



Roles of Lytic Transglycosylases in Biofilm Formation and β -Lactam Resistance in Methicillin-Resistant *Staphylococcus aureus*

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ABSTRACT *Staphylococcus aureus* is responsible for numerous community outbreaks and is one of the most frequent causes of nosocomial infections with significant morbidity and mortality. While the function of lytic transglycosylases (LTs) in relation to cell division, biofilm formation, and antibiotic resistance has been determined for several bacteria, their role in *S. aureus* remains largely unknown. The only known LTs in *S. aureus* are immunodominant staphylococcal antigen A (IsaA) and *Staphylococcus epidermidis* D protein (SceD). Our study demonstrates that, in strains of methicillin-resistant *S. aureus* (MRSA), IsaA and SceD contribute differently to biofilm formation and β -lactam resistance. Deletion of *isaA*, but not *sceD*, led to decreased biofilm formation. Additionally, in *isaA*-deleted strains, β -lactam resistance was significantly decreased compared with that of wild-type strains. Plasmid-based expression of *mecA*, a major determinant of β -lactam resistance in MRSA, in an *isaA*-deleted strain did not restore β -lactam resistance, demonstrating that the β -lactam susceptibility phenotype is exhibited by the *isaA* mutant regardless of the production level of PBP2a. Overall, our results suggest that IsaA is a potential therapeutic target for MRSA infections.

KEYWORDS MRSA, *Staphylococcus aureus*, antibiotic resistance, β -lactams, biofilms, peptidoglycan, transglycosylases

Staphylococcus aureus is an opportunistic pathogen responsible for infections ranging from skin infections to life-threatening diseases (1, 2). Since the 1960s, the emergence of antibiotic-resistant strains, such as methicillin-resistant *S. aureus* (MRSA), has contributed to its spread, and *S. aureus* is now the most common cause of nosocomial infections with significant morbidity and mortality (3). Currently, the emergence of new strains with increased virulence and colonization capacity is responsible for an unprecedented number of community outbreaks in the United States, resulting in the number of deaths caused by *S. aureus* infection to be comparable to those caused by HIV, tuberculosis, and viral hepatitis combined (4, 5). The pathogenicity of *S. aureus* relies on a wide range of virulence factors, including proteins conferring resistance to host antimicrobial peptides (6), secreted toxins (1, 2, 4), and factors enhancing its ability to form matrix-encased biofilms on tissues and medical devices (2, 7). *S. aureus* biofilms have been shown to be resistant to killing by antibiotics at concentrations 10 to 1,000 times greater than the concentration required to kill planktonic cells, leading to the failure of conventional antibiotic therapy and recurrence of infections (3, 8).

It has been reported that bacterial lytic transglycosylases (LTs) are involved in

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various cell functions and phenotypes. LTs cleave the β -1,4-glycosidic bonds between *N*-acetylmuramic and *N*-acetylglucosamine residues of peptidoglycan (9) and affect peptidoglycan turnover and septum cleavage during cell division (10, 11). Additionally, in some Gram-negative bacteria, LTs are involved in virulence mechanisms during infection (11). The peptidoglycan fragments produced by LTs are recycled in the cytoplasm for peptidoglycan biosynthesis (12–14). During infection, released peptidoglycan fragments may also be recognized by host cells, leading to general symptoms, such as fever, lack of appetite, or sleepiness, and inducing inflammation via the production of cytokines (11, 15). This mechanism contributes to virulence in Gram-negative bacteria, such as *Bordetella pertussis* and *Neisseria gonorrhoeae* (11). LTs are known to affect biofilm formation in both Gram-positive and Gram-negative bacteria (16–18). Moreover, deletion of LTs can affect β -lactamase induction (19) and the extent of β -lactam resistance in some Gram-negative bacteria (18, 20, 21).

The only known *S. aureus* LTs are immunodominant staphylococcal antigen A (IsaA) and *Staphylococcus epidermidis* D protein (SceD), both of which have been shown to possess cell wall hydrolytic activity (22). IsaA was identified as a major antigen expressed during sepsis or bacteremia caused by *S. aureus* (23, 24). IsaA is both excreted into the extracellular environment and bound to the cell wall, where it localizes mainly to the septal region of dividing cells (25). Deletion of *isaA* results in increased expression of SceD (22). SceD plays an essential role in nasal colonization in cotton rats, and its inactivation results in impaired cell separation (22). Increased SceD levels in the cell wall fraction of vancomycin-intermediate *S. aureus* strains can partly explain the altered peptidoglycan structure of these strains (26).

Considering the functions LTs reportedly play in the cell cycle, resistance to antibiotics, and virulence in other bacteria, *S. aureus* LTs are likely to have major clinical significance. However, their roles in *S. aureus* have been the subject of only a few investigations and remain largely unknown. The present study was conducted to characterize the roles of LTs in MRSA by investigating their effects on biofilm formation and antibiotic susceptibility.

RESULTS

IsaA is involved in biofilm formation. LT gene mutations have been reported to affect biofilm formation in *Lactococcus lactis* (16), *Salmonella enterica* (17), and *Pseudomonas aeruginosa* (18). Here, we investigated the biofilm-forming capacity of wild-type (WT) and LT mutants of *S. aureus* MR23 (27), a MRSA clinical isolate that produces a large amount of biofilm, as well as the effects of overexpression and complementation of LT genes using a plasmid-based expression system shown in Table 1. The cell growth of WT and LT mutants of MR23 was almost identical, except for the final cell density being slightly higher in the $\Delta isaA\Delta sceD$ mutant than in the WT (Fig. 1A). Note that there was no difference in CFUs between WT and all LT mutants in stationary phase (ca. 2×10^9 CFU/ml). We analyzed the levels of IsaA and SceD in *S. aureus* culture supernatants using Western blotting (Fig. 1B). In agreement with previous findings (22), deletion of *isaA* resulted in an increased level of SceD compared with that of the WT. In the opposite case, no IsaA overproduction was detected in the $\Delta sceD$ mutant. We then confirmed the effect of complementation with plasmids harboring each LT gene on the production of LTs. Notably, the SceD-overproduction phenotype in the $\Delta isaA$ mutant (WT_EV versus $\Delta isaA$ _EV) was abolished by plasmid-based expression of *isaA* ($\Delta isaA$ _EV versus $\Delta isaA$ _pCNisaA). IsaA and SceD protein production in the supernatant fractions was almost identical in whole-cell fractions that contained cell wall proteins and cytoplasmic proteins (see Fig. S1 in the supplemental material).

Deletion of *isaA* significantly decreased biofilm formation but *sceD* deletion did not (Fig. 1C). The biofilm-forming capacity of the $\Delta isaA\Delta sceD$ mutant was nearly identical to that of the $\Delta isaA$ mutant. Plasmid-based expression of *isaA* in the WT strain enhanced biofilm formation. We also confirmed that plasmid-based expression of *isaA* complemented the decreased biofilm phenotype of the $\Delta isaA$ mutant ($\Delta isaA$ _EV versus $\Delta isaA$ _pCNisaA). In contrast, plasmid-based expression of *sceD* did not affect biofilm

TABLE 1 Strains and plasmids used in this study^a

Strain or plasmid	Genotype or characteristic	Source or reference
Strains		
<i>E. coli</i>		
DH5a	F ⁻ , Φ80d <i>lacZ</i> ΔM15, Δ(<i>lacZ</i> YA- <i>argF</i>)U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (r _K ⁻ m _K ⁺), <i>phoA</i> , <i>supE44</i> , λ ⁻ , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	Toyobo
BL21(DE3)	F ⁻ , <i>ompT</i> , <i>hsdS</i> _B (r _B ⁻ m _B ⁻), <i>gal</i> (λcl857, <i>ind1</i> , <i>Sam7</i> , <i>nin5</i> , <i>lacUV5</i> -T7gene1), <i>dcm</i> (DE3)	Promega
DC10B	<i>E. coli</i> K12, Δ <i>dcm</i> mutant	49, 50
<i>S. aureus</i>		
MR23	Clinical isolate of MRSA (ST380)	27
MR23Δ <i>isaA</i>	<i>isaA</i> was deleted from MR23	This study
MR23Δ <i>sceD</i>	<i>sceD</i> was deleted from MR23	This study
MR23Δ <i>isaA</i> Δ <i>sceD</i>	<i>isaA</i> and <i>sceD</i> were deleted from MR23	This study
MR23Δ <i>mecA</i>	<i>mecA</i> was deleted from MR23	This study
MR10	Clinical isolate of MRSA (ST5)	27
MR10Δ <i>isaA</i>	<i>isaA</i> was deleted from MR10	This study
MR10Δ <i>sceD</i>	<i>sceD</i> was deleted from MR10	This study
MR10Δ <i>isaA</i> Δ <i>sceD</i>	<i>isaA</i> and <i>sceD</i> were deleted from MR10	This study
USA300	Clinical isolate of MRSA (ST8)	ATCC BAA1717
USA300Δ <i>isaA</i>	<i>isaA</i> was deleted from USA300	This study
USA300Δ <i>sceD</i>	<i>sceD</i> was deleted from USA300	This study
USA300Δ <i>isaA</i> Δ <i>sceD</i>	<i>isaA</i> and <i>sceD</i> were deleted from USA300	This study
Plasmids		
pET29a	<i>E. coli</i> expression vector; Km ^R	Novagen
pET <i>sceD</i>	<i>sceD</i> cloned in pET29a	This study
pKOR1	An <i>E. coli</i> / <i>S. aureus</i> shuttle vector plasmid for knockout of <i>S. aureus</i> genes by allelic exchange; Ap ^R , Cm ^R	48
pCN51	An <i>E. coli</i> / <i>S. aureus</i> shuttle vector plasmid; Ap ^R , Em ^R	54
pCN51Cm	An <i>E. coli</i> / <i>S. aureus</i> shuttle vector plasmid; Ap ^R , Cm ^R	This study
pCN <i>isaA</i>	<i>isaA</i> cloned in pCN51Cm	This study
pCN <i>sceD</i>	<i>sceD</i> cloned in pCN51Cm	This study
pCN <i>mecA</i>	<i>mecA</i> cloned in pCN51Cm	This study

^aKm^R, kanamycin resistance; Ap^R, ampicillin resistance; Em^R, erythromycin resistance; Cm^R, chloramphenicol resistance; ST, sequence type.

formation in either the WT or Δ*isaA* mutant. These results demonstrate that, of the two LTs, only *IsaA* significantly affects biofilm formation in this strain.

Deletion of *isaA* induces the β-lactam susceptibility phenotype in MRSA. In other bacterial species, deletion of LTs has been shown to affect β-lactam resistance (18, 20, 21); however, no such information is available with regard to *S. aureus* LTs. We determined the MICs of antibiotics against WT and LT mutants of MR23 in Mueller-Hinton II (MH) broth (Table 2). Four-fold or greater differences in MIC values were regarded as significant. No significant increase in MIC was observed for any of the LT mutants. Notably, deletion of *isaA* decreased the MICs of 5/7 β-lactams (oxacillin, cefazolin, flomoxef, ceftiofur, and imipenem), and deletion of *sceD* decreased the MICs of 2/7 β-lactams (ampicillin and ceftiofur). In the assay performed using brain heart infusion broth supplemented with 1% glucose (BHIG), deletion of *isaA* decreased the MICs of 4/7 β-lactams (oxacillin, ampicillin, flomoxef, and ceftiofur), whereas deletion of *sceD* showed no effect (see Table S1 in the supplemental material). Plasmid-based complementation of *isaA* significantly increased the MICs of β-lactams against the Δ*isaA* mutant (Δ*isaA*_EV versus Δ*isaA*_pCN*isaA*). In contrast, plasmid-based expression of *sceD* in the Δ*isaA* mutant did not lead to such an effect (Table 2). These results indicate that *S. aureus* LTs contribute to β-lactam resistance and that *IsaA* is more important in this role than *SceD*.

Correlation between PBP2a production and β-lactam resistance in *isaA* mutants. As β-lactam resistance of MRSA is conferred mainly by *mecA*-encoded PBP2a (28), we investigated the impact of *isaA* and *sceD* deletions on PBP2a production in the presence or absence of oxacillin as an inducer of PBP2a (29). The oxacillin concentrations used were 1/16, 1/8, and 1/4 of the MIC for each strain. Western blotting of WT using an anti-PBP2a antibody revealed two bands of different molecular size (Fig. 2A). Both bands were derived from PBP2a, as they were not detected in the Δ*mecA* mutant

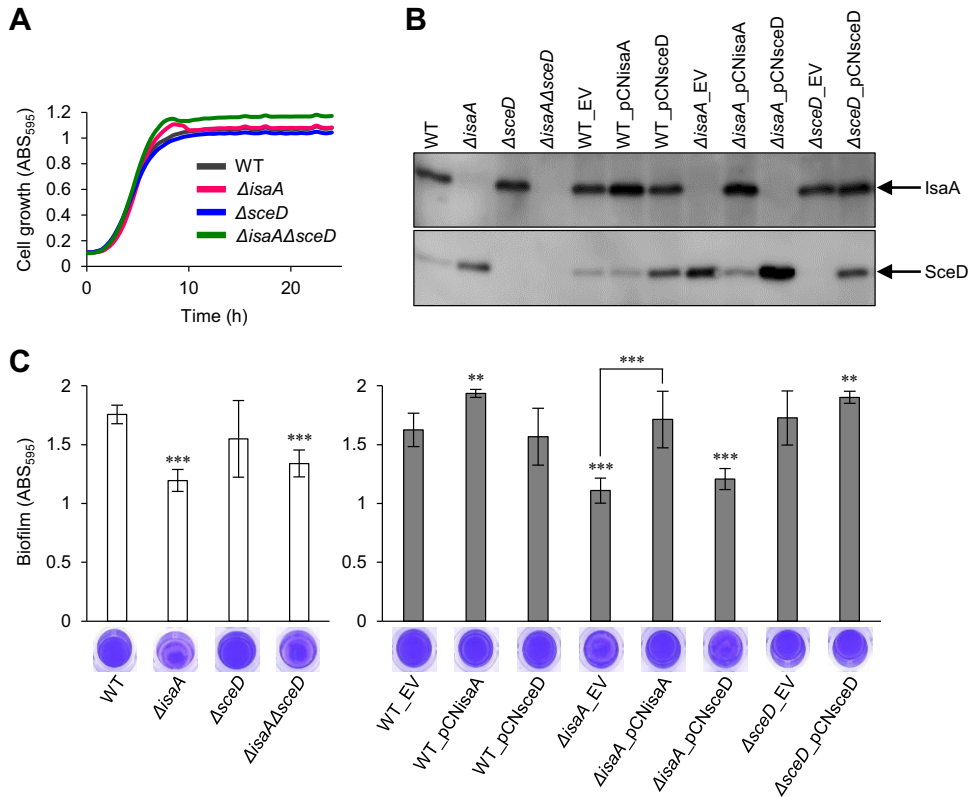


FIG 1 Effect of LT gene deletion on cell growth and biofilm formation of *S. aureus* MR23. (A) Cell growth of wild-type (WT) and LT mutants. The average of three independent cultures is shown. (B) Western blot showing levels of IsaA and SceD in culture supernatants of WT and LT mutants. (C) Biofilm formation of WT and LT mutants without (white bars) or with (gray bars) plasmid. Biofilms formed on the bottom of the 96-well plate were stained with crystal violet; subsequently, absorbance at 595 nm was measured. Representative images of stained biofilms are shown below. The values are the means of six independent cultures. The error bars indicate the standard error of the mean. Differences between WT and mutants were compared using Student's *t* test. ***P* < 0.01, ****P* < 0.001. EV, pCN51Cm (empty vector); pCNisaA, pCN51Cm containing *isaA*; pCNsceD, pCN51Cm containing *sceD*.

in the presence and absence of oxacillin (see Fig. S2 in the supplemental material). The cause of the two bands with different molecular weights corresponding to PBP2a peptides is presently unknown. In WT, expression of the upper PBP2a band (PBP2a_{up}) was oxacillin dependent. Only a weak lower PBP2a band (PBP2a_{low}) was detected in the absence of oxacillin. A similar phenomenon was observed in the $\Delta sceD$ strain. Instead, induction of PBP2a_{up} was decreased in the $\Delta isaA$ strain and dramatically decreased in the $\Delta isaA\Delta sceD$ strain compared with the WT (Fig. 2A). Importantly, the lower production of PBP2a_{up} in the $\Delta isaA$ strain was complemented by plasmid-based expression of *isaA* ($\Delta isaA$ _EV versus $\Delta isaA$ _pCNisaA) (Fig. 2B).

From these results, we hypothesized that the expression of *isaA* is directly or indirectly involved in the production of PBP2a, thereby contributing to the β -lactam resistance of MR23. To establish a cause-effect relationship between the β -lactam susceptibility phenotype and decreased PBP2a production level, pCNmecA, a plasmid containing *mecA* cloned under the control of a cadmium-inducible promoter in pCN51Cm plasmid, was introduced in $\Delta mecA$ and $\Delta isaA$ mutants. In the mutants harboring pCNmecA, PBP2a production was induced depending on the concentration of cadmium added to the culture medium (Fig. 2C). Then, the effects of inducible PBP2a production on β -lactam resistance of the mutants were investigated. The MIC of oxacillin against $\Delta mecA$ _pCNmecA was dramatically increased depending on cadmium concentration (Table 3), confirming that PBP2a produced by the cadmium-inducible system was functional. On the other hand, contrary to our hypothesis, PBP2a induction had no effect on the MIC of oxacillin against $\Delta isaA$ _pCNmecA. Accordingly, these

TABLE 2 MICs of antibiotics for WT and LT mutants of *S. aureus* MR23 cultured in Mueller-Hinton broth^a

Antibiotic	MIC (µg/ml) of:																																																																																																																																																																																																	
	WT	ΔisaA	ΔsceD	ΔisaAΔsceD	WT_EV	WT_pCNisaA	WT_pCNsceD	ΔisaA_EV	ΔisaA_pCNisaA	ΔisaA_pCNsceD	ΔsceD_EV	ΔsceD_pCNsceD																																																																																																																																																																																						
β-lactam													Oxacillin	64	1	32	1	64	64	32	2	16	2	64	64	Ampicillin	4	2	0.5	0.5	8	8	8	4	8	4	0.5	8	Cefazolin	8	1	8	1	16	>16	>16	1	16	2	1	>16	Cefmetazole	8	4	8	2	8	>32	>32	4	16	4	8	32	Flomoxef	8	2	4	1	8	>16	>16	1	8	2	4	>16	Cefoxitin	>16	8	8	8	>16	>16	>16	8	>16	16	8	>16	Imipenem	8	<0.25	8	<0.25	8	>8	>8	<0.25	8	<0.25	8	8	Glycopeptide													Vancomycin	1	2	1	2	2	4	4	2	4	2	1	2	Teicoplanin	4	4	4	2	1	4	4	4	4	4	4	4	Oxazolidinone													Linezolid	8	8	8	2	4	4	4	4	4	4	4	4	Lincomycin													Clindamycin	0.5	0.5	0.12	0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.12	0.5
Oxacillin	64	1	32	1	64	64	32	2	16	2	64	64																																																																																																																																																																																						
Ampicillin	4	2	0.5	0.5	8	8	8	4	8	4	0.5	8																																																																																																																																																																																						
Cefazolin	8	1	8	1	16	>16	>16	1	16	2	1	>16																																																																																																																																																																																						
Cefmetazole	8	4	8	2	8	>32	>32	4	16	4	8	32																																																																																																																																																																																						
Flomoxef	8	2	4	1	8	>16	>16	1	8	2	4	>16																																																																																																																																																																																						
Cefoxitin	>16	8	8	8	>16	>16	>16	8	>16	16	8	>16																																																																																																																																																																																						
Imipenem	8	<0.25	8	<0.25	8	>8	>8	<0.25	8	<0.25	8	8																																																																																																																																																																																						
Glycopeptide													Vancomycin	1	2	1	2	2	4	4	2	4	2	1	2	Teicoplanin	4	4	4	2	1	4	4	4	4	4	4	4	Oxazolidinone													Linezolid	8	8	8	2	4	4	4	4	4	4	4	4	Lincomycin													Clindamycin	0.5	0.5	0.12	0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.12	0.5																																																																																																								
Vancomycin	1	2	1	2	2	4	4	2	4	2	1	2																																																																																																																																																																																						
Teicoplanin	4	4	4	2	1	4	4	4	4	4	4	4																																																																																																																																																																																						
Oxazolidinone													Linezolid	8	8	8	2	4	4	4	4	4	4	4	4	Lincomycin													Clindamycin	0.5	0.5	0.12	0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.12	0.5																																																																																																																																															
Linezolid	8	8	8	2	4	4	4	4	4	4	4	4																																																																																																																																																																																						
Lincomycin													Clindamycin	0.5	0.5	0.12	0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.12	0.5																																																																																																																																																																									
Clindamycin	0.5	0.5	0.12	0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.12	0.5																																																																																																																																																																																						

^aWT, wild type; ΔisaA, isaA-deleted strain; ΔsceD, sceD-deleted strain; ΔisaAΔsceD, isaA and sceD-deleted strain; EV, pCN51Cm (empty vector); pCNisaA, pCN51Cm containing isaA; pCNsceD, pCN51Cm containing sceD.

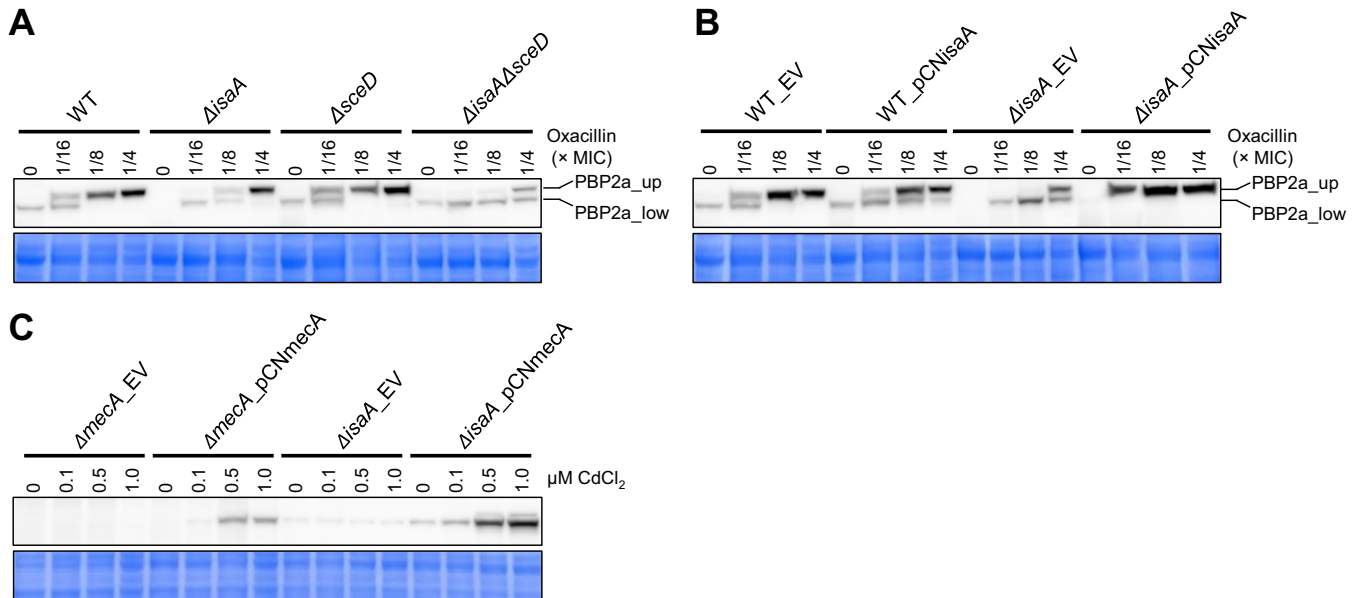


FIG 2 Effect of LT gene deletion on PBP2a production in *S. aureus* MR23. (A and B) Western blot showing levels of PBP2a in wild-type (WT) and LT mutants in the presence (A) and absence (B) of plasmids. (C) Western blot showing levels of PBP2a in *mecA* and *isaA* mutants. Concentrations of oxacillin and CdCl₂ used for induction are indicated above the panels. As loading controls, CBB-stained gels of the same area are shown below the panels. EV, pCN51Cm (empty vector); pCNisaA, pCN51Cm containing *isaA*; pCNmeca, pCN51Cm containing *mecA*.

results indicated that the β -lactam susceptibility phenotype can be exhibited regardless of the production level of PBP2a in the $\Delta isaA$ mutant.

Effect of LT gene deletion in MRSA clones belonging to different sequence types. Here, we showed the effect of LT gene deletion in *S. aureus* MR23. MR23 belongs to multilocus sequence type 380 (ST380) (27). ST380 has been reported to be involved in clonal complex 8 (CC8) (30). CC8 includes *S. aureus* USA300, which is one of the most common and well-characterized community-acquired MRSA (CA-MRSA) clones belonging to ST8 (31). To investigate whether deletion of LT genes caused a similar effect in health care-associated MRSA (HA-MRSA), *S. aureus* MR10 belonging to ST5 (27) was used. ST5 is a widely present HA-MRSA clone (32). Additionally, *S. aureus* USA300 was used to verify the generality of the phenotypes induced by LT mutations.

Deletion of LT genes did not significantly affect cell growth of MR10 and USA300 (see Fig. S3 in the supplemental material), echoing the case of MR23 (Fig. 1A). Deletion of *isaA* decreased the biofilm forming capacity of MR10 and USA300, whereas deletion of *sceD* did not (Fig. 3A). In USA300, the biofilm-forming capacity of the $\Delta isaA\Delta sceD$ strain was almost identical to that of the $\Delta isaA$ strain, as was the case with MR23. Interestingly, double deletion of *isaA* and *sceD* drastically decreased the biofilm-forming capacity of MR10, suggesting that *SceD* has a complementary role in biofilm formation in this strain.

TABLE 3 MICs of oxacillin for *mecA* and *isaA* mutants of *S. aureus* MR23 cultured in Mueller-Hinton broth^a

CdCl ₂ concn (μ M)	MIC (μ g/ml) of:			
	$\Delta mecA$ _EV	$\Delta mecA$ _pCNmeca	$\Delta isaA$ _EV	$\Delta isaA$ _pCNmeca
0	0.5	1	2	2
0.1	0.5	32	2	2
0.5	0.5	128	2	2
1.0	0.5	128	2	4

^a $\Delta mecA$, *mecA*-deleted strain; $\Delta isaA$, *isaA*-deleted strain; EV, pCN51Cm (empty vector); pCNmeca, pCN51Cm containing *mecA*.

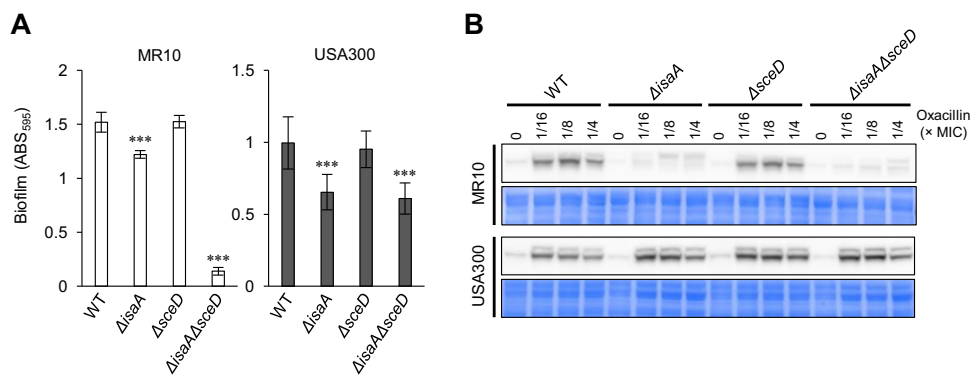


FIG 3 Effect of LT gene deletion in *S. aureus* MR10 and USA300. (A) Biofilm formation of wild-type (WT) and LT mutant. Biofilms formed on the bottom of the 96-well plate were stained with crystal violet; subsequently, absorbance at 595 nm was measured. The values are the means of six independent cultures. The error bars indicate the standard error of the mean. Differences between WT and mutants were compared using Student’s *t* test. ****P* < 0.001. (B) Western blot showing levels of PBP2a in WT and LT mutants. Concentrations of oxacillin used for induction are indicated above the panels. As loading controls, CBB-stained gels of the same area are shown below the panels.

Deletion of *isaA* or both *isaA* and *sceD* resulted in a drastic drop of oxacillin MIC compared with that of the WT in both MR10 and USA300 but deletion of *sceD* did not (Table 4). Next, PBP2a production in MR10 and USA300 and their LT mutants was analyzed. In MR10, PBP2a production was induced by oxacillin in the WT and $\Delta sceD$ mutants but hardly induced in $\Delta isaA$ and $\Delta isaA\Delta sceD$ mutants (Fig. 3B). On the other hand, in USA300, PBP2a production was similarly induced in WT and LT mutants. These results suggest that the β -lactam susceptibility phenotype in $\Delta isaA$ and $\Delta isaA\Delta sceD$ mutants was induced in USA300 regardless of oxacillin-inducible PBP2a production, as was the case with MR23 $\Delta isaA$ overproducing plasmid-derived PBP2a.

DISCUSSION

The roles of *S. aureus* *IsaA* and *SceD* in cellular physiology, cell wall dynamics, and host-pathogen interaction, as well as subcellular localization of *IsaA*, have been already described (22, 25). We show here that the two *S. aureus* LTs *IsaA* and *SceD* contributed differently to biofilm formation; deletion of *isaA* significantly decreased biofilm formation, whereas deletion of *sceD* did not. A similar phenomenon has been described in *Salmonella enterica* serovar Typhimurium, where only 2/7 LTs, namely, *MltE* and *MltC*, were shown to be involved in the regulation of biofilm formation (17). As a double deletion of *isaA* and *sceD* drastically decreased biofilm-forming capacity compared with a single deletion of *isaA* in MR10 but not in MR23 and USA300, the complementary role of *SceD* in biofilm formation varies by *S. aureus* strain.

Deletion of *isaA* resensitized the three tested MRSA strains to β -lactams (Table 2; Table S1; Table 4). To the best of our knowledge, this is the first report to show that the loss of an LT gene alters antibiotic resistance in *S. aureus*. MRSA β -lactam resistance is acquired mainly through the production of PBP2a, which is encoded by *mecA* (28). Therefore, we first hypothesized that PBP2a production was decreased in *isaA*-deleted strains, thereby resulting in a β -lactam-sensitive phenotype. In MR23 and MR10,

TABLE 4 MICs of oxacillin for WT and LT mutants of *S. aureus* MR10 and *S. aureus* USA300 cultured in Mueller-Hinton broth^a

<i>S. aureus</i> strain	MIC (μ g/ml) of:			
	WT	$\Delta isaA$	$\Delta sceD$	$\Delta isaA\Delta sceD$
MR10	512	8	512	2
USA300	256	1	256	1

^aWT, wild type; $\Delta isaA$, *isaA*-deleted strain; $\Delta sceD$, *sceD*-deleted strain; $\Delta isaA\Delta sceD$, *isaA* and *sceD*-deleted strain.

oxacillin-dependent production of PBP2a was remarkably reduced in *isaA*-deleted strains, suggesting that IsaA is involved in the oxacillin-dependent production of PBP2 in these strains. Induction of PBP2a is regulated by a three-component system encoded by *mecR1-mecI-mecR2* regulatory genes consisting of a transcriptional repressor, MecI; a membrane-bound sensor inducer, MecR1; and an anti-repressor, MecR2; in MRSA strains with functional regulatory loci (33, 34). It has been reported that homologous *blaR1-blaI* regulatory genes, which are required for inducible production of β -lactamase encoded by *blaZ*, also regulate the production of PBP2a (35). Inactivation of MecI/Blal through direct interaction with an intracellular peptidoglycan fragment induced by β -lactam has been proposed to constitute another mechanism of induction (36). Peptidoglycan is continuously synthesized, degraded, and recycled during cell growth (37). In Gram-negative bacteria, the peptidoglycan fragment released during peptidoglycan turnover is efficiently transported into the cytoplasm and recycled (12, 38). The presence of β -lactam antibiotics leads to inactivation of PBPs and, consequently, to the cytoplasmic accumulation of peptidoglycan fragments, which can act as a signal or coactivator for β -lactamase induction (39, 40). In mutants lacking LT genes, the inducibility of β -lactamase was greatly diminished (19). Gram-positive bacteria, including *S. aureus*, have been recently reported to recycle the peptidoglycan component *N*-acetylmuramic acid during growth (41). Therefore, it may be possible that the peptidoglycan fragments produced by IsaA are taken up by the cell via an unknown transport mechanism and recycled. In the presence of β -lactam antibiotics, the activity of IsaA may result in intracellular accumulation of a specific peptidoglycan fragment, ultimately leading to induction of *mecA* expression in MRSA through inhibition of MecI/Blal.

The hypothetical mechanism mentioned above may partially explain the reduced β -lactam resistance in MR23 and MR10; however, other data suggested that the loss of resistance in the *isaA*-deleted strains cannot be explained solely by the production level of PBP2a. In USA300, although the β -lactam resistance level of *isaA*-deleted strains was dramatically decreased, PBP2a production was induced by oxacillin in the same manner as in WT. Moreover, plasmid-based expression of *mecA* in the Δ *isaA* strain of MR23 failed to restore β -lactam resistance regardless of successful PBP2a production. Taken together, in *isaA*-deletion mutants, it is considered that the β -lactam susceptibility phenotype is induced regardless of the production level of PBP2a. In previous work by Memmi et al., PBP4 was demonstrated as a key element in β -lactam resistance in CA-MRSA (42). In that report, it was also suggested that constitutive expression of PBP2a did not contribute to oxacillin resistance in the absence of PBP4. Considering the similarity of their data to our data here in terms of deletion of the cell wall-related gene that induces the β -lactam susceptibility phenotype regardless of PBP2a production, these phenomena might be explained by a common molecular mechanism. Further investigation is needed to elucidate the molecular basis of the loss of β -lactam resistance in the mutants lacking *isaA*.

IsaA has been shown to be immunogenic in both humans and mice (43), and a specific antibody response against IsaA has been reported in human sepsis and a murine skin infection model (23, 44). Additionally, it has been reported that monoclonal antibodies targeting IsaA are effective in the treatment of staphylococcal infections (45–47), suggesting it is a promising candidate for antibody-based immunotherapy during *S. aureus* infection. The present study suggests that IsaA is a promising target not only for immunotherapy but also for attenuating biofilm formation and β -lactam resistance, factors which make the treatment of *S. aureus* infections difficult. A better understanding of the role of IsaA in biofilm formation and β -lactam resistance may lead to the development of novel therapeutic strategies for *S. aureus* infections.

MATERIALS AND METHODS

Bacterial strains and mutants. Bacterial strains and plasmids used in this study are listed in Table 1. Bacterial strains were grown overnight in BHI broth (Becton, Dickinson, Franklin Lakes, NJ) unless otherwise mentioned. All mutants were constructed from *S. aureus* MR23 and MR10, which are clinical MRSA isolates from Jikei University Hospital (27), and *S. aureus* USA300 (ATCC BAA1717). Genomic DNA

extracted from *S. aureus* cells was used as a template for PCR. Allelic replacements of the entire sequences of *isaA*, *sceD*, and *mecA* were achieved according to the procedure reported by Bae et al. (48). Briefly, approximately 500-bp upstream and downstream sequences of the relevant gene were amplified by PCR, using KOD-plus v.2 DNA polymerase (Toyobo, Tokyo, Japan) and the primers listed in Table S2 in the supplemental material. The resulting PCR fragments were connected by overlap extension PCR. The generated PCR product was cloned into pKOR1 using the Gateway BP Clonase II enzyme mix (Life Technologies, Palo Alto, CA). The resulting plasmids were amplified in *Escherichia coli* DC10B (49, 50) and then introduced into *S. aureus* by electroporation. After allelic replacement, the generated mutant strains were verified by gene sequencing confirming that the entire open reading frame was accurately deleted.

Antibiotics and antibodies. Ampicillin and gentamicin were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other antibiotics were purchased from Sigma-Aldrich (St. Louis, MO). Antibiotic stock solutions were prepared in sterile distilled water or sterile recommended solvent (51) and then diluted in fresh broth as described below. Anti-IsaA polyclonal antibody against a synthesized peptide fragment of IsaA was raised in rabbits as previously reported (52). Anti-IsaA antibody was purified from rabbit serum with a MAb Trap kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. For overexpression and purification of the SceD protein, *sceD* was amplified from the genome of *S. aureus* MR23 by PCR using the primers listed in Table S2. The amplified PCR fragment was cloned into the pET29a vector (Novagen, Madison, WI) using an In-Fusion HD cloning kit (TaKaRa Bio, Otsu, Japan) according to the manufacturer's protocol. The resulting plasmid pET_sceD was amplified in *E. coli* DH5 α and subsequently introduced into *E. coli* BL21(DE3) by electroporation. *E. coli* BL21(DE3) harboring pET_sceD was cultured in Luria-Bertani broth containing 50 μ g/ml kanamycin at 37°C. When the optical density at 660 nm (OD₆₀₀) reached 0.5, the culture was cooled and kept at 15°C for 30 min. Then, isopropyl β -D-1-thiogalactopyranoside was added to a final concentration of 1 mM to induce expression. Purification of SceD was performed according to the protocol for the purification of *S. aureus* enolase described previously (53). Anti-SceD polyclonal antibody was raised in rabbits using purified SceD as an antigen and was purified in the same way as anti-IsaA antibodies. Mouse anti-PBP2a antibody (product code, 130-10096-200) was purchased from Raybiotech, Inc. (Norcross, GA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA) was used as the secondary antibody.

Plasmid-based expression of genes. For plasmid-based expression of LT genes and *mecA* in *S. aureus* MR23, an expression vector was constructed by replacing the erythromycin resistance gene of pCN51 (54) with a chloramphenicol resistance gene amplified from pKOR1 (48). PCR was performed using KOD-plus v.2 DNA polymerase and the primers listed in Table S2. The two fragments were fused using an In-Fusion HD cloning kit according to the manufacturer's protocol, thereby producing pCN51Cm. For expression of *isaA* and *sceD*, the fragments from the respective native promoter sequences to the stop codon of each gene were cloned. For expression of *mecA*, the fragments from the ribosomal binding site to the stop codon of the *mecA* gene were cloned. Each fragment was amplified using KOD-plus v.2 DNA polymerase and the primers listed in Table S2. The PCR products were subsequently cloned into pCN51Cm, which had been predigested with BamHI and EcoRI using an In-Fusion HD cloning kit. The resulting plasmids were amplified in *E. coli* DC10B and then introduced into *S. aureus* MR23. The generated plasmids were verified by DNA sequencing.

Bacterial growth and biofilm assays. Overnight cultures of *S. aureus* strains were diluted 500-fold in BHIG, and 5 μ g/ml of chloramphenicol (final concentration) was added to BHIG for strains harboring plasmid pCN51Cm and its derivatives. Subsequently, aliquots (200 μ l) of this suspension were transferred to the wells of a 96-well microtiter plate (Corning Inc., Corning, NY) and incubated for 24 h at 37°C under static conditions. Bacterial growth was determined by measuring absorbance at 595 nm using a microplate spectrophotometer (Infinite F200 Pro; Tecan, Mannedorf, Switzerland). For biofilm assays, after removing the supernatant, the biofilms were washed twice with 200 μ l of phosphate-buffered saline (PBS) to remove loosely adherent cells, dried, stained with 100 μ l of 0.05% crystal violet for 1 min, and subsequently washed twice with PBS. Biofilm formation was assessed by measuring absorbance at 595 nm using a microplate spectrophotometer (Tecan). As the biofilms can form on the bottom wells in a patchy manner, multiple reads (4 \times 4) per well function of the Infinite F200 Pro were performed to obtain the average value of the well. The background was not subtracted because the background value was exceedingly low in the dynamic range of this assay. The average value obtained from six wells represented biofilm formation.

Western blotting. IsaA and SceD in culture supernatants and whole-cell fractions were detected by Western blotting. *S. aureus* strains were grown in BHIG for 24 h and then harvested by centrifugation at 5,000 \times g for 10 min. The supernatants (culture supernatant fraction) were stored at -30°C until use. The harvested cells were treated with 25 μ g/ml of lysostaphin (Wako Pure Chemical Industries, Ltd.) in PBS for 30 min at 37°C and subsequently disrupted by sonication. After centrifugation at 10,000 \times g for 10 min, the supernatants (whole-cell fractions) were stored at -30°C until use. Culture supernatants (5 μ l) or whole-cell fractions (5 μ g of protein) were subjected to SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (ATTO Corp., Tokyo, Japan). The membrane was treated for 1 h at room temperature with a blocking buffer composed of 5% Easy Blocker (GeneTex, Irvine, CA) in Tris-buffered saline with 0.05% Tween 20. The membrane was probed with a primary antibody (30 min) followed by a secondary antibody (30 min), both of which were diluted 1:10,000 in Can Get Signal solution (Toyobo). Signals were detected using enhanced chemiluminescence (ECL) prime (GE Healthcare) on an Image Quant LAS 4000 instrument (GE Healthcare).

PBP2a was detected as described previously (50). *S. aureus* cells were cultured in BHIG in the presence or absence of oxacillin (below its MIC) for 24 h. For expression of *mecA* cloned under the control of the

cadmium-inducible promoter, CdCl₂ (final concentration of 0.1, 0.5, or 1.0 μM) was added to BHIG. Cells were harvested by centrifugation, treated with lysostaphin, and disrupted by sonication. To avoid degradation during sample preparation, cOmplete Mini EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN) was added to the buffer. After centrifugation, the supernatants (mixture of cytoplasm and membrane fraction) were collected, and 5 μg of proteins was first run on SDS-PAGE and then electroblotted onto a nitrocellulose membrane. To detect PBP2a, primary (mouse anti-PBP2a, catalog number 130-10096-200; Raybiotech, Inc.) and secondary (HRP-conjugated anti-mouse IgG; Bio-Rad) antibodies were used. Coomassie brilliant blue (CBB)-stained gels were used as loading controls.

Antibiotic susceptibility testing. Overnight cultures of *S. aureus* were diluted in BHIG or MH broth (Becton, Dickinson, Franklin Lakes, NJ) to prepare inocula of 5 × 10⁵ CFU/ml. Five micrograms per milliliter of chloramphenicol (final concentration) was added to the medium for strains harboring plasmids pCN51Cm and its derivatives. MICs were determined by the broth microdilution method following the EUCAST guidelines (<http://www.eucast.org/>) using a commercially available MIC plate (Dry Plate Eiken; Eiken Chemical Co., Tokyo, Japan) or a laboratory-made plate. Three independent replicates were analyzed. Plates were incubated for 24 h at 37°C, with the exception of oxacillin, whose MIC was determined at 35°C. The MIC was defined as the lowest concentration that prevented the appearance of visible growth.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01277-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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