

Low Levels of β -Lactam Antibiotics Induce Extracellular DNA Release and Biofilm Formation in *Staphylococcus aureus*

Jeffrey B. Kaplan,^a Era A. Izano,^a Prerna Gopal,^a Michael T. Karwacki,^a Sangho Kim,^a Jeffrey L. Bose,^b Kenneth W. Bayles,^b and Alexander R. Horswill^c

Department of Oral Biology, New Jersey Dental School, Newark, New Jersey, USA^a; Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska, USA^b; and Department of Microbiology, Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA^c

ABSTRACT Subminimal inhibitory concentrations of antibiotics have been shown to induce bacterial biofilm formation. Few studies have investigated antibiotic-induced biofilm formation in *Staphylococcus aureus*, an important human pathogen. Our goal was to measure *S. aureus* biofilm formation in the presence of low levels of β -lactam antibiotics. Fifteen phylogenetically diverse methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) strains were employed. Methicillin, ampicillin, amoxicillin, and cloxacillin were added to cultures at concentrations ranging from 0 \times to 1 \times MIC. Biofilm formation was measured in 96-well microtiter plates using a crystal violet binding assay. Autoaggregation was measured using a visual test tube settling assay. Extracellular DNA was quantitated using agarose gel electrophoresis. All four antibiotics induced biofilm formation in some strains. The amount of biofilm induction was as high as 10-fold and was inversely proportional to the amount of biofilm produced by the strain in the absence of antibiotics. MRSA strains of lineages USA300, USA400, and USA500 exhibited the highest levels of methicillin-induced biofilm induction. Biofilm formation induced by low-level methicillin was inhibited by DNase. Low-level methicillin also induced DNase-sensitive autoaggregation and extracellular DNA release. The biofilm induction phenotype was absent in a strain deficient in autolysin (*atl*). Our findings demonstrate that subminimal inhibitory concentrations of β -lactam antibiotics significantly induce autolysin-dependent extracellular DNA release and biofilm formation in some strains of *S. aureus*.

IMPORTANCE The widespread use of antibiotics as growth promoters in agriculture may expose bacteria to low levels of the drugs. The aim of this study was to investigate the effects of low levels of antibiotics on bacterial autoaggregation and biofilm formation, two processes that have been shown to foster genetic exchange and antibiotic resistance. We found that low levels of β -lactam antibiotics, a class commonly used in both clinical and agricultural settings, caused significant autoaggregation and biofilm formation by the important human pathogen *Staphylococcus aureus*. Both processes were dependent on cell lysis and release of DNA into the environment. The effect was most pronounced among multidrug-resistant strains known as methicillin-resistant *S. aureus* (MRSA). These results may shed light on the recalcitrance of some bacterial infections to antibiotic treatment in clinical settings and the evolution of antibiotic-resistant bacteria in agricultural settings.

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Address correspondence to Jeffrey B. Kaplan, kaplanjb@umdnj.edu.

Staphylococcus aureus continues to be a major cause of health care-related and community-associated infections. The emergence of multidrug-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) has contributed to the spread of this bacterium (1). In addition, *S. aureus* often forms matrix-encased biofilms on tissues and medical devices, which confer additional drug resistance and further complicate treatment (2).

Although *S. aureus* biofilms have been shown to be resistant to killing by antibiotics at concentrations sufficient to kill planktonic cells, several studies have shown that *S. aureus* biofilm formation can be stimulated by subminimal inhibitory concentrations (sub-MICs) of some antibiotics. The cell wall-active antibiotics oxacillin (3), cephalothin (4), cephalixin (5), and vancomycin (3) and the protein synthesis inhibitor linezolid (6) have been shown to

stimulate *S. aureus* biofilm formation *in vitro* by as much as 4-fold when present at sub-MICs. Enhanced biofilm formation in the presence of sub-MICs of antibiotics is a common phenomenon among bacteria and is thought to result from a global response to cell stress (7). Other studies found an inhibitory effect or no effect on *S. aureus* biofilm formation by sub-MICs of other cell wall-active antibiotics (cefamandole, cefuroxime, nafcillin, teicoplanin, and vancomycin) and protein synthesis inhibitors (tetracycline and roxithromycin) (5, 6, 8, 9). Most of these studies, however, utilized a single strain of *S. aureus* and tested antibiotics at a single or limited number of antibiotic concentrations, usually $\leq 1/2 \times$ MIC.

Antibiotic-induced biofilm formation may be a clinically relevant process because bacteria are exposed to sub-MIC antibiotics

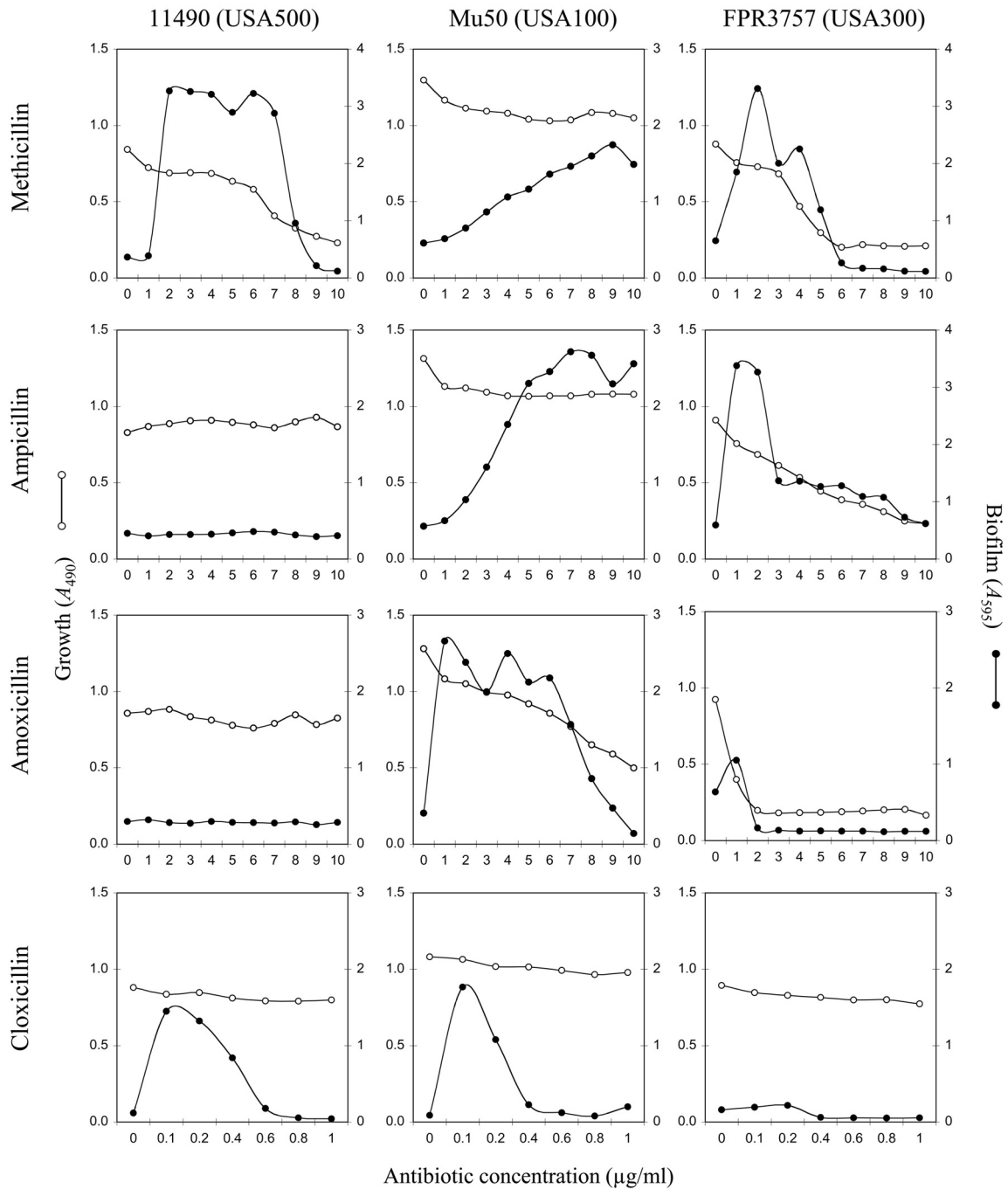


FIG 1 Bacterial growth and biofilm formation by three MRSA strains (strains 11490, Mu50, and FPR3757) in the presence of sub-MICs of four β -lactam antibiotics. Bacterial growth (A_{490}) is indicated on the left-hand y axes, biofilm formation (A_{595}) is indicated on the right-hand y axes, and antibiotic concentration is indicated on the x axes. Values show average absorbance values for duplicate wells. Error bars were omitted for clarity.

during the normal course of antibiotic therapy (10). The widespread use of antibiotics as growth promoters in agriculture may also expose bacteria to low levels of the drugs (11). The goal of the present study was to measure biofilm formation by a variety of *S. aureus* strains *in vitro* in response to sub-MICs of β -lactam antibiotics, a class of antibiotic commonly used in both clinical and agricultural settings.

RESULTS

β -Lactam antibiotics induce *S. aureus* biofilm formation. Figure 1 shows growth (A_{490}) and biofilm formation (A_{595}) by methicillin-resistant *S. aureus* (MRSA) strains 11490 (USA500), Mu50 (USA100), and FPR3757 (USA300) in the presence of methicillin, ampicillin, amoxicillin, and cloxacillin at concentrations ranging from 0 to 10 $\mu\text{g/ml}$. The MIC values for these four

TABLE 1 Induction of *S. aureus* biofilm formation by sub-MICs of methicillin

<i>S. aureus</i> strain (lineage)	Maximum biofilm induction ^a	Basal biofilm formation ^b	Methicillin MIC (μg/ml)	SCCmec genotype ^c	<i>agr</i> genotype	Reference or source
LAC (USA300)	11.8*	15	>8	IV	I	31
11490 (USA500)	10.5*	12	>8	IV	I	PHRI ^e
MW2 (USA400)	8.9*	13	>8	IVa	III	32
Newman	6.2*	19	2	–	I	33
FPR3757 (USA300)	5.8*	16	6	IV	I	34
Mu50 (USA100)	5.1*	12	>8	II	II	35
476	1.8	12	3	–	III	36
COL	1.4	16	>8	I	I	37
RF122	1.2	15	3	–	II	38
252	1.2	61	>8	II	III	36
383	1.1	104	>8	–	ND ^d	39
SH1000	1.0	100	1	–	I	40
MZ100	1.0	122	1	–	I	41
N315	1.0	119	2	II	II	35
SA113	1.0	119	1	–	I	42

^a Maximum fold increase in crystal violet binding in the presence of sub-MICs of methicillin. The method used to calculate the value is described in Materials and Methods. Values are averages from 2 or 3 experiments. Standard errors were $\pm 7\%$ on average. Values that were significantly different ($P < 0.05$ by Student's *t* test) from the values for the no-antibiotic control are indicated by an asterisk.

^b Relative amount of crystal violet binding in the absence of methicillin compared to that of strain SH1000 in the absence of methicillin. The values were calculated as follows: (absorbance of the test strain/absorbance of strain SH1000) \times 100.

^c The staphylococcal cassette chromosome *mec* element (SCC*mec*) genotype is shown. A minus sign indicates the absence of SCC*mec* and that the strain is an MSSA strain.

^d ND, not determined.

^e Barry Kreiswirth at the Public Health Research Institute (PHRI), Newark, NJ, supplied this strain.

antibiotics against these three strains ranged from 2 to >10 μg/ml. All four antibiotics significantly induced biofilm in at least one strain, although the pattern of biofilm induction was strain and antibiotic dependent. Only methicillin induced biofilm formation in all three strains. Most antibiotics that induced biofilm formation exhibited a biphasic dose-response curve characterized by low-dose stimulation of biofilm formation and high-dose inhibition.

Methicillin induces biofilm formation in strains that exhibit a weak biofilm phenotype. Since sub-MICs of methicillin induced biofilm in all three MRSA strains, we tested the ability of sub-MICs of methicillin to induce biofilm formation in a total of 15 MRSA and methicillin-sensitive *S. aureus* (MSSA) strains of diverse origin (Table 1). Biofilm formation was significantly induced in six strains, five of which were MRSA strains. The methicillin concentrations that induced maximum biofilm formation in these five strains (highest A_{595} value) ranged from 1 to 7 μg/ml. The maximum fold level of biofilm induction was inversely correlated to the amount of biofilm produced by the test strain in the absence of methicillin ($P < 0.05$). Figure 2A shows photographs of crystal violet-stained wells that were inoculated with *S. aureus* 11490 (USA500) and FPR3757 (USA300) and cultured in the presence of increasing concentrations of methicillin. Increased crystal violet binding at 2 μg/ml methicillin correlated with a significant increase in biofilm CFU values (Fig. 2B).

Methicillin-induced biofilm formation is inhibited by DNase. Previous studies showed that extracellular DNA (eDNA) constitutes a major adhesive polymer in *S. aureus* biofilms (12). To determine whether eDNA plays a role in methicillin-induced biofilms, we cultured strains 11490 (USA500) and FPR3757 (USA300) in sub-MICs of methicillin in the presence or absence of recombinant human DNase I (rhDNase), a potent inhibitor of *S. aureus* biofilm formation (13). Figure 3A and B show that rhDNase significantly inhibited methicillin-induced biofilm forma-

tion by both strains. Inhibition was more efficient at low methicillin concentrations (0 to 3 μg/ml) than at higher methicillin concentrations (4 to 5 μg/ml). For comparison, we measured the effect of rhDNase on biofilm formation by noninducible *S. aureus* strain 383 cultured in the presence of sub-MICs of methicillin (Fig. 3C). Like strains 11490 (USA500) and FPR3757 (USA300), strain 383 exhibited a biofilm phenotype that was inhibited by rhDNase more efficiently at low methicillin concentrations than at high methicillin concentrations. Dispersin B, an enzyme that degrades *S. aureus* poly-*N*-acetylglucosamine (PNAG) biofilm polysaccharide (12), had no effect on methicillin-induced biofilm formation in any of the three strains, whereas proteinase K caused nearly complete inhibition of biofilm formation by all three strains at all methicillin concentrations (data not shown). These findings are consistent with the observation that MRSA strains produce predominantly protein- and DNA-based biofilms (14). None of the three enzymes had a significant effect on the MIC of methicillin against any of the three test strains (data not shown).

Sub-MICs of methicillin induce autoaggregation and eDNA release. Autoaggregation plays an important role in maintaining the stability of *S. aureus* biofilm colonies (15). We used a visual test tube settling assay to investigate the effects of sub-MICs of methicillin on autoaggregation by *S. aureus* 11490 (USA500) and FPR3757 (USA300) (Fig. 4). Cells cultured in broth supplemented with 2 μg/ml methicillin rapidly settled to the bottom of the tube, whereas control cells cultured without methicillin remained in suspension. Cells cultured in the broth supplemented with 2 μg/ml methicillin and then treated with 10 μg/ml rhDNase remained in suspension, indicating that sub-MICs of methicillin induce eDNA-dependent intercellular adhesion. Supernatants of *S. aureus* FPR3757 (USA300) biofilms cultured in 1 to 3 μg/ml methicillin, as well as supernatants of *S. aureus* 11490 (USA500) biofilms cultured in 3 to 4 μg/ml methicillin, contained high-

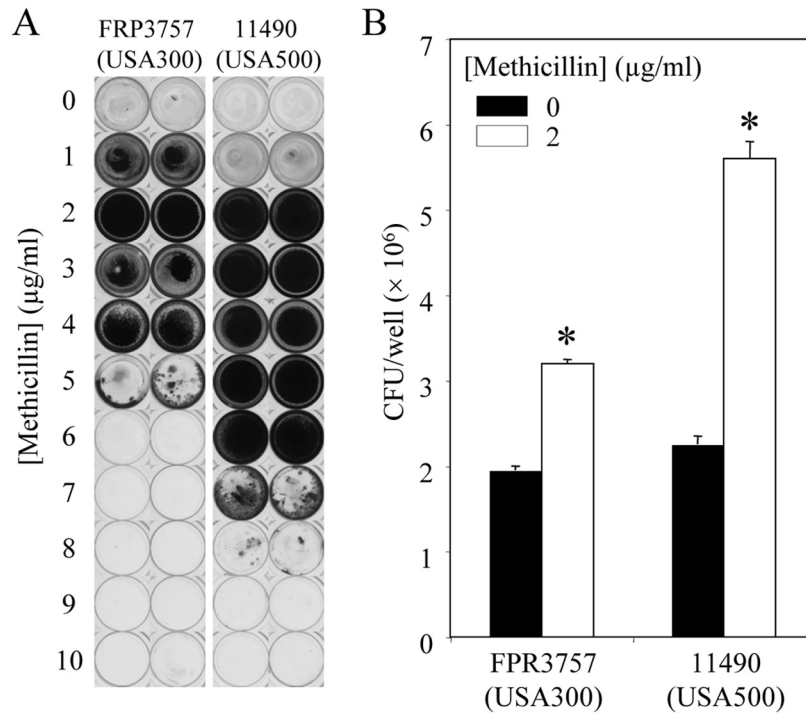


FIG 2 Biofilm formation by *S. aureus* strains FPR3757 (USA300) and 11490 (USA500) in the presence of sub-MICs of methicillin. (A) Photographs of crystal violet-stained microtiter plate wells. Duplicate wells are shown. (B) Average biofilm CFU/well values for biofilms cultured in 0 or 2 μg/ml methicillin. Error bars indicate ranges. Values that were significantly different ($P < 0.05$) from those of the no-methicillin control are indicated by an asterisk.

molecular-weight DNA (Fig. 5), which correlated with biofilm formation (Fig. 2) and is consistent with eDNA release (16, 17).

Methicillin-induced biofilm formation is dependent on autolysin activity. The production of eDNA in *S. aureus* has been shown to result from cell lysis mediated by the autolytic enzyme

AtlA (18). To determine whether AtlA plays a role in methicillin-induced biofilm formation, we cultured *S. aureus* strain LAC and isogenic *atl* mutant strain KB4051 in sub-MICs of methicillin and measured growth and biofilm formation (Fig. 6). We also tested strains deficient in the production of thermonuclease (*nuc*) and

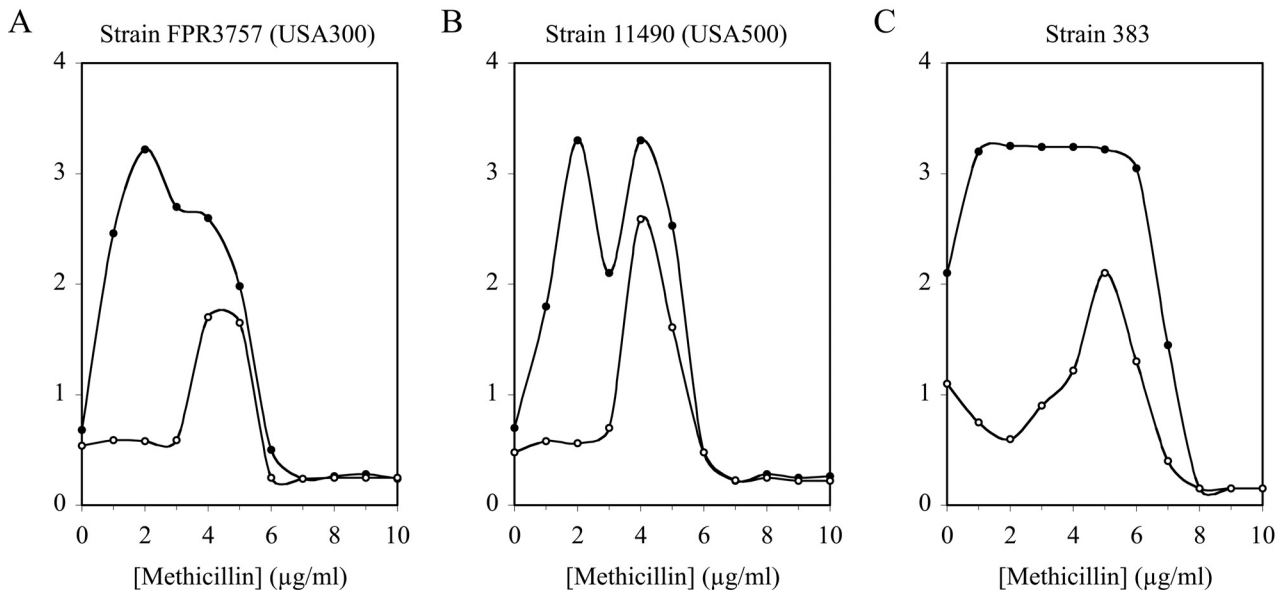


FIG 3 Effect of rhDNase on biofilm formation by *S. aureus* in sub-MICs of methicillin. Strains FPR3757 (USA300), 11490 (USA500), and 383 were cultured in broth supplemented with 10 μg/ml rhDNase (open circles) or no enzyme (filled circles). Values show average absorbance values for duplicate wells. Error bars were omitted for clarity.

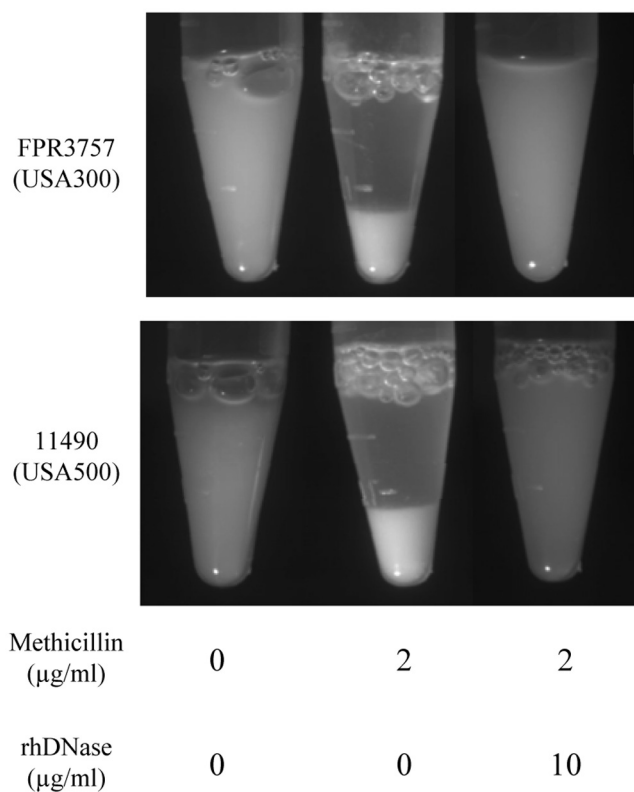


FIG 4 Sub-MICs of methicillin induce autoaggregation in *S. aureus*. Strains FPR3757 (USA300) and 11490 (USA500) were cultured for 18 h in broth supplemented with 0 or 2 $\mu\text{g/ml}$ methicillin and then treated with 0 or 10 $\mu\text{g/ml}$ rhDNase. The cells were rinsed with saline and transferred to a microcentrifuge tube. The tubes were incubated statically for 10 min and then photographed.

the accessory gene regulator (*agr*) quorum-sensing system, both of which have been shown to play a role in *S. aureus* biofilm formation and biofilm dispersal in some strains (19, 20). The biofilm induction phenotype was absent in the *atl* mutant strain, whereas inactivation of the *nuc* locus or *agr* gene had little or no effect on methicillin-induced biofilm formation. All three mutants exhibited the same MIC for methicillin as parental strain LAC (data not shown). We also measured biofilm induction by strain LAC cultured in the presence of polyanethole sulfonate (PAS), an inhibitor of *S. aureus* autolysis (17, 18). PAS completely eliminated the biofilm induction phenotype (Fig. 6B) but did not affect bacterial growth or the methicillin MIC (data not shown).

DISCUSSION

The results of the present study demonstrate that subminimal inhibitory concentrations of four different β -lactam antibiotics significantly induce biofilm formation in some strains of *S. aureus*. We found that sub-MICs of methicillin induced biofilm formation in *S. aureus* strains that exhibited a low basal level of biofilm in the absence of antibiotics, but not in strains that exhibited a high basal level of biofilm. These findings suggest that the antibiotic-induced pathway is turned on in strains that exhibit a strong biofilm phenotype such as strain SH1000, but not in strains that exhibit a weak biofilm phenotype such as strain LAC. A similar inverse relation between basal biofilm production and biofilm in-

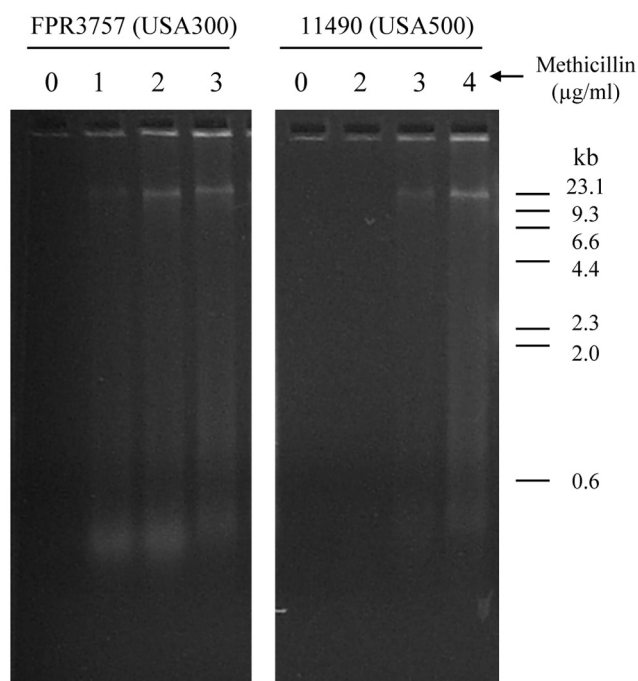


FIG 5 Sub-MICs of methicillin induce eDNA release in *S. aureus*. Biofilms of strains FPR3757 (USA300) and 11490 (USA500) were cultured for 18 h in broth supplemented with increasing concentrations of methicillin. Biofilm supernatants were analyzed by agarose gel electrophoresis. The sizes (in kilobases) of DNA molecular size markers electrophoresed in an adjacent lane are shown to the right of the gels.

ducibility was exhibited by *Staphylococcus epidermidis* in response to sub-MICs of vancomycin, tigecycline, novobiocin, linezolid, and various fluoroquinolones (21, 22). Screening for mutants deficient in the biofilm induction phenotype may be a useful strategy for discovering the effectors of these antibiotic-induced pathways.

The patterns of β -lactam-induced biofilm formation were strain and antibiotic dependent (Fig. 1). When biofilm induction occurred, the fundamental nature of the dose response was biphasic, characterized by low-dose stimulation of biofilm formation and high-dose inhibition. Multiphasic dose-response curves are characteristic of many chemicals, drugs, hormones, biological molecules, and physical stressors (23). In addition, patterns of β -lactam-induced biofilm formation exhibited a bimodal dose response, with each peak exhibiting a different level of sensitivity to inhibition by rhDNase (Fig. 3). These findings suggest that *S. aureus* exhibits two distinct biofilm phenotypes, each with a different biofilm matrix composition.

Our findings demonstrate that β -lactam-induced *S. aureus* biofilm formation is dependent on the production of eDNA. Previous studies showed that eDNA constitutes a major biofilm matrix adhesin in *S. aureus* biofilms cultured in the absence of antibiotics (12, 17–20). The production of eDNA in *S. aureus* has been shown to result from cell lysis mediated by the autolysin AtIA (18). Consistent with these results, we found that an *atl* mutant strain of *S. aureus* exhibited reduced biofilm induction (Fig. 6A) and that polyanethole sulfonate, an inhibitor of autolysis, inhibited the biofilm induction response (Fig. 6B). It is possible that low-level β -lactams induce *S. aureus* biofilm formation by upregulating AtIA production either directly or through modulation of the ex-

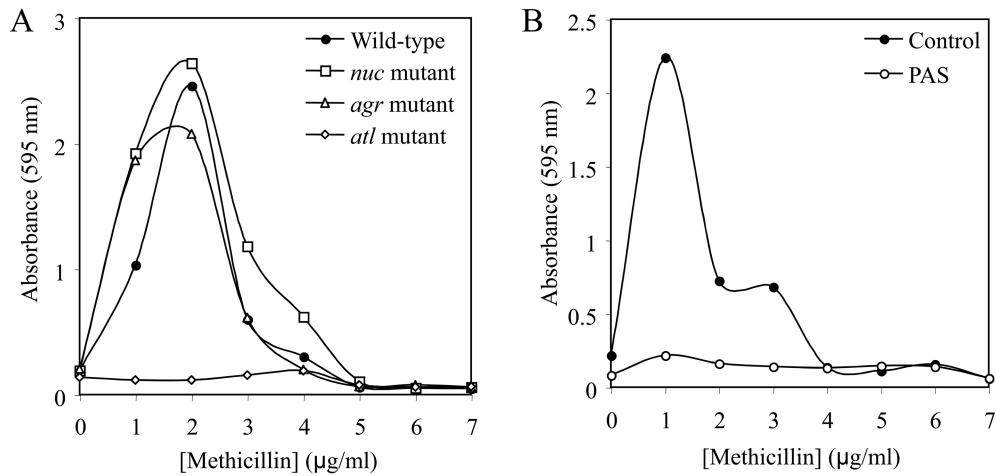


FIG 6 Biofilm formation by *S. aureus* strain LAC in sub-MICs of methicillin. (A) Biofilm formation by wild-type *S. aureus* strain LAC and isogenic *nuc*, *agr*, and *atl* mutant strains in sub-MICs of methicillin. (B) Biofilm formation by strain LAC in sub-MICs of methicillin in the presence or absence of 500 µg/ml of the autolysis inhibitor poly-anethole sulfonate (PAS). Values in both panels show average absorbance for duplicate wells. Error bars were omitted for clarity.

pression of other regulatory proteins. Proteins that regulate *S. aureus atl* expression include the alternative sigma factor σ^B (18), the DNA-binding protein SarA (24), and the glycopeptide resistance-associated two-component system GraRS (25).

S. aureus thermonuclease encoded by the *nuc* gene has been shown to be a negative regulator of biofilm formation (16, 20, 24). Kiedrowski et al. (20) found that an *S. aureus* USA300 strain carrying a mutation in *nuc* exhibited increased biofilm formation and increased high-molecular-weight eDNA, phenotypes identical to those exhibited by strain FPR3757 (USA300) cultured in low-level methicillin (Fig. 2 and 4). These findings suggest that low-level methicillin may suppress *nuc* expression. However, we found that a *nuc* mutation in USA300 strain LAC did not alter the amount of biofilm formed in the absence of antibiotics or the amount of biofilm induction caused by low-level methicillin (Fig. 6). One possible explanation for these findings is that thermonuclease mediates biofilm dispersal, but only under conditions of strong biofilm induction (16).

The *agr* quorum-sensing system has been shown to regulate *S. aureus* biofilm formation through modulation of biofilm dispersal (19). Subrt et al. (4) showed that the β -lactam antibiotic cephalothin, when present at $1/4 \times$ MIC, induced *S. aureus* biofilm formation but did not affect *agr* expression. Consistent with these results, we found that an *agr* mutation in USA300 strain LAC did not affect the amount of biofilm induction caused by low-level methicillin (Fig. 6). Thus, *agr* quorum sensing evidently does not play a role in β -lactam-induced biofilm formation in *S. aureus*.

Biofilm formation by MRSA strains is often dependent on the expression of fibronectin-binding proteins (FnBPs), whereas biofilm formation by MSSA strains usually depends on the production of poly-*N*-acetylglucosamine (PNAG) exopolysaccharide (14) and eDNA (17). Consistent with these observations, we found that the PNAG-degrading enzyme dispersin B had no effect on methicillin-induced biofilm formation by MRSA strains. Bisognano et al. (26) showed that expression of *S. aureus* FnBPs was increased in some highly quinolone-resistant strains when cultured in medium supplemented with $1/4 \times$ MIC ciprofloxacin. It is possible that sub-MICs of methicillin also induce FnBP expres-

sion. Another potential target for sub-MIC β -lactam induction may be *spa* (encoding protein A), which may play a role in biofilm formation (27) and was shown to be induced by low concentrations of cephalothin (4).

Our findings may have clinical relevance because β -lactam antibiotics are commonly used in both clinical and agricultural settings. In the clinic, bacteria may be exposed to sub-MICs of antibiotics at the beginning and end of a dosing regimen, between doses, or continuously during low-dose therapy (10). In addition, cells buried deep within a biofilm colony may be exposed to sub-MICs of antibiotics because of diffusion gradients (28). Given the high level of biofilm induction and the observed variability among antibiotics and strains, it may be useful to test clinical isolates for biofilm inducibility in order to optimize antibiotic chemotherapy in clinical settings. The widespread use of β -lactams as growth promoters in agriculture may also expose bacteria to low levels of these drugs, and there is concern that subtherapeutic antibiotics may promote antibiotic resistance in agricultural settings (29). It is possible that enhanced biofilm formation by *S. aureus* in response to low-dose β -lactams may foster genetic exchange and contribute to the spread of antibiotic resistance genes, which may represent an additional drawback of administering β -lactams to farm animals.

MATERIALS AND METHODS

Antibiotics, enzymes and chemicals. Methicillin was purchased from Sigma-Aldrich (St. Louis, MO), ampicillin was from IBI Scientific (Peosta, IA), and amoxicillin and cloxacillin were from MP Biomedicals (Solon, OH). Stock solutions of antibiotics were prepared in sterile H_2O at a 1-mg/ml concentration. Recombinant human DNase I (rhDNase) was obtained from Genentech (South San Francisco, CA). Dispersin B (DspB), a poly-*N*-acetylglucosamine (PNAG)-specific glycoside hydrolase (12), was obtained from Kane Biotech (Winnipeg, Manitoba, Canada). Proteinase K was purchased from Sigma-Aldrich. Sodium poly-anethole sulfonate was purchased from Polysciences, Inc. (Warrington, PA).

Bacterial strains and growth conditions. The *S. aureus* strains used in this study are listed in Table 1. In some experiments, strains KB4051 (LAC Δ atl), AH1680 (LAC *nuc*::LtrB) and AH1292 (LAC Δ agr::TetM) (20, 30) were also employed. Strains were passaged weekly on blood agar and

stored at 4°C. Bacteria were cultured in filter-sterilized tryptic soy broth (Becton, Dickinson, Heidelberg, Germany) supplemented with 6 g/liter yeast extract and 8 g/liter glucose. All cultures were incubated statically at 37°C.

Biofilm assay. Inocula were prepared in fresh broth from 18-h-old agar colonies as previously described (12). Aliquots of inocula (200 μ l each, ca. 10^4 to 10^5 CFU/ml) were transferred to the wells of a 96-well microtiter plate (catalog no. 353936; Falcon) and incubated for 18 to 24 h. For crystal violet staining, the wells were rinsed with water to remove loosely adherent cells and then stained for 1 min with 200 μ l of Gram's crystal violet. The wells were then rinsed with water and dried. The amount of biofilm biomass was quantitated by destaining the wells with 200 μ l of 33% acetic acid and then measuring the absorbance of the crystal violet solution in a microplate spectrophotometer set at 595 nm. In cases where the absorbance values were above the dynamic range of the instrument (4.0 optical density units [OD]), the samples were diluted with an equal volume of 33% acetic acid prior to the measurements. To quantitate the amount of biofilm induction, strains were cultured in broth containing 0 to 10 μ g/ml of methicillin in 1- μ g increments. The maximum biofilm induction value for each strain (Table 1) was equal to the highest observed absorbance value divided by the absorbance value at 0 μ g/ml methicillin. For biofilm CFU enumeration, the wells were rinsed three times with sterile phosphate-buffered saline (PBS) and then treated with 10 μ g/ml rDNase to detach the biofilm cells. Biofilm CFU values were enumerated by dilution plating on agar.

Autoaggregation assay. Bacteria were cultured in 100-mm-diameter tissue culture-treated petri dishes (catalog no. 353003; Falcon) in 20 ml of broth supplemented with 0 or 2 μ g/ml methicillin. After 18 to 24 h of growth, the broth was carefully removed, and 500 μ l of PBS was added to the dish. The cells were scraped from the surface using a cell scraper and transferred to a 1.5-ml microcentrifuge tube. The cells were resuspended by gentle pipetting, and the tubes were then incubated statically for 10 min and photographed. Some samples were treated with 10 μ g/ml of rDNase for 10 min prior to resuspension.

Extracellular DNA assay. Bacteria were cultured in 6-well tissue culture-treated polystyrene microtiter plate wells (catalog no. 353046; Falcon) in 4 ml of broth supplemented with increasing concentrations of methicillin. After 18 to 24 h of growth, the broth was carefully removed and 1 ml of TE buffer (10 mM Tris, 1 mM EDTA [pH 8]) was added to the wells. The cells were scraped from the surface using a cell scraper, and the cells were transferred to a 1.5-ml microcentrifuge tube. The tubes were centrifuged at 13,000 rpm for 25 s, and the supernatant was removed. The cell pellets were vigorously resuspended in 200 μ l of TE buffer, and the tubes were recentrifuged. A total of 30 μ l of the supernatant was electrophoresed through a 1% agarose gel. DNA was visualized by staining with ethidium bromide.

Reproducibility of results and statistics. All biofilm assays were carried out in duplicate or triplicate wells, which exhibited an average variation in absorbance values of 7%. All assays were repeated 2 to 5 times, and in all cases, the observed patterns of biofilm induction and inhibition were reproducible. The significance of differences between absorbance values was calculated using a Student's *t* test. Correlation was measured using a Pearson correlation coefficient.

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