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MAJOR ARTICLE

The Global Regulon *sarA* Regulates β-Lactam Antibiotic Resistance in Methicillin-Resistant *Staphylococcus aureus* In Vitro and in Endovascular Infections

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Background. The global regulator *sarA* modulates virulence of methicillin-resistant *Staphylococcus aureus* (MRSA) via regulation of principal virulence factors (eg, adhesins and toxins) and biofilm formation. Resistance of *S. aureus* strains to β-lactam antibiotics (eg, oxacillin) depends on the production of penicillin-binding protein 2a (PBP2a), encoded by *mecA*.

Methods. In the present study, we investigated the impact of *sarA* on the phenotypic and genotypic characteristics of oxacillin resistance both in vitro and in an experimental endocarditis model, using prototypic healthcare- and community-associated MRSA parental and their respective *sarA* mutant strain sets.

Results. All *sarA* mutants (vs respective MRSA parental controls) displayed significant reductions in oxacillin resistance and biofilm formation in vitro and oxacillin persistence in an experimental endocarditis model in vivo. These phenotypes corresponded to reduced *mecA* expression and PBP2a production and an interdependency of *sarA* and *sigB* regulators. Moreover, RNA sequencing analyses showed that *sarA* mutants exhibited significantly increased levels of primary extracellular proteases and suppressed pyrimidine biosynthetic pathway, argininosuccinate lyase–encoding, and ABC transporter–related genes as compared to the parental strain.

Conclusions. These results suggested that *sarA* regulates oxacillin resistance in *mecA*-positive MRSA. Thus, abrogation of this regulator represents an attractive and novel drug target to potentiate efficacy of existing antibiotic for MRSA therapy.

Keywords. *sarA*; β-lactam antibiotic resistance; MRSA endocarditis; treatment.

Staphylococcus aureus is a leading cause of endovascular infections, including infective endocarditis [1]. Despite the use of modern antibiotics, morbidity and mortality associated with these infections remain unacceptably high [2–4]. Therefore, there is a critical need to understand the regulation of resistance and virulence determinants driving these treatment failure outcomes to enable development of novel treatment strategies.

Resistance of methicillin-resistant *S. aureus* (MRSA) to β-lactam antibiotics, including nafcillin and oxacillin, depends on elaboration of penicillin-binding protein 2a (PBP2a). PBP2a, encoded by *mecA,* is an alternative cell wall cross-linking enzyme with reduced affinity for virtually all β-lactam antibiotics. Previous studies showed that the expression of *mecA* closely correlated with the level of oxacillin resistance [5]. *mecA* is part of the staphylococcal chromosome cassette (SCC*mec*), which is present in MRSA but absent in methicillin (oxacillin)–susceptible *S. aureus* (MSSA) [6]. Importantly, the SCC*mec* element is transferrable among staphylococci by transduction and conjugation in the laboratory, which contributes to dissemination of resistance [7]. Furthermore, excision of the SCC*mec* element in MRSA strains results in oxacillin susceptibility [8]. Likewise, inactivation of *mecA* in MRSA strains reverses the MRSA phenotype to that of MSSA [5].

The staphylococcal accessory regulator (SarA) protein is a global regulator that governs many of the virulence factors produced by *S. aureus* [9–11]. SarA is also an important positive regulator of biofilm formation, in part because of its reciprocal repressive activity on protease and nuclease production [12]. Importantly, our previous investigation demonstrated that *sarA* mutant strains had significantly slower growth rates in the presence of oxacillin as compared to their respective parental MRSA strains [13]. Thus, we hypothesized that a combination of the impacts of *sarA* on methicillin/oxacillin resistance and biofilm formation may provide a novel strategy to treat MRSA biofilm-related endovascular infections. It is well known that the alternative sigma factor protein, SigB, can influence other regulons, including *sarA*, affording adaptive responses to a variety of environmental stresses [14, 15]. However, the relationship between *sigB* and oxacillin resistance is not well understood.

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The present study is the first to our knowledge to demonstrate that *sarA* mutants exhibit significantly reduced β-lactam (eg, oxacillin) resistance as compared to respective MRSA parental strains in vitro and in experimental infective endocarditis. In addition, we delineate that these effects are based on the impact of *sarA* on *mecA* and also involve regulatory impact on *sigB*. These findings provide potentially unique approaches to overcome intrinsic resistance to existing β-lactam antibiotics (eg, oxacillin) in MRSA strains.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Medium

The bacterial strains and plasmids used in this study are listed in Table 1. The 2 hospital-associated MRSA (HA-MRSA) strains were from patients with persistent bacteremia derived from a multinational clinical trial collection [16, 18, 19]. The 2 community-associated MRSA (CA-MRSA) strains were MW2 (USA400, reported to cause fatal infection in children [13]) and JE2 (USA300 strain LAC, obtained from the National Institutes of Health Network on Antimicrobial Resistance in *Staphylococcus aureus* [NARSA] [17]). These MRSA strains belong to SCC*mec* type IV, in which strains are *mecI* and *mecR2* negative and only have a truncated *mecR1* [20]. In the current studies, the JE2 *sarA* mutant was complemented with *sarA* by transforming it with the plasmid pALC1215, which is pSPT181 carrying the entire *sarA* locus under its native promoter [22]. In addition, we introduced pALC6185, in which *mecA* was expressed from a xylose-inducible promoter into the JE2 *sarA* mutant [21]. These 2 mutant strains were confirmed with polymerase chain reaction (PCR) and sequencing. The other transposon mutants were obtained from NARSA. The *S. aureus* strains were routinely grown in tryptic soy broth (TSB) or TSB agar plates.

Table 1. *Staphylococcus aureus* Strains and Plasmids Used in This Study

Strain or Plasmid	Relevant Characteristic(s)	Source or Reference(s)
Strain		
300-169	MRSA (*agr-I*, SCC*mec* IV, and CC45)	[12, 16]
300-169 Δ*sarA*	300-169 *sarA*::kan	[12]
324-136	MRSA (*agr-I*, SCC*mec* IV, and CC45)	[12, 16]
324-136 Δ*sarA*	324-136 *sarA*::Tn917LTV1	[12]
MW2	MRSA (SCC*mec* IV), USA400	[12, 13]
ALC5415	MW2 *sarA*::kan	[12]
JE2	MRSA (SCC*mec* IV), LAC, USA300	[17]
JE2 Δ*sarA*	JE2 *sarA*::kan with ALC6185	[12]
JE2 Δ*sarA*/p*sarA*	JE2 Δ*sarA* complemented with pALC1215	This study
JE2 Δ*sarA*/p*mecA*	JE2 Δ*sarA* complemented with pALC6185	This study
JE2 Δ*mecA*	Transposon mutant with insertion in *S. aureus* USA300_0032	NTML
JE2 Δ*sigB*	Transposon mutant with insertion in *S. aureus* USA300_2022	NTML
S. aureus ATCC43300	MRSA	ATCC
S. aureus ATCC25923	MSSA	ATCC
Plasmid		
pALC6185	pEPSA5::*mecA* Cm^r	[21]
pALC1215	pSPT181::*sarA* Tet^r	[22]

Abbreviations: ATCC, American Type Culture Collection; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; NTML, Nebraska Transposon Mutant Library; SCC*mec*, staphylococcal chromosome cassette *mec*.

Determination of Oxacillin Minimum Inhibitory Concentrations (MICs)

The oxacillin MICs of study MRSA strain sets were determined by using both the standard Etest method (BioMerieux, La Balme-les-Grottes, France), according to the manufacturer's recommended protocols, and the standard broth microdilution method, as recommended by the Clinical and Laboratory Standards Institute [23].

Population Analyses

Oxacillin population analyses were performed to determine whether the MRSA strains exhibited heterogeneous or homogeneous resistance to oxacillin, using standard protocols [24, 25]. The classification of resistance as heterogeneous versus homogeneous was based on plating findings, defined as the number of colony-forming units (CFU) on oxacillin-containing (50 μg/mL) agar plates divided by the number on drug-free agar plates, multiplied by 100%. Heterogeneous and homogeneous were defined as <1% and ≥1% of CFU growth, respectively [26].

Production of PBP2a

A semiquantitative, rapid and reliable latex agglutination method was used to measure the PBP2a production according to a modified manufacture's instruction (Denka Seiken, Tokyo, Japan) [27]. A positive reaction is indicated by the development of an overt agglutination pattern within 3 minutes, while a negative reaction is indicated by a homogenous suspension appearance. The intensity of agglutination was scored blindly by 2 of us (L. L. and W. A.) as high (+++), moderate (++), or low (+), whereas the negative control, which showed no activity, was scored as negative (−). *S. aureus* ATCC 43300 (MRSA) and ATCC 25923 (MSSA) were used as positive and negative controls, respectively.

Biofilm Formation

Biofilm formation was performed as previously described [12, 16, 25]. Adhering dye (0.1% safranin) was dissolved in 30% acetic acid, and absorption was measured as OD_{490nm} to quantify biofilm formation [12, 16, 25].

Isolation of RNA

Total RNA was isolated from study strain cells after 24 hours of culture at 37°C, using an RNeasy Kit (Qiagen, Valencia, CA) [12]. For the *mecA* transcription assays, total RNA was isolated from all parental MRSA and their respective *sarA* mutant strains in the presence and absence of half the MICs of oxacillin after exposure for 24 hours at 37°C, using the RNeasy kit as described above.

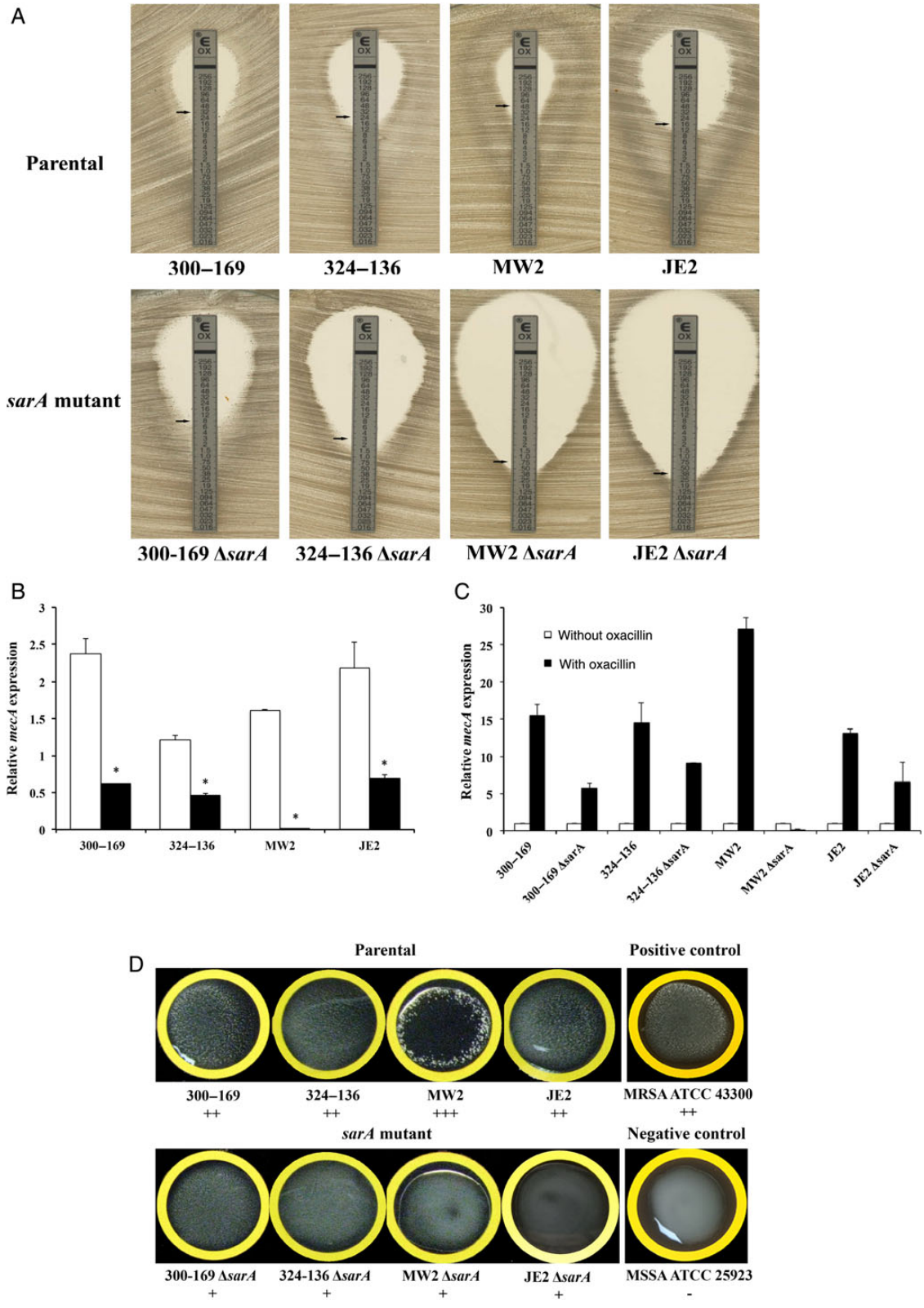

Figure 1. Oxacillin minimum inhibitory concentrations (MICs) determined by the Etest (*A*), relative expression of *mecA* in the absence (*B*), and the presence of half MICs of oxacillin (*C*) and penicillin-binding protein 2a (PBP2a) agglutination (*D*) of study methicillin-resistant *Staphylococcus aureus* parental and *sarA* mutant strain sets. *B*, Relative transcription levels of *mecA* represent the mean (+SD) of 3 biological replicates in vitro. The fold changes represent the normalized *mecA* expression ($2^{-\Delta Ct}$) to the housekeeping gene *gyrB*. **P* < .05, compared with their respective parental strains. Open bars, parental strain; shaded bars, *sarA* mutant. *D*, *mecA*-positive (ATCC 43300) and *mecA*-negative (ATCC 25923) controls. Interpretation of agglutination intensity: +++, high; ++, moderate; +, low; and −, none.

Transcription Analyses by Real-Time Quantitative PCR (qPCR)

Real-time qPCR was performed as described previously [12, 28]. The amplification of *mecA*, *asp23* (surrogate for *sigB* expression), *sarA*, and *gyrB* was performed using primers as described previously [12, 29, 30]. *gyrB* was used to normalize for transcript quantification. Relative quantification was calculated by the $\Delta\Delta C_T$ method.

Whole-Genome Sequencing (WGS)

The MRSA JE2 and its *sarA* mutant strain-pair underwent WGS. Genomic DNA isolation and library preparation were performed as described previously [31, 32]. The DNA library was mapped against published NC_007793 (*S. aureus* USA300_FPR3757) genomes, and the single-nucleotide polymorphism (SNP) and InDels were examined [34, 35].

RNA Sequencing (RNA-seq)

The RNA samples of JE2 and its *sarA* mutant strain-pair described above were used for RNA-Seq. Sequencing was conducted on an Illumina NextSeq platform. The RNA-Seq data were analyzed by CLC Genomics Workbench (V8.5), and the number of reads per kilobase of transcript per million mapped reads for each gene was compared between the parental and *sarA* mutant strains.

Experimental Endocarditis Model in Rabbits

To better define the role of *sarA* in vivo in regard to oxacillin responsiveness in MRSA, a well-characterized rabbit model of catheter-induced aortic valve infective endocarditis was used [12, 28]. After catheterization, animals were infected intravenously (with 10^5 CFU/animal, a 95% infective dose, as established in previous studies [12]) of either JE2 parental, its *sarA* mutant, or complemented strains. Twenty-four hours after infection, animals were randomized to receive no therapy (controls) or oxacillin at 50 mg/kg intramuscularly 3 times daily for 3 days. This oxacillin dose achieves a serum maximum concentration (C_{max}) of approximately 25 μg/mL (unpublished data), which was significantly lower than the C_{max} (43 μg/mL) of the recommended human clinical dose for mild-to-moderate MSSA infections (500 mg intravenously [33]). Twenty-four hours after the last oxacillin treatment, animals were euthanized. The cardiac vegetation, kidney, and spleen were then removed and quantitatively cultured. The limitation of organism density detection in the target tissues is approximately 1 $\log_{10}$ CFU per gram of tissue. This value was assigned to all culture-negative (sterile) tissues as a baseline for the purposes of relative calculation of the mean $\log_{10}$ CFU per gram of tissue (±SD) for statistical comparisons.

The Institutional Animal Care and Use Committee of the Los Angeles Biomedical Research Institute at Harbor–UCLA Medical Center approved all animal study protocols.

Statistics

To compare MRSA counts in target tissues, univariate analyses were performed using the Student *t* test [16]. For other experiments described, unpaired 2-tailed Student *t* tests were performed. *P* values of <.05 were considered statistically significant.

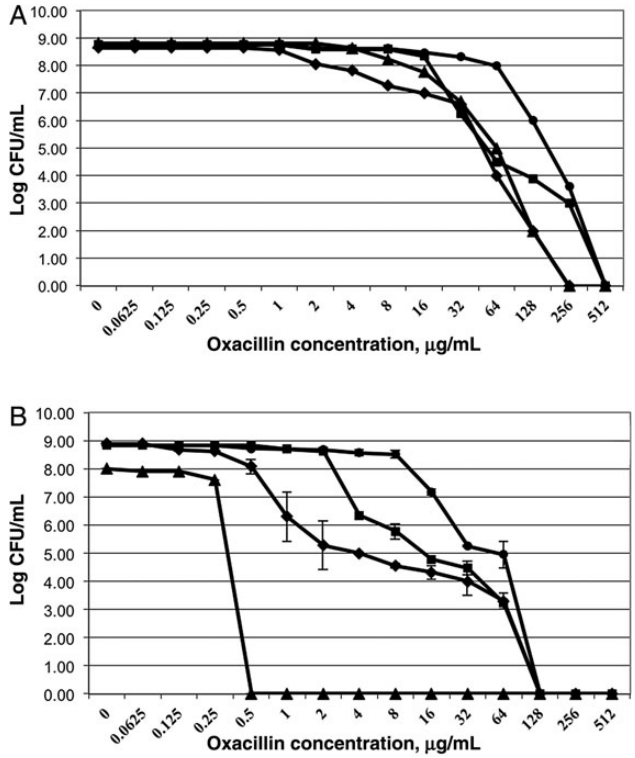

Figure 2. Population analyses of methicillin-resistant *Staphylococcus aureus* parental (*A*) and their respective *sarA* mutant (*B*) strains upon exposure to a range of oxacillin concentrations. These data represent the means (±SD) for 2 separate assays. Strain 300-169, circles; strain 324-136, squares; strain MW2, triangles; and strain JE2, diamonds. CFU, colony-forming units.

RESULTS

Effect of *sarA* on Oxacillin MICs

As expected, all parental MRSA strains were oxacillin resistant, with MICs ranging from 16 to 48 μg/mL (Figure 1*A*). Interestingly, the *sarA* mutants exhibited a 4–64-fold reduction in oxacillin MICs versus their respective parental strains. For instance, the 2 CA-MRSA *sarA* mutants were hypersusceptible to oxacillin (MICs ≤ 1.0 μg/mL). In addition, we found an agreement of overall MICs (within ±2-fold dilutions) between the results of the Etest and broth microdilution in all study strains.

Population Analyses

We demonstrated that only 1 MRSA parental strain (300-169) exhibited oxacillin homogeneous resistance, while the other 3 parental strains showed heterogeneous resistance to oxacillin (Figure 2). In addition, 3 of 4 *sarA* mutants, except *sarA* mutant in the MW2 background, had hetero-oxacillin–resistant subpopulations (Figure 2).

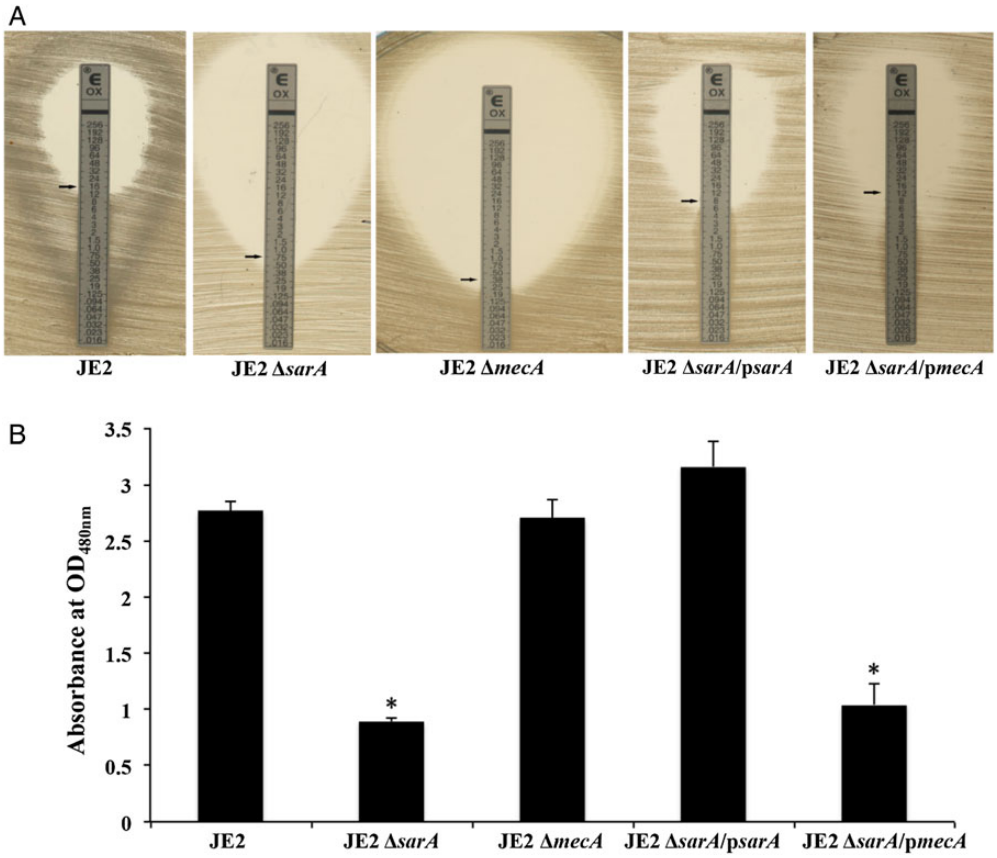

Figure 3. Oxacillin minimum inhibitory concentrations determined by the Etest (*A*) and biofilm formation (*B*) of the methicillin-resistant *Staphylococcus aureus* JE2 parental strain, its *sarA* mutant, *mecA* mutant, Δ*sarA*/p*sarA* (*sarA* complemented in the *sarA* mutant strain), and Δ*sarA*/p*mecA* (*mecA* complemented in the *sarA* mutant strain). Results of biofilm formation are shown as the mean of the OD_{490nm} (+SD) from 3 biological replicates, each of which was done in triplicate. **P* < .05, for the strains, compared with the JE2 parental strain.

Impact of *sarA* on *mecA* Transcription and PBP2a Production

The effect of *sarA* on oxacillin susceptibility suggested that this outcome might be due to its influence on *mecA* expression and PBP2a production. Consistent with the oxacillin MICs, all parental strains had significantly higher *mecA* expression as compared to their respective *sarA* mutants ($P < .005$; Figure 1*B*). Additionally, induced *mecA* expression was observed in the presence of half the MICs of oxacillin versus the absence of oxacillin in all study strains (Figure 1*C*). However, the increased degree of *mecA* expression was substantially lower in the *sarA* mutants as compared to their respective parental strain (Figure 1*C*).

For PBP2a production, as expected, the positive MRSA control strain had strong agglutination, while PBP2a production was not detected in the negative control MSSA strain (Figure 1*D*). In accordance with the *mecA* expression results, a higher intensity of agglutination, indicating higher PBP2a production, was observed in the MRSA parental strains vs their respective *sarA* mutants (Figure 1*D*).

To define the relationship between *sarA* and *mecA* on abrogation of MRSA phenotype, we examined oxacillin MICs using *sarA* and *mecA* mutants, and their complemented strains in the *sarA* mutant strain in the JE2 background. We found that oxacillin MICs were significantly reduced in the *sarA* and *mecA* mutants, thus changing the MRSA phenotype to methicillin susceptible (Figure 3*A*). These reduced oxacillin MICs were restored to MRSA parental levels with complementation of *sarA* or *mecA* in the *sarA* mutant strain (Figure 3*A*). In addition, a similar intensity of agglutination was observed from the PBP2a production assays between the JE2 parental and *ΔsarA/psarA* strains (data not shown).

Biofilm Formation

The Δ*sarA* strain formed significantly less biofilm as compared to its parental JE2 strain and the complemented *sarA* mutant restored formation of biofilm to the level of the parent JE2 strain (Figure 3*B*). Based on the observations of *sarA* on biofilm formation and oxacillin susceptibility, we hypothesized that oxacillin treatment may have good efficacy against biofilm-related MRSA infections. However, there were no significant differences in biofilm formation between the *mecA* mutant and its parental strain, as well as between the JE2 Δ*sarA* and Δ*sarA*/p*mecA*

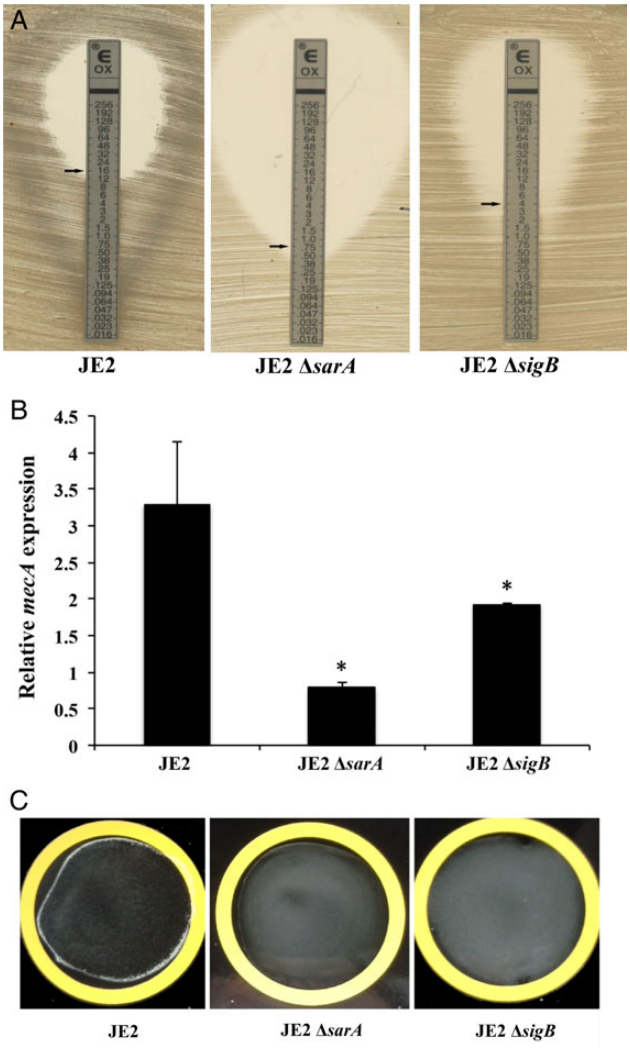

Figure 4. Oxacillin minimum inhibitory concentrations determined by the Etest (*A*), relative expression of *mecA* (*B*), and PBP2a agglutination (*C*) of the JE2 parental strain and its *sarA* and *sigB* mutants. Relative transcript levels of *mecA* represent the mean (+SD) of 3 biological replicates in vitro. The fold changes represent normalized target gene expression ($2^{-\Delta Ct}$) to the housekeeping gene *gyrB*. *$P < .05$, compared with the JE2 parental strain.

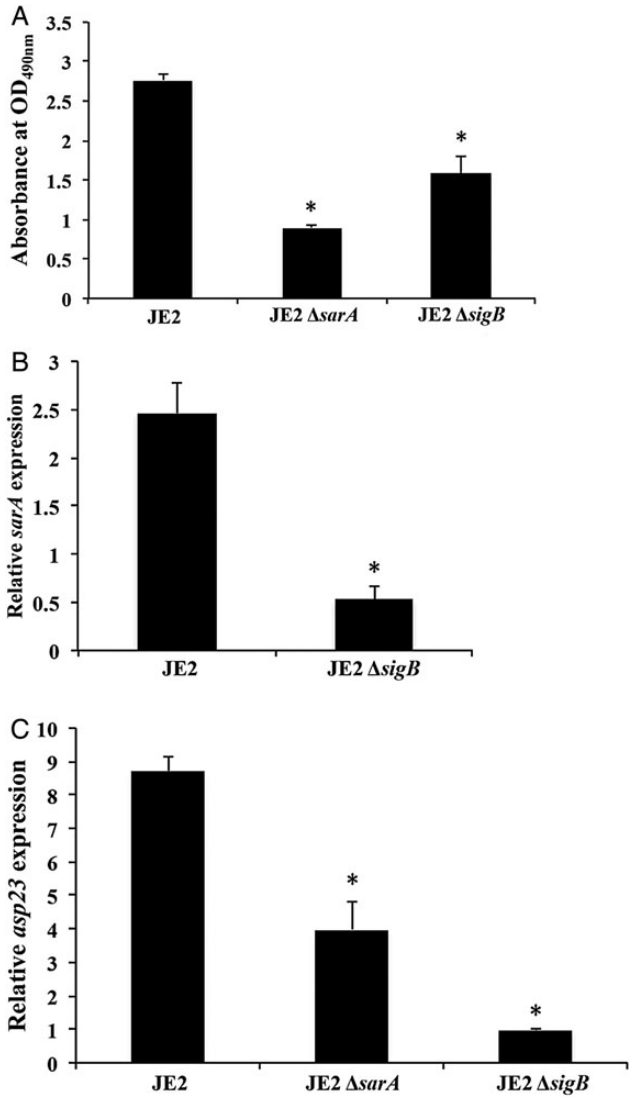

Figure 5. Biofilm formation (*A*) and relative expression of *sarA* (*B*) and *asp23* (*C*) in the methicillin-resistant *Staphylococcus aureus* JE2 parental strains and its *sarA* and/or *sigB* mutant. Relative transcript levels of *sarA* and *asp23* represent the mean (+SD) of 3 biological replicates in vitro. The fold changes represent normalized target gene expression ($2^{-\Delta Ct}$) to the housekeeping gene *gyrB*. *$P < .05$, compared with the JE2 parental strain.

strains, which collectively suggests that *mecA* itself has no effect on biofilm formation (Figure 3*B*).

Influence of *sarA* on *sigB*

It has been demonstrated that modulation of the *sarA* locus is dependent on the stress-induced transcription factor SigB. Thus, the interaction between *sarA* and *sigB* and their impact on oxacillin MICs, PBP2a production, biofilm formation, and *sarA* expression were studied by using *sarA* and *sigB* mutant strains in the JE2 background. As shown in Figure 4*A*, the *sigB* knockout of strain JE2 also had significantly reduced oxacillin MICs (4 μg/mL) versus its parental strain, in part due to the lower expression of *mecA* and lower production of PBP2a (Figure 4*B* and 4*C*). Furthermore, the *sigB* mutant formed significantly less biofilm ($P < .01$; Figure 5*A*). Given that *sigB* is a regulator of *sarA* [15], we found that the *sigB* mutant had significantly less *sarA* expression when compared to the JE2 parental strain ($P < .01$; Figure 5*B*). Interestingly, the *sarA* mutant strain exhibited approximately 2-fold lower *asp23* expression (a marker for *sigB* activity) as compared to the parental strain ($P < .01$; Figure 5*C*). As a control, the *sigB* mutant had very low level of *asp23* expression as compared to JE2 and the *sarA* mutant. Our data suggest interdependency between the *sarA* and *sigB* regulators in terms of the MRSA phenotype and biofilm formation, as well as a positive regulatory effect of *sarA* on *sigB* activity.

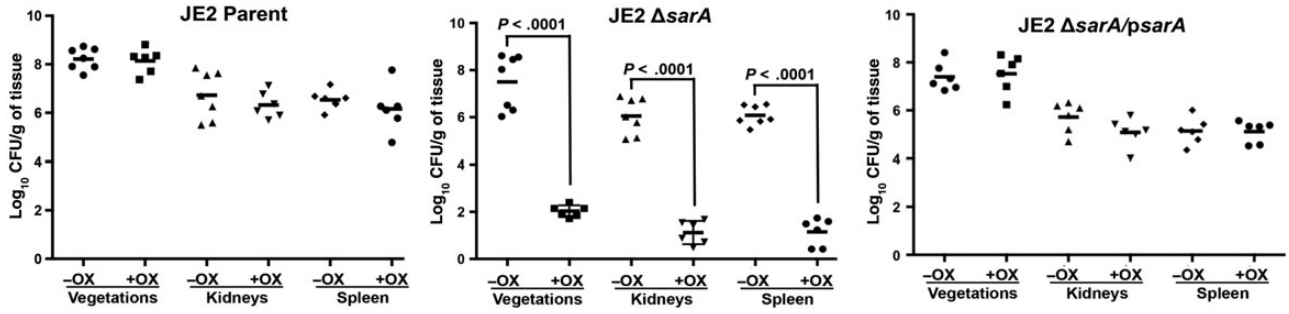

Figure 6. Densities of methicillin-resistant *Staphylococcus aureus* in target tissues in the infective endocarditis model due to challenge with 10^5 colony-forming units (CFU) of the JE2 parental strain, its *sarA* mutant, or the *sarA* complementary strain, with (+) or without (–) oxacillin (OX) therapy (50 mg/kg intramuscularly 3 times daily for 3 days). Each dot represents 1 rabbit. Horizontal black bars indicate mean values for observations.

RNA-seq Findings

We found that 9 and 15 genes were expressed significantly higher and lower, respectively, in the *sarA* mutant versus the JE2 parental strain ($P < .01$; Supplementary Table 1). For instance, 4 major extracellular proteases (*sspB*, *sspA*, *scp*, and *aur*) and proton antiporter genes were expressed higher in the *sarA* mutant versus the parental strain. On the other hand, inactivation of *sarA* decreased the expression of pyrimidine biosynthetic pathway genes (*pyrF*, *pyrE*, *carB*, *pyrB*, *pyrC*, and *pyrP*) and argininosuccinate lyase–encoding genes. In addition, similar RNA-seq results were obtained for MW2 and its *sarA* mutant strain (data not shown). Moreover, 2 *sarA* upregulated (*argH* and SAUSA300-1477) and 2 *sarA* downregulated (*ear* and *sspB*) genes with the most substantial changes were selected for validation of their expression in JE2 and its *sarA* mutant strains by real-time qPCR. Our data demonstrated that the expression of these genes yielded outcomes similar to those observed by RNA-seq (data not shown).

WGS Findings

Interestingly, our data indicated that JE2 and the JE2 *sarA* mutant had identical WGS findings, with the exception of the insertion of *aph(3′)-III* (which confers resistance to kanamycin) within the JE2 *sarA* mutant. There were also approximately 45 SNPs demonstrated between the USA300_FPR3757 and JE2 strains.

Impact of *sarA* on the Efficacy of Oxacillin in a Rabbit Infective Endocarditis Model

Of interest, in the absence of oxacillin therapy, the *sarA* mutant was associated with reduced MRSA densities in vegetations ($P < .05$) but not in other hematogenously seeded target organs (kidneys and spleen), compared with its respective JE2 parental strain (Figure 6). As expected, oxacillin treatment had no efficacy in the model of infective endocarditis caused by the JE2 parental strain. However, of great significance, animals infected with the *sarA* mutant of JE2 were hypersusceptible to oxacillin treatment, with 100% sterile tissue cultures (Figure 6). In addition, animals infected by the *sarA*-complemented variant did not respond to oxacillin treatment; this confirmed the important roles of *sarA* in maintaining the MRSA phenotype and, consequently, the resistance to oxacillin therapeutic outcomes in this model.

DISCUSSION

In the current study, we demonstrated that *sarA* mutants exhibited significantly reduced oxacillin MICs relative to respective parental strains. This finding was attributable in part to decreased *mecA* expression and corresponding reduced PBP2a production, which was consistent with previous reports [36, 37]. For example, Arede et al reported that the inactivation of MecR2, a robust activator of *mecA* transcription that disrupts binding of the repressor MecI to the *mecA* promoter, converted a MRSA strain to methicillin-susceptible phenotype [37]. Previous studies also showed that *blaI* and *blaR1*, 2 genes located upstream of β-lactamase gene (*blaZ*), regulate β-lactamase and PBP2a production in *S. aureus* [38]. Published findings of WGS analysis demonstrated that the β-lactamase genes (*blaI-blaR1-blaZ*) were not found in our 2 study CA-MRSA strains. In addition, our unpublished WGS data showed that, although one of the HA-MRSA strains does have these genes, there are a number of SNPs identified (as compared to a reference strain; data not shown). Therefore, the current study was not designed to address the potential impact of *sarA* on these genes. In addition to *mecA* and *blaZ*, other genes have been reported to influence the MRSA phenotype. For example, inactivation of *llm*, *agr*, and *fmtA* each impart reductions in methicillin resistance without converting the MRSA strain to a *mecA*-negative MSSA strain and with no apparent affect on PBP2a production [39, 40]. In addition, factors essential for methicillin resistance, such as FemA, FemB, and FemC, are also important to the essential resistance phenotype by affecting peptidoglycan precursor synthesis [41, 42]. It has been reported that inactivation of *femAB* renders MRSA hypersusceptible to β-lactam. However, inactivation of *femC* has less effects on methicillin resistance

since highly resistant subpopulations are still present [41]. Moreover, Boyle-Vavra et al reported that VraSR, a 2-component regulatory system, regulates downstream genes that presumably facilitate resistance to cell wall–inhibitory antibiotics, and inactivation of *vraSR* decreases oxacillin resistance without decreasing *mecA* expression [43, 44]. Taken together, these results indicate that, in addition to *mecA* and *blaZ*, the methicillin resistance phenotype is also influenced by other chromosomal factors and that the mechanism(s) of these effects are *mecA* independent.

Our WGS data showed that JE2 and its *sarA* mutant are identical except for the insertion of *aph(3′)-III*, which was used to disrupt *sarA* in the *sarA* mutant. These data underscore the independent role of *sarA* in the MRSA phenotypes observed in our study. Our RNA-Seq data showed that the pyrimidine biosynthetic pathway genes significantly increased in parent versus *sarA* mutants. These findings were consistent with reports by Cordwell et al [45], who demonstrated that the pyrimidine biosynthetic pathway genes had higher expression in MRSA (COL) than MSSA (8325). However, the impact of the perturbation of the pyrimidine biosynthetic pathways on the MRSA phenotype via *sarA-mecA* interactions remains to be elucidated.

Biofilm formation is a key virulence strategy of staphylococci [8, 46]. In the current study, as expected, we found that the *sarA* mutant formed significantly less biofilm than the parental strain and had increased expression of major extracellular proteases [46, 47]. Therefore, it is conceivable that *sarA*-mediated reduction in biofilm formation influences methicillin/oxacillin susceptibility beyond its negative impact on protease genes. For example, a logical hypothesis might posit that the *sarA* mutant derepresses protease expression, leading to the increased proteolytic activity and degradation of PBP2a necessary for oxacillin resistance. Of special interest, we also observed that the *mecA* mutant displayed significantly reduced oxacillin resistance but without a discernible effect on biofilm formation. Like most clinical MRSA strains [48], 3 of the 4 MRSA strains in this study exhibited a heterogeneous pattern of resistance to oxacillin. Pozzi et al recently reported that biofilm formation by *S. aureus* 8325-4 (oxacillin susceptible) and its isogenic strain carrying p*mecA,* which exhibits a heterogeneous oxacillin resistance phenotype, were similar [8]. These findings corroborated our current results, indicating that the heterogeneous oxacillin susceptibility may have no direct impact on biofilm formation.

In parallel to *sarA*, we also investigated another key global regulon, *sigB*, because of its impact on expression of multiple virulence genes and global regulators, including *sarA* [14]. It is instructive to note that the influence of *sigB* on *sarA* is somewhat controversial. Bischoff et al reported a positive regulatory effect of *sigB* on *sarA* [15]. In contrast, Cheung et al [49] demonstrated a negative impact of *sigB* on *sarA* transcription. These contrasting findings may be due to differences in genetic backgrounds of study strains used in the 2 studies. In the present work, we discovered that not only did the *sigB* mutant exhibit decreased *sarA* expression, but also that the *sarA* mutant had reduced *asp23* transcription (a surrogate marker of *sigB* activation). This outcome indicated a positive regulatory effect of *sigB* on *sarA*, as well as a positive, reciprocal feedback influence of *sarA* on *sigB*. The mechanism(s) of this internally amplifying autocrine feedback loop between *sarA* and *sigB* is not well understood. Several observations have suggested that the regulatory effects of SarA might be more complex than initially appreciated [11]. Besides transcriptional regulation, the present results also revealed that the Δ*sigB* null mutation significantly reduced oxacillin resistance and biofilm formation as compared to its JE2 parental strain. This finding was in accordance with previous findings in which inactivation of *sigB* resulted in reduced methicillin resistance [50]. These results support our hypothesis that the *sigB* mutant exhibited reduced *sarA* expression, subsequently resulting in downregulated *mecA*. Consequently, this effect would lead to the reduction of oxacillin resistance, a phenotype potentially amplified by the positive feedback impact imposed by *sarA* on *sigB* activation.

Perhaps most importantly, the genotypic and phenotypic findings described above translated into a significant outcome of *S. aureus* infection in vivo. These data underscored the significance of *sarA* in differential oxacillin resistance in the relevant setting of infective endocarditis. As expected, oxacillin had no therapeutic effect in the model of infective endocarditis caused by MRSA strains. However, animals infected with the *sarA* mutant exhibited a striking degree of hypersusceptibility to oxacillin treatment. The mechanism(s) of this therapeutic effect are thought to occur by inactivation of *sarA*, which subsequently reduces oxacillin resistance and biofilm formation. From a broader perspective, these results raise the exciting possibility that novel compounds targeting *sarA* expression and/or function may add to the future anti-MRSA armamentarium. In addition, these data provide an impetus into further studies to investigate the mechanism(s) of how *sarA* regulates *mecA*, including defining whether *sarA* binds to the *mecA* promoter and/or its messenger RNA and whether increased oxacillin susceptibility in the *sarA* mutants involves other regulatory factors downstream of or beyond the *sarA* network that regulates *mecA*.

Notes

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