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Impact of the functional status of *saeRS* on *in vivo* phenotypes of *Staphylococcus aureus sarA* mutants

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SUMMARY

We investigated the *in vivo* relevance of the impact of *sarA* and *saeRS* on protease production using derivatives of the USA300 strain LAC. The results confirmed that mutation of *saeRS* or *sarA* reduces virulence in a bacteremia model to a comparable degree. However, while eliminating protease production restored virulence in the *sarA* mutant, it had little impact in the *saeRS* mutant. Additionally, constitutive activation of *saeRS* (*saeRS*^{C}) enhanced the virulence of LAC and largely restored virulence in the isogenic *sarA* mutant. Based on these results, together with our analysis of the representative virulence factors alpha toxin, protein A (Spa), and extracellular nucleases, we propose a model in which the attenuation of *saeRS* mutants is defined primarily by decreased production of such factors, while constitutive activation of *saeRS* increases virulence, and reverses the attenuation of *sarA* mutants, because it results in both increased production and decreased protease-mediated degradation of these same factors. This regulatory balance was also apparent in a murine model of catheter-associated infection, with the results suggesting that the impact of *saeRS* on nuclease production plays an important role during the early stages of these infections that is partially offset by increased protease production in *sarA* mutants.

Keywords

Staphylococcus; protease; *saeRS*; *sarA*; bacteremia; biofilm

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INTRODUCTION

Infections caused by *Staphylococcus aureus* are of extreme clinical significance based on both their frequency and severity (Otto, 2012). Moreover, the ability to treat these infections is increasingly compromised by reduced susceptibility of many strains, if not outright resistance, to currently available antibiotics including vancomycin (Holmes *et al.*, 2012). Biofilm-associated infections are a specific concern because they do not respond adequately to antimicrobial therapy irrespective of the resistance status of the offending *S. aureus* strain (Romling and Balsalobre, 2012). One way to overcome these limitations would be to develop new antibiotics with efficacy against the most problematic resistant strains even in the context of a biofilm, but this has proven a difficult task, particularly at a time when pharmaceutical companies have de-emphasized antibiotic development (Boucher *et al.*, 2009). An ancillary approach would be to develop methods to limit biofilm formation itself, thereby enhancing the therapeutic efficacy of both existing and any newly developed antibiotics.

Given the multifactorial nature of *S. aureus* biofilm formation (Archer et al., 2011, Laverty et al., 2013, Otto, 2008), we have placed a primary focus on targeting regulatory elements that modulate the production of critical virulence factors rather than the virulence factors themselves. A number of regulatory loci have been implicated in this regard, but to date few of these have been explored in the context of their therapeutic relevance *in vivo*. One exception is the staphylococcal accessory regulator (*sarA*), mutation of which limits biofilm formation in a murine model of catheter-associated infection to a degree that can be correlated with increased antibiotic susceptibility under both *in vitro* and *in vivo* conditions (Weiss *et al.*, 2009, Weiss *et al.*, 2009). Moreover, with the exception of the 8325-4 strain RN6390 and Newman, both of which can be explained by well-defined regulatory defects, the impact of *sarA* on biofilm formation is consistent in diverse clonal lineages of *S. aureus* including both methicillin-susceptible and methicillin-resistant strains (Beenken *et al.*, 2003, Beenken *et al.*, 2010, Mrak *et al.*, 2012, Zielinska *et al.*, 2011).

Mutation of *sarA* results in the increased production of extracellular proteases, and we have confirmed that this limits the accumulation of multiple virulence factors (Mrak *et al.*, 2012, Tsang *et al.*, 2008, Zielinska *et al.*, 2011, Zielinska et al., 2012). This includes the fibronectin-binding protein FnbA, protein A (Spa), alpha toxin, and phenol-soluble modulins (PSMs) (Mrak *et al.*, 2012, Zielinska *et al.*, 2011), all of which have been implicated in various aspects of *S. aureus* pathogenesis including biofilm formation (Anderson *et al.*, 2012, Caiazza *et al.*, 2003, Cassat *et al.*, 2013, Claro *et al.*, 2013, Edwards *et al.*, 2012, Lower *et al.*, 2011, Kobayashi *et al.*, 2011, Merino *et al.*, 2009, O'Neill *et al.*, 2008, Otto, 2010, Periasamy *et al.*, 2012). A number of reports have also confirmed that proteases, including some derived from sources other than *S. aureus*, limit biofilm formation and promote dispersal from an established biofilm (Boles and Horswill, 2008, Chen *et al.*, 2013, Mootz *et al.*, 2013, Park *et al.*, 2012, Sugimoto *et al.*, 2013).

We also confirmed that eliminating the ability to produce extracellular proteases increases the virulence of *sarA* mutants in a murine bacteremia model (Zielinska *et al.*, 2012). *S. aureus* proteases have been shown to degrade specific components of host defense systems

including complement (Jusko *et al.*, 2014, Kantyka *et al.*, 2013, Zdzalik *et al.*, 2012), but if these host proteins were the relevant *in vivo* targets, it would be anticipated that eliminating protease production would result in decreased rather than increased virulence and, conversely, that the increased production of proteases in *sarA* mutants would result in increased rather than decreased virulence owing to compromised host defenses. Thus, the more likely explanation is that the decreased accumulation of specific *S. aureus* virulence factors accounts for the attenuation of *sarA* mutants and, conversely, that restoration of these factors in protease-deficient *sarA* mutants accounts for this *in vivo* "phenotypic complementation" (Zielinska *et al.*, 2012). Thus, these results suggest that inhibitors of *sarA* could potentially be used in conjunction with conventional antibiotics to enhance the efficacy of conventional antibiotics to overcome the therapeutic recalcitrance that characterizes biofilm-associated infections.

However, *sarA* is part of a large, highly interactive regulatory network, several components of which also modulate protease production (Oscarsson *et al.*, 2006). This raises the possibility that differences in the functional status of other regulatory loci could impact *sarA*-defined *in vivo* phenotypes as well as therapeutic strategies targeting *sarA*. Among these is the *saeRS* two-component regulatory system, mutation of which also enhances protease production. Although this effect is modest by comparison to that associated with mutation of *sarA* (Mrak *et al.*, 2012), the increased production of aureolysin in a LAC *saeRS* mutant was recently shown to have a dramatic impact on bone remodeling in osteomyelitis (Cassat *et al.*, 2013), thus raising the possibility that *saeRS* would also be a viable target for therapeutic intervention.

Perhaps more importantly, the commonly-studied strain Newman has a point mutation in *saeS* that enhances phosphorylation of the SaeR response regulator (Mainiero *et al.*, 2010, Schäfer *et al.*, 2009), and we have confirmed that this "constitutive activation" limits protease activity even in an isogenic *sarA* mutant (Mrak *et al.*, 2012, Zielinska *et al.*, 2011). Indeed, Newman is one of the few *S. aureus* strains in which mutation of *sarA* has relatively little impact on biofilm formation (Beenken *et al.*, 2003). Newman also has other defects of potential relevance in biofilm formation, most notably mutations that prevent anchoring of the fibronectin-binding proteins to the cell surface (Grundmeier *et al.*, 2004), and this compromises the ability to address these issues in derivatives of Newman itself. Indeed, we confirmed that restoration of surface-anchored FnbA greatly enhances biofilm formation in Newman, but that the impact of mutating *sarA* on biofilm formation is still not evident unless the defect in *saeS* was repaired (Mrak *et al.*, 2012). This suggests that the point mutation in *saeS* (L18P), or other changes that result in increased production and/or activity of SaeR, could compromise therapeutic strategies targeting *sarA*. However, these previous studies were limited to *in vitro* experiments, and it remains unclear whether the functional status of *saeRS* impacts *sarA* phenotypes *in vivo*. To avoid the limitation imposed by a lack of surface-anchored fibronectin-binding proteins in Newman, we chose to address this using the contemporary clinical isolate LAC.

RESULTS

To generate derivatives of LAC that differ with respect to the functional status of *saeRS* and *sarA*, we first mutated *saePQRS* (hereinafter referred to as *saeRS*) using the pKOR1 mutagenesis system (Bae and Schneewind, 2006, Mrak *et al.*, 2012). This mutant was then complemented by chromosomal insertion of version of *saeRS* from Newman (Schäfer *et al.*, 2009) as previously described (Mrak *et al.*, 2012). The relevant mutation is a point mutation in *saeS* (L18P), which results in increased phosphorylation of SaeR, a phenotype that we refer to hereinafter as constitutive activation (*saeRS*^{C}). We then introduced a *sarA* mutation the *saeRS* mutant and its *saeRS*^{C} derivative using phage-mediated transduction from an existing LAC *sarA* mutant (Zielinska *et al.*, 2012). Analysis of these mutants by qRT-PCR confirmed that levels of the *saeR* transcript were increased in the *saeRS*^{C} derivative by comparison to the LAC parent strain in both the post-exponential (OD_{560} = 3.0) and stationary (overnight) growth phases (Fig. 1A). Western blot data examining the accumulation of SaeR itself was more difficult to interpret owing to greater variability between repetitive experiments, which we attribute to differences in the background observed in blots done with this antibody (Fig. 1B, lanes 3 and 6), thus making it difficult to achieve statistical significance. Nevertheless, examination of the collective results from these replicates clearly indicated that accumulation of SaeR during the stationary growth phase was higher in the *saeRS*^{C} derivative than the WT parent strain (Fig. 1B, lanes 1 vs. 2). Additionally, the absence of the *saeR* transcript (Fig. 1A), and SaeR itself beyond this background (Fig. 1B), were also confirmed in the *saeRS* mutant.

By comparison to the *saeRS*^{C} derivative, accumulation of the *saeR* transcript was reduced in *sarA* and *saeRS*^{C}*/sarA* mutants irrespective of growth phase, thus suggesting that *sarA* functions at some level upstream of *saeRS*. To the extent that *sarA* is upstream of the accessory gene regulator (*agr*) (Chien *et al*, 1998), while *agr* is upstream of *saeRS* (Novick and Jiang, 2003), one possible explanation for these results is that the impact of *sarA* on accumulation of the *saeR* transcript is due to reduced transcription via an indirect effect mediated through *agr*. However, accumulation of SaeR was higher in the *saeRS*^{C}*/sarA* mutant by comparison to the isogenic *sarA* mutant (Fig. 1B, lanes 4 vs. 5) despite comparable levels of the *saeR* transcript. This suggests that SaeR accumulation is limited in *sarA* mutants owing to some post-transcriptional mechanism, the impact of which is limited by constitutive activation of *saeRS*. This is consistent with the observation that, while accumulation of SaeR in the *saeRS*^{C}*/sarA* mutant was higher by comparison to the *sarA* mutant, it was lower in the *saeRS*^{C}*/sarA* mutant by comparison to the isogenic *saeRS*^{C} derivative itself (Fig. 1B, lanes 4 vs. 1).

One possible explanation for these results is that the relative level of extracellular proteases defines these phenotypes, and further analysis confirmed that overall protease production increases as the functional status of both *saeRS* and *sarA* decreases (Fig. 2A). However, these effects were not equivalent, with the impact of decreased *sarA* function playing the predominant role. Most importantly, protease activity was significantly increased *saeRS*^{C}*/ sarA* mutant by comparison to the isogenic *saeRS* mutant, but significantly decreased by comparison to the isogenic *sarA* mutant (Fig. 2A). This suggests that the increased accumulation of SaeR in the *saeRS*^{C} mutant by comparison to the *saeRS*^{C}*/sarA* mutant (Fig.

1B, lanes 1 vs. 4), and the increased accumulation of SaeR in the *saeRS*^{C}/*sarA* mutant by comparison to its isogenic *sarA* mutant (Fig. 1B, lanes 4 vs. 5), could both be explained by the impact of these loci on the production of extracellular proteases.

To investigate this, we used derivatives of LAC and its *sarA* mutant that are unable to produce any of the 10 recognized extracellular proteases (Zielinska *et al.*, 2012). We also generated a derivative of the *saeRS* and *saeRS*^{C} mutants with additional mutations in the genes encoding aureolysin, SspA, SspB and ScpA. We did not mutate the *spl* operon in these strains for two reasons. First, both the *spl* mutation and the chromosomally inserted constitutively-active variant of *saeRS* are marked by erythromycin resistance, thus precluding transduction of one into the other. Second, and more importantly, mutation of *sarA* results in increased accumulation of all 10 extracellular proteases (Zielinska et al, 2012), while mutation of *saeRS* results in increased accumulation of aureolysin, SspA, SspB, and ScpA but decreased accumulation of the *spl*-encoded proteases (Cassat *et al.*, 2013), thus suggesting that the functional status of the *spl* operon is unlikely to play a distinguishing role between these strains with respect to overall protease activity. This was confirmed by demonstrating that proteolytic activity was reduced to baseline levels in all protease mutants irrespective of the functional status of the *spl* operon (Fig. 2). Importantly, this was assessed using a casein-based protease assay, which is a known substrate of the *spl*-encoded proteases (Reed *et al.*, 2001). Thus, we consider these protease-deficient derivatives equivalent in the context of the experiments we describe.

Western blot comparisons of whole cell lysates from these strains confirmed that the absence of SarA in all *sarA* mutants and that accumulation of SarA was unaffected by the functional status of *saeRS* (Fig. 2B), a result that would also be expected if *saeRS* is downstream of *sarA*. Accumulation of SaeR was higher in the *saeRS*^{C}, *sarA*-positive derivatives than in the isogenic *saeRS*^{C}/*sarA* mutants irrespective of the ability to produce extracellular proteases (Fig. 3A, lanes 1 and 2 vs. lanes 3 and 4), although as with our other SaeR western blots (Fig. 1B) these differences, while readily apparent, did not reach statistical significance. This *sarA*-dependent difference was not apparent in the *saeS*-repaired strains (*saeRS*+) (Fig. 3A, lanes 9 and 10 vs. lanes 11 and 12). Accumulation of SaeR in the *saeRS*+ *sarA* mutant (Fig. 3A, lane 11) appeared to be reduced by comparison to its protease-deficient derivative (Fig. 3A, lane 12), but this difference, while reproducible, did not reach statistical significance. These results demonstrate that, whatever the mechanism involved, the impact of mutating *sarA* on the accumulation of SaeR is limited by constitutive activation of *saeRS*. They also suggest that, while the increased production of extracellular proteases in *sarA* mutants has some impact on the accumulation of SaeR, the primary effect is mediated at the level of transcription and/or mRNA stability.

Based on the current *S. aureus* regulatory paradigm indicating that *sarA* is upstream of *agr* while *agr* is upstream of *saeRS*, we also extended our studies to evaluate accumulation of AgrA as a function of the functional status of *sarA* and *saeRS*. In these experiments, mutation of *saeRS* was found to have no impact on the accumulation of AgrA (Fig. 3B, lanes 5 and 6 vs. 9 and 10), as would expected based on the linear regulatory paradigm outlined above. However, mutation of *sarA* resulted in decreased accumulation of AgrA, and in both the *saeS* mutant and its *saeS*-repaired derivative this could be correlated to a

statistically significant degree with the increased production of extracellular proteases (Fig. 3B, lanes 7 vs. 8 and lanes 11 vs. 12). This is consistent with our previous proteomics comparisons in which the amount of AgrA observed in a LAC *sarA* mutant was reduced by comparison to the parent strain and partially restored in the protease-deficient *sarA* derivative (Zielinska *et al.*, 2012). As with the impact of mutating *sarA* on accumulation of *SaeR*, these effects were also overcome by constitutive activation of *saeRS* (Fig. 3B, lanes 1–4).

Thus, one explanation for these results is that mutation of *sarA* results in reduced accumulation of AgrA owing to protease-mediated degradation, with this in turn resulting in reduced transcription of *saeRS*, both of which are largely overcome as might be expected by constitutive activation of *saeRS*. These results demonstrate that the impact of *sarA* on the accumulation of AgrA is not mediated entirely by its impact on the production of AgrA (Chien *et al*, 1998), but also indirectly by the increased production of extracellular proteases in *sarA* mutants (Fig. 3B, lane 12). This further emphasizes both the complexity of *S. aureus* regulatory circuits and the potential importance of *sarA* in repressing the production of extracellular proteases as a means of maintaining the integrity of these circuits.

To further examine the potential significance of these results, we also examined the accumulation alpha toxin and protein A (Spa) in these mutants. We chose these as representative virulence factors because their production and/or accumulation has been shown to be impacted by both *sarA* and *saeRS* (Mainiero *et al.*, 2010, Mrak *et al.*, 2012). The results confirmed that mutation of *saeRS* alone limits the production and/or accumulation of both of these virulence factors irrespective of the ability to produce extracellular proteases (Fig. 4A and 4B, lanes 5 and 6), with this effect being essentially absolute in the case of alpha toxin (Fig. 4A, lanes 5 and 6). They also demonstrated that mutation of *sarA* has the same phenotypic effect, but in this case the accumulation of both alpha toxin and Spa was restored in the protease-deficient *sarA* mutants (Fig. 4A and 4B, lanes 11 and 12). Accumulation of Spa, but not alpha toxin, was also restored in the protease-deficient *saeRS/sarA* mutant (Fig. 4A and 4B, lanes 7 and 8).

Accumulation of alpha toxin was also restored in the *saeRS*^{C}*/sarA* mutant irrespective of the ability to produce extracellular proteases (Fig. 4A, lanes 3 and 4), thus suggesting that the impact of *saeRS* on the alpha toxin phenotype is in fact mediated at the level of its production. Conversely, the impact of increased protease production in *sarA* mutants on the accumulation of Spa was evident irrespective of the functional status of *saeRS* (Fig. 4B, lanes 3 vs. 4, lanes 7 vs. 8, and lanes 11 vs. 12). We conclude that the impact of *sarA* on the degradation of Spa is phenotypically epistatic to that of *saeRS* on the production of Spa, but that the opposite is true with respect to alpha toxin.

While the results of the experiments discussed above are informative, they are also based on *in vitro* studies, and under such circumstances it would be anticipated that the impact of extracellular proteases would be magnified, perhaps to the point of biological insignificance, owing to the proximity of the proteases on their *S. aureus* targets imposed by the constrained environment of a test tube. Thus, to determine the *in vivo* relevance of these results, we used a murine bacteremia model to examine the relative impact of the functional status of *saeRS*

and *sarA*. While a primary focus of our work is on the impact of these loci in biofilm formation, we chose to use this model first based on the logic that any such physical constraint would be minimized in the dynamic context of blood flow. The results confirmed that mutation of *sarA* or *saeRS* dramatically decreased the virulence of LAC as assessed by almost every *in vivo* parameter examined, the only exception being bacterial burdens in the kidney, which were reduced in the *sarA* mutant but not in the *saeRS* mutant (Figs. 5 and 6). Concomitant mutation of *sarA* and *saeRS* also appeared to have an additive effect, although in most cases this was difficult to assess owing to the impact of mutation of either locus alone.

Conversely, constitutive activation of *saeRS* reversed the attenuation of *sarA* mutants to a statistically significant degree in all tissues (Fig. 6). This was not evident as assessed based on survival curves, but this could be potentially be explained by the time frame of the experiments themselves (Fig. 5). Indeed, in this context, constitutive activation of *saeRS* increased the virulence even of LAC itself. These same general trends were evident when virulence was assessed based the ability to cause secondary bone and joint infection, although the only statistically significant difference as assessed based on overall histopathology scores was the difference between the results observed with the constitutively active variant of LAC and the *sarA/saeRS* double mutant (Fig. 7). Thus, when taken together, these results are consistent with the hypothesis the impact of *sarA* and *saeRS* on virulence in this model is mediated, at least in part, via different mechanisms. This was confirmed by demonstrating that eliminating protease production reversed the attenuation of *sarA* mutants in this model, but had little impact in *saeRS* mutants, with the only significant difference observed between the *saeRS* mutant and its protease-deficient derivative being bacterial burdens in the spleen (Fig. 8).

Mutation of *saeRS* or *sarA* also limited the capacity of LAC to form a biofilm *in vitro*, and in this case the biofilm-deficient phenotype of both mutants was reversed to a comparable degree by eliminating their capacity produce extracellular proteases (Fig. 9A). This was also true in the *saeRS/sarA* and $saeRS^{C}$/*sarA* mutants, although in the latter strain the impact of eliminating protease production had a reduced effect by comparison to all of these other strains. This is consistent with the fact that this strain also produced reduced amounts of extracellular proteases relative to these strains (Fig. 2A). Interestingly, unlike the bacteremia model in which mutation and constitutive activation of *saeRS* had opposite effects as would be expected, constitutive activation of *saeRS* also limited biofilm formation, albeit to a lesser degree than that observed with the isogenic *saeRS* and *sarA* mutants. Additionally, this phenotype was not altered in the protease-deficient $saeRS^{C}$ derivative (Fig. 9A). One possible explanation for this is the observation that extracellular nucleases were recently shown to be produced in reduced amounts in *saeRS* mutants (Olson *et al.*, 2013). Although this previous report did not examine the impact of constitutive activation of *saeRS* on nuclease production, we confirmed that it is increased and that this is not impacted by eliminating the production of extracellular proteases (Fig. 9B). Thus, to the extent that nucleases limit biofilm formation *in vitro* (Beenken *et al.*, 2012, Tsang *et al.*, 2008), this could explain both the reduced capacity of the $saeRS^{C}$ mutants to form a biofilm and the reduced impact of eliminating the protease production in this strain.

We recently demonstrated that the impact of extracellular nucleases differs dramatically under *in vitro* vs. *in vivo* conditions (Beenken *et al.*, 2012), and based on this we also examined the impact of the functional status of *saeRS* and *sarA in vivo* using a murine model of catheter-associated biofilm formation (Weiss *et al.*, 2009). In LAC itself, mutation of *saeRS* resulted in a slight increase in the capacity to form a biofilm by comparison to its isogenic *saeRS*^{C} variant (Fig. 10). These relative effects were not evident in the isogenic *sarA* mutants. In fact, unlike the results observed in our previous study (Zielinska et al., 2012), mutation of *sarA* in LAC had relatively little impact on biofilm formation. However, it did reverse the increase in biofilm formation observed in the isogenic *saeRS* mutant (Fig. 10). To some degree, these results must be interpreted with caution by comparison to our earlier report in that these experiments had to be stopped earlier (3 vs. 5 days) owing to the hypervirulence of the *saeRS*^{C} derivative even by comparison to LAC itself (Fig. 5), which resulted in dramatic skin lesions and the rapid loss of subcutaneously implanted catheters (data not shown). Nevertheless, one potential interpretation of these results, particularly given the impact of the functional status of *saeRS* on nuclease production (Fig. 9B), is that nucleases are an important limiting factor in biofilm formation during the early stages of biofilm formation *in vivo* at a time when the impact of mutating *sarA* on protease production has not yet become fully evident. This is consistent with the observation that biofilm formation was increased, and nuclease production decreased, in the *saeRS* mutant, while the opposite phenotypes were observed in the *saeRS*^{C} mutant (Fig. 9). Presumably, the impact of *sarA* on protease production would predominate relative to that of nucleases at later time points both because they would accumulate to higher levels and because the absence of nucleases would become detrimental has the host mounts a response (Berends *et al.*, 2010). Nevertheless, the fact that mutation of *sarA* reversed the impact of mutating *saeRS* suggests that proteases are playing some role even at this early stage of biofilm formation *in vivo*.

DISSCUSION

We previously demonstrated that mutation of *sarA* attenuates the virulence of the USA300 strain LAC in murine models of both bacteremia and implant-associated infection and that this was due in part to the increased production of extracellular proteases (Zielinska *et al.*, 2012). We also demonstrated that expression of both *saeRS* and *sarA* is associated with reduced production of extracellular proteases (Mrak *et al.*, 2012). Mutation of *saeRS* had a less significant impact on protease activity than that of *sarA*, but it was nevertheless correlated with a reduced capacity to form a biofilm at least under *in vitro* conditions. This suggests that *saeRS* and *sarA* may be viable therapeutic targets based on the common mechanistic theme of their ability to repress protease production, perhaps to the point that therapeutic efficacy could be maximized by developing and exploiting therapeutic strategies that target the intersection of these two regulatory pathways. At the same time, it also raises the possibility that constitutive activation of *saeRS* could repress protease production to an extent that could compromise therapeutic strategies targeting *sarA.* Addressing these issues under *in vivo* conditions was the primary motivation behind the experiments we report.

To these ends, we generated derivatives of the USA300 strain LAC that differ in the functional status of both *saeRS* and *sarA*. We also generated derivatives of each that were unable to produce the most relevant extracellular proteases. These strains were compared

using both *in vitro* methods and *in vivo* models of bacteremia and catheter-associated biofilm formation. The results confirmed that both *saeRS* and *sarA* repress the overall production of extracellular proteases, but they also confirmed that *saeRS* plays a modest role in this regard by comparison to *sarA* (Mrak *et al.*, 2012). Mutation of either locus severely attenuated the virulence of LAC in a bacteremia model, but the results we present suggest that this does not occur via the common mechanism of increased protease production. Specifically, eliminating protease production restored virulence in the *sarA* mutant, but not in the *saeRS* mutant. Rather, the reduced virulence of the *saeRS* mutant appeared to be a function of the reduced production of critical virulence factors, the specific examples we examined being alpha toxin, protein A, and extracellular nucleases. The possibility that the impact of *sarA* and *saeRS* is mediated via different mechanisms is consistent with the observation that concomitant mutation of *sarA* and *saeRS* appeared to have an additive effect. Although this was difficult to discern owing to the dramatic impact of mutating each locus alone, this nevertheless suggests an important regulatory balance between *saeRS* and *sarA*, with the first being defined primarily by the production of such virulence factors, and the second being defined primarily by their protease-mediated degradation.

One caveat in this regard is that we eliminated the production of all 10 extracellular proteases in the *sarA* mutant, but we did not eliminate production of the *spl*-encoded proteases in the LAC *saeRS* mutant or it's constitutively active *saeRS*^{C} variant. This leaves open the possibility that the failure to restore virulence in the *saeRS* mutant could be due to the continued production of the *spl*-encoded proteases. However, while mutation of *saeRS* or *sarA* results in an overall increase in protease production, the regulatory impact of these loci is not equivalent in that mutation of *saeRS* results in decreased accumulation of the *spl*-encoded proteases, while mutation of *sarA* has the opposite effect (Cassat *et al.*, 2013, Zielinska *et al.*, 2012). This is consistent with our demonstration that overall protease production was reduced to baseline levels in all protease-deficient mutants including those in which the spl operon was left intact. Thus, it seems unlikely that the production of *spl*-encoded proteases could account for the failure to restore virulence in the *saeRS* mutant by eliminating the production of aureolysin, SspA, SspB, and ScpA, all of which are produced in increased amounts in both *sarA* and *saeRS* mutants (Cassat *et al.*, 2013).

We would also note that, while we did observe some differences in overall growth rate among these strains, they were minor with respect to both growth rate and overall yield (Fig. 11). Moreover, there was no correlation between these differences and relative virulence. For instance, the *sarA* and protease-deficient *sarA* mutants grew at comparable rates, but differed dramatically with respect to virulence. Similarly, the *saeRS*^{C} derivative grew somewhat slower than the isogenic *saeRS* mutant, but exhibited greater virulence (Fig. 5). Thus, based on these collective results, we propose a model in which the attenuation of *saeRS* and *sarA* mutants occurs via different mechanisms, with the impact of *saeRS* being primarily transcriptional and that of *sarA* being primarily a function of the increased production of extracellular proteases (Fig. 12). However, these are clearly not the exclusive functions of either locus. For example, a recent report demonstrated that mutation of *saeRS* in LAC limits virulence in a murine osteomyelitis model owing in part to the aureolysin-mediated degradation of phenol-soluble modulins (PSMs) and its impact on osteoblast

function and bone remodeling (Cassat *et al.*, 2013), thus suggesting that the impact of *saeRS* as a repressor of protease production does in fact play an important role in this specific clinical context. Nevertheless, our results confirm that these different regulatory functions can be distinguished from each other in the context of bacteremia and, to a lesser extent, catheter-associated biofilm formation *in vivo*.

Indeed, our results suggest that the regulatory balance between *saeRS*-mediated production and *sarA*-mediated degradation can be extended to a tissue-specific context. Specifically, mutation of *saeRS* limited bacterial burdens after systemic infection in the spleen and heart, but had no impact in the kidney. Although the combined effect of mutating *sarA* and *saeRS* was evident in all of these tissues, as well as the relative capacity to cause hematogenous bone and joint infection, it was less evident in the kidney than in the spleen and heart. Similarly, eliminating protease production in a LAC *saeRS* mutant increased virulence as assessed by colony counts in the spleen but had little impact in the heart or kidney. Such tissue-specific results have been reported in other contexts including the relative capacity to produce specific heme-dependent terminal oxidases (Hammer *et al.*, 2013). In fact, to the extent that osteomyelitis is a biofilm-associated infection (Brady *et al.*, 2008), this could explain why we observed relatively little impact of mutating *saeRS* in our murine catheter-model in that the impact of PSMs on osteoblast viability would presumably not be a factor in this model. Thus, these collective results suggest that *saeRS* and *sarA* could both be viable therapeutic targets owing to their impact on protease production. However, given that mutation of *sarA* has a much greater impact on protease production than mutation of *saeRS* (Mrak *et al.*, 2012), it will be important to assess the relative impact of mutating each of these loci, both alone and in combination with each other, in the specific context of osteomyelitis. Given the results we report, it also remains imperative to determine the extent to which constitutive activation of *saeRS* impacts virulence, and whether this has a compromising effect in isogenic *sarA* mutants, in this clinical context.

Finally, while we previously demonstrated that the increased production of extracellular proteases limits the accumulation of a large number of both surface-associated and extracellular virulence factors in *sarA* mutants (Mrak *et al.*, 2012, Zielinska *et al.*, 2012), this is the first report we are aware of suggesting that it also limits accumulation of critical intracellular proteins including the response regulators SaeR and AgrA. Our experiments were done with cell lysates prepared from stationary phase cultures, which leaves open the possibility that this reflects the impact of extracellular proteases on intracellular proteins released into the extracellular environment owing to cell lysis during processing. These lysates were prepared from washed cells, which would presumably limit this possibility, but given our focus on *S. aureus* proteases it was not possible to include protease inhibitors during processing, thus it cannot be eliminated entirely. Nevertheless, these results at least raise the possibility that the increased production of extracellular proteases in *sarA* mutants, or more likely the dysregulation of the protease activation cascade (Shaw et al., 2004), may well impact the accumulation of intracellular as well as extracellular proteins, thus further complicating the regulatory balance between the *saeRS*-mediated production of virulence factors and the *sarA*-mediated protease degradation of these same virulence factors. At the same time, if this is ultimately proven to be true, it suggests that inhibitors of *sarA* could

limit the positive regulatory functions of AgrA and SaeR, both of which make important contributions to the overall virulence of *S. aureus*.

EXPERIMENTAL PROCEDURES

Ethics Statement

All animal experiments were done in accordance with the policies of the Public Health Service (PHS) policy in the Care and Use of Laboratory Animals, the Animal Welfare Act, and the NIH Guide for the Care and Use of Laboratory Animals in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) internationally accredited facility. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences.

Bacterial strains and growth conditions

The *S. aureus* strains used in this study included a plasmid-cured, erythromycin-sensitive derivative of the USA300 strain LAC (Wörmann *et al.*, 2011). To generate derivatives of this strain that differ with respect to the functional status of *saeRS*, we mutated *saeRS* using the pKOR1 mutagenesis system (Bae and Schneewind, 2006, Mrak *et al.*, 2012) then complemented this strain by chromosomal insertion of the constitutively active version of *saePQRS* (*saeRS*^{C}) from Newman (Luong and Lee *et al.*, 2007, Mainiero *et al.*, 2010, Schäfer *et al.*, 2009). Isogenic *sarA* and *agr* mutants were generated by Φ11-mediated transduction from existing mutants (Zielinska *et al.*, 2012, Blevins *et al.*, 1999). Mutations in *aur*, *scpAB*, and *sspABC* were generated using the pKOR1 system, while mutation of the *spl* operon was done by Φ11-mediated transduction from existing mutants (Zielinska *et al.*, 2012 and Wörmann *et al.*, 2011).

Strains were maintained at −80°C in tryptic soy broth (TSB) containing 25% (vol/vol) glycerol. For each experiment, strains were retrieved from cold storage by plating on tryptic soy agar (TSA) with appropriate antibiotic selection. Antibiotics were used at the following concentrations: erythromycin (Erm; 5 μg per ml), tetracycline (Tet; 5 μg per ml), kanamycin (Kan; 50 μg per ml), and neomycin (Neo; 50 μg per ml), with kanamycin and neomycin being used together to avoid selection for spontaneous mutants. For phenotypic assays, strains were inoculated into TSB or biofilm media, as specified, at an initial optical density at 560 nm (OD_{560}) of 0.05 and to the post-exponential (OD_{560} = 3.0) or stationary (16 hr) growth phases. All cultures employed for phenotypic assays were grown without antibiotic selection at 37°C with constant aeration and a medium-to-flask volume ratio of 0.40.

Production of extracellular proteases

Protease activity in standardized samples of conditioned medium from stationary phase cultures was assessed using the casein-based Protease Fluorescent Detection Kit (Sigma, St. Louis, MO) with a 1-hour incubation period (Zielinska *et al.*, 2012).

Western Blotting

Accumulation of alpha-toxin and Spa were assessed using standardized samples of conditioned medium from stationary phase cultures. SaeR and AgrA production were

assessed using whole cell lysates prepared from intact cells harvested from stationary phase cultures. Western blots were done in triplicate with different biological replicates using appropriate rabbit polyclonal IgG antibodies as previously described (Blevins *et al.*, 1999, Mrak *et al.*, 2012, Zielinska *et al.*, 2012,). Blots were blocked with 0.5% skim milk containing 0.1 mg/ml human IgG (Sigma Chemical Co., St. Louis, MO).

Nuclease Activity

Nuclease activity was assessed using a fluorescence resonance energy transfer (FRET)-based assay (Beenken *et al.*, 2012). Briefly, 25 μl sterilized supernatants from stationary phase cultures (16-hour) were mixed with an equal volume of FRET substrate diluted to 2 μM in buffer consisting of 20 mM Tris, pH 8.0, and 10 mM $CaCl_2$. Results were assessed after 15 min at 30°C using a BioTek Synergy 2 microtiter plate reader (BioTek Instruments, Winooski, VT) with an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

Transcriptional analysis

To assess the levels of *saeR*, total bacterial RNA was isolated using the Qiagen RNeasy Mini Kit. Quantitative, real-time RT-PCR was performed using primers and TaqMan probes corresponding to *saeR* as previously described (Mrak *et al.*, 2012). Results were calibrated by comparison to those obtained with the same RNA samples with the 16S ribosomal RNA gene (Zielinska *et al.*, 2011). Results are reported as relative units by comparison to the results observed with the parent strain, the value for which was set to 1.0.

Assessment of biofilm formation *in vitro*

Biofilm formation was assessed *in vitro* using a microtiter plate assay in which the wells were first coated with human plasma proteins and the medium (tryptic soy broth) was supplemented with both salt and glucose (Beenken *et al.*, 2010).

Assessment of biofilm formation *in vivo*

Biofilm formation was assessed *in vivo* using a murine model of catheter-associated biofilm formation (Weiss *et al.*, 2009). Briefly, uncoated catheters were implanted into each flank of NIH Swiss mice and inoculated by direct injection into the lumen of each catheter with 10^5 colony-forming units (cfu) of the test strain in a total volume of 100 μl of phosphate-buffered saline (PBS). Catheters were harvested after 3 days and processed for bacterial counts as previously described (Beenken *et al.*, 2012). Because each mouse had two catheters implanted, and because previous experiments have confirmed the absence of cross-contamination between catheters in opposite flanks of the same mouse (Weiss *et al.*, 2009), each catheter was treated as an independent data point (n = 10).

Bacteremia model

Groups of 10 five to eight week old female outbred NIH-Swiss mice (Harlan, Indianapolis, Ind.) were infected via tail vein injection with 5×10^7 cfu (Blevins *et al.*, 2003) of each strain under study. Tissues were harvested from any mice that died or required euthanasia; otherwise, tissues were harvested after 6 days (Zielinska *et al.*, 2012). Briefly, organs were

removed aseptically and homogenized on ice. Dilutions of each homogenate were then plated on CHROMagar (Blevins *et al.*, 2003) and the number of colony-forming units (cfu) per organ determined following overnight incubation at 37°C. Additionally, the left hind limb was removed and processed for histological analysis as previously described (Zielinska *et al.*, 2012). All tissue sections were evaluated microscopically in a blinded fashion and scored for the degree of inflammation (0–3) based on the extent of acute inflammatory cells seen in the synovial space and bone. The sections were also scored for the absence (0) or presence (1) of Gram-positive cocci/abscess formation, articular cartilage erosion, cortical bone erosion and physis destruction. Statistical analysis was based on a total histopathological score generated for each animal based on these parameters.

Statistical analysis

Western blot data was logarithmically transformed and analyzed using analysis of variance (ANOVA) models with Tukey's Multiple Comparison Test. Bacterial count data from harvested catheters were logarithmically transformed and analyzed using analysis of variance (ANOVA) models to evaluate the effect of each mutation. Pair-wise testing was performed using *t*-tests on the logarithmically transformed data. The significances of the ANOVA and *t*-test analyses were calculated using permutation tests. Wilcoxon rank-sum tests were used to analyze protease activity and histopathology data, while Kaplan-Meier methods were used to calculate survival distributions for lethality studies. Survival distributions were compared using log-rank tests. Statistical analyses were performed using R (version 2.7; The Foundation for Statistical Computing), SigmaPlot and GraphPad Prism 5.0. *P*-values ≤0.05 were considered to be statistically significant.

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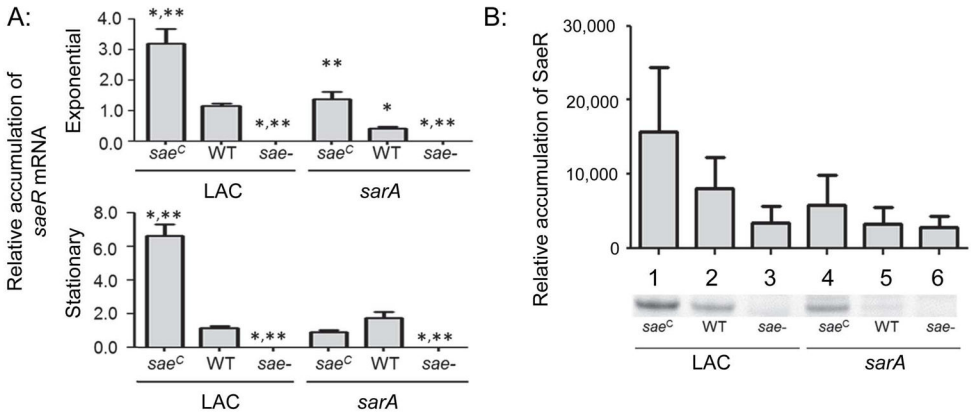

Fig. 1. Phenotypic verification of *sarA* and *saeRS* mutants

A: The abundance of *saeR* mRNA was assessed by qRT-PCR in the exponential and stationary growth phases. Strains on the left of each panel as indicated by the underline are strains derived from LAC itself, while strains on the right are derived from the isogenic LAC *sarA* mutant (e.g. the designation WT on the right indicates results observed with the LAC *sarA* mutant). Results shown represent the average ± standard deviation from two experiments, each of which was repeated in triplicate. Single asterisk indicates statistical significance by comparison to the LAC parent strain (WT). Double asterisks indicate significance by comparison to the *sarA* mutant. **B:** Western blots were performed in triplicate using cell lysates prepared from the same strains and rabbit polyclonal IgG targeting SaeR as primary antibody. Where necessary for legibility, the designation for the constitutively active *saeRS* derivative (*saeRS*^{C}) and *saeRS* mutant were reduced to *sae*^{C} and *sae*-respectively.

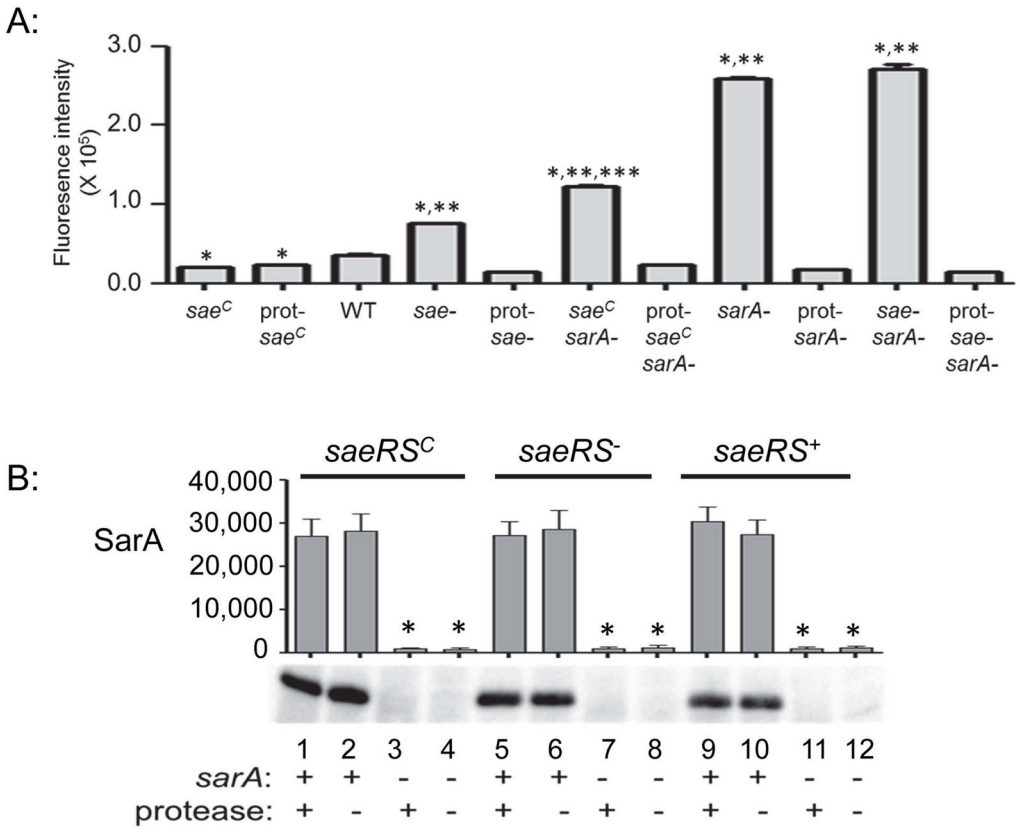

Fig. 2. Protease production and its impact on accumulation of SarA

A: Protease activity was assessed in the indicated strains using a FRET-based assay. The specific nature of each protease mutant (prot-) is described in the text. Single asterisk indicates statistical significance by comparison to the LAC parent strain (WT). Double asterisks indicate significance by comparison to the isogenic protease mutant. Triple asterisk indicates that the results observed with the *sarA* mutant generated in the constitutively active *saeRS* variant (*saeRS*^{C}/*sarA*) were significantly different from those observed with both the *sarA* and *saeRS/sarA* mutants. Strain designations are the same as those defined for Fig. 1.

B: Western blots were used to confirm mutation of *sarA* and assess accumulation of SarA as a function of *saeRS* and the production of extracellular proteases. Single asterisk indicates statistical significance by comparison to the corresponding *saeRS* derivative. The designation *saeRS*+ corresponds to LAC itself. The functional status of *sarA*, and the ability to produce proteases, are indicated by (+) and (−) signs below the blots.

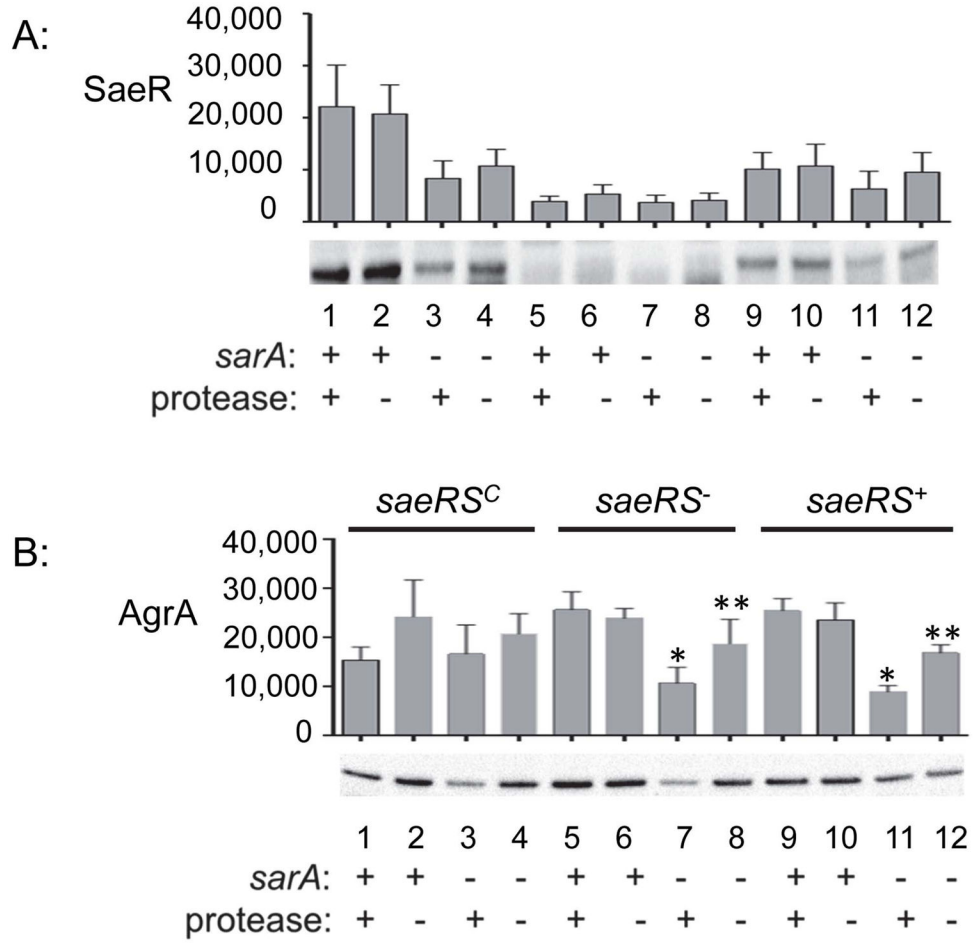

Fig. 3. Protease production and its impact on accumulation of SaeR and AgrA

A: Western blots were used to assess the impact of *saeRS*, *sarA*, and protease production on the accumulation of SaeR. **B.** Western blots were used to assess the impact of *saeRS*, *sarA*, and protease production on the accumulation of AgrA. Single asterisk indicates statistical significance by comparison to the corresponding *saeRS* derivative. Double asterisks indicate significance by comparison to the isogenic *sarA* mutant. In both panels, the designation *saeRS*+ corresponds to LAC itself, with the functional status of *sarA*, and the ability to produce proteases, indicated by (+) and (−) signs below the blots.

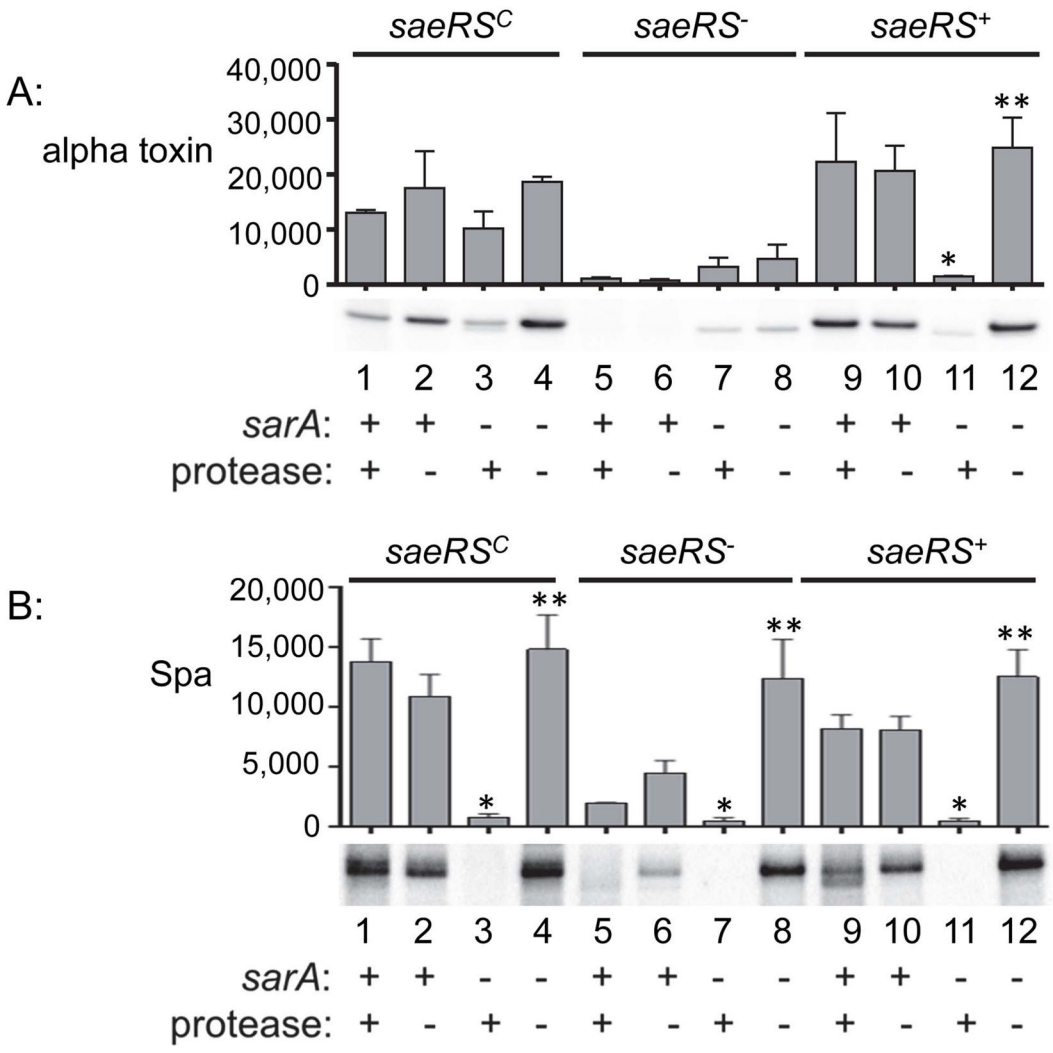

Fig. 4. Protease production and its impact on accumulation of alpha toxin and protein A (Spa)

A: Western blots were used to assess the impact of *saeRS*, *sarA*, and protease production on the accumulation of alpha toxin. **B.** Western blots were used to assess the impact of *saeRS*, *sarA*, and protease production on the accumulation of Spa. Single asterisk indicates statistical significance by comparison to the corresponding *saeRS* derivative. Double asterisks indicate significance by comparison to the isogenic *sarA* mutant. In both panels, the designation *saeRS*+ corresponds to LAC itself, with the functional status of *sarA*, and the ability to produce proteases, indicated by (+) and (−) signs below the blots.

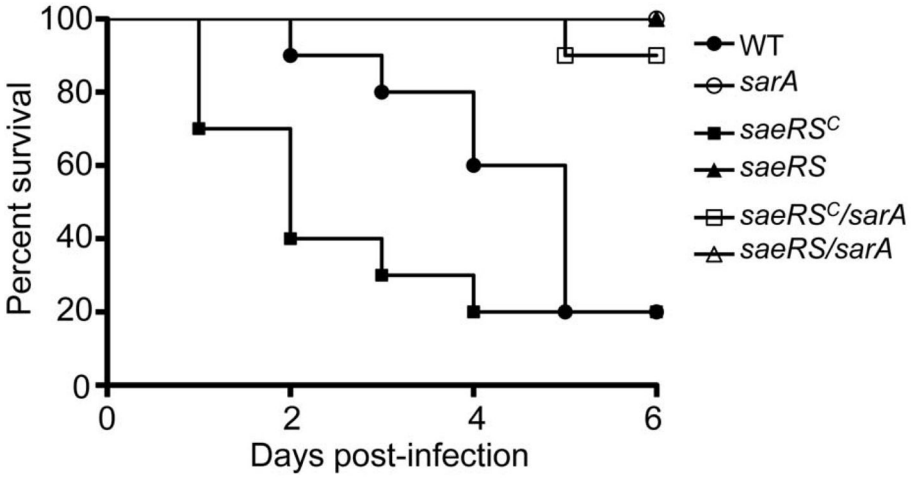

Fig. 5. Survival in bacteremia as a function of the functional status of *saeRS* and *sarA*

Results shown are Kaplan-Meirer survival curves of mice infected by tail vein injection of 5 × 10^7 cfu of the indicated strains. Results observed with all strains other than LAC and its *saeRS*^{C} *and saeRS*^{C}*/sarA* mutants overlapped at 100% survival. Results observed with all *sarA* mutants were statistically significant by comparison to the parent strain irrespective of the functional status of *saeRS*, but they were not significant by comparison to each other. The difference between the parent strain and the *saeRS*^{C} derivative was also significant.

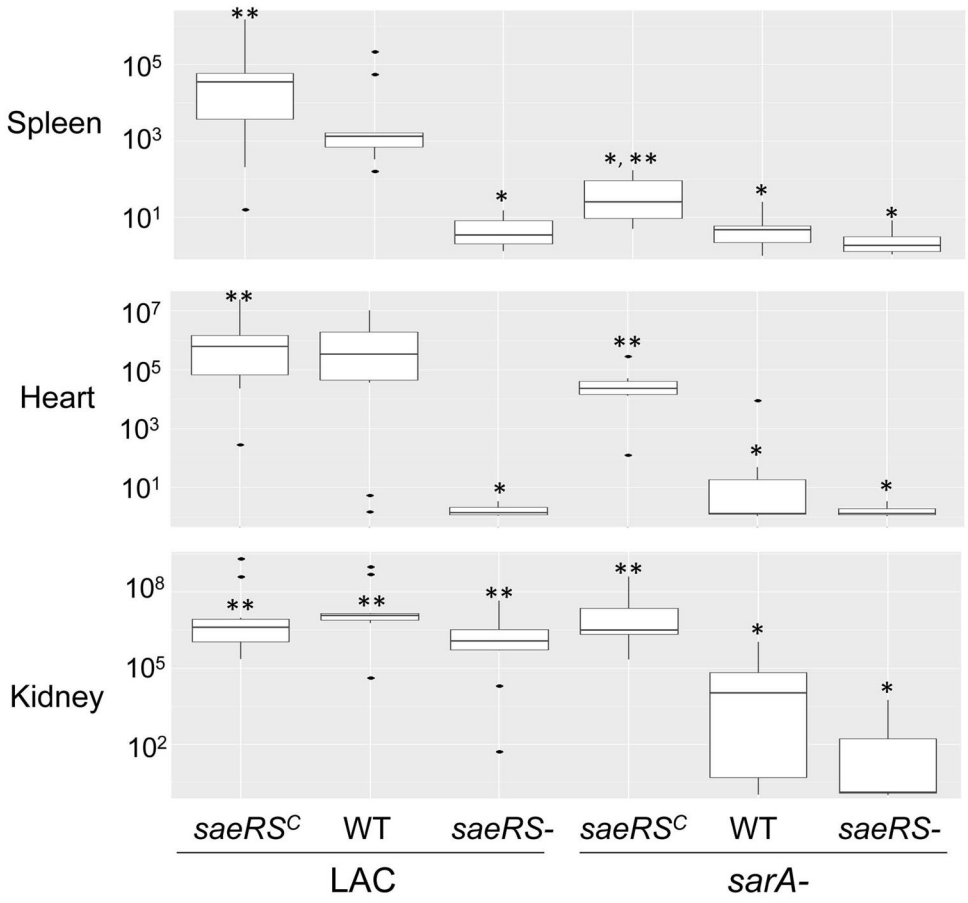

Fig. 6. Impact of *saeRS* vs. *sarA* on virulence in a bacteremia model

Results are shown as colony forming units (cfu) per organ obtained from the indicated tissues 6 days after intravenous injection of the indicated *S. aureus* strains. As in Fig. 1, for simplicity in labeling, the designation WT in the group underlined on the right indicates results observed with the LAC *sarA* mutant. Boxes indicate the 25th and 75th percentiles for each group and define the interquartile range (IQR), with the (+) within each box indicating the mean and the horizontal line indicating the median. Vertical lines define the lowest and highest data points within 1.5 IQR of the lower and higher quartile respectively, with individual dots representing single data points outside this range. A single asterisk indicates significance by comparison to the parent strain, while double asterisks indicate significance by comparison to the isogenic *sarA* mutant.

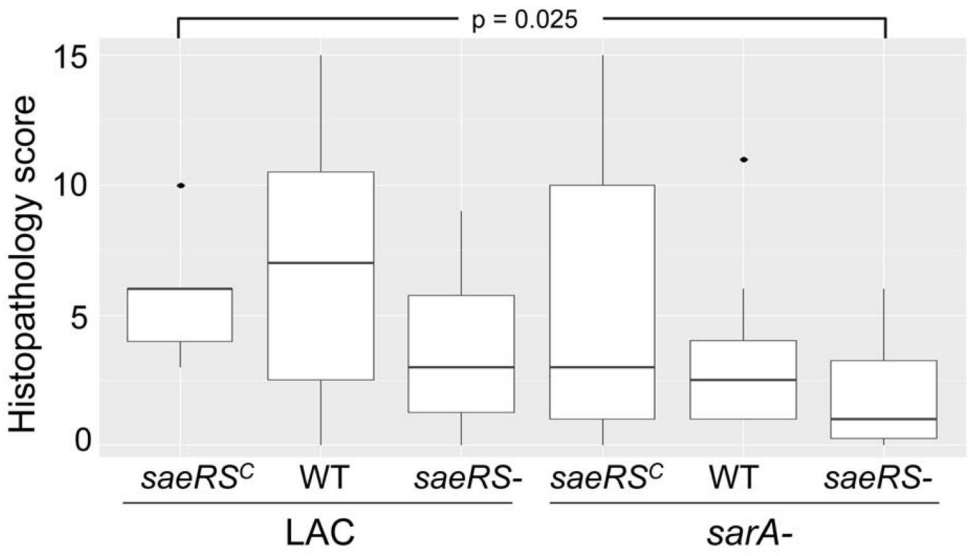

Fig. 7. Impact of *saeRS* and *sarA* on development of secondary musculoskeletal infections

Results illustrate overall histopathological scores for each experimental group. Boxes indicate the 25th and 75th percentiles for each group and define the interquartile range (IQR), with the horizontal line indicating the median. Vertical lines define the lowest and highest data points within 1.5 IQR of the lower and higher quartile respectively, with individual dots representing single data points outside this range. The only statistically significant difference observed was that between the constitutively active *saeRS* derivative (*saeRS*^{C}) and the *saeRS/sarA* mutant.

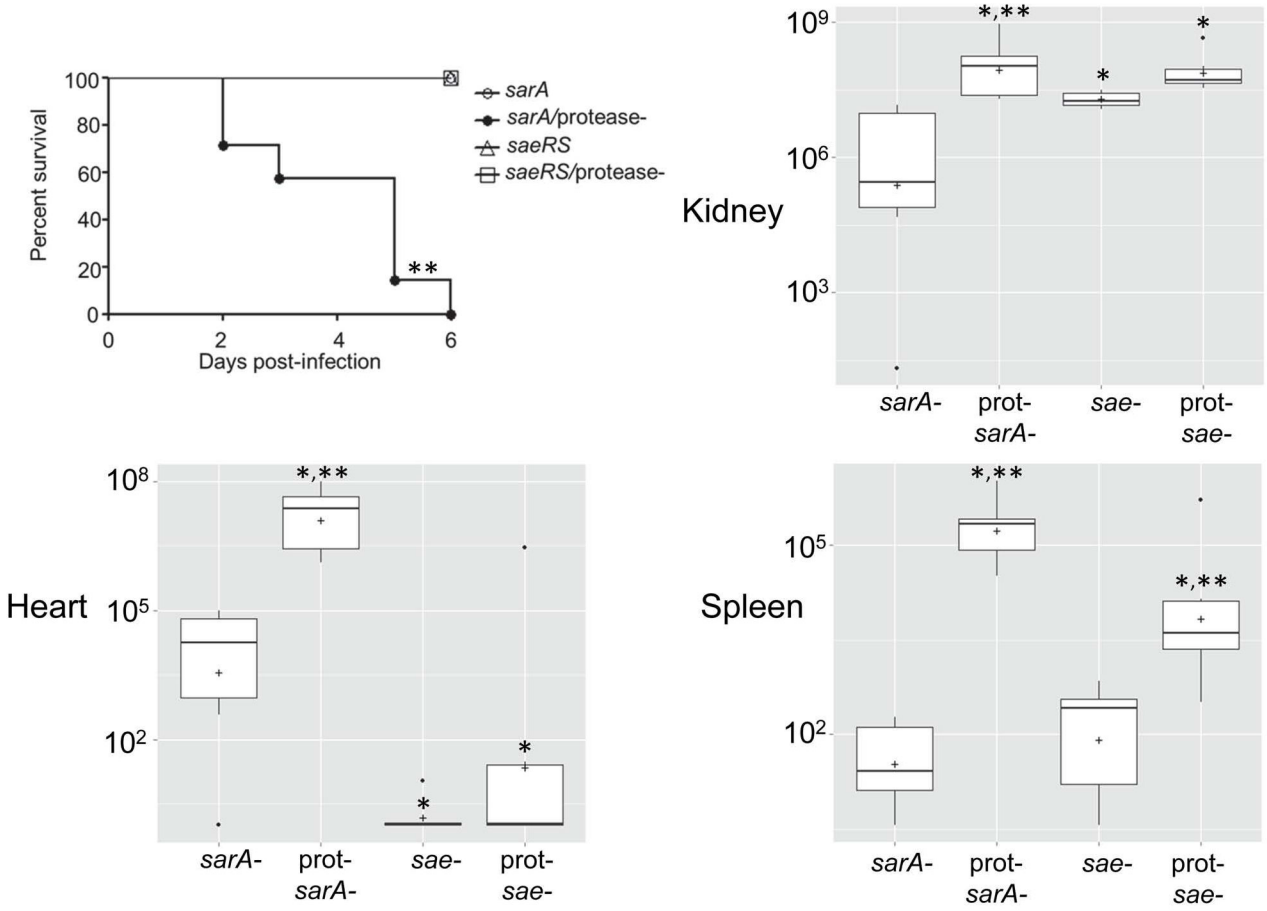

Fig. 8. Impact of protease production on phenotypes of *saeRS* and *sarA* mutants

Upper left illustrates Kaplan-Meirer survival curves of mice infected by tail vein injection of 5×10^7 cfu of the indicated strains. Remaining panels illustrate colony counts obtained from the indicated tissues 6 days after intravenous injection of the indicated *S. aureus* strains. Boxes indicate the 25th and 75th percentiles for each group and define the interquartile range (IQR), with the horizontal line indicating the median. Vertical lines define the lowest and highest data points within 1.5 IQR of the lower and higher quartile respectively, with individual dots representing single data points outside this range. The asterisk indicates significance by comparison to the *sarA* mutant. Double asterisks indicate significance of the protease-deficient mutant by comparison to the isogenic protease-positive regulatory mutant.

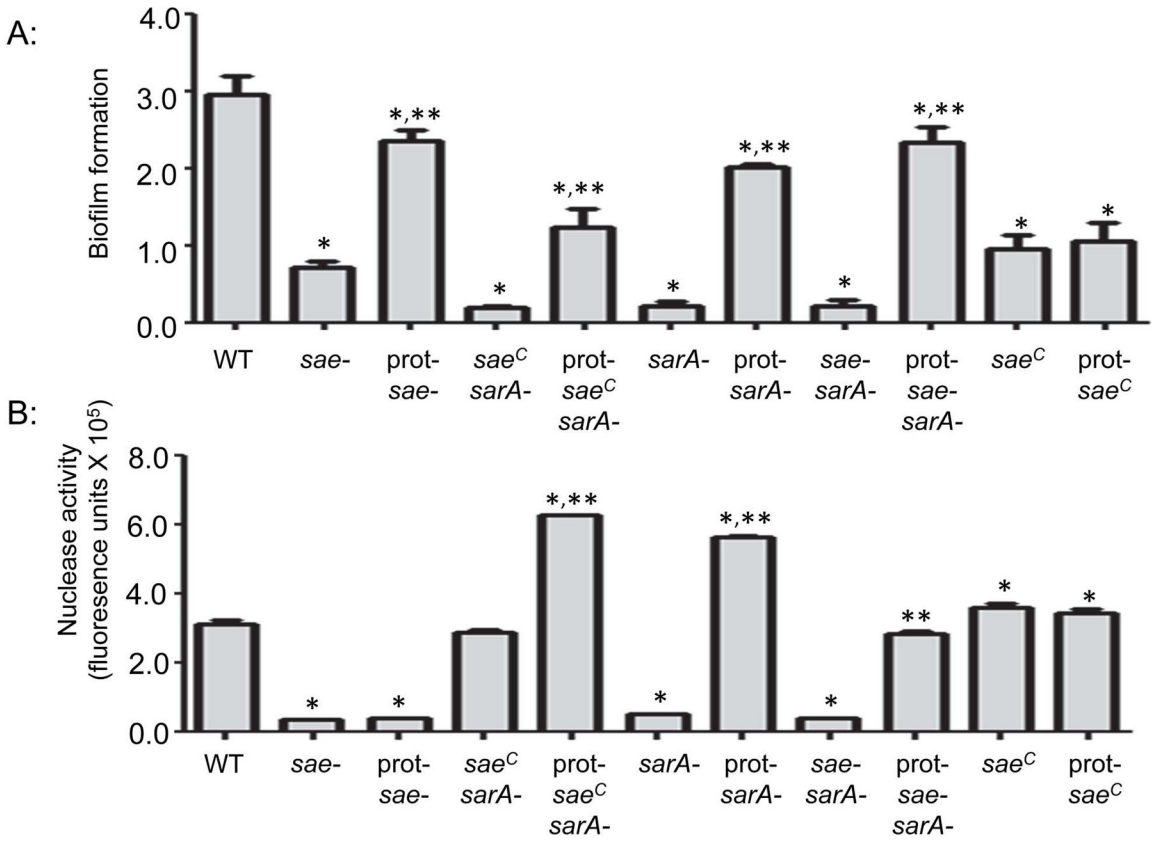

Fig. 9. Impact of *saeRS*, and *sarA* on biofilm formation and nuclease activity *in vitro*

Top: Biofilm formation was assessed using a microtiter plate biofilm. Single asterisk indicates statistical significance by comparison to the LAC parent strain (WT). Double asterisks indicate significance of protease-deficient derivatives by comparison to corresponding protease-producing strain. Note that the results observed with the protease-deficient *saeRS*^{C}*/sarA* mutant were significant, while those observed with the protease-deficient *saeRS*, *sarA*, and *saeRS/sarA* mutants were not. Note also that eliminating protease production had a significant impact in the *saeRS* mutant and all *sarA* mutants irrespective of the functional status of *saeRS*, but had no impact in the *saeRS*^{C} derivative. **Bottom:** Nuclease activity as determined using a FRET-based assay. Strain designations are the same as those indicated in Fig. 2. Single asterisk indicates statistical significance by comparison to the LAC parent strain (WT). Double asterisks indicate significance observed with protease-deficient derivatives by comparison to the same strain with the capacity to produce extracellular proteases.

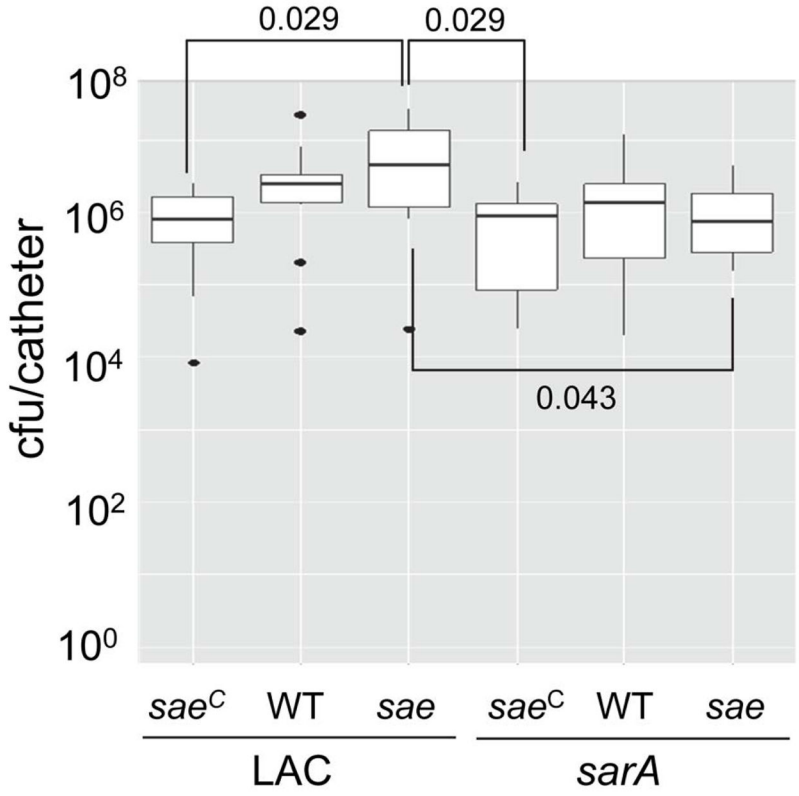

Fig. 10. Impact of *saeRS* and *sarA* on biofilm formation *in vivo*

Biofilm formation was assessed using a murine model of implant-associated biofilm formation (Weiss *et al.*, 2009). Strain designations are the same as in Fig. 1. Boxes indicate the 25th and 75th percentiles for each group and define the interquartile range (IQR), with the horizontal line indicating the median. Vertical lines define the lowest and highest data points within 1.5 IQR of the lower and higher quartile respectively, with individual dots representing single data points outside this range. Numbers above and within the graph indicate statistical significance between the designated groups.

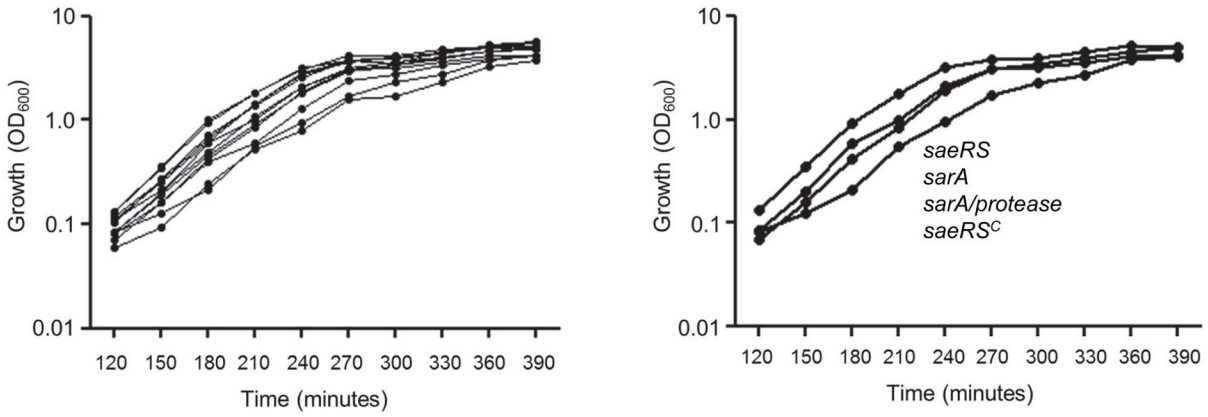

Fig. 11. Impact of *saeRS* and *sarA* on growth *in vitro*

Growth was assessed in tryptic soy broth based on optical density (OD_{600}) at the indicated time points. The panel on the left illustrates the results observed with all strains included in these studies and is intended only to illustrate that the differences in growth rate and overall yield were similar in all strains. The panel on the right breaks out the growth curves of the indicated strains because these were considered the most relevant in the overall context of this report. The strains are listed vertically to correspond to the order of the growth curves observed with each strain (i.e. the greatest difference observed among all strains was that between the *saeRS* mutant and its constitutively active variant).

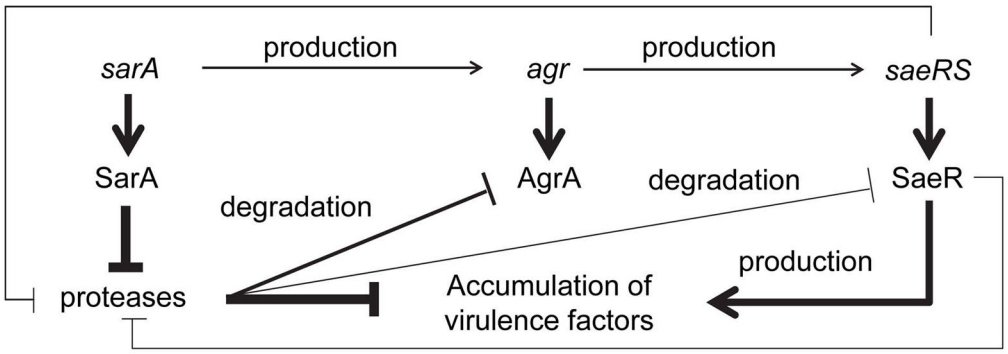

Fig. 12. Model for the relative impact of *saeRS* and *sarA in vivo*

The model proposes that the primary phenotypic impact of *sarA* under *in vivo* conditions is due to the increased production of extracellular proteases and the impact of these proteases with respect degradation of critical targets, potentially even including intracellular regulatory proteins. In contrast, while the functional status of *saeRS* impacts protease production even in an isogenic *sarA* mutant, the impact of this is small by comparison to the impact of *saeRS* on the production of these same virulence factors. The end result is a balance between *saeRS*-mediated production and *sarA*-mediated degradation, the outcome of which can have a dramatic impact on clinical outcome depending on the context in which it is evaluated. The weight of connecting lines indicates the relative impact of each component of these proposed regulatory interactions.