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Impact of *Staphylococcus aureus* regulatory mutations that modulate biofilm formation in the USA300 strain LAC on virulence in a murine bacteremia model

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ABSTRACT
Staphylococcus aureus causes acute and chronic forms of infection, the latter often associated with formation of a biofilm. It has previously been demonstrated that mutation of *atl, codY, rot, sarA*, and *sigB* limits biofilm formation in the USA300 strain LAC while mutation of *agr, fur*, and *mgrA* has the opposite effect. Here we used a murine sepsis model to assess the impact of these same loci in acute infection. Mutation of *agr, atl*, and *fur* had no impact on virulence, while mutation of *mgrA* and *rot* increased virulence. In contrast, mutation of *codY, sarA*, and *sigB* significantly attenuated virulence. Mutation of *sigB* resulted in reduced accumulation of AgrA and SarA, while mutation of *sarA* resulted in reduced accumulation of AgrA, but this cannot account for the reduced virulence of *sarA* or *sigB* mutants because the isogenic *agr* mutant was not attenuated. Indeed, as assessed by accumulation of alpha toxin and protein A, all of the mutants we examined exhibited unique phenotypes by comparison to an *agr* mutant and to each other. Attenuation of the *sarA, sigB* and *codY* mutants was correlated with increased production of extracellular proteases and global changes in extracellular protein profiles. These results suggest that the inability to repress the production of extracellular proteases plays a key role in attenuating the virulence of *S. aureus* in acute as well as chronic, biofilm-associated infections, thus opening up the possibility that strategies aimed at the de-repression of protease production could be used to broad therapeutic advantage. They also suggest that the impact of *codY, sarA*, and *sigB* on protease production occurs via an *agr*-independent mechanism.

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Introduction

The production of *Staphylococcus aureus* virulence factors is modulated by a complex and highly interactive regulatory circuit.[1] This affords the bacterium tremendous flexibility with respect to its ability to respond to changing conditions within the host including the influence of an ongoing host immune response.[2-4] This flexibility is reflected in the ability of *S. aureus* to cause a diverse array of infections. In general, these can be characterized as acute infections, the clinical characteristics of which are often defined by toxin production, and chronic infections, the clinical characteristics of which are often associated with formation of a biofilm.[5] To some extent a general theme of the overall *S. aureus* regulatory circuitry is to modulate the production of specific virulence factors that contribute to these alternative forms of infection.[6] A primary example is the accessory gene regulator (*agr*), expression of which limits biofilm formation but at the same time promotes toxin production.[7,8]

The treatment of all forms of *S. aureus* infection is complicated by the persistent emergence of antibiotic resistant strains, which accounts for its inclusion among the ESKAPE pathogens.[9] The treatment of chronic biofilm-associated infections is further complicated by the presence of the biofilm itself, which confers a therapeutically-relevant level of intrinsic resistance to conventional antibiotics and host defenses.[10] This has created an

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urgent need for new antibiotics that are both effective against the most problematic antibiotic-resistant strains and retain a therapeutically-relevant level of efficacy in the context of a biofilm. New antibiotics have been and continue to be developed,[11] but given the remarkable intrinsic resistance conferred by the presence of a biofilm, accomplishing both of these goals has proven to be a formidable task.[12]

This has led to the suggestion that strategies targeting *S. aureus* regulatory circuits and/or specific virulence factors produced under the control of these circuits could be used to therapeutic benefit either alone or in combination with conventional antibiotics.[13,14] Our approach has been to investigate the regulatory basis for biofilm formation itself, the goal being to identify those regulatory loci that offer the greatest opportunity for therapeutic intervention. These efforts have led us to focus on the staphylococcal accessory regulator (*sarA*), mutation of which limits biofilm formation to a greater extent than mutation of any other regulatory locus we have examined.[15] Moreover, this limitation can be correlated with increased antibiotic susceptibility in biofilm-associated infections caused by diverse strains of *S. aureus* including methicillin-resistant strains.[16] This suggests that inhibitors of *sarA* expression and/or function could be used to therapeutic advantage in the context of biofilm-associated *S. aureus* infections.

However, biofilms are highly dynamic structures in which changes in gene expression promote the development, maturation, and ultimately the dissemination of bacterial cells from the biofilm, at which point they can enter the systemic circulation and cause infection at secondary sites.[17,18] Thus, inhibition of *sarA* as a means of limiting biofilm formation could have the adverse consequence of promoting acute, systemic infection. Conversely, inhibitors of *agr* expression and/or function may be of therapeutic benefit in the context of acute infection, but could also have the adverse consequence of promoting chronic, biofilm-associated infection. Indeed, *agr*-defective strains are often isolated from patients suffering from diverse forms of infection, perhaps owing at least in part to the advantage gained by the intrinsic antibiotic resistance afforded to the bacterium in the context of a biofilm.[19] Moreover, one report that examined the clinical history of 814 patients with *S. aureus* sepsis found that *agr* dysfunction was associated with a statistically significant increase in mortality.[20]

Such results emphasize the need to consider the contribution of individual regulatory loci in diverse forms of *S. aureus* infection. To this end, we also examined the role of *sarA* in acute models of *S. aureus* infection, and the results confirmed that *sarA* mutants are attenuated in murine models of bacteremia and acute, post-traumatic osteomyelitis.[21-23] This suggests that *sarA* may be a viable therapeutic target in diverse forms of *S. aureus* infection. However, many other regulatory loci have also been shown to impact various forms of infection.[1] Defining the relative impact of these loci in diverse forms of *S. aureus* infection is difficult because most reports focused on individual regulatory loci in the context of a single form of *S. aureus* infection. This precludes the ability to determine which of these loci offer the greatest therapeutic promise in diverse forms of *S. aureus* infection. We have begun to address this by making direct comparisons between regulatory loci in the context of biofilm-associated infection,[15,16] but we have not done so in the context of acute infection. Thus, in this report we extended previous experiments focusing on regulatory loci that impact biofilm-associated infection to directly assess the relative impact of these same regulatory loci on virulence in a murine bacteremia model of acute *S. aureus* infection.

Results

Owing to its current prominence as a cause of *S. aureus* infection,[24-26] the experiments we report were done with a derivative of the USA300 strain LAC cured of its resident erythromycin-resistance plasmid.[23] We initially focused on isogenic derivatives of this strain with mutations in *codY, fur, mgrA, sarA*, and *sigB*. These regulatory loci were chosen to allow direct comparisons with the results observed in previous biofilm studies in which mutation of these same loci was shown to either enhance (*fur, mgrA*) or limit (*codY, sarA* and *sigB*) biofilm formation.[7,15,16] We also included a *rot* mutant based on a recent report concluding that mutation of *rot* limits biofilm formation in LAC.[27] Strains were introduced into NIH-Swiss mice by tail vein injection of 5×10^7 colony-forming units (cfu) as previously described.[23] Over the 7 day period of this experiment, this resulted in the death of 60% of mice infected with the LAC parent strain (Fig. 1). The mutants evaluated in this experiment were found to fall into one of three groups, with mutation of *fur* having no statistically significant effect on virulence, mutation of *codY, sarA*, and *sigB* significantly attenuating virulence, and mutation of *mgrA* and *rot* resulting in a significant increase in virulence relative to LAC (Fig. 1). These results were consistent with our studies examining the relative capacity of these mutants to escape the bloodstream and colonize soft tissues. Specifically, mutation of *codY, sarA*, and *sigB* attenuated virulence as assessed based on colony counts in the spleen (Fig. 2A), heart (Fig. 2B), and peripheral blood (Fig. 2C). Results observed in the kidney were less discriminatory in that the only significant difference was that between LAC and its isogenic *sarA* mutant (Fig. 2D).

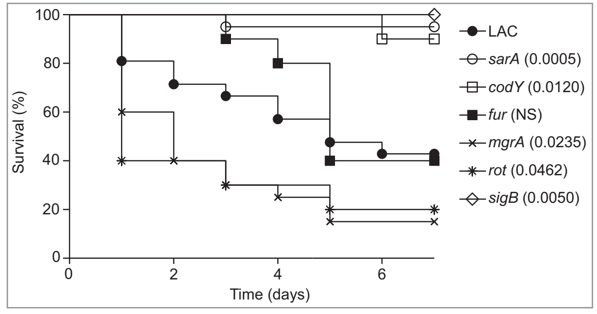

Figure 1. Relative virulence of *S. aureus* regulatory mutants in acute sepsis. Kaplan-Meier survival curves are shown for the USA300 strain LAC and the indicated isogenic mutants. Numbers in parenthesis indicate p values for each mutant by comparison to the results observed with LAC. NS = not significant.

To investigate the mechanistic basis for the virulence phenotypes we observed, we performed western blots of whole cell lysates using antibodies targeting AgrA and SarA as previously described.[23] Mutation of *sarA* and *sigB* resulted in a significant decrease in the accumulation of AgrA (Fig. 3A), which is the response regulator of the two component quorum-sensing system encoded by *agr*.[28] Additionally, mutation of *sigB* resulted in a significant decrease in the accumulation of SarA (Fig. 3B). This suggests that the decreased virulence observed with the *sarA* and *sigB* mutants may be at least partially attributable to the impact of these mutations on expression of *agr*. However, comparison of *agr, sarA*, and *sigB* mutants revealed that all three had distinct phenotypes as defined by the relative accumulation of alpha toxin and protein A (Spa), which are prototype virulence factors known to be inversely regulated by *agr*.[28] Specifically, as assessed by western blot of conditioned medium from each strain, and as expected based on previous reports,[28] accumulation of Spa was increased in the LAC *agr* mutant while accumulation of alpha toxin was decreased (Fig. 3C and D). In contrast, accumulation of both Spa and alpha toxin was decreased in the *sarA* mutant, while accumulation of Spa was decreased, and accumulation of alpha toxin increased, in the *sigB* mutant (Fig. 3C and D).

Additionally, if the attenuation of *sarA* and *sigB* mutants is defined by the impact of these loci on expression of *agr*, then it would also be anticipated that an isogenic *agr* mutant would be attenuated to a comparable degree by comparison to *sarA* and *sigB* mutants. To examine this issue, we used our murine bacteremia model to compare LAC with its isogenic *agr* mutant. Interestingly, we found that mutation of *agr* had little impact on virulence (Fig. 4). These results were surprising given that mutation of *agr* in LAC as well as other *S. aureus* strains has been shown to limit virulence in animal models of *S. aureus* infection.[29-32] However, most of these models focused on some form of localized infection, primarily of the skin, and such models do not necessarily mimic the systemic infection modeled here. Indeed, Cameron *et al.* recently examined a number of clinical isolates of *S. aureus* and concluded that *agr* expression was not essential for

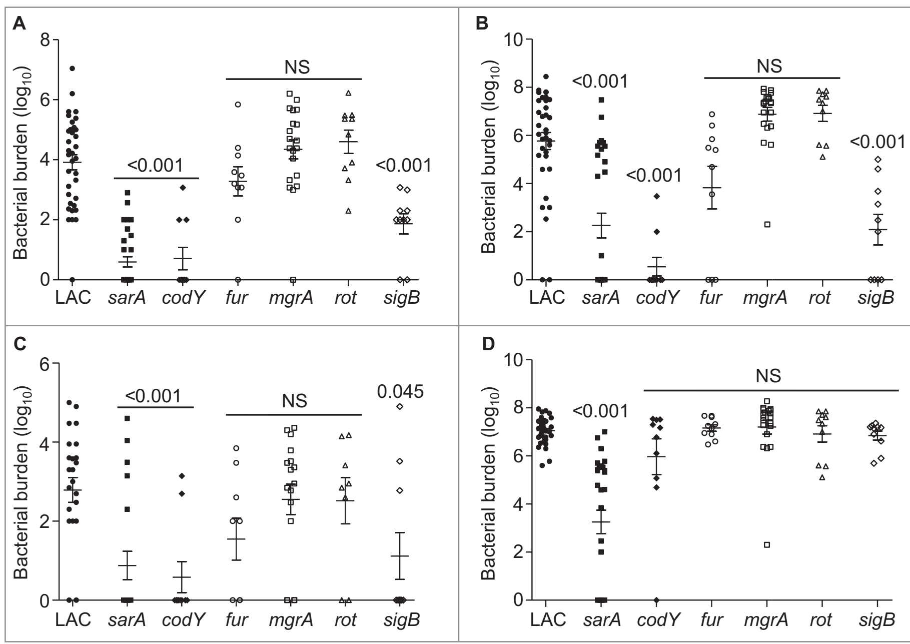

Figure 2. Relative virulence of *S. aureus* regulatory mutants as assessed by colonization. The number of colony-forming units (cfu) in the (A) spleen, (B) heart, (C) peripheral blood, and (D) kidney are shown by scatter plot. Numbers above each plot indicate p values for each mutant by comparison to the results observed with LAC. NS = not significant. Bars represent the mean ± SEM of $\log_{10}$ transformed values.

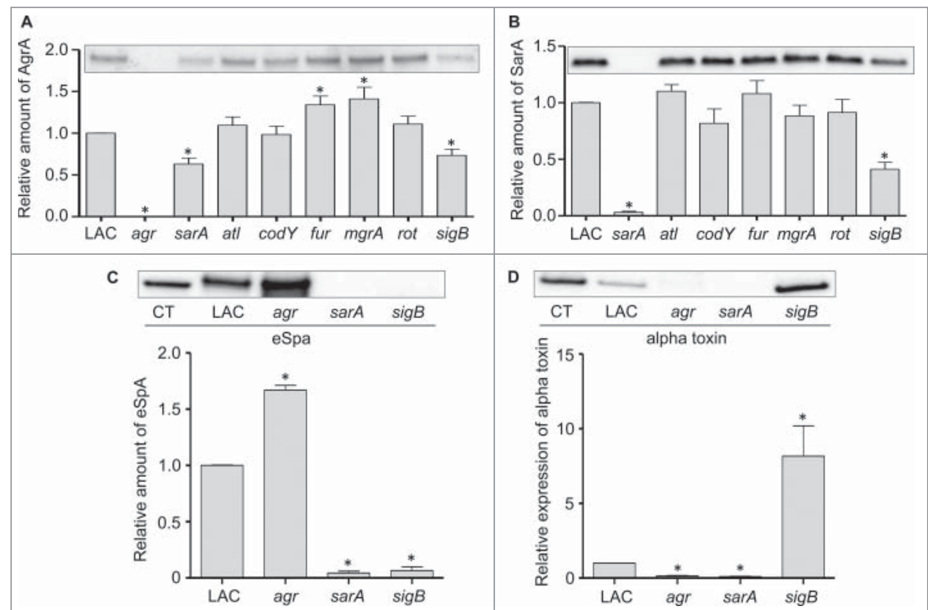

Figure 3. Relative accumulation of AgrA, SarA, eSpa, and alpha toxin in *S. aureus* regulatory mutants as assessed by immunoblot analysis. Representative western blots of cell lysates (A and B) or conditioned medium (C and D) prepared from LAC and the indicated isogenic mutants were analyzed by western blots using (A) anti-AgrA antibody, (B) anti-SarA antibody, (C) anti-Spa antibody, or (D) anti-alpha toxin antibody. Graphs indicate cumulative densitometric values obtained from all biological and experimental replicates. Asterisks indicate statistical significance ($p \leq 0.05$) by comparison to values obtained with the LAC parent strain.

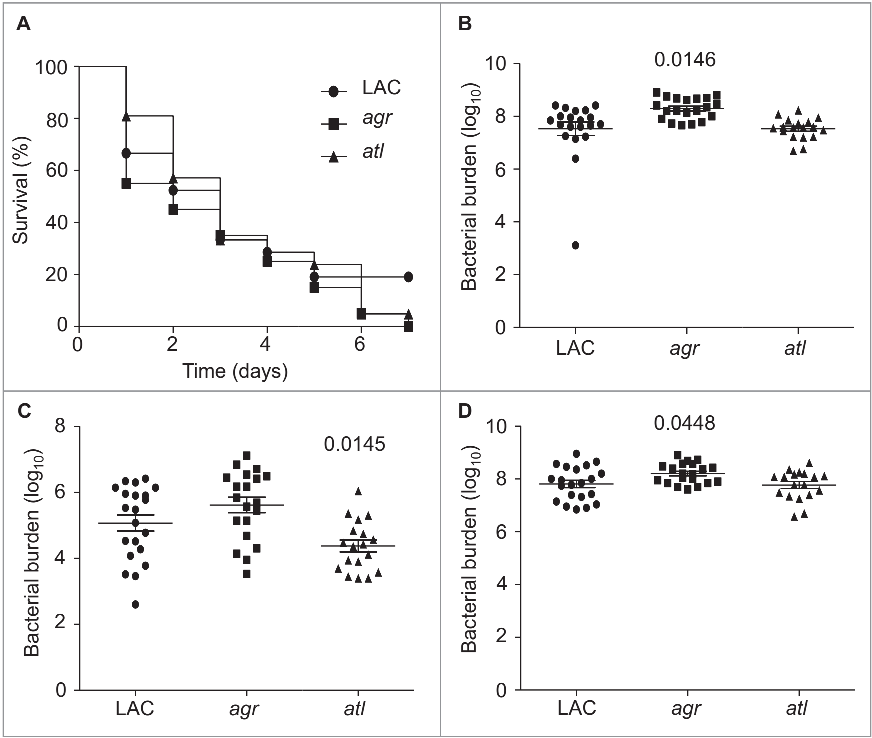

Figure 4. Relative virulence of *S. aureus agr* and *atl* mutants in acute sepsis. (A) Kaplan-Meier survival curves are shown for LAC and the indicated isogenic mutants. Number of cfu observed in homogenates prepared from (B) heart, (C) spleen, and (D) kidney. The number above each scatter plot cluster indicates the p value for each mutant found to significantly different by comparison to LAC. Bars represent the mean ± SEM of log_{10} transformed values.

virulence in a murine bacteremia model.[33] However, the more important point in the context of this report is that this finding, together with the alpha toxin and Spa results discussed above, effectively rules out the possibility that the attenuation observed with the LAC *sarA* and *sigB* mutants is a function of their impact on expression of *agr*.

While the focus of the experiments we report was on regulatory loci, we also examined the impact of mutating *atl* in this experiment based on the observation that mutation of *atl* has been shown by several laboratories, including our own, to limit biofilm formation.[15,34,35] This experiment was complicated by the fact that a characteristic phenotype of *atl* mutants grown *in vitro* is the formation of large aggregates.[36] To address this, we first carried out studies in which the apparent number of colony-forming units (cfu) was assessed before and after sonication. The number of detectable cfu increased in all strains after sonication, and in the case of four mutants (*atl, sarA, fur*, and *rot*) the number as assessed before sonication was significantly lower than the number observed with the LAC parent strain (Fig. 5A). However, with the exception of the *atl* mutant, all of the differences we observed were well within an order of magnitude ($2.6 \times 10^9 - 8.9 \times 10^9$). In contrast, statistical analysis confirmed that the number of cfu as assessed before sonication was significantly higher in every strain we examined by comparison to the *atl* mutant (Fig. 5A). These experiments also confirmed that there was no difference between any of the strains we examined, including the *atl* mutant, after sonication.

Based on these results, *in vivo* analysis of the *atl* mutant was done using an inoculum prepared after sonication. As with *agr*, mutation of *atl* was also found to have no significant impact on overall virulence (Fig. 4). Mutation of *atl* also had no impact on the accumulation of AgrA (Fig. 3A) or SarA (Fig. 3B). Although most studies focusing on the role of *atl* in *S. aureus* pathogenesis have focused on biofilm formation, the results we observed with the *atl* mutant in our bacteremia model are consistent with those of Takahashi *et al.*, who found that mutation of

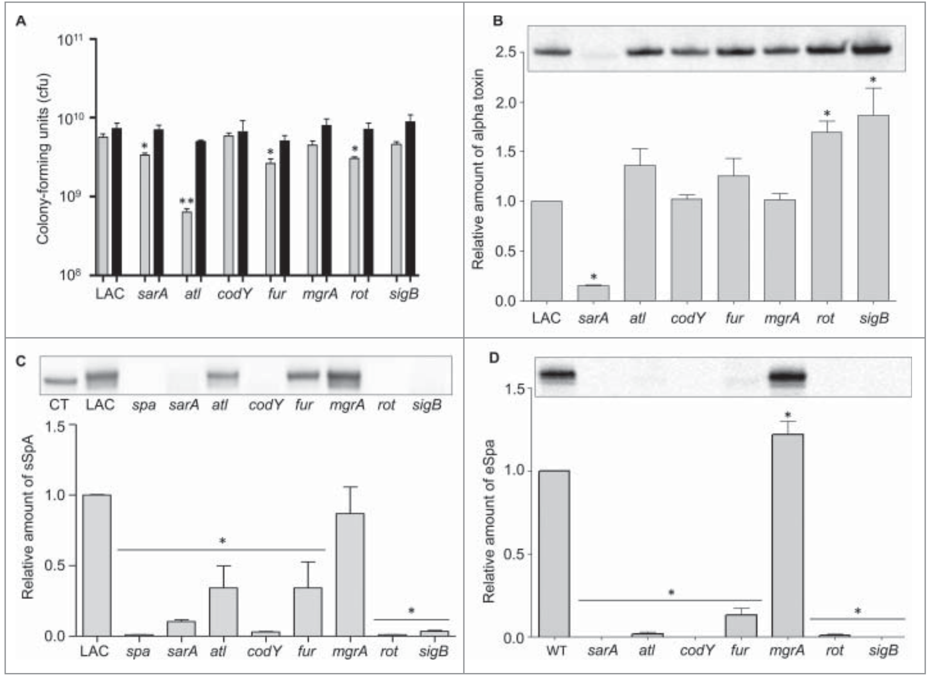

Figure 5. Analysis of mutant aggregative phenotypes and their respective production of alpha toxin and Spa. (A) Bars indicate the number of cfu observed before (grey) and after sonication (black). Single asterisk indicates statistical significance prior to sonication by comparison to LAC. Double asterisk indicates statistical significance of the *atl* mutant by comparison to all other strains prior to sonication. No significant differences were observed after sonication. (B) Representative western blot of conditioned medium from LAC and the indicated isogenic mutants using anti-alpha toxin antibody (top). Graph indicates cumulative densitometric values obtained from all biological and experimental replicates (bottom). (C) Representative western blot of surface protein preparations from LAC and the indicated isogenic mutants using anti-Spa antibody (top). CT = purified Spa control. Graph indicates cumulative densitometric values obtained from all biological and experimental replicates (bottom). (D) Representative western blot of conditioned medium from LAC and the indicated isogenic mutants using anti-Spa antibody (top). Graph indicates cumulative densitometric values obtained from all biological and experimental replicates (bottom). Asterisks in each graph indicate statistical significance ($p \leq 0.05$) by comparison to values obtained with the LAC parent strain.

atl had no impact on virulence in a murine model of intraperitoneal infection.[36]

In contrast to *sarA* and *sigB*, mutation of *fur* and *mgrA* resulted in a modest (<2-fold) but statistically significant increase in the accumulation of AgrA (Fig. 3A). However, this was not reflected in the alpha toxin phenotype of these mutants in that neither produced alpha toxin at significantly greater levels than the isogenic LAC parent strain (Fig. 5B). In contrast, mutation of *rot* and *sigB* resulted in a significant increase in the accumulation of alpha toxin, while mutation of *sarA* had the opposite effect. Indeed, alpha toxin was essentially absent in conditioned medium from LAC *agr* and *sarA* mutants (Fig. 3D). The possibility that the decrease in alpha toxin observed in the *sarA* mutant is at least partially attributable to the impact of mutating *sarA* on *agr* cannot be completely discounted, but previous studies from our laboratory have demonstrated that the accumulation of alpha toxin can be restored to wild-type levels in a *sarA* mutant by eliminating the production of extracellular proteases.[37,38] The relative abundance of alpha toxin in conditioned media was correlated with virulence in *rot* and *sarA* mutants, with both being increased in a *rot* mutant and both being decreased in a *sarA* mutant, but this was not the case with the *sigB* mutant in that the accumulation of alpha toxin was increased (Fig. 5B) while overall virulence was decreased (Fig. 1).

We also assessed the impact of each mutation on the accumulation of protein A (Spa). Because *S. aureus* naturally produces Spa in both extracellular and surface-associated forms, these experiments were done by western blot of both cell extracts enriched for surface-associated proteins (sSpa) and extracellular Spa (eSpa).[39,40] With the exception of the *mgrA* mutant, the amount of both sSpa (Fig. 5C) and eSpa (Fig. 5D) was reduced relative to LAC. In contrast, accumulation of Spa was increased in a LAC *mgrA* mutant, particularly when assessed in its extracellular form (Fig. 5D). These results are consistent with a previous study demonstrating by RNA-seq that the amount of *spa* transcripts was dramatically increased in a LAC *mgrA* mutant.[41]

In western blots done with surface protein-enriched cell extracts, sSpa was essentially absent in *codY*, *rot*, *sarA*, and *sigB* mutants, and present in significantly reduced amounts *atl* and *fur* mutants (Fig. 5C). When assessed using conditioned medium, eSpa was essentially absent in every strain except LAC and its *agr* (Fig. 3C) and *mgrA* mutant (Fig. 5D). To the extent that Spa in both of these forms contributes to the virulence of *S. aureus* by promoting immune evasion, its virtual absence could contribute to the reduced virulence of the *codY*, *sarA* and *sigB* mutants.[42] Conversely, its increased abundance could contribute to the increased virulence of a LAC *mgrA* mutant. However, it is difficult to envision how reduced accumulation of Spa would contribute to increased virulence of a LAC *rot* mutant.

As noted above, we focused on alpha toxin and Spa because they are prototype virulence factors in the context of the pathogenic versatility of *S. aureus* and are differentially regulated by *agr* relative to each other.[6,43] A number of the loci we examined are also known to impact expression and/or function of *agr*. Although it was not true of the *sarA* or *sigB* mutants, it might therefore be anticipated that mutation of other regulatory loci would result in alpha toxin and Spa phenotypes comparable to those observed in the *agr* mutant (e.g. increased production of Spa and decreased production of alpha toxin). However, all of the mutants we examined exhibited unique alpha toxin and Spa phenotypes relative to the *agr* mutant and relative to each other (Table 1). This provides support for the hypothesis that the virulence changes we observed are largely *agr*-independent. It also emphasizes the overall complexity of *S. aureus* regulatory circuits and suggests that additional virulence factors that remain to be identified are involved in defining the virulence phenotypes of each of these mutants. Additional studies will be required to identify these virulence factors, but one clear correlation

Table 1. Summary of phenotypes. Table summarizes whether the accumulation of AgrA, SarA, alpha (α) toxin, Spa, and virulence was increased, decreased, or unchanged (NC) in each of the indicated LAC regulatory mutants. A dash (-) indicates the indicated protein was absent. Accumulation of SarA in an *agr* mutant was not tested (NT).

Mutation	AgrA	SarA	α toxin	Spa	Virulence
agr	—	NT	Down	Up	NC
atl	NC	NC	NC	Down	NC
codY	NC	NC	NC	Down	Down
fur	Up	NC	NC	Down	NC
mgrA	Up	NC	NC	Up	Up
rot	NC	NC	Up	Down	Up
sarA	Down	—	Down	Down	Down
sigB	Down	Down	Up	Down	Down

we did observe, and one that could potentially be exploited to help identify such virulence factors, was that attenuation of the *sarA, codY*, and *sigB* mutants was in all cases correlated with the increased production of extracellular proteases and an altered exoprotein profile (Fig. 6). Mutation of *rot* was also previously shown to result in an increase in protease production to an extent that could be correlated with a reduced capacity to form a biofilm, but in our comparisons we did not observe a significant increase in the accumulation of extracellular proteases in the *rot* mutant (Fig. 6).[27] This is consistent with the observation that, unlike *codY, sarA* and *sigB* mutants, the LAC *rot* mutant exhibited increased rather than decreased virulence (Fig. 1).

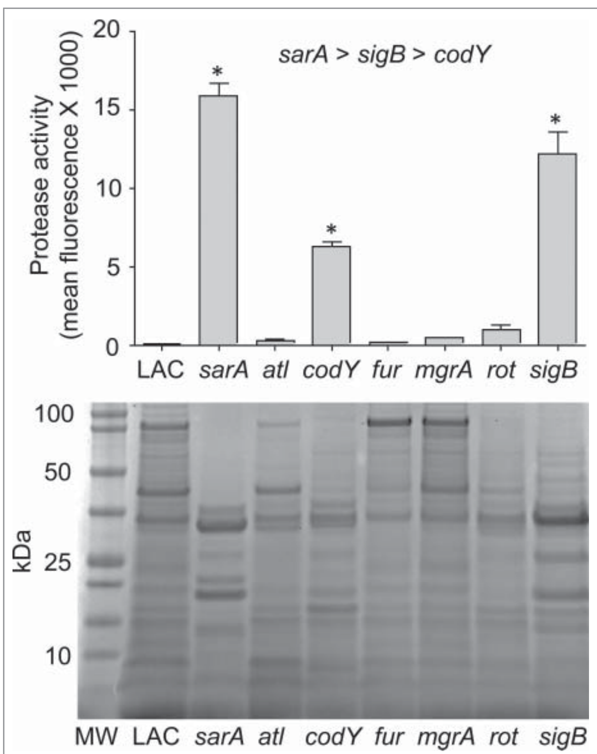

Figure 6. Protease activity in LAC regulatory mutants. Top: Total protease production in LAC and the indicated regulatory mutants was assessed using a commercially available FITC-casein cleavage hydrolysis assay. Results are reported as mean fluorescence values ± the standard error of the mean. Asterisks indicate statistically significant differences ($p \leq 0.05$) between the indicated mutants relative to the results observed with LAC. As indicated above the graph, individual comparisons also confirmed that the amount of total protease activity observed in the *sarA* mutant was increased to a statistically significant extent by comparison to the results observed with both the *sigB* and *codY* mutants and that total protease activity in the *sigB* mutant was increased to a statistically significant extent by comparison to those observed with the *codY* mutant. Bottom: Extracellular protein profiles for LAC and each regulatory mutant were assessed by SDS-PAGE. MW = molecular weight markers, with the molecular weight in kilodaltons (kDa) of representative markers shown to the left.

Interestingly, mutation of *sarA* and *sigB* resulted in a comparable increase in the overall production of extracellular proteases (Fig. 6) but had opposite effects on the accumulation of alpha toxin (Fig. 3D). One possible explanation that we are currently exploring is that, while *sarA* and *sigB* repress the overall production of extracellular proteases, they do not repress the production of the specific protease(s) that degrade alpha toxin to the same degree. The alternative is that mutation of *sigB* has a much greater impact on the production of alpha toxin than mutation of *sarA*, thus resulting in a net increase in the accumulation of alpha toxin in a LAC *sigB* mutant despite its increased production of extracellular proteases.

Finally, we did not include complementation studies in our *in vivo* experiments for two reasons. The first is the number of mice this would have required would have been very large given the number of mutants we examined, particularly since all of our *in vivo* experiments were repeated at least twice. The second is that such studies are sometimes inconclusive owing to plasmid instability *in vivo*. However, we did confirm that all mutations that had a significant impact on Spa and/or alpha toxin phenotypes could be complemented under *in vitro* conditions (Fig. S1).

Discussion

The continuing increase in antibiotic resistance has led to the suggestion that strategies targeting *S. aureus* virulence factors and/or the regulatory circuits that control the production of these virulence factors could be therapeutically beneficial.[13,44-50] The two *S. aureus* regulatory loci that have been explored to the greatest extent as therapeutic targets are *agr* and *sarA*.[51-53] These loci interact with each other, with SarA being required for full expression of *agr*.[54] Although we did not assess this at the level of gene expression, we did confirm that AgrA is present in reduced amounts in a LAC *sarA* mutant. However, it is also clear that *sarA* and *agr* serve independent regulatory functions. This is evident in the observation that mutation of *agr* enhances biofilm formation while mutation of *sarA* has the opposite effect.[7,50,55-57] Thus, the therapeutic focus on these loci has generally been targeted toward different forms of infection, with *agr* being proposed as a target for acute, toxin-mediated diseases and *sarA* as a target for chronic, biofilm-associated infections.[51,52,58]

Our results confirm that mutation of *sarA* limits the virulence of LAC in a murine sepsis model to a

significant degree relative to the isogenic parent strain, but also demonstrate that mutation of *codY* and *sigB* attenuate virulence in this clinical context to a comparable degree. Mutation of these same three loci also limited the ability of LAC to form a biofilm which suggests that these loci are all viable therapeutic targets in the context of both acute and chronic forms of *S. aureus* infection.[15] This is particularly true since mutation of all of these loci could be correlated with enhanced susceptibility to daptomycin *in vivo* in a murine catheter model.[16] However, when assessed using ceftaroline, only mutation of *sarA* and *sigB* had a significant effect. This suggests that *sarA* or *sigB* would be preferable therapeutic targets by comparison to *codY*.

Further support for this hypothesis comes from the observation that, while mutation of *codY* has been shown to limit biofilm formation in LAC and other strains of the USA300 clonal lineage, it has also been shown to enhance biofilm formation in UAMS-1 and the clinical isolate SA564.[15,59, 60] Interestingly, mutation of *codY* in these latter strains was also correlated with increased expression of *agr*,[59] which would be expected to decrease rather than increase biofilm formation.[7,50,55,57] At present, the mechanistic basis for these strain-dependent biofilm phenotypes is not fully understood, but it has been suggested that it may be related to the relative contribution of the polysaccharide intercellular adhesion (PIA) to biofilm formation in different strains of *S. aureus*.[15,59, 60]

There are also conflicting reports regarding the role of *codY* in defining the virulence of *S. aureus* in acute infections. For instance, our results are consistent with a report demonstrating that mutation of *codY* in LAC limited virulence in a neutropenic murine model of pulmonary infection,[61] but they are in contrast to a report demonstrating that a *codY* mutant generated in the USA300 strain 923 exhibited increased virulence in murine models of skin infection and necrotizing pneumonia.[62] These disparate results may simply reflect the use of different animal models and the importance of the microenvironment *in vivo*, or perhaps differences between strains of *S. aureus*. However, the more important point in the context of this report is that, when viewed collectively, such conflicting reports in the context of both biofilm-associated and acute infection further diminish enthusiasm for *codY* as a therapeutic target. They also emphasize the need to extend the studies we report here to include evaluation of these same loci in alternative animal models and additional strains of *S. aureus*.

Similarly, while mutation of *sigB* in LAC did have a significant impact on daptomycin susceptibility in the context of an established biofilm, it did not have a significant impact *in vivo* in the methicillin-sensitive, USA200 strain UAMS-1.[16] This would suggest that, in the context of coverage for diverse clinical isolates of *S. aureus, sarA* would be the preferable target even by comparison to *sigB*. Moreover, much as with *codY*, there are conflicting reports regarding the phenotypic impact of mutating *sigB*. For example, Bischoff *et al.* concluded that mutation of *sigB* results in decreased levels of *sarA* expression.[63] In contrast, Cheung *et al.* concluded that mutation of *sigB* in the 8325-4 strain RN6390 results in increased production of SarA.[64] These authors also concluded that this accounts for the increased production of alpha toxin in a *sigB* mutant. We did not include studies assessing gene expression in the context of *sarA*, but we did find that the accumulation of SarA is reduced in a LAC *sigB* mutant. This suggests that mutation of *sigB* and *sarA* in LAC would result in similar phenotypes, and in fact we found that this was generally the case.

However, one notable exception to this is that mutation of *sarA* essentially abolished the accumulation of alpha toxin while mutation of *sigB* had the opposite effect. Despite the fact that both mutants were attenuated in our sepsis model, this provides further support for the hypothesis that *sarA* would be the preferred therapeutic target by comparison to *sigB*, particularly when combined with the observation that mutation of *sarA* limits biofilm formation to a greater extent than mutation of *sigB*.[15] At the same time, it has been reported that *sigB* is required for intracellular persistence and development of small colony variants (SCV), both of which are considered key elements in the development of chronic *S. aureus* infections, and that this is not the case with *sarA*.[65,66] Thus, the possibility that *sigB* would be the preferred target in the context of chronic infections cannot be ruled out without additional experimentation that includes direct comparisons between these loci in an appropriate animal model.

From a mechanistic point of view, the increased accumulation of alpha toxin in a LAC *sigB* mutant is consistent with a report concluding that mutation of *sigB* results in increased levels of *agr* expression.[63] To the extent that *rsbU* is required for maximum *sigB* activity, this is also consistent with the observation that repair of the *rsbU* defect in the 8325-4 strain RN6390 resulted in increased *sigB* activity and reduced expression of *agr*.[67] Repair of the *rsbU* defect in RN6390 also resulted in decreased hemolytic activity, increased production of Spa, and an increased capacity to form a biofilm,[68] all of which are consistent with decreased levels of *agr* expression. Additionally, to the extent that mutation of *sigB* would be expected to result in the opposite phenotypes, they are also consistent with the observation that a LAC *sigB* mutant exhibited increased accumulation of alpha toxin, decreased production of Spa, and a decreased

capacity to form a biofilm as was observed both here and in our previous reports.[15,16] However, none of these phenotypes are consistent with the observation that mutation of *sigB* in LAC was correlated with decreased accumulation of AgrA.

One possible explanation for this apparent disparity is related to the experimental methodologies employed. Specifically, previous reports focused on *agr* at a transcriptional level, generally with a specific focus on RNAIII, while we focused on the accumulation of AgrA. Indeed, the mechanistic basis by which *sigB* impacts *agr* is unclear,[27] and it is possible that mutation of *sigB* impacts the production of RNAIII differently than it does production of AgrA. An alternative possibility is that the increased production of extracellular proteases in the LAC *sigB* mutant results in increased degradation of AgrA, which would not be apparent in assays focusing on transcription. Indeed, we previously demonstrated that the accumulation of AgrA is limited in a *sarA* mutant owing to protease-mediated degradation.[38] Although it remains to be determined whether this is also the case in a *sigB* mutant, the studies we report confirm that protease activity is increased in a LAC *sigB* mutant to a degree that approaches that observed in an isogenic *sarA* mutant.

Irrespective of the mechanism(s) involved, none of these results can explain the attenuation of a LAC *sigB* mutant despite the increased accumulation of alpha toxin. At the same time, while repair of *rsbU* in RN6390 was previously reported to result in reduced expression of *agr*, it was also correlated with what the authors described as a "surprising increase in mouse lethality" as assessed using a bacteremia model.[68] To the extent that repair of *rsbU* results in increased expression of *sigB*, this is consistent with the observation that mutation of *sigB* resulted in reduced lethality in the studies we report. The fact that mutation of *sigB* limited the accumulation of AgrA suggests that this attenuation could be at least partially *agr*-dependent, although as noted above this seems unlikely given that the isogenic *agr* mutant was not attenuated in our model. This was surprising in light of the many reports demonstrating that *agr* mutants are attenuated in animal models of infection.[31,69-71] However, most of these other reports focused on models other than bacteremia, and it has been shown that serum apolipoprotein B, including that from mice, binds and effectively inactivates the quorum-sensing pheromone of the *agr* system.[72] Additionally, serum lipoproteins have been shown to inactivate phenol soluble modulins (PSMs), which have been shown in turn to be a primary determinant of the virulence of community-associated, methicillin-resistant *S. aureus* strains like LAC.[22,26,73,74] Thus, one possible explanation for the fact that mutation of *sigB* limited virulence in a murine bacteremia model while mutation of *agr* did not is that the functionality of *agr* and/or PSMs is decreased owing to the presence of serum lipoproteins. Such a scenario would also suggest that the attenuation we observed in a LAC *sigB* is independent of its impact on *agr* expression.

Mutation of *rot* was previously reported to result in increased virulence and increased production of both alpha toxin and extracellular proteases.[75] We also observed increased virulence and increased accumulation of alpha toxin. However, we did not observe a significant increase in the production of extracellular proteases in our LAC *rot* mutant. This suggests that the increased virulence we observed with a *rot* mutant is likely due to changes in the production of important virulence factors relative to the rate of their protease-mediated degradation. This is consistent with the observation that *rot* was originally identified as a repressor of *S. aureus* toxin production.[75] This was subsequently shown to involve an interaction between the *agr*-encoded RNAIII and *rot* mRNA that limits translation of the latter.[76,77] Thus, it would be anticipated that mutation of *agr* and mutation of *rot* would have opposite effects on virulence. This is consistent with the observation that mutation of *rot* enhanced the virulence of LAC while mutation of *agr* did not.

In contrast, our results demonstrating that mutation of *mgrA* enhances virulence are not consistent with reports demonstrating that mutation of *mgrA* attenuates virulence in murine models of sepsis and septic arthritis as well as rabbit models of sepsis and endocarditis.[41,78] We have no explanation for this disparity, but would note that these collective studies were also done using different strains of *S. aureus*. Indeed, none of the *mgrA* mutants employed in these earlier virulence studies were generated in LAC. Rather, they were generated in the commonly-studied *S. aureus* strain Newman, which has a recognized mutation in the *saeRS* regulatory system,[68] or in MW2 and 502A.[41] Even aside from recognized regulatory defects like those present in Newman, different strains of *S. aureus* exhibit a great deal of genetic and phenotypic diversity. In fact, a recent report confirmed that there is as much phenotypic diversity within different clonal lineages of *S. aureus* as there is between these clonal lineages.[79] It is also virtually certain that all strains of *S. aureus* carry mutations,[68] thus making it essentially impossible to define a definitive wild-type strain. Although no specific mutations have been identified in LAC that we are aware of, this is presumably true of USA300 strains. Nevertheless, the clinical predominance of such strains makes them worthy of investigation, and this is the primary reason we chose LAC for our studies. Thus, we believe the possibility that the impact of

mutating *mgrA* on virulence in acute infection is strain-dependent diminishes enthusiasm for *mgrA* as a therapeutic target, particularly when viewed in light of the fact that mutation of *mgrA* enhances biofilm formation.[15,41]

While much remains to be explored regarding the virulence phenotypes of the mutants we examined, one common phenotype observed with the attenuated *codY*, *sarA* and *sigB* mutants is that they all produced extracellular proteases at significantly increased levels relative to LAC. However, the impact of mutating these loci was not equivalent. Specifically, protease production was highest in the *sarA* mutant and decreased progressively in the *sigB* and *codY* mutants, respectively. As discussed above, mutation of *sigB* results in increased accumulation of alpha toxin, and to the extent that mutation of *sarA* resulted in a greater increase in protease production than mutation of *sigB*, one possible explanation for the disparate alpha toxin phenotypes we observed in LAC *sigB* and *sarA* mutants may be due to the relative impact of these loci on the production vs. protease-mediated degradation of alpha toxin. Alternatively, our protease assays did not allow us to distinguish between the activity of different proteases, and it is also possible that mutation of *sigB* vs. *sarA* has a differential impact on the production of specific proteases that contribute to the degradation of alpha toxin. Nevertheless, the correlation between the increased production of extracellular proteases and decreased virulence suggests that the inability to repress the production of extracellular proteases may play a key role in attenuating the virulence of *S. aureus* in acute as well as chronic, biofilm-associated infections. The observations that mutation of *sarA* results in a greater increase in protease production than any other mutant we have examined, and that this can be correlated with a reduced capacity to cause both acute and biofilm-associated infections suggests that *sarA* may be the best target by which this observation can be exploited to therapeutic advantage.[21,23]

Materials and methods

Bacterial strains and growth conditions

The strains used in these experiments are summarized in Table S1. With the exception of the *spa* mutant and the complemented *rot* mutant, the methods used to generate and confirm all mutants and the corresponding complemented strains were described in previous reports.[15,34,80-82] The Nebraska Transposon Mutant Library (NTML) was utilized to generate the *spa* mutant by phage transduction from the original JE2 mutant into our strain of LAC. The *rot* complementation strain was constructed similarly by transducing a previously described *rot* complementation plasmid into the *rot* mutant.[15,83] All strains were maintained at −80°C in a suspension containing tryptic soy broth (TSB) and 25% (v/v) glycerol. For each experiment, strains under study were retrieved from cold storage by plating on tryptic soy agar (TSA) with appropriate antibiotic selection. Antibiotics were incorporated into the culture media as appropriate at the following concentrations: erythromycin, 10 μg ml^{-1}; chloramphenicol 10 μg ml^{-1}; tetracycline, 5 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; and neomycin, 50 μg ml^{-1}; spectinomycin, 1 mg ml^{-1}. Kanamycin and neomycin were always used together to avoid selection of spontaneously resistant strains.

Murine bacteremia model

Bacterial strains were retrieved from cold storage and grown at 37°C to stationary phase (16-17 hrs) in TSB with appropriate antibiotic selection. Cultures were standardized to an OD_{560} of 0.05 in TSB without antibiotics and grown to an OD_{560} of 1.0. Bacterial cells were harvested by centrifugation and separate aliquots were resuspended in an equal volume of sterile phosphate-buffered saline (PBS) containing 10% DMSO and 5% bovine serum albumin (BSA) which were stored at −80°C. The number of colony-forming units (cfu) in aliquots prepared from each strain was confirmed by plate count after 20 hrs incubation at 37°C.

The bacteremia model used in this study was previously described by Zielinska et al. (2012).[23] Briefly, the strains under study were removed from cold storage, washed with PBS, and standardized in PBS to a cell density of 5×10^8 cfu per ml. For each experiment, groups of ten 5–8 week-old female NIH-Swiss mice were infected via tail vein injection with 5×10^7 cfu of LAC or one of its isogenic mutants. Organs and tissues were harvested from any mice found dead or which required compassionate euthanasia; otherwise, tissues were harvested at 7 days post-infection. Organs were removed aseptically and homogenized. Serial dilutions of each homogenate were then plated on TSA without selection, and the number of cfus per organ determined following overnight incubation at 37°C. To rule out the possibility of contaminants skewing the results, replicate samples were also plated on CHROMagar (BBL™, Cat. # 254102/215081). All experiments were repeated at least twice, with the total number of mice infected with each strain indicated in the scatter plots.

Western blotting

Samples for western blots and the primary and secondary antibodies used were all prepared and used as previously described.[23,38] Western blots included at least two

biological replicates with at least two experimental replicates of each. Densitometric values were obtained with a Bio-Rad ChemiDocMP Imaging System and Image Lab Software (Bio-Rad Laboratories, Inc., Irvine, CA).

Sonication assay

To ensure that the results were not skewed by the impact of any given mutation on cellular clumping, all strains were grown in overnight cultures with appropriate selection and standardized to an OD_{560} of 10.0 in a volume of 5 ml. Serial dilutions of a 100 μl aliquot were performed on ice and plated on TSA without selection. The remaining cultures were kept on ice and sonicated (QSonica S4000, Newtown, CT) for a period of 4 minutes at 6 watts. Serial dilutions were then prepared post-sonication and plated on TSA. The number of cfu before and after sonication was determined by plate count after 20 hrs incubation at 37°C.

Characterization of exoprotein profiles

Exoprotein profiles were examined as described by Zielinksa et al. (2012).[23] Briefly, overnight (16-17 hrs) cultures were standardized to an OD_{560} of 10.0. Conditioned medium from each culture was then harvested by centrifugation and the supernatant filter sterilized. Samples were resolved by SDS-PAGE using 4–12% gradient Novex Bis-Tris Plus gels (Life Technologies, cat. # NW04125BOX). Proteins were visualized by staining with SimplyBlue™ SafeStain (Life Technologies, cat. # LC6060) and imaging using Bio-Rad ChemiDocMP Imaging System (Bio-Rad Laboratories, Inc., Irvine, CA).

Total protease activity

Total protease activity was assessed using conditioned media prepared as described above and the Protease Fluorescent Detection Kit (Sigma Chemical Co., cat # PF0100). MFI values for conditioned medium from LAC were set to a value of 100% activity, with the activity observed in each mutant shown relative to this value. All assays included two biological replicates with at least three experimental replicates of each.

Cell lysis procedure

Bacterial cells from overnight cultures standardized as described above were harvested by centrifugation and resuspended in 750 μl of ice-cold TEG buffer (25 mM Tris at a pH of 8 and 25 mM EGTA). Cell suspensions were then transferred to 2 ml RNase/DNase free Fastprep Lysing Matrix B tubes (MP Biomedicals, cat. # 116911050). Cell suspensions were then lysed in a FastPrep®-24 benchtop homogenizer (MP Biomedicals, Solon, OH) for two separate 40 second intervals at a rate of 6.0 m/sec (interrupted by a 5 minute interval in which the homogenates were chilled on ice). After centrifugation at 15,000 X *g* at 4°C for 10 minutes, supernatants were aliquoted and stored at -20°C until use.

Preparation of samples for analysis of surface-associated Spa (sSpa)

To examine relative amounts of sSpa, samples enriched for surface-associated proteins were prepared as previously described.[23] Here, bacterial cells from overnight cultures standardized as described above were harvested by centrifugation. Cell pellets were then resuspended to a density 1×10^9 cells per ml. Cells were then washed in distilled water before resuspending in 200 μl of filter-sterilized digestion buffer consisting of 100 μl of 1M Tris-HCl (pH 7.5), 50 μl of 5M NaCl, 675 mg of sucrose, 50 μl of 1M $MgCl_2$, 25 μl of lysostaphin (10 mg/ml), 100 μl mutanolysin (1.25 mg/ml), 5 units of DNase I, 50 μl of 100 mM PMSF, 2.5 μl of 1M benzamidine, 50 μl of 100 mM N_α-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME), 25 μl leupeptin (1 mg/ml), and 12.5 μl pepstatin (1 mg/ml). Samples were then brought to a final volume of 2.5 ml using distilled water. Cell suspensions were then incubated at 37°C for 4 hours. The lysis reactions were then centrifuged at 6000 X *g* for 20 minutes at 4°C and the supernatants aliquoted and stored at -80°C until used for western blot.

Ethics statement

All experiments involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences and performed according to NIH guidelines, the Animal Welfare Act, and US Federal law.

Statistical analysis

To allow for statistical comparison across biological and experimental replicates from all *in vitro* assays, the results obtained for each mutant were averaged across all replicates. This average was then plotted relative to the results observed with LAC after setting the value observed with LAC either to 1.0 (western blots) or 100% (protease assay). Analysis of variance (ANOVA) models were then used to assess the statistical significance of the results observed with each mutant relative to LAC (Bonferroni correction). ANOVA methods were also used to analyze cfu data. Specifically, Dunnett's procedure was

used to compare each mutant mean to the mean of LAC. The cfu data were $\log_{10}$-transformed prior to analysis, and P-values were calculated using permutation methods. P-values less than or equal to 0.05 were considered to be statistically significant. Statistical analyses were performed using the statistical programming language R version 3.3.3 (Vienna, Austria), SAS 9.4 (Cary, NC) and GraphPad Prism 5.0 (La Jolla, CA).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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