Exploiting Correlations between Protein Abundance and the Functional Status of \( \text{saeRS} \) and \( \text{sarA} \) To Identify Virulence Factors of Potential Importance in the Pathogenesis of \( \text{Staphylococcus aureus} \) Osteomyelitis

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ABSTRACT: We used a murine model of postsurgical osteomyelitis (OM) to evaluate the relative virulence of the \( \text{Staphylococcus aureus} \) strain LAC and five isogenic variants that differ in the functional status of \( \text{saeRS} \) and \( \text{sarA} \) relative to each other. LAC and a variant in which \( \text{saeRS} \) activity is increased (\( \text{saeC} \)) were comparably virulent to each other, while \( \Delta \text{saeRS}, \Delta \text{sarA}, \Delta \text{saeRS}/\Delta \text{sarA}, \text{and } \text{saeC}/\Delta \text{sarA} \) mutants were all attenuated to a comparable degree. Phenotypic comparisons including a mass-based proteomics approach that allowed us to assess the number and abundance of full-length proteins suggested that mutation of \( \text{saeRS} \) attenuates virulence in our OM model owing primarily to the decreased production of \( \text{S. aureus} \) virulence factors, while mutation of \( \text{sarA} \) does so owing to protease-mediated degradation of these same virulence factors. This was confirmed by demonstrating that eliminating protease production restored virulence to a greater extent in a LAC \( \text{sarA} \) mutant than in the isogenic \( \text{saeRS} \) mutant. Irrespective of the mechanism involved, mutation of \( \text{saeRS} \) or \( \text{sarA} \) was shown to result in reduced accumulation of virulence factors of potential importance. Thus, using our proteomics approach we correlated the abundance of specific proteins with virulence in these six strains and identified 14 proteins that were present in a significantly increased amount (log2 ≥ 5.0) in both virulent strains by comparison to all four attenuated strains. We examined biofilm formation and virulence in our OM model using a LAC mutant unable to produce one of these 14 proteins, specifically staphylocoagulase. The results confirmed that mutation of coa limits biofilm formation and, to a lesser extent, virulence in our OM model, although in both cases the limitation was reduced by comparison to the isogenic \( \text{sarA} \) mutant.

KEYWORDS: \text{Staphylococcus aureus}, osteomyelitis, virulence factors, biofilm, protease, proteomics

\( \text{Staphylococcus aureus} \) is the principal cause of osteomyelitis (OM) and other forms of orthopedic infection including those associated with the presence of an indwelling prosthesis. The medical treatment of these infections is complicated by a compromised localized vasculature, the presence of a biofilm, and the presence of bacterial variants (e.g., persister cells) with reduced metabolic activity and consequently increased antibiotic tolerance. The continued emergence of antibiotic resistant strains, most notably methicillin-resistant \( \text{S. aureus} \) (MRSA), further complicates the success of traditional antibiotic-based therapies. For these reasons, the majority of...
OM cases caused by *S. aureus* require surgical intervention in addition to long-term, intensive antibiotic therapy. Even after such intensive medical and surgical intervention the recurrence rate remains unacceptably high. Identifying critical *S. aureus* virulence factors, and improving our understanding of how these factors impact pathogenesis in OM, is key to potentially finding new therapeutic targets that can be exploited to better address this clinical problem.

One approach to identifying such virulence factors is to exploit the impact of regulatory loci in the specific context of the pathogenesis of OM. Regulatory circuits in *S. aureus* are complex and highly interactive, thus allowing the bacterium to adjust the production of its many virulence factors to diverse microenvironments within the host. In the specific case of OM, it has been demonstrated that mutation of the staphylococcal accessory regulator (*sarA*) or the *S. aureus* exoprotein (*saeRS*) regulatory locus attenuates virulence in a murine model of postsurgical OM as assessed by both cortical bone loss and reactive bone (callus) formation. Mutation of both loci has also been shown to result in the increased production of extracellular proteases and decreased accumulation of specific virulence factors including alpha toxin, phenol-soluble modulins (PSMs) and protein A (Spa). In fact, the increased production of extracellular proteases, specifically aureolysin, has been shown to play a significant role in defining the attenuation of a *LAC* saeRS mutant owing to the decreased accumulation of PSMs.

Mutation of *sarA* also results in reduced accumulation of PSMs owing to protease-mediated degradation, and in fact mutation of *sarA* results in a much greater increase in protease production than mutation of *saeRS*. This suggests that mutation of *sarA* may attenuate the virulence of *LAC* to an even greater extent than mutation of *saeRS*. However, the accumulation of any protein is a function of its production vs its degradation, and our studies suggest that the primary impact of mutating *saeRS* on virulence is due to reduced production of *S. aureus* virulence factors, while that of mutating *sarA* is due to protease-mediated degradation of these virulence factors. Thus, the relative impact of these two regulatory loci in the context of OM remains unknown. To assess this experimentally, we evaluated the virulence of *LAC* and five isogenic derivatives that differ with respect to the functional status of *saeRS* and *sarA* relative to each other. Comparisons were made using a murine model of postsurgical OM. We then took advantage of the results of these studies to identify and prioritize specific virulence factors of potential relevance by correlating relative virulence with the accumulation of full-length proteins present in conditioned medium (CM) from stationary phase cultures of the same strains.

### RESULTS

**Mutation of saeRS or sarA Attenuates the Virulence of LAC in OM to a Comparable Degree.** We previously generated five derivatives of *LAC* that vary with respect to the functional status of *saeRS* and *sarA* relative to each other. To assess the relative virulence of these strains, we employed a murine model of postsurgical osteomyelitis. Briefly, a unicortical defect was created in the femur of mice. *LAC* or one of these five isogenic derivatives was then inoculated directly into the medullary canal. After 14 days, infected bones were harvested and analyzed by microcomputed tomography (µCT). Duplicate samples were also harvested and processed to determine bacterial burdens in the femur. By comparison to uninfected mice subjected to the same surgical procedure (sham), the femurs of all infected mice showed marked callus formation adjacent to the inoculation site and extending to the proximal and distal ends of the femur (Figure 1). In sham mice, the unicortical defect was almost completely sealed, while this was not the case with any of the infected mice irrespective of the strain used to initiate the infection.

To quantitatively assess virulence differences between these strains, µCT images were analyzed to assess relative levels of callus formation and cortical bone destruction. As assessed based on both parameters, *LAC* and its derivative with increased activation of *saeRS* (*saeC*) were comparably virulent (Figure 2). As previously reported, mutation of *saeRS* (*ΔsaeRS*) and/or *sarA* (*ΔsarA*) resulted in decreased callus formation and cortical bone destruction. The functional status of *saeRS* did not have a statistically significant effect in the *sarA* mutant with respect to either of these parameters, but there did...
appear to be a trend suggesting that reactive bone formation decreased as the combined activity of both sae and sarA decreased (Figure 2). Similarly, while the difference did not reach statistical significance, mutation of saeRS appeared to limit callus formation to a greater extent than mutation of sarA.

In addition to μCT analysis, we also quantified bacterial burdens in the femur. By comparison to LAC, we did not observe a statistically significant difference in bacterial burdens in the femurs of mice infected with the saeC derivative, the ΔsarA mutant, or the saeC/ΔsarA mutant (Figure 3). Significantly reduced bacterial burdens were observed in the femurs of mice infected with the ΔsaeRS mutant. Specifically, no bacteria were recovered from the femurs of 60% of the mice infected with this strain. The number of bacteria recovered from the remaining 40% of these mice ranged from 10^4 to 10^5 cfu per femur. The reasons for this variability are unclear. However, these experiments were done as two independent biological replicates, and most, but not all, of the mice in which no viable bacteria were recovered were included in the first replicate. Nevertheless, these results are consistent with a previous report that also found that bacterial burdens were reduced in a LAC Δsae mutant. Moreover, no viable bacteria were recovered from any of the femurs of mice infected with the ΔsaeRS/ΔsarA double mutant (Figure 3). This suggests that sarA, which had not been previously examined in this regard, also contributes to the ability of S. aureus to persist in the bone as defined by the 14-day postinfection period we employed and that concomitant mutation of saeRS and sarA has an additive effect in this regard. Taken together with the μCT data, our results indicate that saeRS and sarA contribute to the pathogenesis of OM to a comparable degree.

**Correlations between Virulence, Protease Production, and Protein Abundance.** Mutation of saeRS or sarA has been shown to result in the increased production of extracellular proteases, and this has been correlated with reduced accumulation of specific virulence factors and reduced virulence. We confirmed that mutation of saeRS or sarA results in increased protease activity and that mutation of sarA has a much greater impact in this regard than mutation of saeRS (Figure 4). The impact of mutating saeRS and sarA on protease production was additive in that a statistically significant difference was observed between the sarA and saeRS/sarA mutants. Conversely, protease production was reduced in the ΔsaeC/sarA mutant relative to the isogenic ΔsarA mutant. Although the difference in virulence between the ΔsaeC/sarA and sarA mutants did not reach statistical significance (Figure 2), this is consistent with the trends we observed in our OM comparisons, and in this case the...
difference between the \( \Delta \text{sae}^+/\text{sarA} \) and \( \Delta \text{sarA} \) mutants was statistically significant (Figure 4).

The relative levels of protease production were inversely correlated with the accumulation of high-molecular weight proteins as assessed by SDS-PAGE analysis of conditioned medium (CM) from stationary phase cultures of each of these strains. CM samples from stationary phase cultures were chosen because protease production is highest in this growth phase. We also believe that stationary phase cultures are most likely to be representative of in vivo growth conditions. Evidence to support this hypothesis comes from the observation that protease-deficient mutants have been shown to be hypervirulent in vivo in diverse animal models of infection.\(^{10} \) The abundance of high molecular weight proteins was dramatically reduced in CM from the \( \Delta \text{sarA} \) mutant, with a corresponding increase in the abundance of lower molecular weight proteins (Figure S1). This was true irrespective of the functional status of \( \text{saeRS} \), although overall protein profiles of CM from the \( \text{sae}^+/\Delta \text{sarA} \) and \( \Delta \text{saeRS}/\Delta \text{sarA} \) mutants did differ from each other and from that observed in the isogenic \( \Delta \text{sarA} \) mutant. This is consistent with the relative level of protease production in these strains, and it provides an additional indication that the functional status of \( \text{saeRS} \) has an impact on the phenotype of a LAC \( \Delta \text{sarA} \) mutant. In contrast, the abundance of many proteins, including high molecular weight proteins, was reduced in CM from a \( \Delta \text{saeRS} \) mutant, but the overall distribution of these proteins was largely unaffected (Figure S1). These observations are consistent with the hypothesis that mutation of \( \text{saeRS} \) impacts the abundance of \( S. \text{aureus} \) exoproteins primarily at the level of their production, while mutation of \( \text{sarA} \) does so primarily at the level of their accumulation owing to protease-mediated degradation.

To further examine this hypothesis, we carried out gel-based proteomic studies employing a novel mass-based approach that allowed us to focus specifically on spectral counts derived from full-length proteins to the exclusion of spectral counts derived from degradation products of those proteins.\(^{11} \) On the basis of triplicate samples, and irrespective of the abundance of each protein, we identified an average of 1090 full-length proteins in CM from LAC and 1007 in CM from its \( \text{sae}^+ \) derivative (Figure 6). An average of 763 (\( \geq 70\% \)) of these were detectable in CM from the \( \Delta \text{saeRS} \) mutant. In contrast, an average of 145 and 160 full-length proteins (\( \leq 15.9\% \)) were detected in CM from the isogenic \( \Delta \text{sarA} \) and \( \Delta \text{saeRS}/\Delta \text{sarA} \) mutants, respectively. This number was more than doubled to an average of 349 in the \( \text{sae}^+/\Delta \text{sarA} \) mutant (Figure 5), likely owing to increased protein production associated with the \( \text{sae}^+ \) allele.

To assess the abundance of individual proteins, we carried out an analysis based on total spectral counts derived from full-length proteins rather than the total number of detectable proteins. The number of spectral counts was highest in the \( \text{sae}^+ \) derivative (average = 21 356), slightly lower in LAC (18 709) and decreased progressively through the \( \Delta \text{saeRS} \) (8485), \( \text{sae}^+/\Delta \text{sarA} \) (5478), \( \Delta \text{sarA} \) (3223), and \( \Delta \text{saeRS}/\Delta \text{sarA} \) mutants (1746) (Figure 5). The fact that \( \geq 70\% \) of full-length proteins that were detectable in LAC and its \( \text{sae}^+ \) derivative were also detectable in the \( \Delta \text{saeRS} \) mutant, while this proportion was reduced to \( \leq 45\% \) when comparisons were made based on total spectral counts, is consistent with the hypothesis that the primary mechanism by which \( \text{saeRS} \) impacts exoproduct accumulation is at the level of production. Similarly, the fact that the decrease observed with the \( \Delta \text{sarA} \) mutant was comparable whether assessed by total proteins (\( \leq 14\% \)) or spectral counts (\( \leq 17\% \)) is consistent with the hypothesis that the impact of mutating \( \text{sarA} \) occurs primarily at the level of protease-mediated degradation. However, irrespective of the mechanism responsible, the association between relative virulence (Figure 2) and the number of spectral counts derived from full-length proteins (Figure 5) suggests that defining correlations among these strains between relative virulence and protein abundance as defined based on spectral counts derived from full-length proteins has the potential to identify \( S. \text{aureus} \) virulence factors that are potentially important in the pathogenesis of OM.

To this end, we explored two different methods of data analysis to identify proteins that were increased in abundance in LAC and its \( \text{sae}^+ \) by comparison to all four of the attenuated strains (\( \Delta \text{saeRS}, \text{sae}^+/\Delta \text{sarA}, \Delta \text{sarA}, \Delta \text{saeRS}/\Delta \text{sarA} \)). In the first approach, we did individual pairwise comparisons (\( t \) test) between each of the two most virulent strains and each of the four attenuated strains. Comparisons were made on the basis of statistical significance (\( p \leq 0.05 \)) using a log2 fold-change (FC) cutoff of \( \geq 2 \), which corresponds to an absolute FC \( \geq 4 \). The list of proteins meeting these criteria in each pairwise comparison was then compared, using Venny 2.1,\(^{12} \) to identify proteins that were increased in both virulent strains by comparison to all four attenuated mutants. This resulted in the identification of a common set of 114 proteins (Figure 6 and Table S1).

To prioritize among these 114 proteins, we increased the stringency to a log2 FC \( \geq 5 \), which corresponds to an absolute FC \( \geq 32 \). This narrowed the list of high priority targets that differed between virulent and attenuated strains from 114 to 10. To validate these results, we also analyzed the entire...
proteome data set using the edgeR generalized linear model quasi-likelihood (glmQLT) method. This statistical analysis allowed us to compare spectral counts obtained from full-length proteins present in CM from the virulent (LAC and its saeRS derivative) vs attenuated (saeC and LAC vs ΔsaeRS, saeC/ΔsarA, ΔsarA, ΔsaeRS/ΔsarA). Using this approach, we identified 333 proteins that were significantly increased (p ≤ 0.05; log2 FC ≥ 2) in both virulent strains by comparison to all four attenuated strains (Figure 6 and Table S2). To further prioritize among these, we then selected those proteins exhibiting a log2 FC ≥ 5. Using this approach, 11 proteins were identified that differed in abundance between virulent and attenuated strains.

The list of proteins prioritized with each analysis method were similar but not identical. Thus, using both analysis methods we identified a total of 14 proteins that differed in abundance by a log2 FC ≥ 5 in both virulent strains vs all four attenuated strains (Table 1). Of these, 3 were identified using the pairwise analysis method but not the quasi-likelihood GLM method (Table 1). Similarly, 4 were identified using the quasi-likelihood GLM method but not the pairwise analysis method. The other 7 proteins were identified using both data analysis methods (Table 1, Figure 7). These 7 proteins were the fibronectin-binding proteins FnbA and FnbB, Sbi, staphylocoagulase, an FtsK/SpoIII family protein, alanine dehydrogenase 1, and an uncharacterized putative surface protein encoded by SAUSA300_0408. All 7 of these were also identified in our previous study focusing solely on identifying proteins that are present in reduced amounts in a LAC sarA mutant owing to protease-mediated degradation, an observation that we believe further validates our experimental approach. It is also interesting to note that the only two proteins found to be present at a level log2 FC ≥ 5 in all four attenuated strains vs both of the more virulent strains were the extracellular proteases aureolysin and SspA (Figure 7).

**Investigating the Role of Staphylocoagulase.** As a first step toward ultimately examining the hypothesis that the specific proteins identified in our studies play a role in the pathogenesis of OM, we began the process of generating mutations in the genes encoding these proteins. We initially employed transduction from existing mutants in the Nebraska Transposon Mutant Library (NTML), and among the first of our successful transductions was the mutation in the gene encoding staphylocoagulase (coa). We chose to move forward with these mutants based on previous reports suggesting that coagulase plays an important role in immune evasion, biofilm formation, and osteoblast physiology. We confirmed that all 7 LAC Δcoa mutants generated by transduction from the NTML coa mutant exhibited a reduced capacity to form a biofilm by comparison to LAC, albeit to a lesser extent than was observed in the isogenic sarA mutant (Figure 8). We also assessed the relative virulence of one of these Δcoa mutants in our OM model, and while trends were evident with respect to a reduction in both new bone formation and cortical bone

Figure 6. Venn diagram indicating overlap between proteins present in conditioned medium from LAC and its saeRS and sarA mutants. Conditioned medium (CM) from three independent stationary phase cultures of each strain were resolved by SDS-PAGE and stained with Coomassie Blue (Figure S1). The number of proteins identified as significantly differing (p ≤ 0.05; log2 FC ≥ 2) between both of the virulent strains (saeC and LAC) compared to each attenuated strain are shown.

Figure 7. Differential protein accumulation in virulent versus attenuated strains. Volcano plot showing the log2 fold change (x-axis) and −log10 FDR-adjusted p-value (y-axis) of each protein identified in each strain. Inner vertical lines indicate a log2 fold change of 2.0. Outer vertical lines indicate a log2 fold change of 5.0. Proteins that were not found to differ significantly (as defined by an FDR corrected p-value ≥0.05 and a fold change ≤2) between virulent and attenuated strains using the quasi-likelihood analysis method are shown as open circles. Proteins in which the abundance was statistically significant (p ≤ 0.05) and the log2 fold change ≥2.0 but ≤5.0 as defined by both data analysis methods are shown in black. Proteins in which the log2 fold change was ≥5.0 as defined by at least one data analysis method are shown in gray. The 7 proteins identified as present in significantly increased amounts in both virulent strains by comparison to all four attenuated strains by both analysis methods are labeled in the upper right quadrant.
Table 1. Proteins Selected as Priority Targets for Further Studies

| protein | gene | accession number | localization | molecular weight (kDa) | method |
|---------|------|------------------|--------------|------------------------|--------|
| Immunoglobulin-binding protein sbi | sbi | SBI_STA3 | unknown | 50 | both |
| Staphylocoagulase | coa | A0A0H2XHF9_STA3 | extracellular | 69 | both |
| Fibronectin binding protein B | fnbB | A0A0H2XGG3_STA3 | cell wall | 104 | both |
| Alanine dehydrogenase 1 | ald1 | DHA1_STA3 | cytoplasmic | 40 | both |
| FtsK/SpolII family protein | SAUSA300_1687 | | membrane | 145 | both |
| Fibronectin-binding protein A | fnbA | FNBA_STA3 | cell wall | 112 | both |
| Putative surface protein | SAUSA300_0408 | | unknown | 57 | both |
| Uncharacterized leukocidin-like protein 2 | SAUSA300_1975 | LUKL1_STA3 | extracellular | 40 | pairwise |
| Uncharacterized leukocidin-like protein 1 | SAUSA300_1974 | LUKL1_STA3 | extracellular | 39 | pairwise |
| Putative staphylocoagulase | SAUSA300_0773 | A0A0H2XEN7_STA3 | extracellular | 59 | pairwise |
| Transcriptional regulatory protein WalR | walR | WALR_STA3 | cytoplasmic | 27 | GLM |
| Uncharacterized protein | SAUSA300_0198 | A0A0H2XFU2_STA3 | unknown | 36 | GLM |
| Serine protease HtrA-like | SAUSA300_0923 | HTRAL_STA3 | unknown | 86 | GLM |
| Protein RecA | recA | A0A0H2XFW9_STA3 | cytoplasmic | 35 | GLM |

![Figure 8. Impact of staphylocoagulase on biofilm formation and osteomyelitis. The top panel illustrates the relative levels of biofilm formation in LAC, its isogenic sarA mutant, and each of 7 independently generated LAC coa mutants. Assays were performed in 3 replicates and the average observed with LAC set to 100%. All other results are shown relative to this value. The bottom panel illustrates quantitative assessment of reactive bone formation (left) and cortical bone destruction (right) in ΔsarA and Δcoa mutants relative to the LAC parent strain. Statistical analysis was done by one-way ANOVA with Dunnett’s correction. A single asterisk (*) indicates a significant difference relative to LAC. Double asterisks (**) indicate statistical significance relative to the ΔsarA mutant.](image)

![Figure 9. Impact of the functional status of saeRS and sarA on biofilm formation. Biofilm formation was assessed in each of the indicated strains. Assays were performed in 6 replicates and the average observed with LAC set to 100%. All other results, including each of the 6 individual LAC replicates, are shown relative to this value. Statistical analysis was done by one-way ANOVA with Dunnett’s correction. A single asterisk (*) indicates a significant difference relative to LAC. Double asterisks (**) indicate statistical significance relative to the ΔsarA and ΔsaeRS/ΔsarA mutants.](image)

destruction, neither of these differences were found to be statistically significant by comparison to LAC (Figure 8).

**Investigating Potential Mechanisms of Attenuation Associated with Mutation of saeRS and/or sarA.** The pathogenesis of OM is complex and incompletely understood, but two phenotypes that have been implicated as important contributing factors are biofilm formation and cytotoxicity for osteoblast and/or osteoclasts. These are difficult phenotypes to assess directly in vivo, but they can be readily assessed in vitro. Thus, we examined each of these to determine whether the impact of saeRS and sarA on these phenotypes could be correlated with relative virulence. As previously demonstrated, we found that mutation of sarA limited biofilm formation to a much greater extent than mutation of saeRS, and this was true irrespective of the functional status of saeRS (Figure 9). However, biofilm formation was increased to a statistically significant extent in the saeC/ΔsarA mutant relative to the ΔsarA and ΔsaeRS/ΔsarA mutants. Similar trends were observed in the context of osteoblast and osteoclast cytotoxicity. Specifically, CM from stationary phase cultures of LAC, its saeC derivative, and its ΔsaeRS mutant were comparably cytotoxic for both cell types, while mutation of sarA largely eliminated this cytotoxicity (Figure 10).

A primary reason we carried out these in vitro studies was to determine whether any of these phenotypes could be definitively correlated with differences in virulence we observed in our OM model. If so, this would greatly facilitate the ability to examine a large number of potential targets prior to proceeding to in vivo analysis. However, while biofilm formation and cytotoxicity were significantly reduced in 3 of the 4 attenuated strains, neither was significantly reduced in the ΔsaeRS mutant. One possible explanation for this is that the magnitude of the impact of mutating sarA on protease production as assessed under in vitro conditions is sufficient to be apparent in the context of biofilm formation and cytotoxicity, while the impact of mutating saeRS on protease production is not. However, this does not preclude the
An abundance was statistically significant compared to LAC. Double asterisks (**) indicate a log2 fold change of 5.0. Proteins that were present in increased amounts in a ΔsaeRS mutant relative to a ΔsarA mutant (Figure 11, Table S3).

Of these, 36 were present in equivalent amounts in CM from the ΔsaeRS mutant and LAC, thus suggesting that Spl-mediated degradation may be a limiting factor in the accumulation of these proteins in a ΔsarA mutant. This also suggests that these proteases, or specific targets of these proteases that are present in decreased amounts in CM from a ΔsarA mutant, may contribute to biofilm formation and/or cytotoxicity for osteoblasts and osteoclasts as assessed under in vitro conditions. The remaining 55 proteins were present in decreased amounts in the ΔsaeRS mutant relative to LAC and its sarC derivative. This leaves open the possibility that they contribute to the attenuation of both the ΔsaeRS and ΔsarA mutants in our murine OM model, but are unlikely to contribute to the attenuation of the ΔsaeRS mutant and not the ΔsarA mutant.

Finally, to further examine the hypothesis that mutation of saeRS limits virulence in our OM model owing primarily to its impact on protein production, while sarA does so owing primarily to its impact on protease production and the degradation of S. aureus proteins, we generated derivatives of LAC and each of these mutants with a limited capacity to produce extracellular proteases. Specifically, protease-deficient derivatives of LAC and its sarA mutant were unable to produce aureolysin, ScpA, SpdB, or any of the spl-encoded proteases, while the saeRS mutant retained the capacity to produce the spl-encoded proteases. However, as discussed above, mutation of saeRS does not result in the increased production of these proteases. Eliminating protease production restored biofilm formation and cytotoxicity in the ΔsarA mutant, but had little impact in the ΔsaeRS mutant (Figure 12). This was also true in a LAC ΔsaeRS/ΔsarA mutant. Moreover, as evidenced by visual assessment of μCT images, eliminating protease production restored virulence to a greater extent in the ΔsarA mutant than in the ΔsaeRS mutant, and enhanced the virulence of LAC itself (Figure 13). In fact, the increased virulence observed in the protease-deficient derivatives of LAC and its ΔsarA mutant resulted in broken bones to an extent that precluded accurate quantitative analysis of these μCT images.

### DISCUSSION

Osteomyelitis is a relatively infrequent form of S. aureus infection, but it is one that presents a unique clinical problem that demands an equally unique, multidisciplinary clinical approach. This also applies to infections associated with indwelling orthopedic devices, and in this respect, it is important to recognize that the number of such infections is predicted to increase dramatically in the immediate future. Indeed, it has been estimated that the number of periprosthetic joint infections associated with total hip and knee arthroplasty in the United States will surpass 60,000 by 2020 at an annual cost that will exceed $1.62 billion. This makes it imperative to develop prophylactic and therapeutic strategies that can be used to combat these infections either alone or as a means of enhancing the efficacy of conventional antibiotic therapy.

The studies we report are based on the scientific premise that a key component required for the development of such
strategies is a clear understanding of the pathogenesis of orthopedic infections that takes into consideration the specific microenvironment of bone. In this respect it is important to note that *S. aureus* is overwhelmingly the primary clinical concern based on both the frequency and severity of the infections caused by this bacterial pathogen.\(^{18,20}\) It has been demonstrated that expression of *sarA* and *saeRS* is increased during the acute and chronic phases of osteomyelitis,\(^{21,22}\) and previous reports have demonstrated that mutation of *saeRS* or *sarA* attenuates virulence in a murine model of postsurgical OM.\(^{4,5}\) This accounts for our experimental focus on these regulatory loci in this report.

In addition, the attenuation of a LAC Δ*saeRS* mutant has been correlated with the increased production of extracellular proteases, specifically aurolysin, and the resulting decrease in the accumulation of phenol-soluble modulins (PSMs), although this could not fully explain the attenuation of a LAC Δ*saeRS* mutant.\(^{4,5}\) As demonstrated in previous reports,\(^{7,23}\) and confirmed in the studies reported here, mutation of *sarA* results in a much greater increase in protease production than mutation of *saeRS*. This suggests that mutation of *sarA* would attenuate virulence in OM even by comparison to a Δ*saeRS* mutant.

To address this, we took advantage of our previous studies demonstrating that mutation of *saeRS* or *sarA* attenuates virulence to a comparable degree in a murine bacteremia model\(^6\) to define the relative virulence of LAC and five isogenic derivatives that differ with respect to the functional status of *saeRS* and *sarA* in a murine model of postsurgical OM. The results demonstrated that mutation of *saeRS* or *sarA* also attenuates virulence in this model to a comparable degree (Figure 2). The attenuation observed with the LAC Δ*sarA* mutant was reversed to a limited extent in the *saeC/sarA* mutant, but the difference was not statistically significant in the

Figure 12. Impact of protease production in Δ*sarA* and Δ*saeRS* mutants in vitro. Biofilm formation (top) and osteoblast cytotoxicity (bottom) were assessed in each of the indicated strains (+) and their protease-deficient derivatives (−). Biofilm assays were performed in 6 replicates and the average observed with LAC set to 100%. All other results, including each of the 6 individual LAC replicates, are shown relative to this value. Cell viability was determined using a Live/Dead assay kit (Molecular Probes) in which mean fluorescence intensity (MFI) is an indication of cell viability. Statistical analysis was done by one-way ANOVA with Dunnett’s correction. A single asterisk (*) indicates a significant difference relative to LAC. Double asterisks (**) indicate statistical significance relative to the Δ*sarA* and Δ*saeRS*/Δ*sarA* mutants.

Figure 13. Impact of protease production in Δ*sarA* and Δ*saeRS* mutants in vivo. A murine model of post-traumatic osteomyelitis was used to assess the relative virulence of LAC, its Δ*sarA* and Δ*sae* mutants, and protease-deficient derivatives of each strain (Δprotease). All images from all mice in each experimental group are shown for comparison along with the percentage of femurs from all animals within each group in which the femur was broken.
context of either μCT analysis or bacteriological burdens in the femur. Mutation of *saeRS* did have a greater impact than mutation of *sarA* on bacterial burdens in the femur (Figure 3). However, mutation of *saeRS* and *sarA* had an additive effect in this regard, thus suggesting that both loci contribute to the ability of *S. aureus* to colonize and persist in the bone.

Although mutation of *saeRS* and mutation of *sarA* had a comparable impact on virulence but not on protease production, the accumulation of any protein is a function of its production vs its degradation. Indeed, we previously proposed that the primary impact of mutating *saeRS* on the virulence of *S. aureus* is mediated at the level of virulence factor production while that of mutating *sarA* is mediated at the level of the protease-mediated degradation of these virulence factors. The results we report here provide further support for this hypothesis. Specifically, mutation of *saeRS* resulted in a protein profile that included the majority of proteins present in LAC and its *saeC* derivative, albeit in reduced amounts, while the protein profile of the isogenic ΔsarA mutant was characterized by a lack of high-molecular weight proteins (Figure S1). However, differences in the relative impact of extracellular proteases in a ΔsarA mutant vs a ΔsaeRS mutant do not preclude the possibility that mutation of these loci impacts the accumulation of an overlapping set of proteins that are relevant in the pathogenesis of OM. Indeed, there are reports describing transcriptional changes associated with OM, but the results we report suggest that a better approach would be to consider virulence differences in the context of protein accumulation rather than transcriptional changes alone.

To address this, we utilized a novel mass-based proteomic approach recently developed and validated in our laboratories that allows us to focus on spectral counts derived from full-length proteins to the exclusion those derived from degradation products of those proteins. The results confirmed that the accumulation of full-length proteins is significantly reduced in all four of the strains found to be attenuated in our murine OM model compared to the virulent strains LAC and its *saeC* derivative (Figure 5). Using a stringent cutoff of a log, fold-change of ≥5.0 (absolute fold-change ≥32) and each of two data analysis methods, we identified 14 proteins that were more abundant in both virulent strains by comparison to all four attenuated strains (Table 1, Figure 7). This suggests to us that these proteins are of potential interest in the pathogenesis of OM. However, we are not suggesting that these 14 proteins are the only proteins of potential interest. For instance, staphylooccolocal protein A (Spa) was not included among the priority list of 14 proteins, and it has been implicated in the pathogenesis of OM. Moreover, the abundance of Spa was reduced to a statistically significant extent in all four attenuated strains (Figure S2) and did not meet the highly stringent standards we chose to employ only because of its relatively high abundance in the ΔsaeRS mutant. Thus, it could be argued that these standards are too stringent. However, we believe that the methods we employed are appropriate in that they increase the likelihood of identifying high-priority targets that warrant further examination. In fact, we used two different data analysis methods to further increase the stringency of our approach, and this reduced this group of high-priority targets from 14 to 7 based on the fact that they were identified using both methods.

Included among these 7 proteins were the fibronectin-binding proteins FnbA and FnbB. This is potentially relevant in that these proteins have been implicated in biofilm formation, which is a key component of many types of *S. aureus* infection including OM and anilane dehydrogenase 1. The latter is a cytoplasmic protein, but this does not preclude the possibility that it may act as a ‘moonlighting’ virulence factor, particularly given that other dehydrogenases have been reported to moonlight on the cell surface promoting adhesion to extracellular matrix proteins.

Also included were the immunoglobulin binding protein Sbi and staphylocoagulase, both of which have been implicated as important components of immune evasion. Other reports have concluded that coagulase production contributes to biofilm formation and, at least as assessed under in vitro conditions, osteoblast physiology and bone destruction. As further validation of our experimental approach, we demonstrated that LAC Δcoa mutants have a reduced capacity to form a biofilm and exhibit a modest reduction in virulence in our OM model (Figure 8). The fact that mutation of *coa* had less impact on biofilm formation and virulence in our OM model than mutation of *sarA* is not unexpected given that mutation of *saeRS* limits the accumulation of many *S. aureus* proteins of potential relevance. This was also true with respect to osteoblast and osteoclast cytotoxicity, which was significantly reduced in a ΔsarA mutant but not in a Δcoa mutant (Figure S3). Nevertheless, these results suggest that coagulase does play a role in OM as previously suggested. They also suggest that the impact of mutating *saeRS* or *sarA* on the pathogenesis of OM is likely to be multifactorial.

From a mechanistic point of view, there are two considerations that we tried to take into account. The first is whether we could identify any in vitro phenotypes that could be directly correlated with virulence. This was based on the hope such phenotypes could be used to further prioritize *S. aureus* proteins of potential interest before pursuing in vivo studies. However, while there were clear correlations, none of the in vitro phenotypes we examined could be definitively correlated with relative virulence. This includes protease production, biofilm formation and cytotoxicity for osteoblasts and osteoclasts. The second consideration is the manner by which mutation of *saeRS* and *sarA* limits virulence in our OM model, and in this respect we believe the results we report provide further support for the hypothesis that mutation of *saeRS* does so by limiting the production of important virulence factors, while *sarA* does so by limiting their accumulation owing to the increased production of extracellular proteases. Thus, in effect mutation of *saeRS* vs *sarA* represent two distinct means to the same end, that being reduced virulence in the specific clinical context of osteomyelitis.

This is consistent with the observation that eliminating protease production restored virulence in the ΔsarA mutant to a greater extent than was observed in the isogenic ΔsaeRS mutant (Figure 13). In fact, eliminating the production of extracellular proteases in the ΔsarA mutant and even in LAC itself enhanced virulence in our OM model to an extent to

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which the proportion fractured bones precluded accurate quantitative μCT analysis (Figure 13). However, protein production vs degradation are not mutually exclusive functions, and this does not mean that increased protease production is irrelevant in a LAC ΔsaeRS mutant. Rather, it just suggests that the relatively modest impact of mutating saeRS on protease production may be phenotypically apparent only because the amount of many S. aureus proteins is already limited in the Δsae mutant. Nevertheless, this does not preclude the possibility that mutation of saeC and/or sarA results in the reduced accumulation of common S. aureus proteins that contribute to the pathogenesis of OM either alone or in combination with each other, and we believe the results of the experiments we report have allowed us to identify and prioritize specific proteins of interest in this regard.

At the same time, it is also possible that the attenuation of LAC ΔsarA and ΔsaeRS mutants can be attributed to the impact of these mutations on different S. aureus proteins, and the experimental approach we describe would preclude the identification of such proteins. This possibility prompted us to make proteomic comparisons between the Δsara and ΔsaeRS mutants themselves (Figure 11). The results confirmed that the abundance of 91 proteins was elevated in the ΔsaeRS mutant by comparison to the Δsara mutant. However, the abundance of the majority of these was still reduced by comparison to LAC itself. The extent to which the abundance of any given protein must be reduced to have a phenotypic impact in vivo is not known, thus leaving open the possibility that the reduced abundance of these proteins contributes to the attenuation of the ΔsaeRS mutant by comparison to LAC and its ΔsaeC derivative. However, since these 91 proteins were more abundant in ΔsaeRS than Δsara mutants, it seems unlikely they would contribute to the attenuation of the ΔsaeRS mutant but not the Δsara mutant.

In contrast, very few proteins were present in increased amounts in the Δsara mutant by comparison to the ΔsaeRS mutant. Interestingly, this did include all six of the spl-encoded proteases. This is consistent with previous reports demonstrating that the abundance of these proteases is increased in a Δsara mutant but not in a ΔsaeRS mutant.5,9 This suggests that specific targets of these proteases, or the proteases themselves, may contribute to the reduced biofilm formation and cytotoxicity observed with the Δsara mutant as assessed under in vitro conditions.

Finally, the proteomic approach we described can also be used to identify S. aureus proteins that are less likely to be involved in the pathogenesis of OM (Table S4). For instance, LukD, LukF, and LukS were all present in increased amounts in a LAC Δsara mutant by comparison to the isogenic ΔsaeRS mutant. The abundance of these proteins in the ΔsaeRS mutant was comparable to LAC itself. This suggests that these exotoxins are unlikely to contribute to the attenuation of the Δsara or ΔsaeRS mutants. With respect to LukP and LukS, this is consistent with the observation that mutation of sarA also limits virulence in the methicillin-sensitive strain UAMS–1,1, which does not encode either of these genes.

**CONCLUSION**

The results we report demonstrate that mutation of saeRS or sarA in the USA300 strain LAC attenuates virulence to a comparable degree in a murine model of postsurgical OM to a comparable degree. Our results also support the conclusion that the primary impact of mutating saeRS is mediated at the level of protein production, while that of mutating sarA is mediated at the level of protease-mediated protein degradation. Irrespective of the underlying mechanism that limits their accumulation, this opens up the possibility of identifying and prioritizing S. aureus virulence factors of potential relevance in the specific context of OM based on a correlation between their relative abundance in S. aureus strains that are demonstrably different with respect to virulence in this important clinical context. Because mutation of saeRS or sarA impacts the accumulation of a large number of possible virulence factors, prioritization is a key element of our approach, and in this regard, we purposefully applied a very stringent standard in the analysis of our proteomic comparisons. This accounts for our primary focus on 7 proteins, but it certainly does not preclude the possibility that other proteins not among this primary group are also important. Nevertheless, we believe the results we report clearly indicate that these proteins warrant direct examination as virulence factors of potential relevance in the pathogenesis of OM.

**EXPERIMENTAL SECTION**

**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 United States federal law.

**Bacterial Strains and Growth Conditions.** The bacterial strains used in this study were previously described.6,39,40 Briefly, an erythromycin-sensitive derivative of the USA300 strain LAC was used as the parent strain from which the isogenic derivatives ΔsaeC, ΔsaeRS, ΔsaeC/Δsara, Δsara, and ΔsaeRS/Δsara were generated. The ΔsaeRS/Δprotease mutant was generated by transduction of the saeRS mutation into a LAC derivative containing mutations in sspaB, scpA, and the gene encoding aureolysin (aur). These mutations were generated using the pKOR derivative pJB38 for sspaB and scpA and the pKOR1::aur construct for aur. The Δsara/Δprotease mutant was made by transduction of the sara mutation20 into a LAC derivative unable to produce these same proteases as well as those encoded by the spl operon.40 Strains were maintained at −80 °C in tryptic soy broth (TSB) containing 25% (v/v) glycerol. For each experiment, strains were retrieved from cold storage by plating on tryptic soy agar41 with appropriate antibiotic selection. Antibiotics used were erythromycin (5 μg/mL), tetracycline (5 μg/mL), kanamycin (50 μg/mL), and neomycin (50 μg/mL).

**Murine Model of Osteomyelitis.** Induction of OM was done as previously described.5,6 Briefly, 6–8 week-old C57BL/6 female mice were anesthetized and an incision made in the right hind limb to expose the femur. Using a precision needle, a unicortical defect was created at the midfemur. The intramedullary canal was inoculated via the unicortical defect with 2 μL of a bacterial suspension containing 1 × 10⁶ cells harvested from midexponential phase (OD₆₀₀ = 1.0) cultures. Muscle and skin were sutured, and the infection allowed to proceed for 14 days. After this time, mice were euthanized and the infected femurs harvested for microcomputed tomography (μCT) analysis or quantitation of the bacterial burden.

**Microcomputed Tomography (μCT).** Image acquisition and analysis were done according to protocols described elsewhere with minor modifications.6,10 Briefly, imaging was performed with the Skyscan 1174 X-ray Microtomograph.
(Bruker, Kontich, Belgium) using an isotropic voxel size of 6.7 μm, an X-ray voltage of 50 kV (800 μA) and a 0.25 mm aluminum filter. Reconstruction was carried out using the Skyscan Nrecon software. The reconstructed cross-sectional slices were processed using the Skyscan CT-analyzer software as follows: first, bone tissue was isolated from the soft tissue and background using a global thresholding (low = 85; high = 255). Using the bone-including binarized images a semi-automated protocol was run to delineate regions of interest where the reactive new bone (callus) was isolated from the cortical bone (this protocol is a morphological escalator that separates the reactive bone structures using multiple rounds of opening and closing of gaps using increasing preset diameters for each round). The resulting images were loaded as ROI and corrected by drawing inclusive or exclusive contours on the periosteal surface to keep only and strictly the cortical bone. Using these defined ROI, the volume of cortical bone was calculated, and the amount of cortical bone destruction estimated by subtracting the value obtained from each bone from the average obtained from sham operated bones inoculated with PBS. New bone formation was quantified using the subtractive ROI function on the previously delineated cortical bone-including ROI images and calculating the bone volume included in the newly defined ROI. Statistical analysis of data from each experimental group was done by one-way ANOVA with Dunnett’s correction. Separate comparisons were made with all strains relative to LAC or to its ΔsarA mutant. A p-value ≤ 0.05 was considered statistically significant.

**Bacterial Burdens in the Femur.** Bacterial loads in each femur were determined as previously reported. Briefly, femurs were separated from surrounding soft tissue, frozen in liquid nitrogen, and homogenized. Homogenized bones were resuspended in 1 mL PBS. Subsequently, homogenates were sonicated, vortexed, serially diluted and plated on TSA solidified with 1.5% agar. Colony forming units (cfu) were counted and differences between groups of mice assessed using a one-way analysis of variance (ANOVA) model. B Briefly, cfu data was logarithmically transformed prior to analysis. For samples with no bacterial counts, a number near 1 was added to each cfu value before the transformation was applied. Contrasts were defined to assess the comparisons of interest. Adjustments for multiple comparisons were made using simultaneous general linear hypothesis testing procedures. Adjusted p-values ≤ 0.05 were considered significant. Analyses were done using R (version 3.4.3, R Foundation for Statistical Computing, Vienna, Austria). Multiple comparison procedures were implemented using the R library multcomp.

**Extracellular Protease Activity.** Overnight cultures grown in 5.0 mL of TSB without antibiotic selection were standardized relative to each other based on optical density (OD_{560} = 10) and cells removed by centrifugation. Supernatants were then filter sterilized (0.2 μm) to obtain conditioned media (CM). Protease activity in these samples was assessed using the EnzChek Gelatinase/Collagenase Assay Kit (Thermo). Fluorescence was measured after 2 and 16 h of incubation. Statistical analysis was done by one-way ANOVA with Dunnett’s correction. Separate comparisons were made with all strains relative to LAC or to its ΔsarA mutant. A p-value ≤ 0.05 was considered statistically significant.

**Biofilm Formation.** These assays were done as previously described. Briefly, overnight cultures grown in biofilm media (TSB supplemented with glucose and sodium chloride) without antibiotic selection were standardized (OD_{490} = 0.05) and inoculated into a microtiter plate where the wells were coated with human plasma proteins beforehand. Biofilm formation was then assessed after 24 h. Statistical analysis was done by one-way ANOVA with Dunnett’s correction. Separate comparisons were made with all strains relative to LAC or to its ΔsarA mutant. A p-value ≤ 0.05 was considered statistically significant.

**Cytotoxicity Assay.** These assays were done according to a previously reported protocol. Briefly, MC3T3-E1 and RAW 264.7 cells were seeded into black 96 well microtiter plates with a clear bottom at densities of 10,000 and 50,000 cells/well, respectively. After 24 h the media was replaced with a 1:1 mixture of cell media and CM standardized as described above (OD_{540} = 10). Plates were incubated for 24 h. Cytotoxicity was determined using the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Thermo Fisher Scientific). Statistical analysis was done by one-way ANOVA with Dunnett’s correction. Separate comparisons were made with all strains relative to LAC or to its ΔsarA mutant. A p-value ≤ 0.05 was considered statistically significant.

**Exoprotein Profile Analysis.** Assessment of the secreted proteome was performed in triplicate as previously described. Briefly, an equal volume of standardized CM from each sample was resolved by one-dimensional SDS-PAGE and visualized by Coomassie-staining. Each gel lane was sliced into 24 equiv bands of 2 mm each. Gel bands were destained, reduced, alkylated, dehydrated, and trypsin digested. Acidified tryptic peptides were separated using reverse phase UPLC. Eluted peptides were ionized by electrospray (2.15 kV) followed by MS/MS analysis using higher-energy collisional dissociation (HCD) on an Orbitrap Fusion Tribrid mass spectrometer (Thermo) in top-speed data-dependent mode. MS/MS data were acquired using the ion trap analyzer and proteins were identified by database search using Mascot (Matrix Science, version 2.5.1) against the USA300 S. aureus database (2653 entries, GenBank accession JTJK01000002). A decoy database (based on the reverse of the protein sequences) was used in the search to calculate the FDR for the search algorithm. Scaffold (Proteome Software) was used to verify MS/MS based peptide and protein identifications (FDR < 1%; identified peptides ≥ 2). Total spectral counts for each replicate were exported from Scaffold into Microsoft Excel and R for further analysis.

Data analysis was done as previously described. Briefly, spectral count data collected from wild-type was used to locate the gel band with the maximum spectral count for a given protein. Spectral count observed in this band were added to spectral count observed in the gel bands immediately above and below to obtain total spectral count in a 3-band continuous window corresponding to the overall spectral peak for each full-length protein. A counts matrix for all samples including each of the replicates was generated based on this 3-band window. For the first analysis method the spectral count for each identified protein in each of the virulent strains was compared to the spectral count in each of the attenuated strains using two tailed t tests. Proteins with p > 0.05 were filtered out from each comparison. For the proteins with p ≤ 0.05, the fold change was determined, first with a cutoff of log2 FC ≥ 2 and then with a cutoff of log2 FC ≥ 5. The resulting lists of the proteins meeting these criteria in each pairwise comparison were then compared using Venny (version 2.1) to identify commonalities and differences.
between each set of comparisons. For the second analysis method, the spectral counts were imported into R for statistical analysis using the EdgeR Bioconductor package.\textsuperscript{13,14} The spectral counts were normalized using Trimmed Mean of M-values (TMM) prior to performing the generalized linear model quasi-likelihood ratio test. Data visualization images were generated using R studio.

**Mutation of coa.** The mutated coa gene was moved to LAC via transduction from a donor strain obtained from the Nebraska Transposon Mutant Library (NTML)\textsuperscript{13} through BEI Resources (Manassas, VA; http://www.beiresources.org). The isogenic LAC Δcoa mutants were validated with a PCR validated by PCR using primers specific for the coa gene (5′ GCTAGGCGCATTAGCAGTTG and 3′ TCGTAACCTCT- TTCCGCGTGCT). These oligos bind to sites flanking the transposon insertion site. The genetic background of these mutants was also verified with a PCR specific for the small cryptic plasmid present in LAC and absent in the plasmid curated LAC derivative strain, JE2, in which the NTML was generated (data not shown). The primers used for this PCR were 5′ CCGAGGCCAAGGTCAAATA, 3′ GCAGT-TGGTGGAACTCAAA.

**ASSOCIATED CONTENT**

\* Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.9b00291.

Tables S1–S4; Figures S1–S3 (PDF)

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Conceived and designed the experiments: AR, KB, MS. Performed the experiments: AR, KB, SB, CW, AJ. Analyzed the data: AR, SB, CW, AJ, HS, MS. Wrote the paper: AR, KB, MS.

**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS**

OM, osteomyelitis; sarA, staphylococcal accessory regulator; saeRS, S. aureus exoprotein regulatory locus; PSMs, phenol-soluble modulins; Spa, taphylococcal protein A; CM, conditioned media; μCT, microcomputed tomography; FC, fold-change; gmlQLT, generalized linear model quasi-likelihood; NTML, Nebraska Transposon Mutant Library; coa, staphylocoagulase.

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