The flasks were cultured at 37°C with continuous shaking. Aliquots of 100 μ l were withdrawn at the following time points: 0 min, 15 min, 30 min, and 60 min. The aliquots were 10-fold serially diluted in test tubes of sterile saline (0.9% NaCl), and diluted samples were spread onto TSB agar plates. After overnight incubation, agar plates with 20 to 200 colonies were used to determine colony counts, and the bacterial density in the corresponding culture was calculated. Time-kill kinetic curves were plotted from the bacterial density and time data. Each assay was performed three times; the mean and standard deviation of bacterial counts are reported.

Bacterial binding affinity assay. One-hundred-microliter aliquots of purified RFP-CBD fusion proteins were serially diluted in 20 mM phosphate buffer (pH 7.5). Each well was then inoculated with 100 μ l of heat-killed (121°C, 30 min) MRSA strain USA400 at an optical density at 600 nm (OD_{600}) of 10, yielding a total volume of 200 μ l per well. After a 10-min incubation at ambient temperature, the heat-killed USA400 cells were spin down and resuspended in fresh buffer, and captured RFP-CBD fusion proteins were quantified with a fluorescent plate reader using an excitation wavelength of 560 nm and emission wavelength of 610 nm. All assays were performed in triplicate.

Murine bacteremia challenge model. The protocols for animal infection, treatment, and immunization were approved by the Institutional Animal Care and Use Committee of Dartmouth College (Hanover, NH), in accordance with the Association for the Assessment and Accreditation of Laboratory Animal Care guidelines. All efforts were made to minimize animal suffering.

Seven-week-old C57BL/6J mice were given, by intraperitoneal injection, a dose of 2×10^8 CFU of *S. aureus* strain USA400 in a 3% suspension of porcine gastric mucin and 1 h later were treated by subcutaneous injection of 200 μ g of LST or F12 in 100 μ l of PBS. Control mice received 100 μ l of PBS. Mice were observed regularly for a minimum of 3 days following treatment. The head-to-head LST-versus-F12 experiments (Fig. 1B) included 5 mice per group, the domain-level chimeric experiments (Fig. 2D) were performed with 11 mice per group, and the charge-engineered-variant experiments (Fig. 4A) had 6 mice per group.

Molecular modeling. Molecular models of the LST variant sequences were built using the Swiss Model workspace (49). Chain A of the X-ray crystal structure under PDB code 4LXC was used as the template for all models (19). The electrostatic potential field of each variant was computed in Swiss-PdbViewer 4.1.0 (50) using the Coulomb method with atomic partial charges.

Statistical analysis. Statistical analysis of Kaplan-Meier curves was performed in GraphPad Prism 6.0 using the Gehan-Breslow-Wilcoxon test.

Data availability. All data needed to evaluate the conclusions in the paper are presented in the paper. Additional data are available from the authors upon request.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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H.Z. designed and conducted the *in vitro* assays, with the exception of time-kill assays that were performed by H.C. K.E.G., H.Z., and S.E. designed the *in vivo* experiments, which were conducted solely by S.E. H.Z., S.E., J.F., and K.E.G. analyzed the data. K.E.G. and H.Z. wrote the manuscript, and all authors provided input and comments for the manuscript.

K.E.G. and H.Z. are coinventors on multiple patents relating to engineered variants of lysostaphin and therapeutic use thereof, and these patents have been licensed to Lyticon LLC. K.E.G. is a cofounder and executive officer of Lyticon LLC. No other authors have a conflict of interest. Potential conflicts of interest for K.E.G. are under management at Dartmouth. We declare that the work presented here is free of any bias.

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