5-24-2010

Epistatic Relationships Between sarA and agr in Staphylococcus Aureus Biofilm Formation

Karen E. Beenken
University of Arkansas

Lara N. Mrak
University of Arkansas

Linda M. Griffin
University of Arkansas

Agnieszka K. Zielinska
University of Arkansas

Lindsey N. Shaw
University of South Florida, shaw@usf.edu

See next page for additional authors

Follow this and additional works at: http://scholarcommons.usf.edu/bcm_facpub

Part of the Medical Sciences Commons

Scholar Commons Citation
Beenken, Karen E.; Mrak, Lara N.; Griffin, Linda M.; Zielinska, Agnieszka K.; Shaw, Lindsey N.; Rice, Kelly C.; Horswill, Alexander R.; Bayles, Kenneth W.; and Smeltzer, Mark S., "Epistatic Relationships Between sarA and agr in Staphylococcus Aureus Biofilm Formation" (2010). Cell Biology, Microbiology, and Molecular Biology Faculty Publications. 1.
http://scholarcommons.usf.edu/bcm_facpub/1

This Article is brought to you for free and open access by the Cell Biology, Microbiology, and Molecular Biology at Scholar Commons. It has been accepted for inclusion in Cell Biology, Microbiology, and Molecular Biology Faculty Publications by an authorized administrator of Scholar Commons. For more information, please contact scholarcommons@usf.edu.
Authors
Karen E. Beenken, Lara N. Mrak, Linda M. Griffin, Agnieszka K. Zielinska, Lindsey N. Shaw, Kelly C. Rice, Alexander R. Horswill, Kenneth W. Bayles, and Mark S. Smeltzer

This article is available at Scholar Commons: http://scholarcommons.usf.edu/bcm_facpub/1
Epistatic Relationships between sarA and agr in Staphylococcus aureus Biofilm Formation

Karen E. Beenken1,9, Lara N. Mrak1,9, Linda M. Griffin1, Agnieszka K. Zielinska1, Lindsey N. Shaw2, Kelly C. Rice3, Alexander R. Horswill4, Kenneth W. Bayles5, Mark S. Smeltzer1*

1 Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, Arkansas, United States of America, 2Department of Biology, University of South Florida, Tampa, Florida, United States of America, 3 Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida, United States of America, 4 Department of Microbiology, University of Iowa, Iowa City, Iowa, United States of America, 5 Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska, United States of America

Abstract

Background: The accessory gene regulator (agr) and staphylococcal accessory regulator (sarA) play opposing roles in Staphylococcus aureus biofilm formation. There is mounting evidence to suggest that these opposing roles are therapeutically relevant in that mutation of agr results in increased biofilm formation and decreased antibiotic susceptibility while mutation of sarA has the opposite effect. To the extent that induction of agr or inhibition of sarA could potentially be used to limit biofilm formation, this makes it important to understand the epistatic relationships between these two loci.

Methodology/Principal Findings: We generated isogenic sarA and agr mutants in clinical isolates of S. aureus and assessed the relative impact on biofilm formation. Mutation of agr resulted in an increased capacity to form a biofilm in the 8325-4 laboratory strain RN6390 but had little impact in clinical isolates S. aureus. In contrast, mutation of sarA resulted in a reduced capacity to form a biofilm in all clinical isolates irrespective of the functional status of agr. This suggests that the regulatory role of sarA in biofilm formation is independent of the interaction between sarA and agr and that sarA is epistatic to agr in this context. This was confirmed by demonstrating that restoration of sarA function restored the ability to form a biofilm even in the corresponding agr mutants. Mutation of sarA in clinical isolates also resulted in increased production of extracellular proteases and extracellular nucleases, both of which contributed to the biofilm-deficient phenotype of sarA mutants. However, studies comparing different strains with and without proteases inhibitors and/or mutation of the nuclease genes demonstrated that the agr-independent, sarA-mediated repression of extracellular proteases plays a primary role in this regard.

Conclusions and Significance: The results we report suggest that inhibitors of sarA-mediated regulation could be used to limit biofilm formation in S. aureus and that the efficacy of such inhibitors would not be limited by spontaneous mutation of agr in the human host.

Introduction

Biofilm formation is an important aspect of many Staphylococcus aureus infections including endocarditis, osteomyelitis, and infections of implanted medical devices. This is true not only with respect to the pathogenesis of the infection itself but also with respect to antimicrobial therapy. Indeed, the presence of a biofilm limits the efficacy of antimicrobial therapy to the point that surgical intervention is often required to remove infected tissues and/or implanted devices [1]. For this reason, a considerable research effort has been aimed at defining the mechanistic basis of S. aureus biofilm formation. These studies have focused on the role of both individual components and the regulatory factors that modulate the production of these components. To date, over 20 genes have been implicated, with approximately half of these serving a regulatory role [2]. We chose to focus on the accessory gene regulator (agr) and the staphylococcal accessory regulator (sarA) because both of these loci have been shown to play central roles in S. aureus regulatory circuits that includes important but generally opposing roles in biofilm formation. Specifically, while there is one report to the contrary [3], most studies have concluded that expression of agr limits biofilm formation [4–6], and it has been suggested that this may serve as a means for the dispersal of S. aureus from an established biofilm [7–9]. In contrast, expression of sarA has consistently been shown to promote biofilm formation in both S. aureus and S. epidermidis [4–6,10,11]. There are also reports demonstrating that the opposing roles of sarA and agr in biofilm formation are therapeutically relevant. For instance, we demonstrated that mutation of sarA can be correlated with increased susceptibility to the functionally-diverse antibiotics...
daptomycin, linezolid and vancomycin in the specific context of an established biofilm [12,13]. At least under in vitro conditions, this increased susceptibility was evident even after taking into account the reduced capacity of a sarA mutant to form a biofilm [12]. In contrast, induction of agr expression was shown to result in the detachment of S. aureus cells from an established biofilm resulting in increased susceptibility to diverse antibiotics including rifampicin and levofloxacin [7,8,14]. Other reports have demonstrated that agr mutants accumulate within a biofilm and ultimately become the predominant subpopulation [6]. There is also a report demonstrating that the loss of agr function may confer a selective advantage in vivo, particularly under the pressure of vancomycin therapy [15]. Taken together, these results suggest that the opposing roles of sarA and agr in biofilm formation can be directly correlated with antibiotic susceptibility, with expression of the first leading to biofilm-associated intrinsic resistance and expression of the second having the opposite effect. This makes it important to define the epistatic relationships between sarA and agr in the context of biofilm formation.

Because both sarA and agr play global regulatory roles in S. aureus [16–18], it is not obvious why either would have an impact on biofilm formation. Moreover, expression of sarA is generally associated with increased expression of agr [16,19–21], and based on this it might be anticipated that these two loci would play consistent rather than opposing roles with respect to each other. However, sarA also modulates expression of many genes independently of agr, examples of which include the genes encoding extracellular proteases and nucleases [22,23]. Specifically, mutation of agr results in reduced production of these enzymes while mutation of sarA has the opposite effect [17,23,24]. Additionally, extracellular DNA (eDNA) has been shown to contribute to biofilm formation in S. aureus [25], and limiting nuclease or protease production by mutagenesis or the use of inhibitors of protease activity has been shown to promote biofilm formation and to partially restore biofilm formation in a sarA mutant [8,14,23,26].

Collectively, these studies suggest that the opposing roles of agr and sarA in biofilm formation are due to the fact that the first induces while the second represses the production of extracellular proteases and/or nucleases. This is consistent with our results comparing the clinical isolate UAMS-1 with the commonly-studied 8325-4 laboratory strain RN6390. Specifically, by comparison to RN6390, UAMS-1 expresses agr at much lower levels, produces reduced amounts of extracellular proteases, and forms a more robust biofilm [10,24,27]. Additionally, mutation of agr enhances biofilm formation in RN6390 but has little impact in UAMS-1 and, conversely, mutation of sarA results in increased protease production and decreased biofilm formation in UAMS-1 but has little impact in RN6390 [10]. All S. aureus strains derived from 8325, including the 8325-4 strain RN6390, have genetic defects that have been shown to contribute to its high level of agr expression [29–30], and restoration of the rsbU defect in RN6390 has been correlated with decreased expression of agr, decreased production of extracellular proteases, and an increased capacity to form a biofilm [8–9]. However, community-acquired, methicillin-resistant S. aureus (CA-MRSA) isolates of the USA300 clonal lineage, which are increasingly prominent worldwide [31], are closely related to 8325-derived strains and also express agr at high levels [17,32,33]. As a result, they produce extracellular toxins at high levels [34], and this contributes to their ability to cause invasive disease even in otherwise healthy individuals [35,36].

The production of extracellular proteases is also under the regulatory control of agr, and this suggests that the increased toxin production in USA300 isolates would be correlated with increased protease production and may therefore come at the expense of the ability to form a biofilm. Support for this hypothesis comes from a recent report demonstrating that mutation of agr, the inclusion of inhibitors of extracellular protease activity, and the mutation of specific genes encoding extracellular proteases can all be correlated with an enhanced capacity to form a biofilm in the USA300 isolate LAC [7,8,14]. However, to date, this issue has not been examined in a comprehensive manner, and it remains unclear whether expression of agr has a significant impact on biofilm formation in USA300 isolates in general and, if so, whether this limits the regulatory role of sarA in this respect. Based on this, we examined the impact of sarA and agr on biofilm formation in diverse clinical isolates with a specific emphasis on CA-MRSA isolates of the USA300 clonal lineage.

Results and Discussion

The impact of agr on biofilm formation

We previously demonstrated that clinical isolates of S. aureus generally form a more robust biofilm than the 8325-4 laboratory strain RN6390 and that biofilm formation in the latter is enhanced by mutation of agr [10]. This suggests that the level of agr expression in RN6390 can be functionally defined as excessive at least in the context of biofilm formation. The fact that clinical isolates of the USA300 clonal lineage are genotypically related to isolates of the 8325 lineage [32] and also express agr at high levels [8] led us to question whether they might also have a reduced capacity to form a biofilm and, if so, whether this could also be overcome by mutation of agr. To examine this issue, we chose three USA300 isolates, the genomes of two of which (FPR3757 and TCH1516, designated here as UAMS-1782 and UAMS-1790) have been sequenced [32,37] while the third (UAMS-1625) was isolated from a patient with a fatal brain abscess [30].

The levels of RNAIII produced by each strain were dependent on both growth phase and growth medium. RNAIII levels were higher in TSB than in biofilm medium (BM) in all strains except RN6390, which produced comparable levels of RNAIII whether grown in BM or TSB (Fig. 1). The reduced production of RNAIII in BM is consistent with reports demonstrating that RNAIII production is repressed in medium supplemented with glucose [7,24]. Nevertheless, all clinical isolates produced RNAIII in the expected growth-phase dependent pattern when grown in BM in that, by comparison to exponential growth, the production of RNAIII was increased in all strains in the post-exponential growth phase while, by comparison to the post-exponential growth phase, RNAIII levels were decreased in all strains in stationary-phase cultures (Fig. 1). RN6390 was once again an exception in that the levels of RNAIII observed in this strain were generally consistent across all growth phases.

Of the planktonic growth conditions examined in this report, the most applicable by comparison to our biofilm assays is a stationary-phase culture in biofilm medium, and under these conditions the targeted strains could be divided into three groups. The first consisted solely of RN6390, which produced higher levels of RNAIII by comparison to all other strains. The second consisted of the USA300 isolates UAMS-1782 and UAMS-1790, both of which produced RNAIII at levels that exceeded those observed in UAMS-1 or the USA300 isolate UAMS-1625 to a statistically-significant degree (Fig. 1). These results demonstrate that, while USA300 isolates generally produced RNAIII at levels that exceeded those observed in UAMS-1, they were nevertheless below those observed in RN6390. More importantly, this difference appears to be biologically relevant in that, as defined by our microtiter plate assay, all USA300 isolates formed a biofilm...
that was comparable to that observed with UAMS-1 and significantly greater than that observed with RN6390 (Fig. 2).

The fact that all three USA300 isolates formed a biofilm in our assay is in contrast to an earlier report demonstrating that the USA300 isolate LAC did not form a biofilm in a microtiter plate assay [8]. Because we did not include LAC in our experiments, one possible explanation for this difference is strain-dependent variability even among USA300 isolates. However, this seems unlikely in that one of the USA300 isolates we included (UAMS-1782) is essentially indistinguishable from LAC as defined by both genotypic and phenotypic characteristics [39]. An alternative explanation is that we employed a different growth medium in our microtiter plate assay and, unlike the earlier study [8], coated the substrate with human plasma. With regard to the first, one possible explanation for our disparate results is that expression of agr was reduced in USA300 isolates under our growth conditions to the point that it had no impact on biofilm formation. To the extent that RNAIII levels remained relatively high in RN6390 even under these conditions, this is consistent with the observation that RN6390 was the only strain we examined in which mutation of agr had an impact on biofilm formation (see below).

Coating with plasma was also shown to play an important role. Specifically, we repeated our experiments using the same microtiter plate assay but without plasma coating and found that, as with the other clinical isolates we have examined [10], plasma coating enhanced biofilm formation in all USA300 isolates (Fig. 3). Thus, it seems likely that our use of supplemented TSB as a growth medium and plasma coating of the substrate both contributed to the ability of USA300 isolates to form a biofilm in our assay. While it is difficult to correlate in vitro results using any assay conditions with in vivo conditions, implanted medical devices are coated with plasma proteins in vivo [40–43]. Moreover, the results we have observed with our microtiter plate biofilm assay have in all cases been consistent with those obtained using a murine model of catheter-associated biofilm formation [13,23,25,44]. This includes experiments done with the USA300 isolate UAMS-1625. Specifically, while we found that this strain had a reduced capacity to form a biofilm in vivo by comparison to UAMS-1, it was nevertheless capable of doing so to an extent that could be correlated with reduced antibiotic susceptibility [13]. We also confirmed the negative impact of mutating sarA on biofilm formation, and the positive impact of this on antibiotic susceptibility, in both UAMS-1 and UAMS-1625 under both in vitro and in vivo conditions [12,13].

We also found that mutation of agr did not enhance biofilm formation in any of the USA300 isolates irrespective of whether plasma coating was employed (Fig. 4) and this is consistent with the results of Lauderdale et al. [8] who found that mutation of agr in LAC had little impact on biofilm formation as assessed using flow cells. However, this certainly does not preclude an important role for agr in S. aureus biofilm formation, particularly given the relatively low levels of RNAIII production observed in USA300 isolates under our biofilm growth conditions. For instance, O’Neill...
et al. [45] demonstrated that mutation of \(agr\) enhanced biofilm formation in 5 of 13 methicillin-resistant clinical isolates even when the medium was supplemented with glucose to a degree that has been associated with reduced expression of \(agr\) [7]. Additionally, mutation of \(sigB\) in LAC limited biofilm formation even in flow cells, and concomitant mutation of \(agr\) reversed this effect [8].

The impact of \(sarA\) on biofilm formation and its relationship to the production of extracellular proteases

Unlike \(agr\), mutation of \(sarA\) was found to limit biofilm formation in all USA300 isolates irrespective of the functional status of \(agr\) (Fig. 4). O’Neill et al. [45] also found that mutation of \(sarA\) inhibited biofilm formation in all strains but suggested that this involved two different mechanisms, with the negative impact of mutating \(sarA\) in methicillin-sensitive strains being due primarily to the decreased expression of the \(ica\) operon leading to reduced production of the polysaccharide intracellular adhesin (PIA) while in methicillin-resistant strains the more important consideration was the impact of \(sarA\) on the production of surface-associated protein adhesins. We previously demonstrated that mutation of \(sarA\) in the methicillin-sensitive strain UAMS-1 does result in reduced PIA production, but an isogenic \(ica\) mutant retained the capacity to form a biofilm [27]. This demonstrates that decreased PIA production cannot account for the biofilm-deficient phenotype of a UAMS-1 \(sarA\) mutant. Rather, our results to date suggest that the more important consideration even in UAMS-1 is the increased production of extracellular proteases [23]. Production of these proteases has a negative impact on the presence of several surface-associated adhesins. These include the fibronectin-binding proteins (FibA and FibB) and protein A [24,46], both of which contribute to biofilm formation in at least some clinical isolates of \(S. aureus\) [43,47,48]. Additionally, expression of \(agr\) both represses the production of these adhesins and induces the production of extracellular proteases [21,49], either or both of which could contribute to the negative correlation between \(agr\) and biofilm formation. At the same time, transcription of the genes encoding extracellular proteases, including aureolysin \((aur)\) and \(sspA\), is directly repressed by both SarA and Rot [22]. Moreover, the regulatory impact of \(sarA\), \(rot\) and \(agr\) appears to be dependent on the relative concentrations of their products with respect to each other rather than the concentration of any individual product alone [50]. This suggests that USA300 isolates may not produce extracellular proteases at the levels that might be expected based on their relatively high overall levels of \(agr\) expression, and we found that this was in fact the case. Specifically, by comparison to RN6390, all three USA300 isolates produced reduced amounts of all extracellular proteases that could be detected using either casein or gelatin zymography (Fig. 5). In fact,
USA300 protease levels were more comparable to UAMS-1 than RN6390 despite the relatively low levels of RNAIII production in UAMS-1. As was observed in assays examining the production of RNAIII (Fig. 1), this was true whether protease production was assessed using supernatants from cultures grown in TSB or in biofilm medium (Fig. 5).

These results also suggest that the enhanced ability of USA300 isolates to form a biofilm by comparison to RN6390 might be a function of their decreased production of extracellular proteases. To further address this issue, we examined the relative impact of mutating sarA and agr on both biofilm formation and protease production, and in all cases we found a direct and inverse relationship between these two phenotypes. Specifically, the only strain derived from RN6390 that produced a biofilm in our microtiter plate assay was an agr mutant, which also exhibited decreased production of extracellular proteases (Fig. 6). Although mutation of sarA in RN6390 had relatively little impact of either of these phenotypes, concomitant mutation of sarA and agr in RN6390 reversed both phenotypes by comparison to the corresponding agr mutant (Fig. 6). This demonstrates that sarA is epistatic to agr even in RN6390 in the context of both protease production and biofilm formation. The same inverse relationships were also observed in UAMS-1 and all three of the USA300 isolates we examined, the difference being that the impact of sarA

**Figure 5. Production of extracellular proteases in USA300 isolates.** Supernatants were harvested from overnight (15 hr) cultures grown in TSB (left) or biofilm medium (right) and standardized with respect to each other prior to zymographic analysis using both casein (top) and gelatin gels (bottom).
doi:10.1371/journal.pone.0010790.g005

**Figure 6. Relationships between sarA, agr, protease production and biofilm formation.** Biofilm formation and the production of extracellular proteases was assessed in the indicated wild-type (WT) strains and their isogenic sarA (S), agr (A), and sarA/agr (SA) mutants with and without complementation with a functional copy of sarA (S\(^{C}\)). Results for biofilm assay (top) are shown as the mean ± the standard deviation of 6 replicate samples. Statistical analysis confirmed a significant difference between the RN6390 agr and sarA-complemented RN6390 sarA/agr mutants and all other RN6390 derivative and between the sarA and sarA/agr mutants and their sarA-complemented derivatives in both UAMS-1 and UAMS-1782. No significant differences were observed the sarA-complemented derivatives of UAMS-1 or UAMS-1782 and their respective parent strains.
doi:10.1371/journal.pone.0010790.g006
was evident in these strains irrespective of the functional status of *agr* (Fig. 6). These results confirm that the impact of *sarA* on protease production and biofilm formation is at least partially independent of *agr*. This was confirmed by demonstrating that the *sarA* defect was complemented in all strains, including RN6390, by introducing a functional, plasmid-borne copy of *sarA* even into a *sarA/agr* double mutant (Fig. 6).

While suggestive, the inverse relationship between biofilm formation and the production of extracellular proteases does not prove a cause-and-effect relationship. However, we subsequently demonstrated that a cocktail of three protease inhibitors that is capable of limiting the activity of multiple *S. aureus* extracellular proteases [23] enhanced biofilm formation in *sarA* mutants generated in UAMS-1, RN6390, and in two of the three USA300 isolates, the only exception being the USA300 isolate UAMS-1625 (Fig. 7). The overall levels of both RNAIII (Fig. 1) and extracellular proteases (Fig. 8) were comparable between UAMS-1625 and the other USA300 isolates, and the impact of mutating *sarA* on the production of extracellular proteases was comparable in all strains other than RN6390 (Fig. 8). This suggests that one possible explanation for the differential impact of protease inhibitors in UAMS-1625 is that this strain produces either a unique protease that was not inhibited by any component of the inhibitor cocktail or a common protease that is produced in elevated amounts by comparison to the other strains such that the inhibitor cocktail had a reduced effect in this strain. In this respect it is important to note that the inclusion of protease inhibitors did not fully restore biofilm formation (Fig. 7) or protease production (Fig. 8) in any of the *sarA* mutants. However, the relative impact of the inhibitor cocktail also appeared to be comparable among all USA300 isolates including UAMS-1625 (Fig. 8).

Interestingly, while the overall impact of the inhibitor cocktail was consistent in all strains we did observe a strain-dependent effect with respect to both the impact of mutating *sarA* and the relative efficacy of the protease inhibitor cocktail. Specifically, in UAMS-1, the impact of mutating *sarA* was most evident in the increased production of aureolysin and SspA, and the impact of the protease inhibitor cocktail was most evident in the reduced activity of SspA and SspB (Fig. 8). In contrast, the impact of mutating *sarA* in USA300 isolates was most evident in the production of aureolysin, SspA and SspB, with the inhibitor cocktail having the greatest impact on the activity of SspB. The *spl*-encoded proteases have also been implicated in biofilm formation [8], but these were not detectable in either of our zymograms. These results are consistent with the hypothesis that multiple proteases contribute to the biofilm-deficient phenotype of *S. aureus* *sarA* mutants [7,8,23].

The impact of *sarA* on production of the polysaccharide intercellular adhesin (PIA)

UAMS-1625 can also be distinguished from the other USA300 isolates included in this study by the absence of the arginine catabolite metabolic element (ACME) [38]. We are unaware of a correlation between ACME and biofilm formation, but there are reports describing the role of the *arc* operon itself in this context. Specifically expression of the *arc* operon was induced in UAMS-1 in a biofilm by comparison to both exponential and stationary-phase planktonic cultures [27]. Additionally, mutation of *arcD*, which encodes the arginine/ornithine antiporter of the arginine deiminase pathway, resulted in the reduced production of PIA in UAMS-1 [44]. However, as with the *ica* operon itself [27], this was not associated with a decreased capacity to form a biofilm. Nevertheless, the possibility that ACME contributes to biofilm formation by virtue of its impact on the production of PIA cannot be ruled out. Indeed, mutation of *sarA* results in reduced production of PIA [7,11], and based on this one possible explanation for our results is that PIA plays a more important role in biofilm formation in UAMS-1625 than in the other USA300 isolates and that, in the absence of ACME, a UAMS-1625 *sarA* mutant cannot produce enough PIA to sustain biofilm formation irrespective of any other factor including extracellular proteases. To explore this possibility, we examined the relative

![Figure 7. The impact of protease inhibitors on biofilm formation in *S. aureus* *sarA* mutants. Biofilm formation was assessed in each of the indicated strains (WT) and their isogenic *sarA* mutants with (SPI) and without (S) the inclusion of protease inhibitors. Results are shown as the mean ± the standard deviation of 6 replicate samples. Statistical analysis confirmed a significant difference between each wild-type strain and its *sarA* mutant and, with the exception of UAMS-1625, between the *sarA* mutants assayed in the presence or absence of protease inhibitors. In RN6390 and UAMS-1782, the difference between the wild-type strain and its isogenic *sarA* mutant assayed in the presence of protease inhibitors was also significant. doi:10.1371/journal.pone.0010790.g007](#)
levels of PIA produced by each strain using an anti-PIA immunoblot. These studies demonstrated that all of the USA300 isolates produced almost undetectable amounts of PIA irrespective of the presence of ACME or the functional status of sarA [23]. This not only suggests that the relative levels of PIA production do not account for the difference between UAMS-1625 and other USA300 isolates but also that PIA plays little role in USA300 strains [45].

The impact of sarA on nuclelease production and its impact on biofilm formation

Overall, our results are consistent with the hypothesis that the inability of sarA mutants to repress the production of extracellular proteases plays a predominant role with respect to their dominant-negative phenotype by comparison to agr. However, the inclusion of protease inhibitors did not fully restore biofilm formation in any of the sarA mutants (Fig. 7). One explanation for this partial effect is that the concentration of each inhibitor, which was chosen based on the highest concentration that did not inhibit growth [23], did not fully inhibit the activity of all extracellular proteases in UAMS-1 or any of the USA300 sarA mutants by comparison to their respective parent strains (Fig. 8). The alternative although not mutually exclusive explanation for the partial impact of protease inhibitors is that other factors also contribute to the biofilm-deficient phenotype of sarA mutants. Because extracellular DNA (eDNA) also contributes to biofilm formation in S. aureus [25,26], one possibility in this regard is the production of extracellular nucleases. Indeed, we previously demonstrated that mutation of sarA in UAMS-1 resulted in increased production of nuclease and that nuclease partially restored the ability of a USA300 sarA mutant to form a biofilm [23]. In this report, we found that mutation of sarA also results in increased production of extracellular nucleases in USA300 isolates (Fig. 10). Moreover, the same epistatic relationships between sarA and agr that were observed in the context of biofilm formation and protease production were also observed in the context of nuclease production (Fig. 11). This demonstrates that the impact of sarA on nuclelease production is also independent of the interaction between sarA and agr and that nuclelease production and biofilm formation are also inversely correlated.

Concomitant mutation of sarA and agr resulted in increased nuclelease activity in all strains even by comparison to the isogenic sarA mutant (Fig. 11). In this respect it is important to note that, while protease production remained elevated in sarA/agr mutants by comparison to the parent strains, it was reduced by comparison to the corresponding sarA mutants (Fig. 6). Thus, one possible explanation for these results is that the increased production of extracellular proteases limits the accumulation of extracellular nucleases and that the negative impact of mutating agr on protease production attenuates this effect. Additionally, UAMS-1 and all three isolates of the USA300 clonal lineage produced extracellular nucleases at levels that exceeded those observed in RN6390 (Figs. 10 and 11). Given its high level of protease production by comparison to clinical isolates, one possible explanation for this is that the increased production of extracellular proteases limits nuclelease activity in RN6390 irrespective of the functional status of sarA. However, the important point is that, because RN6390 was the only strain that did not form a biofilm even with plasma coating, these results are an exception to the inverse relationship between nuclelease production and biofilm formation, and to the extent that no such exceptions were observed with respect to protease production, they are also consistent with the hypothesis
that the production of extracellular proteases plays a predominant role by comparison to extracellular nucleases in S. aureus biofilm formation. Moreover, RN6390 was also found to produce more PIA than either UAMS-1 or the USA300 isolates (Fig. 9). Thus, of the three sarA-regulated components known to contribute to biofilm formation, RN6390 produces two (PIA and nuclease) at levels that would be expected to promote biofilm formation and one (protease) at levels that would be expected to limit biofilm formation. Taken together, these results provide further support for the hypothesis that the sarA-mediated repression of extracellular proteases plays a predominant role in S. aureus biofilm formation.

At the same time, our previous results focusing on the impact of nuclease production on biofilm formation in UAMS-1 were done using a mutant generated in the nuc gene designated as open-reading frame SA0746 in the N315 genome, mutation of which was shown to abolish nuclease production even in a sarA mutant and that mutation of nuc2 had no effect as assessed using DNase agar (Fig. 12). Even so, mutation of either or both of these genes was shown to enhance biofilm formation in UAMS-1 albeit to a limited degree and only in the absence of plasma coating (Fig. 13). This was also true in a UAMS-1 sarA mutant although in this case the effect was statistically significant only when both nuc1 and nuc2 were mutated and only when the assay was done with plasma coating. These results suggest that our failure to detect a nuclease-deficient phenotype in our nuc2 mutant may have been due primarily to our use of a relatively insensitive assay. They also confirm that the increased production of nucleases contributes to biofilm formation in S. aureus at least under some circumstances and that the agr-independent, sarA-mediated repression of extracellular nuclease production may also be important in this regard.

**Summary**

Taken together, the results discussed above demonstrate that sarA is epistatic to agr in the context of biofilm formation and that this is true irrespective of the level of agr expression or the overall genetic relatedness among clinical isolates. The results also suggest that the primary regulatory role of sarA in this context is to repress the production of extracellular enzymes including proteases and nucleases. They also suggest that in the specific context of these phenotypes the regulatory events observed in RN6390 reflect an imbalance by comparison to clinical isolates including those of the USA300 clonal lineage. This imbalance is defined both by the absolute level of agr expression and the inability of RN6390 to modulate expression of agr, and consequently the predominance of agr relative to sarA, and this is directly reflected in the increased production of extracellular proteases in RN6390. This presumably reflects the fact that all 8325-4 strains, including RN6390, carry mutations in at least two genes (rsbU and tcaR) known to impact S. aureus regulatory circuits [22,29]. This is consistent with the observation that restoration of rsbU in an 8325-4 strain (SH1000) resulted in reduced production of extracellular proteases [28]. To the extent that RN6390 was the only strain we examined in which mutation of agr resulted in an enhanced capacity to form a biofilm (Fig. 6), it is also consistent with the observation that the impact of mutating agr on biofilm formation in LAC was most evident in an agr/sigB mutant [8]. Other reports have suggested that the impact of the tcaR mutation on expression of sarS also has a profound impact on S.

![Figure 10. Impact of sarA on extracellular nucleases.](image)

Figure 11. Epistatic relationship between sarA and agr in nuclease production. Biofilm formation and production of extracellular nucleases was assessed using DNase agar in the indicated wild-type (WT) strains and their isogenic sarA (S), agr (A), and sarA/agr (SA) mutants with (S') and without complementation of the sarA defect.

doi:10.1371/journal.pone.0010790.g011
aureus regulatory circuits [22, 50]. As evidenced by expression levels of asp23, which is indicative of the functional status of rsbU (or, more precisely, sigB) and sarS, neither of these mutations is present in any of the clinical isolates examined in this report (Fig. 14). However, to the extent that RN6390 has these defects, the more important observation is that mutation of sarA resulted in a significantly reduced capacity to form a biofilm in all of the isolates we examined other than RN6390. Moreover, we extended our experiments to include other clonal lineages of S. aureus, and in every case mutation of sarA resulted in a reduced capacity to form a biofilm to a degree that was comparable to that observed in UAMS-1 and isolates of the USA300 clonal lineage (Fig. 15).

We recently demonstrated that mutation of sarA in UAMS-1 and the USA300 isolate UAMS-1625 can be correlated with increased antibiotic susceptibility both in vitro and in vivo [12,13], and our results demonstrating a comparable effect in other clinical isolates suggest that this would be the case irrespective of strain identity. This is consistent with the hypothesis that inhibitors of sarA-mediated regulation would have broad therapeutic utility in the specific context of biofilm-associated staphylococcal infection. While such inhibitors would have neither bacteriostatic or bactericidal properties and thus would have limited therapeutic utility in and of themselves, many S. aureus infections are recalcitrant to antimicrobial therapy even in the absence of issues related to acquired resistance. This includes orthopaedic and catheter-associated infections, the resolution of which often requires surgical intervention to debride infected tissues and/or removal of the infected device [42,43,52,53]. Based on this, such inhibitors would potentially be a viable alternative for the development of adjunct therapies that could be used to significantly enhance the efficacy of more conventional antimicrobial agents in the specific context of S. aureus biofilm-associated infection. The results we report demonstrate that the impact of sarA on biofilm formation is epistatic to agr, and this implies that, irrespective of the specific mechanism involved, such inhibitors would maintain their efficacy even in the context of the accumulation of agr mutants that occurs not only within biofilms [6] but also in vivo during the course of antimicrobial therapy [15].

Materials and Methods

Bacterial strains and growth conditions

The S. aureus strains used in this study are listed in Table 1. Generation of agr, sarA and agr/sarA mutants in each of the targeted strains was done by Φ11-mediated transduction of the agr::tet, sarA::tet or sarA::kan mutations from existing strains [24]. Complementation of the sarA mutation was done as previously described [24]. Mutagenesis of the SA0746-encoded nuclease gene (designated here as nuc1) in UAMS-1 and its isogenic sarA mutant was done using pKOR1 as previously described [7]. The UAMS-1

Figure 12. Activity of UAMS-1 nuclease genes. Nuclease activity was assessed using DNase agar in UAMS-1 (WT) and its sarA mutant and in derivatives of each of these strains carrying mutations in SA0746 (nuc1) and/or SA1160 (nuc2).

doi:10.1371/journal.pone.0010790.g012

Figure 13. Impact of nuclease genes on biofilm formation. Biofilm formation was assessed in UAMS-1 (WT) and its sarA mutant (S) and in derivatives of each of these strains carrying mutations in nuc1 (n1), nuc2 (n2), or both nuc1 and nuc2 (n12) with and without plasma coating of the substrate. Results are shown as the mean ± the standard deviation of 6 replicate samples. Statistical analysis confirmed a significant difference between UAMS-1 (WT) and its isogenic nuc1 and nuc2 mutants in the absence of plasma coating and between the sarA mutant (S) and its isogenic nuc1/nuc2 mutant in the presence of plasma coating.

doi:10.1371/journal.pone.0010790.g013
SA1160 nuc mutant (designated here as nuc2) was generated by allele replacement mutagenesis. Specifically, 498 and 469 bp fragments were independently amplified from the upstream and downstream regions of the targeted gene using the primers shown in Table 1. These fragments were cloned into pGD647 on either side of the \textit{erm} gene using the EcoRI and KpnI restriction sites for the upstream fragment and XbaI and PstI restriction sites for the downstream fragment. The resulting \textit{nuc2-erm-nuc2} cassette, which contains all of the SA1160 gene except the 115 bp region that was replaced with the 1.3 kb \textit{erm} gene, was then excised by EcoRI/PstI digestion and subcloned into pCL52.2 [55]. After passage through RN4220 and electroporation into UAMS-1, allele replacement was accomplished by growth and 45°C followed by repetitive culture without antibiotic selection at 30°C. Colonies were plated on tryptic soy agar (TSA) without antibiotic and then patched to TSA containing erythromycin or tetracycline. Colonies resistant to erythromycin and sensitive to tetracycline were then screened by PCR to confirm allele replacement using the 5′

Figure 14. Expression of \textit{asp23} and \textit{sarS} in clinical isolates. RNA was isolated from each of the indicated strains during the exponential (E) and post-exponential (PE) growth phases and the amounts of the \textit{asp23} and \textit{sarS} transcripts determined by qRT-PCR. Values obtained with RN6390 exponential phase cultures were set to 1.0 with the results observed with other strains shown relative to this value. Results are shown as the mean ± the standard deviation of duplicate samples. Statistical analysis confirmed a significant difference between RN6390 and each of the other strains in the context of both \textit{asp23} and \textit{sarS} during both the exponential and post-exponential growth phases.

doi:10.1371/journal.pone.0010790.g014

Figure 15. Impact of \textit{sarA} on biofilm formation in isolates of other USA clonal lineages. Biofilm formation was assessed in wild-type strains (WT) from each of eight USA clonal lineages (numerical designations refer to USA clonal lineage) and their corresponding \textit{sarA} mutants (S). Results are shown as the mean ± the standard deviation of 6 replicate samples. Statistical analysis confirmed a significant difference between each wild-type strain and its isogenic \textit{sarA} mutant.

doi:10.1371/journal.pone.0010790.g015
Table 1. Bacterial strains used in this study.

| Strain       | Description                                      | Reference   |
|--------------|--------------------------------------------------|-------------|
| UAMS-1       | MSSA, osteomyelitis isolate                      | [56]        |
| UAMS-128     | RN6390 (8325-4)                                  | [57]        |
| UAMS-155     | UAMS-1agr::tetM                                  |             |
| UAMS-240     | UAMS-128, sarA::tetK                             | [24]        |
| UAMS-929     | UAMS-1, sarA::kan                                | [24]        |
| UAMS-930     | UAMS-929, agr::tetM                              | [24]        |
| UAMS-959     | UAMS-983, agr::tetM                              | [24]        |
| UAMS-969     | UAMS-929, (pLI50::sarA)                          | [24]        |
| UAMS-970     | UAMS-930, (pLI50::sarA)                          | [24]        |
| UAMS-979     | UAMS-983, (pLI50::sarA)                          | [24]        |
| UAMS-980     | UAMS-959, (pLI50::sarA)                          | [24]        |
| UAMS-982     | UAMS-128, agr::tetM                              | [24]        |
| UAMS-983     | UAMS-128, sarA::kan                              | [24]        |
| UAMS-1039    | USA400 isolate (MW2)                             | NRS1231     |
| UAMS-1454    | UAMS-1 nuc2::erm                                 | This study  |
| UAMS-1471    | UAMS-1 nuc1                                     | [23]        |
| UAMS-1477    | UAMS-929 nuc1                                   | [23]        |
| UAMS-1478    | UAMS-1 nuc1, nuc2::erm                           | This study  |
| UAMS-1484    | UAMS-929 nuc2                                   | This study  |
| UAMS-1485    | UAMS-929, nuc1, nuc2::erm                        | This study  |
| UAMS-1625    | USA300 isolate                                  | [38]        |
| UAMS-1653    | UAMS-1625, sarA::tetK                            | [12]        |
| UAMS-1660    | UAMS-1625, agr::tetM                             | This study  |
| UAMS-1782    | USA300 isolate FPR3757                          | NRS482      |
| UAMS-1790    | USA300 isolate                                  |             |
| UAMS-1796    | UAMS-1790, sarA::tetK                            | This study  |
| UAMS-1804    | UAMS-1782, sarA::kan                             | This study  |
| UAMS-1819    | UAMS-1782, agr::tetM                             | This study  |
| UAMS-1820    | UAMS-1790, agr::tetM                             | This study  |
| UAMS-1836    | UAMS-1660, sarA::tetK                            | This study  |
| UAMS-1837    | UAMS-1819, sarA::kan                             | This study  |
| UAMS-1838    | UAMS-1820, sarA::tetK                            | This study  |
| UAMS-1893    | USA100 isolate                                  | NRS642      |
| UAMS-1894    | USA200 isolate                                  | NRS651      |
| UAMS-1895    | USA500 isolate                                  | NRS685      |
| UAMS-1896    | USA600 isolate                                  | NRS648      |
| UAMS-1898    | USA800 isolate                                  | NRS653      |
| UAMS-1899    | USA1000 isolate                                 | NRS676      |
| UAMS-1900    | USA1100 isolate                                 | NRS484      |
| UAMS-1901    | UAMS-1804, (pLI50::sarA)                         | This study  |
| UAMS-1904    | UAMS-1837, (pLI50::sarA)                         | This study  |
| UAMS-1930    | UAMS-1899, sarA::kan                             | This study  |
| UAMS-1931    | UAMS-1900, sarA::kan                             | This study  |
| UAMS-1938    | UAMS-1039, sarA::kan                             | This study  |
| UAMS-1941    | UAMS-1893, sarA::tetK                            | This study  |
| UAMS-1942    | UAMS-1895, sarA::tetK                            | This study  |
| UAMS-1943    | UAMS-1896, sarA::tetK                            | This study  |
| UAMS-1944    | UAMS-1898, sarA::tetK                            | This study  |
| UAMS-1945    | UAMS-1894, sarA::tetK                            | This study  |

1NRS isolates were obtained from the repository at the Network for Antimicrobial Resistance in Staphylococcus aureus (NARSA), Eurofins Medinet, Inc., Chantilly, VA 20151. doi:10.1371/journal.pone.0010790.t001

Table 1. Cont.

primer from the upstream PCR and the 3' primer from the downstream PCR. The resulting nuc2 mutation was subsequently introduced into the isogenic nuc1, sarA and sarA/nuc1 mutants by phage-mediated transduction [7].

All strains were maintained as stock cultures at −80°C in tryptic soy broth (TSB) containing 25% (v/v) glycerol. For each experiment, each strain was retrieved from cold storage by plating on tryptic soy agar (TSA) with appropriate antibiotic selection. Antibiotics were used at the following concentrations: erythromycin (Erm; 10 μg per ml), tetracycline (Tet; 5 μg per ml), kanamycin (Kan; 50 μg per ml), neomycin (Neo; 50 μg per ml), and chloramphenicol (Cm; 10 μg per ml). Kanamycin and neomycin were always used together to avoid the spontaneous generation of resistant strains. To ensure that the results of all phenotypic assays were consistent, unless otherwise noted all assays were done using cultures grown in TSB supplemented with 0.5% glucose and 3.0% sodium chloride (biofilm medium) without antibiotic selection as previously described [7,10,27]. Culture conditions in all cases were 37°C with constant aeration and a medium-to-flask volume ratio of ≤0.50. In experiments evaluating the impact of growth phase, the exponential and post-exponential growth phases were defined based on optical densities (OD) of 1.0 and 3.0 respectively. Stationary phase samples were defined by overnight (24 hr) growth.

Transcriptional analysis

To assess levels of asp23, sarS and RNAIII expression, total bacterial RNA was isolated using the Qiagen RNeasy Mini Kit as previously described [23]. Quantitative, real-time RT-PCR (qRT-PCR) was then performed using RNAIII-specific primers and a corresponding TaqMan probe (Table 2). Results were calibrated by comparison to the results obtained with the same RNA samples using primers and a TaqMan probe corresponding to a 16S ribosomal RNA gene (Table 2). Results are reported as relative units by comparison to the results observed with the lowest sample in any given experiment, with the latter being set to a value of 1.0.

Assessment of biofilm formation

Biofilm formation was assessed in vitro using a static, microtiter plate biofilm assay as previously described [7,10]. Unless otherwise indicated, the microtiter plate substrate was first coated overnight with 20% human plasma.

Production of extracellular proteases

Protease activity was assessed by zymography using 4–16% Zymogram (Blue Casein) Gels and 10% Zymogram (Gelatin) Gels (Invitrogen, Carlsbad, CA). In both cases, supernatants were harvested from overnight (15 hr) cultures and normalized with respect to each other prior to filter sterilization. Sterile supernatants were then concentrated 15-fold using Centricon YM-3 filter units (Millipore, Bedford, MA) before loading equivalent samples using a buffer containing DTT but not β-mercaptoethanol. After electrophoresis, gels were first incubated for 30 min at room temperature (RT) in renaturing buffer (2.5% TritonX-100) and then overnight at 37°C in developing buffer (0.2 M Tris, 5 mM CaCl2, 1 mM DTT).
To visualize protease bands, gels were then stained with SimplyBlue SafeStain (Invitrogen, Carlsbad, CA). Briefly, supernatants were harvested from cultures and the amount of PIA was assessed using anti-PIA antiserum and the WesternBreeze chemiluminescence immunodetection kit (Invitrogen Corp., Carlsbad, CA).

### Production of the polysaccharide intercellular adhesin (PIA)

To assess the production of PIA, overnight cultures were normalized with respect to each other before harvesting cells by centrifugation. Cells were resuspended in 50 μl of 0.5 M EDTA (pH 8.0) and boiled for 5 min. Cellular debris was removed by centrifugation before incubating the supernatant with proteinase K (20 mg/ml) at 37°C for 60 minutes. After the addition of 10 μl of Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl [pH 7.4]), each extract was spotted onto a nitrocellulose membrane using a BIO-Dot microfiltration apparatus (Bio-Rad Laboratories, Inc., Hercules, CA). After drying, the presence and amount of PIA was assessed using anti-PIA antiserum and the WesternBreeze chemiluminescence immunodetection kit (Invitrogen Corp., Carlsbad, CA).

### Statistical analysis

Statistical analysis of results comparing wild-type strains was done using the Students t-test. Statistical analysis of results comparing different strains with their isogenic sarA and agr mutants and their sarA-complemented derivatives was done by ANOVA based on all pairwise comparisons. In both cases p values <0.05 were considered significant.

### Acknowledgments

The authors thank Drs. Kim Jefferson (Virginia Commonwealth University, Richmond, VA) and Gerald Pier (Channing Laboratory, Harvard Medical School) for the generous gift of anti-PIA antibody.

### Author Contributions

Conceived and designed the experiments: KEB LNM AKZ MSS. Performed the experiments: KEB LNM LMG AKZ. Analyzed the data: KEB LNM AKZ MSS. Contributed reagents/materials/analysis tools: KCR ARH KWB. Wrote the paper: MSS.

### References

1. Brady RA, Leid JG, Calhoun JH, Costerton JW, Shirtliff ME (2008) Nuclease production was assessed using D’NASE Test Agar. PLoS Pathog 4: e1000052.

2. O’Gara JP (2007) sar and beyond: biofilm mechanisms and regulation in Staphylococcus epidermidis and Staphylococcus aureus. FEMS Microbiol Lett 270: 179–189.

3. Coelho LR, Souza RR, Ferreira FA, Guimaraes MA, Ferreira-Carvalho BT, et al. (2008) Impact of the sarA and sarB systems on adherence to polystyrene in Staphylococcus aureus. Biofilm Med Microbiol 52: 13–22.

4. Kong KF, Vuong C, Otto M (2006) Staphylococcus quorum sensing in biofilm formation. Antimicrob Agents Chemother 53: 183–200.

5. Yarwood JM, Paquette KM, Tikh IB, Volper EM, Greenberg EP (2004) Quorum sensing in Staphylococcus aureus biofilms. J Bacteriol 186: 1838–1850.

6. Weiss EC, Zielinska A, Beenken KE, Spencer HJ, Daily SJ, et al. (2009) Impact of sarA and not sigmaB is essential for biofilm development by Staphylococcus epidermidis in a catheter-associated in vivo model of biofilm formation. Antimicrob Agents Chemother 53: 2475–2482.

7. Valle J, Toledo-Arana A, Berasain C, Ghigo JM, Amorena B, et al. (2003) SarA and sarB are required for biofilm development by Staphylococcus aureus in a catheter-associated model of biofilm formation. Antimicrob Agents Chemother 48: 1073–1087.

8. Weiss EC, Ziebinska A, Beenken KE, Spencer HJ, Daily SJ, et al. (2009) Impact of sarA on daptomycin susceptibility of Staphylococcus aureus in a catheter-associated in vivo model of biofilm formation. Antimicrob Agents Chemother 53: 4096–4102.

9. Yarwood JM, Bartels DJ, Volper EM, Greenberg EP (2004) Quorum sensing in Staphylococcus aureus biofilms. J Bacteriol 186: 11381–11385.

10. Beenen KE, Blevis JS, Smeltzer MS (2003) Mutation of sarC in Staphylococcus aureus limits biofilm formation. Infect Immun 71: 4206–4211.

11. Valle J, Toledo-Arana A, Berasain C, Ghigo JM, Amorena B, et al. (2003) SarA and not sigmaB is essential for biofilm development by Staphylococcus epidermidis. Mol Microbiol 48: 1073–1087.

12. Weiss EC, Ziebinska A, Beenken KE, Spencer HJ, Daily SJ, et al. (2009) Impact of sarA on daptomycin susceptibility of Staphylococcus aureus in a catheter-associated in vivo model of biofilm formation. Antimicrob Agents Chemother 53: 4096–4102.

13. Lauderdale KJ, Boles BR, Marcuende J, Horswill AR (2010) Biofilm dispersal of community-associated methicillin-resistant Staphylococcus aureus in a catheter-associated in vivo model of biofilm formation. Antimicrob Agents Chemother 53: 2475–2482.

14. Lauderdale KJ, Boles BR, Marcuende J, Horswill AR (2010) Biofilm dispersal of community-associated methicillin-resistant Staphylococcus aureus in a catheter-associated in vivo model of biofilm formation. Antimicrob Agents Chemother 53: 2475–2482.

15. Bronner S, Monteil H, Prevost G (2004) Regulation of virulence determinants in Staphylococcus aureus complex and applications. FEMS Microbiol Rev 28: 183–200.
17. Cassat JE, Duman PM, Murphy E, Projan SJ, Benenk KE, et al. (2006) Transcriptional profiling of a Staphylococcus aureus clinical isolate and its isogenic agr and sarA mutants reveals global differences in comparison to the laboratory strain RN6390. Microbiology 152: 3075–3090.

18. Duman PM, D Elevated RNase H, Haney S, Palacios D, Tsuker-Kelloff G, et al. (2001) Transcription profiling-based identification of Staphylococcus aureus genes regulated by the agr and/or sarA loci. J Bacteriol 183: 7341–7353.

19. Cheung AL, Bayer AS, Zhang G, Gershman H, Xiong YQ (2004) Regulation of virulence determinants in vitro and in vivo in Staphylococcus aureus. FEBS Immunol Med Microbiol 40: 1–9.

20. Chien Y, Manna AC, Cheung AL (1998) SarA level is a determinant of virulence in Staphylococcus aureus. In: Third International Conference on Biofilm Research and Applications.

21. Novick RP (2003) Autoinduction and signal transduction in the regulation of virulence determinant expression and stress resistance: characterization of a functional r抒 strain derived from Staphylococcus aureus 8325-4. J Bacteriol 185: 4547–4567.

22. Kottik J, Giczy J, Fuchs T (1998) Deletion of the alternative sigma factor sigmaB in Staphylococcus aureus reveals its function as a global regulator of virulence gene expression. J Bacteriol 180: 4814–4820.

23. Tenover FC, Georger RV (2009) Methicillin-resistant Staphylococcus aureus strain USA300: origin and epidemiology. J Antimicrob Chemother 64: 1–19.

24. Blevins JS, Beenken KE, Elasri MO, Hurlburt BK, Smeltzer MS (2002) Strain-dependent differences in the regulatory roles of sarA and agr in Staphylococcus aureus. In: Third International Conference on Biofilm Research and Applications.

25. Rice KC, Mann EE, Endres JL, Weiss EC, Cassat JE, et al. (2007) The murein hydrolase regulator contributes to DNA release and biofilm development in Staphylococcus aureus. J Bacteriol 189: 8325–8333.

26. Mann EE, Rice KC, Bolis BR, Endres JL, Ranjit D, et al. (2009) Modulation of agr-dependent differences in the regulatory roles of sarA and agr. J Bacteriol 187: 4665–4684.

27. Beenken KE, Duman PM, McAleece F, Macapagal FD, Murphy E, et al. (2004) Global gene expression in Staphylococcus aureus biofilms. J Bacteriol 186: 4676–4684.

28. Hoch JH, Aish JL, White J, Shaw L, Lithgow JK, et al. (2002) sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional r抒 strain derived from Staphylococcus aureus 8325-4. J Bacteriol 184: 5457–5467.

29. McCallum N, Buchel M, Mak H, Wada A, Berger-Bachi B (2004) TcaR, a putative sarA-like regulator of the agr virulence system in Staphylococcus aureus. J Bacteriol 186: 2966–2972.

30. Tenover FC, Georger RV (2009) Methicillin-resistant Staphylococcus aureus strain USA300: origin and epidemiology. J Antimicrob Chemother 64: 1–19.

31. Diep BA, Gill SR, Chang RF, Phan TH, Davidson MG, et al. (2006) Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant Staphylococcus aureus. Lancet 367: 731–739.

32. Wang R, Braughton KR, Kretschmer D, Bach TH, Quack SY, et al. (2007) Identification of novel cytoytic peptides as key virulence determinants for community-associated MRSA. Nat Med 13: 1310–1314.

33. Li M, Diep BA, Villaruz AE, Braughton KR, Jiang X, et al. (2009) Evolution of virulence in epidemic community-associated methicillin-resistant Staphylococcus aureus. Proc Natl Acad Sci USA 106: 5803–5808.

34. Miller LG, Kaplan SL (2009) Staphylococcus aureus: a community pathogen. Infect Dis Clin North Am 23: 35–52.

35. Montgomery CP, Boyle-Vavra S, Adem PV, Lee JC, Husain AN, et al. (2008) Comparison of virulence in community-associated methicillin-resistant Staphylococcus aureus pulsortypes USA300 and USA400 in a rat model of pneumonia. J Infect Dis 198: 561–570.

36.oker RF, Ross HF, Projan SJ, Kornblum J, Kreiswirth B, et al. (1993) Identification of plasma proteins adsorbed on hemodialysis tubing that promote Staphylococcus aureus adhesion. J Lab Clin Med 135: 32–42.

37. Csetek SK, Hultén KS, Qin X, Jiang H, Yerraapragada S, et al. (2007) Subtle genetic changes enhance virulence of methicillin resistant and sensitive Staphylococcus aureus. BMC Microbiol 7: 99.

38. Sifri CD, Park J, Helmg WA, Perup AW, Shaik SK (2007) Fatal brain abscess due to community-associated methicillin-resistant Staphylococcus aureus strain USA300. Clin Infect Dis 45: 113–117.

39. Kenny AD, Otto M, Braughton KK, Whitney AR, Chen L, et al. (2008) Epidemic community-associated methicillin-resistant Staphylococcus aureus: recent clonal expansion and diversification. Proc Natl Acad Sci USA 105: 1327–1332.

40. Francois P, Letourneur D, Lew DP, Jozefonwicz J, Vaudaux P (2000) Identification of plasma proteins adsorbed on hemodialysis tubing that promote Staphylococcus aureus adhesion. J Lab Clin Med 135: 32–42.

41. Francois P, Vaudaux P, Lew PD (1998) Role of plasma and extracellular matrix proteins in the physiopathology of foreign body infections. Ann Vase Surg 12: 34–40.

42. Viale P, Stefania S (2006) Vascular catheter-associated infections: a microbiological and therapeutic update. J Chemother 18: 235–249.

43. Vanger DC, Einblich JM (2005) Device-related infections: a review. J Long Term Eff Med Implants 15: 467–488.

44. Zhu Y, Weiss EC, Otto M, Fey PD, Smeltzer MS, et al. (2007) Staphylococcus aureus Biofilm metabolism and the influence of arginine on polysaccharide intercellular adhesion synthesis, biofilm formation, and pathogenesis. Infect Immun 75: 4129–4127.

45. O'Neill E, Pozzi C, Houston P, Smyth D, Humphreys H, et al. (2007) Association between methicillin susceptibility and biofilm regulation in Staphylococcus aureus isolates from device-related infections. J Clin Microbiol 45: 1379–1383.

46. Karlsson A, Saravia-Otten P, Tegmark K, Morfesd E, Arvidsson S (2001) Decreased amounts of cell wall-associated protein A and fibronectin-binding proteins in Staphylococcus aureus sarA mutants due to up-regulation of extracellular proteases. Infect Immun 69: 4742–4748.

47. Mevio N, Toledo-Arana A, Vergara-Irigaray M, Valde J, Solano C, et al. (2009) Protein A-mediated multicellular behavior in Staphylococcus aureus. J Bacteriol 191: 832–843.

48. O'Neill E, Pozzi C, Houston P, Humphreys H, Robinson DA, et al. (2008) A novel Staphylococcus aureus biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. J Bacteriol 190: 3805–3815.

49. Shaw L, Golouka E, Potemza J, Foster SJ (2004) The role and regulation of the extracellular proteases of Staphylococcus aureus. Microbiology 150: 217–220.

50. Gratwison E, Karlsson S, Oscarson J, Sogled P, Nilsson P, et al. (2009) Mathematical modelling of the regulation of spa (protein A) transcription in Staphylococcus aureus. Int J Med Microbiol 299: 63–74.

51. Tang J, Zhou R, Shi X, Kang M, Wang H, et al. (2008) Two thermostable nucleases encoded in Staphylococcus aureus: evidence from mutant analysis and in vitro expression. FEBS Microbiol Lett 247: 176–183.

52. Calbous JH, Manring MG (2005) Adult osteomyelitis. Infect Dis Clin North Am 19: 763–786.

53. Eggersmann P, Sax H, Pitter D (2004) Catheter-related infections. Microbes Infect 6: 1033–1042.

54. Guerout-Fleuray AM, Shazand K, Frandsen N, Strager P (1995) Antibiotic resistance cassettes for Bacillus subtilis. Gene 167: 335–336.

55. Sore S, Sun J, Lee CV (1997) Molecular characterization and transcriptional analysis of type 8 capsule gene in Staphylococcus aureus. J Bacteriol 179: 1624–1631.

56. Cassat JE, Duman PM, McAleece F, Murphy E, Projan SJ, et al. (2005) Comparative genomics of Staphylococcus aureus mucosakelal isolates. J Bacteriol 187: 576–592.

57. Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth B, et al. (1993) Synthesis of staphyloccocal virulence factors is controlled by a regulatory RNA molecule. EMBO J 12: 3967–3973.