

# Modulation of *Staphylococcus aureus* Biofilm Matrix by Subinhibitory Concentrations of Clindamycin

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***Staphylococcus aureus* biofilms are extremely difficult to treat. They provide a protected niche for the bacteria, rendering them highly recalcitrant toward host defenses as well as antibiotic treatment. Bacteria within a biofilm are shielded from the immune system by the formation of an extracellular polymeric matrix, composed of polysaccharides, extracellular DNA (eDNA), and proteins. Many antibiotics do not readily penetrate biofilms, resulting in the presence of subinhibitory concentrations of antibiotics. Here, we show that subinhibitory concentrations of clindamycin triggered a transcriptional stress response in *S. aureus* via the alternative sigma factor B ( $\sigma^B$ ) and upregulated the expression of the major biofilm-associated genes *atlA*, *lrgA*, *agrA*, the *psm* genes, *fnbA*, and *fnbB*. Our data suggest that subinhibitory concentrations of clindamycin alter the ability of *S. aureus* to form biofilms and shift the composition of the biofilm matrix toward higher eDNA content. An understanding of the molecular mechanisms underlying biofilm assembly and dispersal in response to subinhibitory concentrations of clinically relevant antibiotics such as clindamycin is critical to further optimize antibiotic treatment strategies of biofilm-associated *S. aureus* infections.**

*Staphylococcus aureus* is a major cause of both health care-related and community-associated (CA) infections. The Gram-positive human-pathogenic bacterium produces and secretes a range of toxins and enzymes leading to acute infections such as bacteremia and skin abscesses (1, 2). In addition, most *S. aureus* strains are capable of biofilm formation and can persist in host tissues such as the bone, leading to chronic osteomyelitis, or on implanted medical devices such as vascular catheters, vascular grafts, heart valves, and prosthetic joints (3–5). Biofilm-associated infections are extremely difficult to treat, and these chronic or relapsing infections typically require prolonged antibiotic treatment or removal of the device (6–8). Antibiotic resistance of bacteria within a biofilm may result from slow growth, phenotypic heterogeneity, persister cell formation, and inactivation or reduced penetration of the antibiotic (9, 10). Diffusion of the antibiotic through biofilm cell clusters is dependent on the thickness and the composition of the extracellular polymeric matrix (9, 11). The slow transport within biofilms suggests that the bacteria may encounter subinhibitory concentrations of antibiotics. Previous studies have shown that low doses of different antibiotics trigger biofilm formation (12, 13) and lead to dramatic alterations in bacterial gene expression in *S. aureus* (14).

Biofilm formation proceeds in at least three phases: initial attachment, biofilm maturation, and dispersal (15, 16). Initial surface attachment is dependent on bacterial surface molecules such as the *S. aureus* murein hydrolase AtlA, teichoic acids, and fibronectin-binding proteins (FnBPs) (17–20). After attachment to the surface, the bacteria multiply and produce the extracellular polymeric matrix. Various studies showed that the biofilm matrix of many laboratory and clinical staphylococcal isolates is composed of substantial quantities of extracellular DNA (eDNA) (21–23) which is counterbalanced by the effect of the bacterial nuclease Nuc1 (24, 25). The negatively charged DNA plays an important role during attachment and biofilm maturation, potentially through its electrostatic properties (26). The release of eDNA into the biofilm matrix is dependent on murein hydrolases (19, 27). AtlA, the major autolytic enzyme in *S. aureus*, is required for cell division, cell wall turnover, and bacterial lysis (28, 29). Autolysis

in *S. aureus* biofilms is also regulated by the activity of the Cid/Lrg holin-antiholin system. CidA oligomerizes in the bacterial cell membrane and increases the activity of murein hydrolases, possibly by exporting them into the extracellular space (22, 30, 31). In the dispersal phase, bacteria detach from the biofilm and spread to new infection sites. A quorum-sensing system (Agr) is responsible for the switch between biofilm formation and detachment (32, 33). Recently, it was shown that the quorum-sensing-controlled phenol-soluble modulins (PSMs) (34, 35) are present in biofilms as large amyloid fibers which, under certain conditions, stabilize *S. aureus* biofilms (36) but also play a role in biofilm dispersal due to their surfactant-like properties (34, 37).

There is growing evidence that *S. aureus* specifically responds to subinhibitory antibiotic concentrations by inducing biofilm formation and changing its biofilm matrix composition (38). Clindamycin (CLI), a protein synthesis inhibitor used in the treatment of Gram-positive bacterial infections, modulates the production of several toxins and virulence factors in *S. aureus* (14, 39–43). Recently, it was also shown that the production and expression of PSMs are significantly increased in planktonic *S. aureus* cultures by subinhibitory concentrations of clindamycin (44, 45). Clindamycin achieves high intracellular levels in bones and phagocytic cells and potentiates opsonization and phagocytosis at subinhibitory concentrations (46, 47). However, the impact of

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subinhibitory concentrations of clindamycin on the biofilm formation and matrix composition of clindamycin-sensitive *S. aureus* has not been investigated so far.

It is evident that during antibiotic treatment, host tissues, wounds, and biofilm-infected medical devices are exposed to a range of antibiotic concentrations. Here, we examined whether the exposure of *S. aureus* to subinhibitory concentrations of clindamycin modulates biofilm formation and changes the matrix composition in the community-acquired methicillin-resistant *S. aureus* (CA-MRSA) USA300 LAC strain (LAC wild type [wt]) and its isogenic mutants as well as clinical isolates (CIs). This study provides a deeper understanding of the modulation and regulation of *S. aureus* matrix components in response to subinhibitory concentrations of clindamycin, a commonly used antibiotic in clinical settings.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** All bacterial strains and plasmids used in this study are listed in Tables S1 to S3 in the supplemental material. Bacterial strains were grown on blood agar plates (Columbia agar plus 5% sheep blood; bioMérieux SA). Brain heart infusion (BHI) broth (BD Difco) supplemented with 0.4% glucose (wt/vol; BHIg) was used for all overnight cultures (220 rpm, 37°C) and static biofilm assays with clindamycin hydrochloride (AppliChem GmbH) or amoxicillin (Sigma-Aldrich). The MIC of clindamycin in BHIg broth was determined as previously described (41). The MIC for the LAC wt, the isogenic mutants, and the clinical isolates CI-1 to CI-8, CI-11, and CI-12 was 0.125 mg/liter in BHIg broth. The MIC for CI-9 was 0.06 mg/liter, and for CI-10 it was 0.03 mg/liter. All clindamycin-resistant clinical isolates (CI-13 to CI-18) showed visible growth up to 256 mg/liter of clindamycin in BHIg broth (MIC > 256 mg/liter). Antibiotics were used at the following concentrations: erythromycin at 10 µg/ml (Erm), chloramphenicol at 10 µg/ml (Cm), and ampicillin at 100 µg/ml (Amp).

To assess any effect that might be due to the genetic background of the strains, sequence typing of the polymorphic repeat region of the protein A gene (*spa*) (48) was performed using Ridom StaphType software (Ridom GmbH, Würzburg, Germany).

**Construction of *S. aureus* mutants.** Phage  $\phi$ 11 was used to transduce all knockout mutations into the LAC wt background. The *agr::tet(M)* mutation was transduced from *S. aureus* strain RN6911 (49) into LAC. The generated strain LAC *agr::tet(M)* had a complete deletion of the *agr* locus, which was confirmed by sequencing using the primers *agrlucos-F* and *agrlucos-R* (see Table S2 in the supplemental material). The strains NE1438, NE1692, and NE460 from the Nebraska Transposon Mutant Library were obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) repository (50). The mutants carry the *bursa aurealis* mariner-based erythromycin resistance-expressing transposon (Tn) within the *lrgA* (SAUSA300\_0256), *cidA* (SAUSA300\_2479), or *atIA* (SAUSA300\_0955) gene and were used to introduce mutations into LAC wt by phage transduction. To delete the *erm* cassette of the *S. aureus* LAC transposon mutants, we used allelic exchange via homologous recombination of the *bursa aurealis* Tn insertions (51). Briefly, the transposon mutants were transformed with the plasmid pTnT carrying a 500-bp homologous region 5' and 3' of the *bursa aurealis* Tn and a chloramphenicol resistance marker. Strains carrying the plasmid were incubated overnight on chloramphenicol (Cm) agar plates. The next day the colonies were restreaked, and after an additional overnight incubation on Cm agar plates at 44°C, the colonies representing single recombinants were used for further subculturing steps at 30°C without antibiotic. Anhydrotetracycline (ATc; 100 ng/ml) was used as counterselection because it inhibits growth of cells still carrying the plasmid. Colonies which had lost the plasmid (Cm sensitive) were screened on Erm agar plates for double recombination. The *erm* cassette deletion was confirmed by PCR using the primer pairs *cidA/Buster*, *atIA/Upstream*, and *lrgA/lrgA-R* and

by sequencing with the primers listed in Table S2 in the supplemental material.

**Biofilm experiments.** Bacterial strains were grown overnight on fresh blood agar plates, resuspended in phosphate-buffered saline (PBS; Kantonsapotheke Zurich, Switzerland), and diluted in BHIg broth. A total of 180 µl of BHIg broth with or without clindamycin or amoxicillin was transferred to a polystyrene 96-well plate (Cellstar; Greiner), and 20 µl of diluted bacteria (final concentration, about  $5 \times 10^5$  CFU/ml) or medium (blank) was added before incubation for 18 h at 37°C in a wet chamber unless otherwise indicated. After incubation, the wells were rinsed three times with 200 µl of PBS, air dried, and stained with 0.1% crystal violet for 30 min. Unbound dye was removed by three washes with distilled water. The adhering dye was dissolved with 30% acetic acid, and the absorption was measured at 570 nm with a VersaMax microplate reader and Soft Max Pro, version 4.3.1 LS, software (Molecular Devices). Biofilm assays in 12-well polystyrene plates (Cellstar; Greiner Bio-One) used for RNA isolation, viable bacteria count (CFU/ml), and eDNA analysis were done as described above with 7.5-fold more volume per well.

**qRT-PCR analysis.** Transcripts from LAC wt and *atIA* mutant biofilms cultured with or without subinhibitory concentrations of clindamycin were assayed by quantitative reverse transcription-PCR (qRT-PCR). After 18 h of incubation in a 12-well polystyrene plate, *S. aureus* biofilm of LAC wt was washed three times with PBS and scraped off with a cell scraper. An aliquot was used for determination of CFU counts per milliliter. The biomass from two wells was pooled and spun down at  $12,000 \times g$  at 4°C for 5 min. RNA isolation and qRT-PCR were performed as previously described (41). All primers used for qRT-PCR are listed in Table S2 in the supplemental material. Differences in mRNA levels were calculated as follows: the  $\Delta C_T$  (where  $C_T$  is cycle threshold) values of *gyrB* and the genes of interests were calculated by normalization of the mean  $C_T$  from each biological replicate to the median of the biological replicates without CLI. For each sample, the fold change was calculated according to the Pfaffl analysis method (52). PCR experiments were performed using at least three biological replicates, each tested in technical triplicates.

**Isolation and quantification of extracellular DNA.** Bacteria were cultured in 12-well plates in BHIg broth with or without subinhibitory concentrations of clindamycin (0.06 µg/ml). After 18 h of incubation, the supernatant was removed, and the biofilm was washed three times with PBS to remove planktonic cells. Five hundred microliters of PBS was added, and the cells were scraped from the surface using a cell scraper. The cells were transferred to a 1.5-ml microcentrifuge tube and sonicated in an ultrasonic bath (XUBA3; Grant). To degrade components of the biofilm matrix to release eDNA, the samples were incubated for 30 min at 37°C with 1 µl of peptide-N-glycosidase F ([PNGase F] 500 U/µl; NEB) and then with proteinase K (final concentration, 5 µg/ml; Sigma-Aldrich). An aliquot was used for determination of the CFU count per milliliter. After filtration (0.22-µm-pore-size polyvinylidene difluoride [PVDF] filter; Millipore), 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1; Sigma-Aldrich) was added, and the mixture was vortexed and centrifuged at  $10,000 \times g$  for 10 min. The upper phase was collected, and eDNA was precipitated by addition of 1/10 volume of 3 M sodium acetate (Sigma-Aldrich) and 2.5 volumes of 100% ethanol (Sigma-Aldrich). After overnight precipitation at -20°C, centrifugation at  $20,000 \times g$  for 10 min at 4°C, and washes with ice-cold 70% ethanol, the eDNA pellet was air dried, resuspended in Tris-EDTA (10:1) buffer, and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The eDNA amount was calculated relative to the mean biofilm mass of a 12-well polystyrene plate. eDNA was separated on a 0.8% agarose gel (0.5 µg/ml ethidium bromide) and visualized with a Benchtop UV Transilluminator (UVP, Axon Lab) and TS Image software (UVP).

**Zymogram assays.** *S. aureus* LAC wt and *atIA* overnight cultures grown in BHIg broth without or with (0.03 and 0.06 µg/ml) clindamycin were set to the same optical densities at 600 nm ( $OD_{600}$ ) and centrifuged at  $4,000 \times g$  at 4°C for 10 min. The supernatant was filtered (0.45-µm-pore-size PVDF filter; Millipore) and stored at -80°C. Pellets were resus-

pended in PBS (pH 8) containing protease inhibitor (cOmplete protease inhibitor cocktail tablet; Roche). After incubation at 4°C for 1 h, cells were pelleted as described above and resuspended in 200  $\mu$ l of PBS (pH 8) plus 2% SDS. Cells were incubated at 37°C for 20 min, followed by centrifugation (10,000  $\times$  g, 4°C). Protein concentrations of the crude cell wall extracts (SDS extracts) were determined by a bicinchoninic acid (BCA) protein assay (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. Murein hydrolase activities in the crude cell wall extract and the supernatant were analyzed by zymographic analysis. Ten micrograms of total protein from crude cell wall extracts and 13  $\mu$ g of total protein from supernatants were separated using a 10% acrylamide gel containing about 100 mg of pellet (wet weight) of heat-inactivated *Micrococcus luteus* or *S. aureus* RN4220 with an electrophoresis system (Bio-Rad). After electrophoresis, the gels were incubated in the reaction buffer (25 mM Tris-HCl [pH 8], 1% Triton X-100) for at least 18 h, statically at 37°C. Gels were imaged with an Epson Perfection V700 photo scanner (Epson). Contrast and brightness were enhanced with IrfanView software.

**Extraction of staphyloxanthin.** LAC wt and clinical isolates were grown in BHlg broth at 37°C in petri dishes (94 by 16 mm, Cellstar; Greiner) for 18 h. The supernatant was removed and after washes with PBS, the biofilm was scraped off with a cell scraper, followed by centrifugation (10,000  $\times$  g for 10 min) and washing with double-distilled H<sub>2</sub>O (ddH<sub>2</sub>O). Pictures of pelleted bacteria were enhanced for contrast and brightness with IrfanView software. Methanol (Sigma-Aldrich) extraction of staphyloxanthin was performed as described by Morikawa et al. (53). Absorption was measured at 465 nm with a VersaMax microplate reader and Soft Max Pro, version 4.3.1 LS, software (Molecular Devices).

**Surfactant activity.** A drop-collapse assay was used as semiquantitative measurement of surfactant activity. The reduction of surface tension causes a collapse of the drop on a hydrophobic surface (Parafilm). Bacteria (LAC wt, *agr*, and *atlA* strains) were cultured with shaking or under static conditions in a 96-well plate in BHlg broth with or without clindamycin (0.03 and 0.06  $\mu$ g/ml) at 37°C for different time periods. After incubation, shaking cultures or the supernatant of the 96-well plate were spun down at 8,000  $\times$  g for 15 min. Fifty-microliter drops were spotted on Parafilm and imaged with an Epson Perfection V700 photo scanner (Epson).

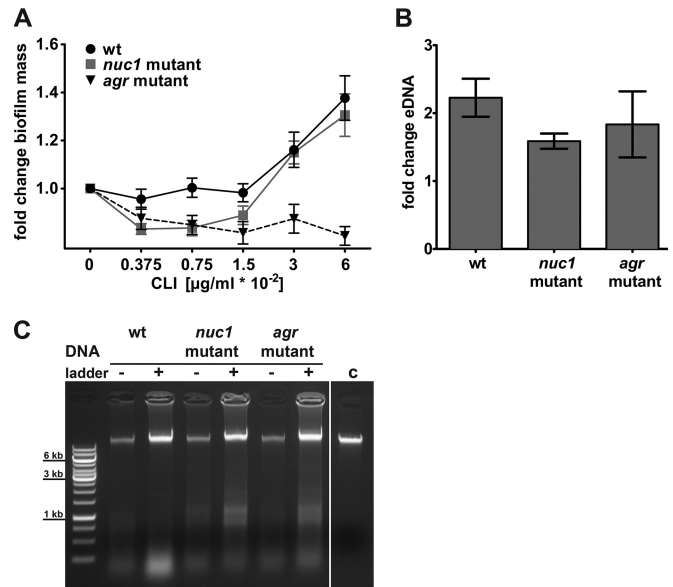
**Electron microscopy.** LAC wt and the *atlA* mutant were grown in BHlg broth with and without clindamycin (0.06  $\mu$ g/ml) for 18 h at 37°C. Bacteria were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide and then 1% aqueous uranyl acetate. After stepwise dehydration in 70, 80, and 100% ethanol followed by propylene oxide, samples were embedded in Epon 30 and thin sectioned. Samples were poststained according to the Reynolds lead staining procedure. Images were taken with a 100 kV transmission electron microscope (TEM) with a digital charge-coupled-device (CCD) camera (TEM Philips CM100).

***spa* typing of clinical isolates.** Sequence typing of the polymorphic repeat region of the protein A gene (*spa*) (48) was performed using Ridom StaphType software (Ridom GmbH, Würzburg, Germany). Primers for *spa* typing are listed in Table S2 in the supplemental material.

**Statistics.** Data were analyzed by using a Mann-Whitney U test for single comparisons or a Kruskal-Wallis test followed by *post hoc* Dunn's multiple comparisons. *P* values were calculated by using GraphPad Prism (version 5), and differences were considered significant for *P* values of <0.05.

## RESULTS

**Increased amount of biofilm formation and eDNA by *S. aureus* USA300 LAC in the presence of subinhibitory concentrations of clindamycin.** We assessed the impact of subinhibitory concentrations of clindamycin on biofilm formation. Growth of *S. aureus* LAC wt in the presence of subinhibitory concentrations of clindamycin led to an increase in the static biofilm mass after 18 h of growth in a microtiter plate. The most pronounced increase in

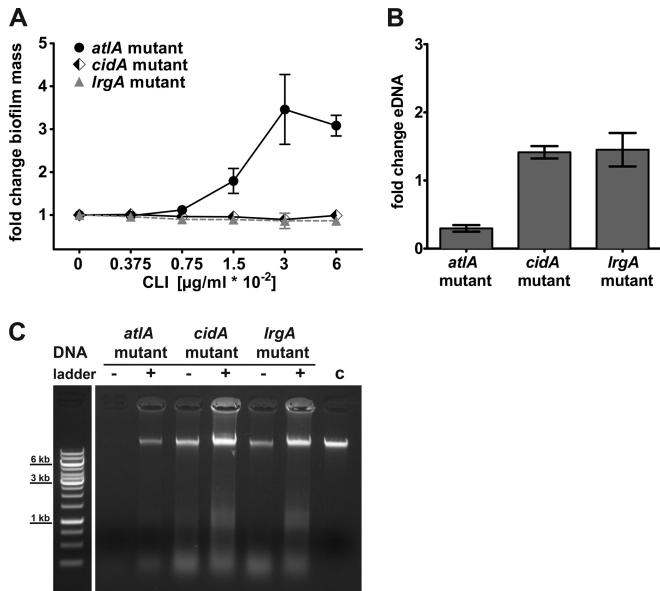


**FIG 1** Modulation of biofilm formation and eDNA release in *S. aureus* LAC in response to subinhibitory concentrations of clindamycin. (A) Quantification of the biofilm mass of the LAC wt, *nuc1*, and *agr* strains with crystal violet in the absence and presence of subinhibitory concentrations of clindamycin (CLI) after 18 h of static incubation. Biofilm mass without clindamycin was set to 1, and fold changes are presented. Results presented are average data from at least three replicates  $\pm$  standard error of the mean. (B) eDNA isolated from biofilms grown for 18 h from wt and mutants (*nuc1* and *agr* strains) grown in the presence of clindamycin were normalized to the biofilm mass, and results are presented as fold change relative to the level of the control without clindamycin. Results are average data from three replicates  $\pm$  standard error of the means. (C) Representative agarose gel of isolated eDNA from biofilms incubated with (+; 0.06  $\mu$ g/ml CLI) and without (-) clindamycin. Lane c, *S. aureus* wt chromosomal DNA control ( $\sim$  60 ng). A Gene Ruler 1-kb DNA ladder is shown in the leftmost lane.

biofilm mass occurred in the presence of 0.06  $\mu$ g/ml clindamycin (Fig. 1A). The viable LAC wt bacteria counts (numbers of CFU per milliliter) from the biofilm mass after 18 h showed no significant difference when bacteria were grown with various subinhibitory concentrations of clindamycin (see Fig. S1A in the supplemental material). As eDNA is a major matrix component and might contribute to the increased biofilm mass, we analyzed the amount of eDNA in clindamycin-exposed biofilm cultures and compared the amount to that of controls grown without clindamycin. When normalized to the total biofilm mass, larger amounts of eDNA were detected in biofilms cultured with clindamycin than in the respective control without clindamycin (Fig. 1B). The isolated eDNA contained high-molecular-weight DNA (Fig. 1C), as described previously (12).

Since the Nucl nuclease and the Agr quorum-sensing system are important for biofilm development and dispersal, we also tested isogenic mutants deficient in *nuc1* and the *agr* locus (25, 33). The *nuc1* and *agr* mutants showed a higher total biofilm mass than the wt after 18 h of incubation (see Fig. S1B in the supplemental material). In a next step, we evaluated the influence of subinhibitory clindamycin concentrations on biofilm formation in both mutants. In the *nuc1* strain, the clindamycin-induced increase in biofilm mass was similar to the level of the wt strain, whereas no increase was observed in the *agr* mutant (Fig. 1A). Viable bacteria counts of both mutants grown with or without





**FIG 2** Subinhibitory concentrations of clindamycin affect *S. aureus* autolysin mutant. (A) Quantification of the biofilm mass of the LAC *atlA*, *cidA*, and *lrgA* mutants with crystal violet in the absence and presence of subinhibitory concentrations of clindamycin (CLI) after 18 h of static incubation. Biofilm without clindamycin was set to 1, and fold changes are presented. Results presented are average data from at least three replicates  $\pm$  standard error of the means. (B) eDNA isolated from biofilms grown for 18 h from LAC mutants (*atlA*, *cidA*, and *lrgA* strains) grown in the presence of clindamycin were normalized to the biofilm mass and presented as fold change relative to the control without clindamycin. Results are average data from three replicates  $\pm$  standard error of the mean. (C) Representative agarose gel of isolated eDNA from biofilms incubated with (+; 0.06  $\mu\text{g/ml}$  CLI) and without (–) clindamycin. Lane c, *S. aureus* wt chromosomal DNA control ( $\sim 60$  ng). A Gene Ruler 1-kb DNA ladder is shown in the leftmost lane.

clindamycin (0.06  $\mu\text{g/ml}$ ) remained stable (see Fig. S1C). Similar to the wt strain, both mutants had larger amounts of eDNA in their biofilms when cultured with clindamycin than the biofilms of the respective controls without clindamycin (Fig. 1B and C). As the *agr* mutant did not show a clindamycin-induced increase in biofilm mass in the static biofilm assay (Fig. 1A), we concluded that the observed increased biofilm mass of the wt and *nuc1* mutant in response to subinhibitory concentrations of clindamycin did not result only from increased amounts of eDNA in the extracellular matrix.

**Elevated biofilm formation by the *S. aureus* USA300 LAC autolysin mutant *atlA* in the presence of clindamycin.** The observed increase in eDNA led us to investigate whether *S. aureus* autolysin systems (AtlA and CidA/LrgA) are involved in clindamycin-induced changes of the biofilm matrix. We confirmed that deletion of *atlA* significantly impaired biofilm formation (see Fig. S1B in the supplemental material) (19). When we cultured the isogenic *atlA* mutant in the presence of subinhibitory concentrations of clindamycin, we observed an up to 3.5-fold increase in the biofilm mass with 0.03 and 0.06  $\mu\text{g/ml}$  of clindamycin (Fig. 2A). In addition, the *atlA* mutant strain cultured with clindamycin (0.06  $\mu\text{g/ml}$ ) showed an approximately 3-fold increase in the CFU count per milliliter in the biofilm mass (see Fig. S1C). The *lrgA* mutant grown without clindamycin resulted in increased biofilm accumulation compared to the level in the wt strain (see Fig. S1B). In contrast to results in a previous study by Rice et al. (22), dele-

tion of *cidA* did not impair biofilm formation in our biofilm setting after 18 h but did produce an increase in biofilm mass (see Fig. S1B). No clindamycin-induced alteration of the biofilm phenotype was observed in the *cidA* and *lrgA* mutants (Fig. 2A).

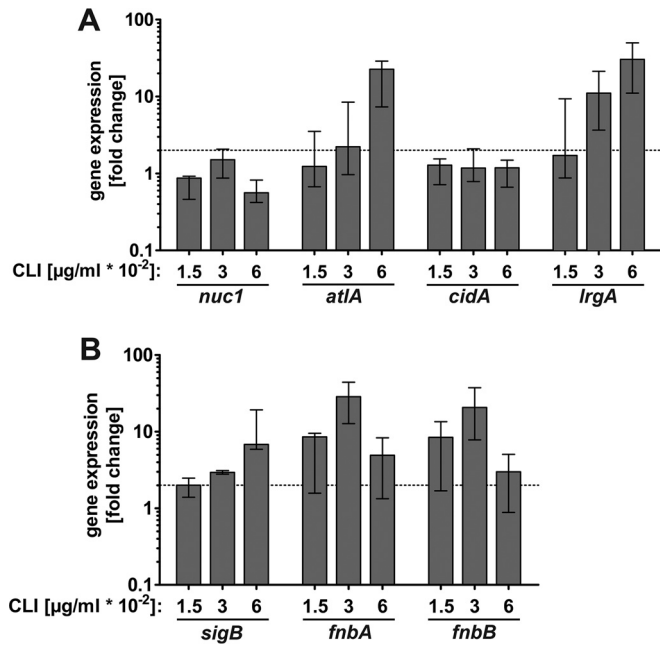
When we calculated the amount of eDNA relative to the biofilm mass, the *atlA* mutant showed a negative fold change of eDNA after exposure to subinhibitory concentrations of clindamycin compared to the level in the control (Fig. 2B). In contrast, a modest increase in eDNA in the extracellular biofilm matrix was observed in the *lrgA* and *cidA* mutants (Fig. 2B), and the isolated eDNA of all three mutant strains contained high-molecular-weight DNA (Fig. 2C). We concluded that CidA and LrgA are not essential for clindamycin-induced eDNA release in our biofilm model.

Mlynek and colleagues showed that subinhibitory concentrations of the  $\beta$ -lactam antibiotic amoxicillin induced biofilm formation in the USA300 wt and a nuclease-deficient mutant, while no alteration of biofilm formation was observed in an *atlA* mutant (54). To compare our biofilm model with these previously published data on antibiotic-induced biofilm alterations, we also tested the influence of amoxicillin on biofilm formation. In agreement with Mlynek and colleagues, we observed amoxicillin-mediated induction of biofilm formation for the USA300 LAC wt as well as the *nuc1* background, and no induction was observed for the *atlA* mutant background (see Fig. S2 in the supplemental material).

**Upregulation of genes involved in stress response, attachment, and eDNA release by clindamycin.** To corroborate our functional biofilm assay, we investigated the effect of subinhibitory clindamycin concentrations on the expression of genes involved in autolysis, stress response, and attachment. cDNA levels derived from RNA of 18-h biofilms grown with and without clindamycin were quantified by real-time PCR (qRT-PCR). As shown in Fig. 3A, expression of *atlA* and *lrgA* was upregulated in response to subinhibitory concentrations of clindamycin while *cidA* and *nuc1* expression was not affected.

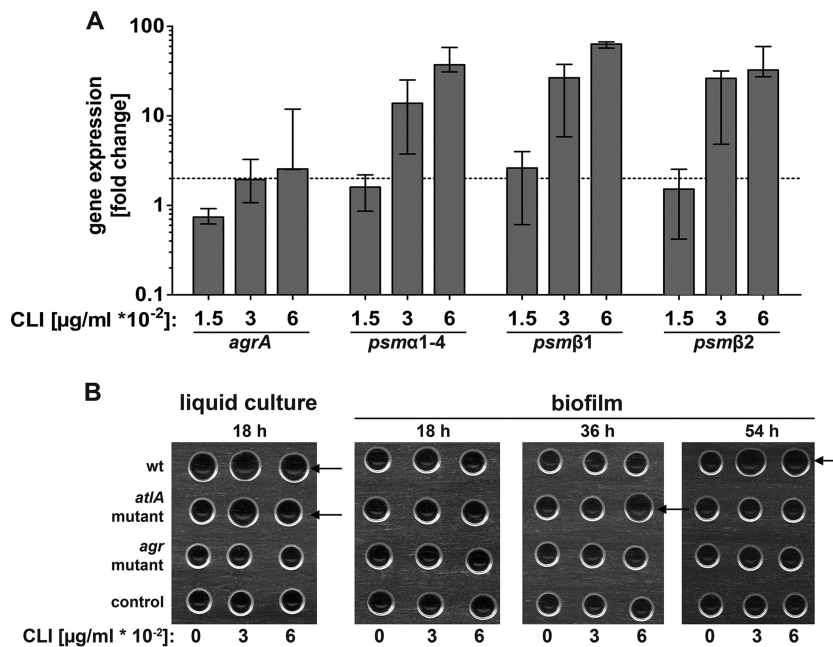
As subinhibitory concentrations of antibiotics can induce a general stress response (38, 55), gene expression of the alternative sigma factor B ( $\sigma^B$ ) was investigated in our biofilm model and showed an upregulation of 3- and 7-fold in response to 0.03 and 0.06  $\mu\text{g/ml}$  clindamycin, respectively (Fig. 3B). Since the *S. aureus* carotenoid pigment staphyloxanthin is positively regulated by  $\sigma^B$  (56, 57), we quantified the amount of staphyloxanthin of the LAC wt in response to subinhibitory concentrations of clindamycin and amoxicillin in our biofilm model. We found that the amount of staphyloxanthin increased with subinhibitory concentrations of clindamycin but not with addition of amoxicillin (see Fig. S3A and B in the supplemental material). As  $\sigma^B$  upregulates adhesion factors (58–60), we investigated the expression of the genes encoding the fibronectin binding proteins A (FnbA) and B (FnbB). Transcript levels of *fnbA* and *fnbB* were clearly higher in biofilms grown in the presence of 0.03  $\mu\text{g/ml}$  of clindamycin (Fig. 3B).

**Subinhibitory concentrations of clindamycin induce *psm* gene expression and surfactant activity.** In addition to eDNA and the FnBPs, the recently discovered PSMs are important biofilm matrix components which are able to stabilize *S. aureus* biofilms but also play a role in biofilm dispersal (34, 36, 37). Transcription of the *psm* genes is under the control of the AgrA DNA binding protein (35). Thus, we studied the effect of subinhibitory

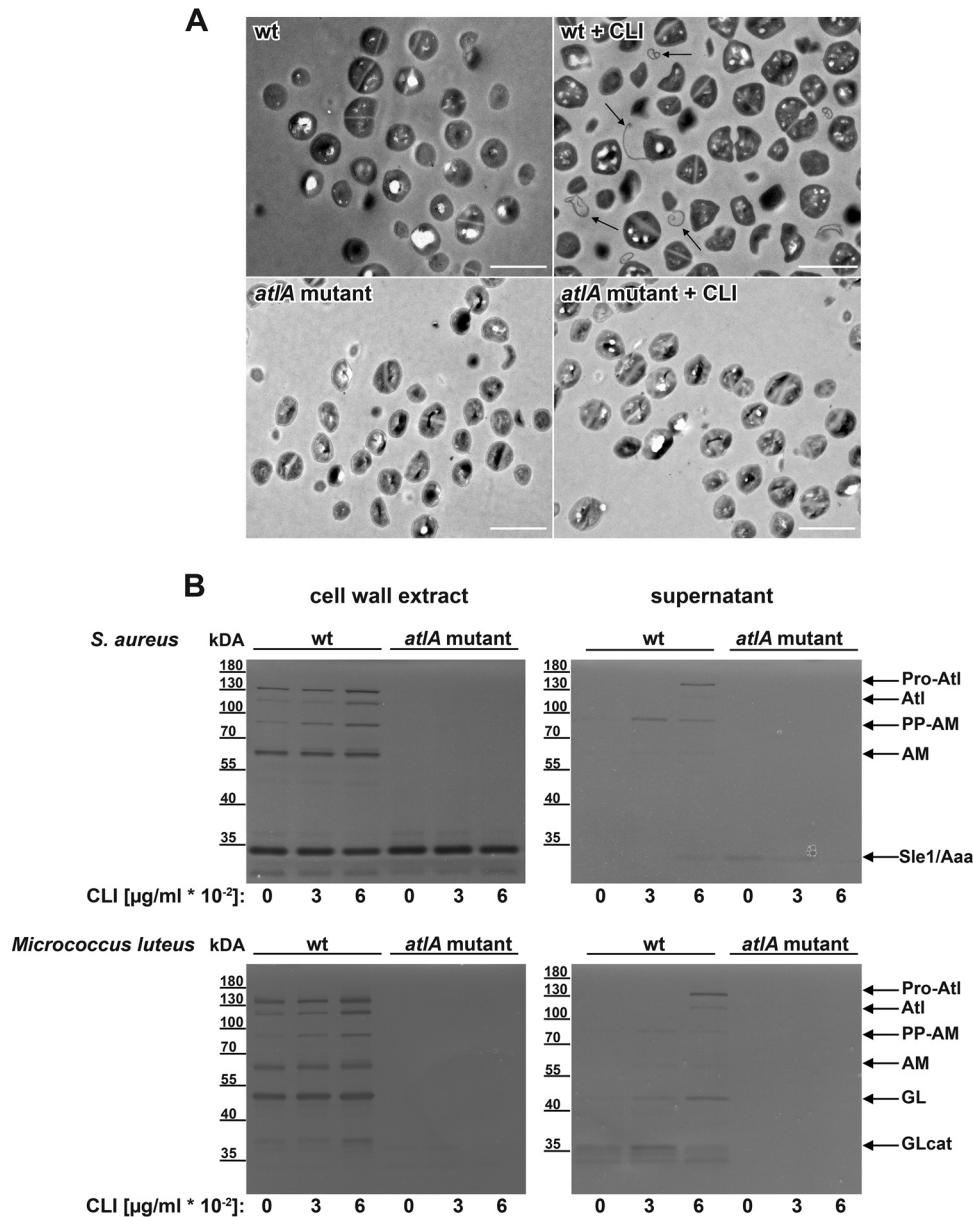


**FIG 3** Impact of subinhibitory concentrations of clindamycin on the expression of biofilm-associated genes. Relative transcript levels of genes important for eDNA and autolysis (A) or stress response and adhesion (B) derived from LAC wt biofilm mass with and without clindamycin after 18 h as determined by qRT-PCR. Fold change ratios were calculated by normalizing cDNA levels of the gene of interest against the housekeeping gene *gyrB*. Data are presented as fold change compared to levels in biofilms grown without clindamycin (set to 1), and error bars indicate interquartile range of at least three independent experiments. The dotted line indicates a 2-fold upregulation.

concentrations of clindamycin on gene expression of *agrA*, the *psmA* operon (*psmA1-psmA4*), and the two genes of the *psmβ* operon (*psmβ1* and *psmβ2*) in our biofilm system. The expression of *agrA* was upregulated 2.5-fold in the presence of clindamycin (0.06 μg/ml), and the upregulation of *psmA1-psmA4* as well as of *psmβ1* and *psmβ2* was even more pronounced (up to 60-fold) (Fig. 4A). We also tested the induction of *psm* transcription in an *atIA* mutant background, which showed the same clindamycin-induced upregulation of *psm* genes as observed in the wt strain (see Fig. S4 in the supplemental material). PSMs were shown to exhibit surfactant-like activities (61), which lower the surface tension of liquids. In order to assess the functional effect of the highly upregulated *psm* transcription, we used a drop-collapse assay to illustrate the differences in surface tension of supernatants from biofilms or bacterial cultures grown with and without clindamycin. Surface tension was low in supernatants from bacterial overnight cultures of LAC wt and the *atIA* mutant, irrespective of clindamycin treatment (Fig. 4B). In contrast, surface tension was high in biofilm supernatants after 18 h. After 36 h the biofilm supernatant of the *atIA* mutant showed a decrease in surface tension in response to 0.06 μg/ml of clindamycin. In the supernatant of a 54-h old biofilm, the surface tension of the wt strain was clearly decreased in response to clindamycin compared to that of the control. As PSM transcription was highly induced in the wt strain and the *atIA* mutant by clindamycin, the observed decrease in surface tension in these two strains might derive from PSM overproduction. No surfactant activity was detected in the supernatants of the *agr* mutant strain, indicating that the observed de-



**FIG 4** Overexpression of the *psm* genes and increase in surfactant activity in *S. aureus* biofilms in response to clindamycin. (A) Relative transcript levels of *agrA* and the *psm* genes derived from LAC wt biofilm mass after 18 h with and without clindamycin as determined by qRT-PCR. Fold change ratios were calculated by normalizing cDNA levels of the gene of interest against the housekeeping gene *gyrB*. Data are presented as fold change compared to biofilms grown without clindamycin (set to 1), and error bars indicate interquartile range of at least three independent experiments. The dotted line indicates a 2-fold upregulation. (B) Surface tension of supernatants of a bacterial liquid culture and of biofilms grown for indicated time with and without clindamycin is illustrated by a drop-collapse assay. If surfactants were present in the drop, the surface tension was reduced, and the drops spread or collapsed (indicated by arrows). Surfactant activity was high in the LAC wt and the *atIA* mutant in shaking cultures after 18 h. When grown as a biofilm, the *atIA* mutant showed an increase in surfactant activity in response to clindamycin after 36 h while the LAC wt showed a similar effect after 54 h. Representative images of three independent experiments are shown.

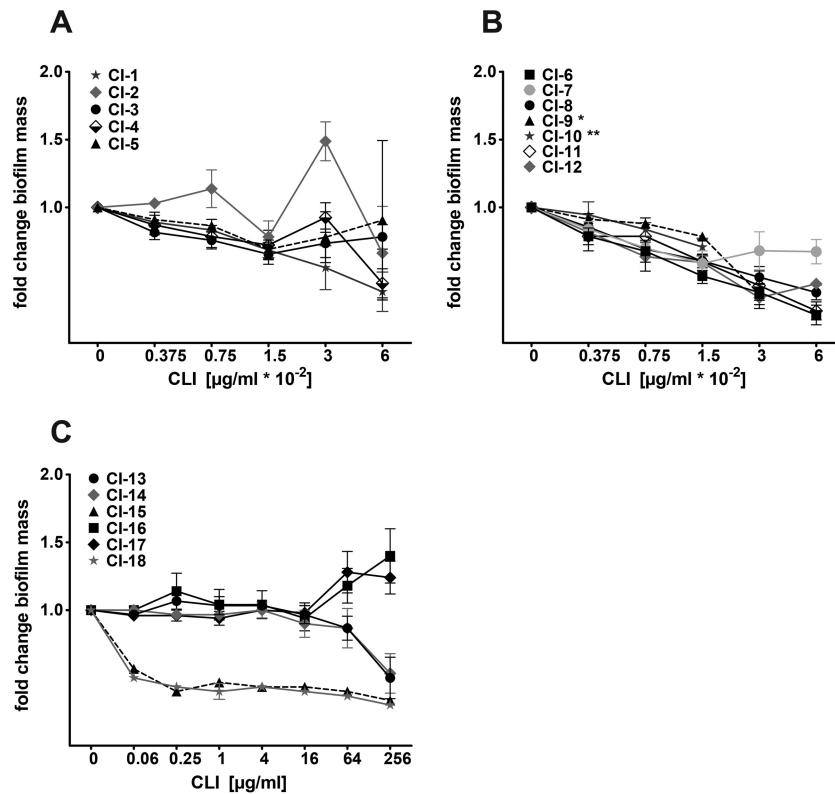


**FIG 5** Subinhibitory concentrations of clindamycin change *S. aureus* cell morphology. (A) Representative transmission electron images of the LAC wt and *atIA* mutant from cultures grown without and with clindamycin (0.06 µg/ml CLI). Arrows highlight membrane-like fibers. Scale bar, 2 µm. (B) Zymogram analysis of the LAC wt and *atIA* mutant autolytic activity from cultures grown for 18 h without and with clindamycin (0.03 to 0.06 µg/ml CLI). Crude cell wall extract (left panel) or filtered supernatants (right panel) were loaded on gels containing *S. aureus* RN4220 or *Micrococcus luteus* as the substrate. Bacteriolytic activity is indicated by the dark bands on the opaque gel. Bands are assigned as follows (27, 77): Pro-Atl (~138 kDa), Atl with full-length propeptide; Atl (~115 kDa), amidase plus glucosaminidase; PP-AM (~84 kDa), amidase with propeptide; AM (62 to 65 kDa), amidase; GL (<55 kDa), glucosaminidase; Atl-GLcat (~36 kDa and 38 kDa), glucosaminidase catalytic domain lacking repeat domain; Sle1/Aaa (~32 kDa), *S. aureus* autolysin. Band size was estimated based on the PageRuler prestained protein ladder. Representative gels of at least three independent experiments are shown.

crease in surface tension is dependent on the quorum-sensing system.

**Difference in *S. aureus* USA300 LAC morphology due to subinhibitory concentrations of clindamycin.** Previous studies showed that the cell walls of staphylococci are thickened when exposed to high concentrations of clindamycin (62, 63). Therefore, we examined the effect of subinhibitory concentrations of clindamycin on *S. aureus* cell morphology by transmission electron microscopy (TEM). Cells grown in the presence of clindamycin

(0.06 µg/ml) lost their spherical cell shape and appeared inflated, potentially reflecting a stress response phenotype. We did not see a thickening of the cell wall after 18 h of incubation with clindamycin. However, we observed a “peeling off” of the cell wall along with membrane-like fibers protruding from the bacterial cells (Fig. 5A). As *AtlA* was shown to play an important role in cell division and separation (28, 29), clindamycin-induced *atIA* expression (Fig. 3A) may contribute to the observed phenotype. Therefore, we also examined the cell morphology of the *atIA* mu-



**FIG 6** Impact of subinhibitory concentrations of clindamycin on *S. aureus* clinical isolates. Biofilm mass quantification of MSSA clinical isolates CI-1 to CI-5 (A), clindamycin-sensitive MRSA clinical isolates CI-6 to CI-12 (B), and clindamycin-resistant MRSA clinical isolates CI-13 to CI-18 (C) with crystal violet in the absence and presence of subinhibitory concentrations of clindamycin (CLI) after 18 h of static incubation. Biofilm without clindamycin was set to 1, and fold changes are presented. Results are average data from at least three replicates  $\pm$  standard error of the mean. \*, highest tested clindamycin concentration, 0.03  $\mu\text{g/ml}$  (MIC, 0.06  $\mu\text{g/ml}$ ); \*\*, highest tested clindamycin concentration, 0.015  $\mu\text{g/ml}$  (MIC, 0.03  $\mu\text{g/ml}$ ).

tant grown with and without clindamycin by TEM. In the *atlA* mutant no apparent peeling-off phenotype was observed in response to clindamycin (Fig. 5A). In conclusion, the *AtIA* upregulation by clindamycin might be responsible for the observed peeling-off phenotype of the cell wall. To investigate the influence of clindamycin on *AtIA* hydrolase activity in more detail, we performed zymogram analysis of the wt and the *atlA* mutant in response to clindamycin. *Micrococcus luteus* or *Staphylococcus aureus* cells were used to differentiate between glucosaminidase (GL) and amidase (AM) enzyme activity, respectively. Several autolytic bands were seen with the wt strain because *AtIA* undergoes multiple proteolytic cleavage events which are absent in the *atlA* mutant (Fig. 5B). The additional 32-kDa murein hydrolase Sle1 (64), also referred to as Aaa (65), was present in the wt as well as in the *atlA* mutant, as seen when *Staphylococcus aureus* was used as a substrate within the gel. The crude cell wall extracts showed minimal enhanced autolytic activity in response to clindamycin (0.03 and 0.06  $\mu\text{g/ml}$ ) compared to that of the control (Fig. 5B, left panel). A more profound effect of clindamycin was observed for the extracellular autolytic activity in the culture supernatants, in particular, the GL activity seen with *Micrococcus* as a substrate (Fig. 5B, right panel).

**Alteration of biofilm formation by clindamycin in clinical isolates.** Since clindamycin is routinely used in clinics to treat *S. aureus* infections, we investigated whether subinhibitory concentrations of clindamycin also altered biofilm formation in *S. aureus*

clinical isolates (strains are listed in Table S3 in the supplemental material) in our static biofilm model. All 18 tested clinical isolates formed a static biofilm (see Fig. S5). One out of five clindamycin-sensitive, methicillin-sensitive *S. aureus* (MSSA) strains (CI-1 to CI-5) showed a slight induction of biofilm formation when subjected to clindamycin at subinhibitory concentrations (CI-2) (Fig. 6A). This strain was isolated from a foreign-body-associated infection (vascular prosthesis infection), while the other four strains were isolated from native tissue-associated infections (endocarditis, panaritium, abscess, and wound infection). All seven clindamycin-sensitive MRSA strains (CI-6 to CI-12) showed a reduction of biofilm-forming capacity upon clindamycin exposure (Fig. 6B). We also tested six clindamycin-resistant MRSA strains for their biofilm phenotype when subjected to clindamycin at concentrations just below their MICs. Two out of six strains showed clindamycin-dependent biofilm induction when challenged with 64 and 256  $\mu\text{g/ml}$  clindamycin (Fig. 6C). These two clinical isolates (CI-16 and CI-17) were USA300 clones (*spa* type t008) (66), like our model strain LAC wt, and had been isolated from skin and soft tissue infections. The other four clindamycin-resistant strains were of *spa* types t586 ( $n = 2$ ) and t032 ( $n = 2$ ) and showed no clindamycin-dependent biofilm induction, but strains from each of the two *spa* types showed similar behavior in response to clindamycin. This indicates that the clindamycin-dependent biofilm phenotype might be more pronounced in certain *S. aureus* subgroups, like the USA300 clonal lineage.



We next investigated whether the biofilm phenotype of the clinical isolates in response to clindamycin correlated with staphyloxanthin production and was thereby associated with a  $\sigma^B$  response to clindamycin. We chose five representative strains to quantify staphyloxanthin production, including two MSSA strains, one showing a clindamycin-induced biofilm phenotype (CI-2) and one which did not show a response to clindamycin (CI-4). Furthermore, we tested two clindamycin-sensitive MRSA strains, neither of which showed biofilm induction by clindamycin (CI-6 and CI-8), and one clindamycin-resistant MRSA strain which showed induction of biofilm formation upon clindamycin exposure (CI-16). As shown in Fig. S3C and D in the supplemental material, four out of five tested clinical isolates showed an increase in staphyloxanthin production. Strains CI-4 and CI-6 showed clear induction of staphyloxanthin production upon clindamycin exposure but did not show an increase in biofilm formation. The clindamycin-resistant MRSA CI-16 (*spa* type t008; USA300) showed the same biofilm and staphyloxanthin phenotype as our model strain LAC. These data indicate that the observed clindamycin-induced biofilm phenotype of our tested clinical isolates correlates with a  $\sigma^B$  response to clindamycin in the USA300 background but not in the other tested clinical isolates that had distinct *spa* types.

## DISCUSSION

In this study, we show that subinhibitory concentrations of the frequently clinically used protein synthesis inhibitor clindamycin induced biofilm formation and altered the biofilm matrix composition of the CA-MRSA strain USA300 LAC. Although previous studies have investigated the effect of several antibiotics at subinhibitory concentrations on staphylococcal biofilm formation and matrix composition (12, 13, 38, 54, 67), the effect of subinhibitory concentrations of clindamycin, an important antibiotic to treat *S. aureus* infections, had not been investigated so far. Clindamycin-mediated biofilm induction was observed in the wt strain as well as in the *nuc1* and *at1A* mutants. In addition, clindamycin had no effect on *nuc1* expression. Taking these findings together, this shows that the observed biofilm induction by clindamycin is not due to transcriptional *nuc1* repression. The *at1A* mutant was deficient in biofilm formation, which is in agreement with the role of At1A in primary attachment (19, 28), and showed a 3.5-fold induction of biofilm formation by clindamycin. The presence of clindamycin also resulted in an increase of the viable bacteria count in the *at1A* mutant. Therefore, the clindamycin-induced increase in biofilm mass might derive from a combination of the higher viable bacteria count in the biofilm and an increase in primary attachment to the plastic surface.

Glucose-induced biofilms in the USA300 LAC strain are mainly polysaccharide intercellular adhesin (PIA) independent (16, 68), and a major component of these biofilms is eDNA (19, 22, 23). In *S. aureus* biofilms, the eDNA is likely produced through the autolysis of a subpopulation of cells which is mediated by the murein hydrolase At1A (27). Isolation of eDNA from biofilms grown with and without clindamycin confirmed the presence of a small amount of eDNA in the *at1A* mutant relative to the biofilm mass. In contrast to results in the *at1A* mutant, clindamycin increased the amount of eDNA within the biofilm matrix in the wt as well as in the other mutant strains (*nuc1*, *agr*, *cidA*, and *lrgA* strains) relative to the biofilm mass. This suggests that the increase in eDNA of the wt and *nuc1* mutant only partially contributes to

the increase in biofilm formation. Clindamycin had no effect on biofilm formation of the *cidA* and the *lrgA* mutants. In the absence of clindamycin, we observed increased biofilm formation in the *lrgA* mutant compared to that in the wt strain, as previously reported for an *S. aureus* UAMS-1 *lrgAB* mutant (24). Expression of *lrgAB* is induced by the LysSR two-component system, which senses a reduction in the membrane potential ( $\Delta\Psi$ ) (69). Although in an earlier study a *cidA* mutant in *S. aureus* UAMS-1 exhibited decreased lysis resulting in smaller amounts of eDNA and impaired biofilm formation (22, 24), the LAC *cidA* mutant showed the same biofilm phenotype as the wt strain in our biofilm model after 18 h. Transcription of *cidA* in UAMS-1 biofilms steadily increased over time (up to 90-fold after 72 h) (70). Therefore, its effect on biofilm formation might play only a minor role in our 18-h-old biofilm system while LrgAB might be activated by changes in the membrane potential at an earlier time point.

The *at1A* gene expression was approximately 25-fold upregulated in response to subinhibitory concentrations of clindamycin, which is the most likely cause of the observed increase of eDNA in the biofilm. We did not detect a difference in *cidA* expression but an upregulation of *agrA* and *lrgA* in response to clindamycin. The *lrgAB* operon is positively regulated by the Agr system and by the transcriptional regulator SarA (71), which was shown to be upregulated in response to clindamycin (14). Therefore, the observed upregulation of *lrgA* is likely due to the clindamycin-derived upregulation of these regulators. Since subinhibitory concentrations of clindamycin highly induced biofilm formation in the *at1A* mutant strain, we speculate that additional factors, likely fibronectin-binding proteins (FnBPs), are upregulated that might compensate for the impaired attachment of the *at1A* mutant strain. FnBPs mediate intercellular cell-cell adhesion via homophilic bonds (72) and are required for primary attachment and biofilm accumulation in the USA300 LAC strain (20). Corroborating this, we observed an upregulation of *fnbA* and *fnbB* expression by clindamycin in the biofilm. It has previously been shown that the *S. aureus* strain Newman showed increased amounts of *fnbB* transcripts due to selective mRNA stabilization in response to clindamycin (73).

The observed alteration in the expression profile in response to subinhibitory concentrations of clindamycin might be due to the activation of a general stress response via the alternative sigma factor B ( $\sigma^B$ ). We confirmed that *sigB* expression was upregulated and that significantly larger amounts of  $\sigma^B$ -dependent staphyloxanthin were isolated in response to clindamycin.  $\sigma^B$  controls a large regulon which includes the expression of *fnbA* and *fnbB* (58–60) as well as *at1A* in certain *S. aureus* strains (19).

In contrast to the results obtained with our model strain USA300 LAC, the  $\sigma^B$ -dependent staphyloxanthin production correlated with the observed biofilm phenotypes in only two out of five selected clinical isolates. This indicates that the clindamycin-induced  $\sigma^B$  stress response does not necessarily translate into elevated biofilm formation. In addition, the observed amoxicillin-dependent biofilm induction was not associated with staphyloxanthin production. These experiments indicate that antibiotic-induced biofilm formation might derive from a  $\sigma^B$ -dependent stress response in only certain clonal lineages (USA300). At the same time, a  $\sigma^B$ -dependent stress response is not a necessary prerequisite for antibiotic-induced biofilm formation.

Other important matrix components which were recently discovered to play a role in *S. aureus* biofilm formation and dispersal



are PSMs (74). The formation of amyloid fibers is thought to contribute to *S. aureus* biofilm integrity under certain conditions and might serve as a storage mechanism until the surfactant activity of the monomeric PSM peptides is required (36, 37). The *psm $\alpha$*  and *psm $\beta$*  genes are highly induced by subinhibitory concentrations of clindamycin in our biofilm model. In addition, surfactant activity increased in the supernatant of a 36-h-old *atlA* mutant biofilm and after 54 h also in the wt strain in response to clindamycin. The presence of eDNA in the biofilm matrix facilitates PSMs to form amyloid fibers (75). As we observed induction of *psm* gene expression and eDNA release in our biofilm model, we speculate that the formation of amyloid fibers might be increased at early time points (18 h) when we observed a thick biofilm with large amounts of eDNA. At later time points, degradation of eDNA and decomposition of the fibers might lead to biofilm dispersal via surfactant activity of the PSM monomers. Deficient release of eDNA in the *atlA* mutant might therefore hinder eDNA-mediated amyloid fiber formation of PSMs and thereby contribute to the observed surfactant activity at early time points in the mutant.

In summary, we showed that subinhibitory concentrations of clindamycin have diverse effects on *S. aureus* biofilm formation which highly depend on the strain background. In-depth analysis of the model strain LAC wt from the USA300 clonal lineage showed that subinhibitory concentrations of clindamycin alter the biofilm matrix composition by changing autolysis and eDNA release, increasing adhesion factors and secreted proteins, which likely leads to an interaction between the matrix components. Previous studies showed that biofilm matrix components bind to each other and act as an electrostatic network by connecting cells within the biofilm (26, 76). We hypothesize that the clindamycin-induced matrix components such as eDNA, PSMs, and FnbBs are highly interconnected within the biofilm matrix, which might result in a more compact and stable biofilm. Since the increase in biofilm formation and alteration of the matrix composition might impact *S. aureus* biofilm-associated infections, clindamycin should be dosed as high as possible in order to prevent subinhibitory clindamycin concentrations in affected tissues and biofilms.

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