In Vitro Effects of Antimicrobial Agents on Planktonic and Biofilm Forms of *Staphylococcus lugdunensis* Clinical Isolates[▽]

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Staphylococcus lugdunensis is an atypically virulent coagulase-negative staphylococcal species associated with acute and destructive infections that often resemble *Staphylococcus aureus* infections. Several types of infection caused by *S. lugdunensis* (e.g., native valve endocarditis, prosthetic joint infection, and intravascular catheter infection) are associated with biofilm formation, which may lead to an inability to eradicate the infection due to the intrinsic nature of biofilms to resist high levels of antibiotics. In this study, planktonic MICs and MBCs and biofilm bactericidal concentrations of 10 antistaphylococcal antimicrobial agents were measured for 15 *S. lugdunensis* isolates collected from patients with endocarditis, medical device infections, or skin and soft tissue infections. Planktonic isolates were susceptible to all agents studied, but biofilms were resistant to high concentrations of most of the drugs. However, moxifloxacin was able to kill 73% of isolates growing in biofilms at ≤ 0.5 $\mu\text{g/ml}$. Relative to the effect on cell density, subinhibitory concentrations of nafcillin substantially stimulated biofilm formation of most isolates, whereas tetracycline and linezolid significantly decreased biofilm formation in 93 and 80% of isolates, respectively. An unexpected outcome of MBC testing was the observation that vancomycin was not bactericidal against 93% of *S. lugdunensis* isolates, suggesting widespread vancomycin tolerance in this species. These data provide insights into the response of *S. lugdunensis* isolates when challenged with various levels of antimicrobial agents in clinical use.

Coagulase-negative staphylococci (CNS), a major cause of device-related infections, generally demonstrate resistance to a number of antimicrobial agents (10). The ability of CNS to colonize and subsequently form biofilms on the surfaces of medical devices is the primary contributing factor in the pathogenesis of such infections (51). Biofilms often show inherent resistance to high levels of antimicrobial agents, which makes treatment of biofilm infections a difficult and costly endeavor that may be further confounded if the causative organism is resistant to multiple drugs.

Antimicrobial concentrations below planktonic MICs may be encountered clinically during the treatment and prevention of biofilm-related device infections. Subinhibitory concentrations of various antibiotics have been shown to both stimulate and impede CNS biofilm formation (3, 12, 41). These effects appear to depend on the particular strain and antimicrobial agent combination under investigation. For instance, low levels of the ribosome-targeting drugs tetracycline and quinupristin-dalfopristin were shown to upregulate transcription of the *Staphylococcus epidermidis* genes responsible for intercellular adhesion in the biofilm matrix, thereby leading to increased biofilm formation (41). On the other hand, subinhibitory concentrations of the β -lactam agent dicloxacillin reduced the amount of biofilm formed by *Staphylococcus haemolyticus* and *S. epidermidis* (4). Thus, broadening our current understanding

of the effects that subinhibitory concentrations of antimicrobial agents elicit on biofilms formed by CNS is of clinical interest.

The coagulase-negative species *Staphylococcus lugdunensis*, first described in 1988 (16), has established a niche as a formidable pathogen. *S. lugdunensis* is a skin commensal that is particularly found in the inguinal region (49). Accordingly, the highest incidence of infections caused by *S. lugdunensis* include those of wounds, skin, and soft tissues (21). Additional types of *S. lugdunensis* infections are similar to those of other pathogenic CNS and include urinary tract infection (17), vascular catheter-related infection (21), and prosthetic joint infection (43). However, unlike other CNS, *S. lugdunensis* has a propensity to cause native valve endocarditis, and its associated infections tend to mimic those of *Staphylococcus aureus* on the basis of their highly destructive and potentially serious nature (38, 48). Further, isolates may produce bound coagulase, resulting in the misidentification of *S. lugdunensis* as *S. aureus* in clinical laboratories that rely on rapid coagulase testing (16, 48). In general, most *S. lugdunensis* isolates appear to be more susceptible to a wider array of antimicrobial agents than are other staphylococci (13, 20, 48, 49).

Biofilms play a role in the pathogenesis of many *S. lugdunensis* infections, but studies on this topic are lacking. Since biofilm formation perturbs the efficacy of antimicrobial agents, and this is an important point of consideration in determining the clinical course of treatment, we sought to define the response of *S. lugdunensis* biofilms to a variety of antimicrobial agents. To that end, we measured the activities that inhibitory and subinhibitory concentrations of antistaphylococcal drugs displayed against planktonic and biofilm forms of *S. lugdunensis* clinical isolates.

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TABLE 1. *Staphylococcus lugdunensis* isolates

Isolate	Source	Test result ^a			Source or reference
		Staphaurex assay ^b	Synergistic hemolysis	β-Lactamase	
IDRL-856	Endocarditis	—	+	+	38
IDRL-2394	Prosthetic joint infection	—	+	—	This study
IDRL-2414	Endocarditis	—	+	+	38
IDRL-2492	Endocarditis	—	+	—	38
IDRL-2526	Prosthetic joint infection	—	—	—	This study
IDRL-2554	Prosthetic joint infection	—	+	+	This study
IDRL-2588	Infected hematoma	—	+	+	This study
IDRL-2622	Prosthetic joint infection	—	+	+	This study
IDRL-2639	Paronychia	—	+	+	This study
IDRL-2640	Folliculitis	+	+	—	This study
IDRL-2664	Prosthetic joint infection	—	+	—	This study
IDRL-5204	Prosthetic joint infection	—	—	—	This study
IDRL-5254	Intravascular catheter	—	+	—	This study
IDRL-5256	Prosthetic joint infection	+	+	—	This study
IDRL-5258	Prosthetic joint infection	—	+	—	This study

^a —, Not detected; +, detected.^b That is, the Staphaurex latex agglutination assay for detection of bound coagulase (clumping factor) and/or protein A.

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MATERIALS AND METHODS

Microorganisms and growth conditions. *S. lugdunensis* isolates are listed in Table 1. Use of all study isolates was approved by the Mayo Clinic Institutional Review Board. Isolates were collected at Mayo Clinic between 1983 and 2003 and were identified as *S. lugdunensis* using conventional biochemical tests, including the detection of tube coagulase, ornithine decarboxylase, and pyrrolidonyl arylamidase (Remel, Lenexa, KS). Eight isolates were also verified as *S. lugdunensis* by partial 16S rRNA gene PCR amplification and sequencing as previously described (data not shown) (47).

Latex agglutination detection of clumping factor and/or protein A (Staphaurex; Remel) was performed on all isolates. Isolates were assayed for production of delta-like hemolysin by testing for the presence of synergistic hemolytic activity with the *S. aureus* RN4220 beta-hemolysin on Trypticase soy agar with 5% sheep blood, as previously described (11, 18).

S. aureus SA113 (ATCC 35556) and *S. epidermidis* RP62A (ATCC 35984), well-characterized biofilm-forming strains (6, 8), were purchased from the American Type Culture Collection (Manassas, VA) for use as positive controls in microtiter plate biofilm assays. Organisms stored at -70°C were freshly streaked on sheep blood or Trypticase soy agar before each assay replicate. For biofilm formation and biofilm susceptibility assays, single colonies from 16- to 24 h-old plates were grown in Trypticase soy broth (BD BBL, Franklin Lakes, NJ) sup-

plemented with 1% (wt/vol) sterile-filtered glucose (TSB_{gluc1%}) with shaking at 130 rpm. All experiments were performed at 37°C in ambient air.

Antimicrobial agents. Cefazolin, tetracycline, vancomycin, trimethoprim, and sulfamethoxazole (Sigma-Aldrich, St. Louis, MO), daptomycin (Cubist Pharmaceuticals, Lexington, MA), linezolid (Pharmacia & Upjohn, Kalamazoo, MI), moxifloxacin (Bayer Pharmaceuticals, West Haven, CT), quinupristin-dalfopristin (King Pharmaceuticals, Bristol, TN), and rifampin (Bedford Labs, Bedford, OH) powders were prepared for use in susceptibility assays and microtiter plate biofilm assays as described in the Clinical and Laboratory Standards Institute (CLSI) susceptibility testing guidelines (34). Trimethoprim and sulfamethoxazole were prepared separately and mixed immediately before use.

β-Lactamase screening. Cefinase disks (BD BBL) were used to screen isolates for β-lactamase production.

mecA PCR. DNA prepared by alkaline wash (15) was tested for the presence of *mecA* by PCR as previously described (27).

Planktonic antimicrobial susceptibility testing. MIC and MBC assays against the 10 antimicrobial agents listed in Table 2 were performed in cation-adjusted Mueller-Hinton broth (CAMHB) according to CLSI guidelines for broth microdilution susceptibility testing (33, 34). *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used as quality control strains for MICs and MBCs.

Biofilm antimicrobial susceptibility testing. Biofilm bactericidal concentrations (BBCs) were determined by using an adaptation of a previously published biofilm susceptibility testing method (32). Briefly, isolates grown for 22 h in TSB_{gluc1%} were adjusted to a turbidity of 1.0 McFarland (corresponding to ca.

TABLE 2. In vitro antimicrobial susceptibilities of *S. lugdunensis* isolates

MIC or MBC type ^a	MICs and MBCs (μg/ml) of the following antimicrobial agents ^b :									
	CFZ	DAP	LZD	MXF	NAF	Q-D	RIF	TET	SXT ^c	VAN
MIC^c										
MIC ₅₀	0.25	0.125	1	0.125	0.5	0.25	≤0.03	0.25	0.25/4.8	1
MIC ₉₀	0.5	0.25	1	0.25	0.5	2	≤0.03	1	1/19	2
Range	≤0.125–0.5	0.06–1	0.5–1	0.125–0.25	0.25–0.5	0.06–2	≤0.03	0.06–1	0.125/2.4–16/304	0.5–2
MBC^c										
MBC ₅₀	0.5	0.5	>128	0.125	0.5	2	8	>32	>16/304	>128
MBC ₉₀	1	1	>128	0.25	2	>32	>32	>32	>16/304	>128
Range	0.125–2	0.06–1	>128	0.125–1	0.25–2	0.5–>32	0.03–>32	>32	1/38–>16/304	4–>128

^a Sample size for MIC and MBC, $n = 15$.^b CFZ, cefazolin; DAP, daptomycin; LZD, linezolid; MXF, moxifloxacin; NAF, nafcillin; RIF, rifampin; Q-D, quinupristin-dalfopristin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; VAN, vancomycin.^c The values shown represent trimethoprim/sulfamethoxazole concentrations, respectively.

1×10^8 to 2×10^8 CFU/ml [data not shown]) and diluted 1:50 in TSB_{gluc1%}. 200- μ l aliquots were placed into each row of a 96-well flat-bottom microtiter plate (Nunc Delta, Nunc, Denmark), covered with a 96-peg lid (Nunc-TSP; Nunc), and statically incubated for 24 h to allow for biofilm formation on the pegs. To remove nonadherent cells, biofilms were rinsed by dipping the peg lid once into a series of three 96-well plates filled with 200 μ l of sterile phosphate-buffered saline. The peg lid was placed on a new flat-bottom plate prepared for broth microdilution susceptibility testing as described by CLSI guidelines (34), except that the wells contained a final volume of 200 μ l of antimicrobial agent diluted in CAMHB (instead of the 100 μ l typically used for broth microdilution testing) or 200 μ l of CAMHB without drug for the positive growth controls. Biofilms were challenged with antimicrobial agents overnight, and the peg lid was rinsed three times in phosphate-buffered saline as described above and placed on a final 96-well plate containing 200 μ l of TSB_{gluc1%}. Biofilm cells were dislodged from the pegs into recovery media by bath sonicating the microtiter plate for 5 min at 40 kHz (Zenith Ultrasonics, Norwood, NJ). The peg lid was discarded and replaced with a normal lid, and the optical density at 600 nm (OD₆₀₀) was measured on a plate reader. Wells containing uninoculated TSB_{gluc1%} were used for sterility controls and served as spectrophotometric blanks. The plate was incubated for 24 h, and a second OD₆₀₀ measurement was taken. The BBC was defined as the lowest drug concentration that exhibited a change in OD₆₀₀ between 0 and 24 h that was $\leq 10\%$ of the equivalent reading for the positive growth control well (32).

Microtiter plate biofilm assays. The effect of subinhibitory concentrations of antimicrobial agents on biofilm formation was measured by using a modification of a previously described assay (6, 19). Isolates grown for 22 h in TSB_{gluc1%} were adjusted to a turbidity of 1.0 McFarland and diluted 1:50 in TSB_{gluc1%} with or without subinhibitory concentrations of antimicrobial agents. Then, 200- μ l aliquots of each diluted isolate were placed into four wells of a 96-well flat-bottom microtiter plate and statically incubated for 24 h. To determine the cell and biofilm density, the biofilm from one well per isolate was scraped with a pipette tip, resuspended in the culture medium, and 100 μ l of the resuspended culture was transferred to a new microtiter plate to measure the OD₆₀₀ on a microtiter plate reader (Multiskan; Thermo Electron, Waltham, MA). The culture medium was discarded from the biofilm microtiter plates, and the biofilms were washed twice by fully submerging the plates in deionized water, air dried overnight, stained with 0.1% safranin for 1 min, rinsed with tap water, and air dried again overnight. The stained biofilm was resuspended to homogeneity in 200 μ l of 30% glacial acetic acid, and the OD₄₉₂ was measured. Wells containing uninoculated TSB_{gluc1%} served as sterility controls; the OD₆₀₀ and OD₄₉₂ of these wells were used as spectrophotometric blanks. All assays were repeated three times on different days with similar results.

We compared the effect of antimicrobial agents on the ability of the tested isolates to form biofilm, independent of effects on cell growth. We used previously described normalization approaches that compensate for differences in growth rate (9, 12, 28, 39). Briefly, data for each experiment were analyzed by dividing the mean biofilm OD₄₉₂ measurement (three wells per isolate) by the cell and biofilm density OD₆₀₀ measurement (one well per isolate). The coefficients of variation of the OD₆₀₀ measurement ($n = 10$) for *S. lugdunensis* IDRL-5204, a poor biofilm former, and *S. lugdunensis* IDRL-5258, a strong biofilm former, were 4.9 and 5.9%, respectively (data not shown).

Each isolate was tested against the drugs listed in Table 2 at subinhibitory concentrations of 0 $\times$, 0.0625 $\times$, 0.25 $\times$, and 0.5 $\times$ the MIC₅₀ of the 15 *S. lugdunensis* isolates; quinupristin-dalfopristin was tested at subinhibitory concentrations of 0 $\times$, 0.03 $\times$, 0.125 $\times$, and 0.25 $\times$ the MIC₅₀. The exact *S. lugdunensis* MIC₅₀ of rifampin, as determined by broth microdilution in CAMHB according to CLSI guidelines (34) in an experiment separate to that reported in Table 2, was determined to be 0.0039 μ g/ml.

Statistical analysis. Biofilm formation data were analyzed with the Student *t* test using JMP 5.1.2 software (SAS Institute, Inc., Cary, NC).

RESULTS

Characterization of *S. lugdunensis* isolates. Fifteen tube CNS strains isolated in the clinical microbiology lab at our institution were positively identified as *S. lugdunensis* using a simple biochemical testing scheme (17, 38). All isolates were positive for production of ornithine decarboxylase and pyrrolidonyl arylamidase. The isolates originated from a variety of sources typical of infections caused by this species (Table 1). Several of the infection types represented (e.g., endocarditis,

prosthetic joint infection, and intravascular catheter infection) are presumably of biofilm origin, suggesting that the majority of our isolates were capable of forming biofilm in vivo.

Two phenotypic characteristics of *S. lugdunensis* that resemble properties of *S. aureus* are the presence of bound coagulase and production of a synergistic hemolysin that interacts with beta-hemolysins to potentiate complete clearing of sheep erythrocytes (18). Bound coagulase is detected among *S. lugdunensis* at variable rates (13, 29). Only 2 of 15 (13%) of our isolates were Staphaurex positive (Table 1), indicating that the presence of bound coagulase (clumping factor) and/or protein A in our collection of isolates was relatively low. Synergistic hemolytic activity, mediated by-products of the *slush* locus (11), was observed in 13 of 15 (87%) isolates (Table 1).

Planktonic antimicrobial susceptibility testing. We determined the in vitro susceptibilities of the *S. lugdunensis* isolates to a panel of 10 antimicrobial agents, representing different drug classes, used to treat staphylococcal infections in clinical practice. The MICs and MBCs are shown in Table 2.

With a single exception, the MICs for each isolate fell below the CLSI-defined *S. lugdunensis* susceptibility breakpoints (7). The only resistance noted was in IDRL-5204, which had a trimethoprim-sulfamethoxazole MIC of trimethoprim (16 μ g/ml)-sulfamethoxazole (304 μ g/ml). All isolates were exquisitely susceptible to rifampin (MIC < 0.03 μ g/ml). Thirteen of fifteen (87%) isolates had moxifloxacin MICs of 0.125 μ g/ml.

Six of the fifteen (40%) *S. lugdunensis* isolates reacted positively in the cefinase assay for β -lactamase production (Table 1). This finding is somewhat higher than previously reported frequencies (24 to 29%) of β -lactamase-positive *S. lugdunensis* isolates in the United States (18, 22). All isolates were *mecA* negative by PCR, which is concordant with previous reports (24, 29). Accordingly, the nafcillin MICs ranged from 0.25 to 0.5 μ g/ml.

Cefazolin, daptomycin, moxifloxacin, and nafcillin exhibited bactericidal activity against all *S. lugdunensis* isolates, with MBC ranges within two doubling dilutions of their respective MIC ranges. Linezolid and tetracycline, both of which inhibit protein synthesis, showed bacteriostatic activity against all isolates.

Although all isolates were susceptible to vancomycin (MIC range, 0.5 to 2 μ g/ml), the vancomycin MBC₅₀ was >128 μ g/ml. Two isolates (IDRL-2394 and IDRL-5254) had vancomycin MBCs of 64 μ g/ml. IDRL-5256 was the only isolate with a vancomycin MBC (4 μ g/ml) within two doubling dilutions of the MIC (1 μ g/ml), a range representative of the bactericidal activity against staphylococci that is conventionally associated with vancomycin. Tolerance is typically defined as an MBC/MIC ratio of ≥ 32 (34); all isolates except IDRL-5256 had vancomycin MBC/MIC ratios of ≥ 64 .

Antimicrobial susceptibilities of *S. lugdunensis* biofilms. In order to assess the antimicrobial susceptibility of *S. lugdunensis* biofilms, we tested antimicrobial agents at traditional MIC concentrations in a modified protocol originally proposed for clinically determining *Pseudomonas aeruginosa* biofilm antimicrobial susceptibility levels (32). Biofilms formed on polystyrene pegs were challenged with antimicrobial agents for 24 h, after which time the bacteria were transferred by sonication from the pegs into recovery media and incubated for an additional 24 h. The endpoint of this assay was a spectrophotomet-

TABLE 3. In vitro antimicrobial susceptibilities of *S. lugdunensis* biofilms

Isolate	BBC ($\mu\text{g/ml}$) of the following antimicrobial agents ^a :									
	CFZ	DAP	LZD	MXF	NAF	Q-D	RIF	TET	SXT ^b	VAN
IDRL-856	>128	16	>128	0.25	16	32	8	128	>8/152	>128
IDRL-2394	>128	64	>128	2	>128	0.5	0.25	128	>8/152	>128
IDRL-2414	>128	>128	>128	0.5	>128	4	0.25	128	>8/152	>128
IDRL-2492	>128	8	>128	0.25	8	4	64	128	>8/152	>128
IDRL-2526	>128	8	>128	0.5	4	8	32	128	>8/152	>128
IDRL-2554	>128	4	>128	≤ 0.125	2	1	≤ 0.125	128	0.25/4.8	4
IDRL-2588	>128	128	>128	0.5	8	>128	64	128	>8/152	>128
IDRL-2622	2	1	>128	2	8	8	64	128	>8/152	>128
IDRL-2639	4	32	>128	0.25	1	128	32	16	2/38	>128
IDRL-2640	>128	>128	>128	2	>128	32	64	128	>8/152	>128
IDRL-2664	8	32	>128	≤ 0.125	2	128	≤ 0.125	128	0.125/2.4	8
IDRL-5204	1	8	4	0.5	0.5	≤ 0.125	32	1	>8/152	4
IDRL-5254	>128	128	>128	0.5	>128	32	64	128	>8/152	>128
IDRL-5256	>128	64	>128	1	>128	4	32	128	>8/152	>128
IDRL-5258	>128	16	>128	≤ 0.125	0.5	2	8	64	>8/152	>128
Range	1->128	1->128	4->128	≤ 0.125 -2	0.5->128	≤ 0.125 ->128	≤ 0.125 -64	1-128	0.125/2.4->8/152	4->128
BBC ₅₀	>128	32	>128	0.5	8	8	32	128	>8/152	>128
BBC ₉₀	>128	128	>128	2	>128	128	64	128	>8/152	>128

^a CFZ, cefazolin; DAP, daptomycin; LZD, linezolid; MXF, moxifloxacin; NAF, nafcillin; RIF, rifampin; Q-D, quinupristin-dalfopristin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; VAN, vancomycin.

^b The values shown represent trimethoprim/sulfamethoxazole concentrations, respectively.

ric reading that quantitated the amount of bacterial regrowth in recovery media after exposure to increasing concentrations of antimicrobial agents. Since the assay output was defined by the percent regrowth compared to the positive growth controls, the reported susceptibility levels represent killing concentrations, and were thus termed biofilm bactericidal concentrations (i.e., BBCs).

Growth in each of the positive control wells of the recovery plate following sonication from the pegs indicated that all isolates were able to form biofilms in this model system. Table 3 lists the BBCs of 10 antimicrobial agents against each isolate. Each drug's BBC₉₀ was above the CLSI-defined planktonic MIC breakpoint for resistance (where available for *S. lugdunensis*) (7). Rifampin and moxifloxacin were the only agents for which at least one isolate did not have a BBC greater than or equal to the highest concentration tested. The moxifloxacin BBC range (≤ 0.125 to $2 \mu\text{g/ml}$) was overall much lower than other BBC ranges, and the BBCs of 11 of 15 (73%) isolates would be considered susceptible if conventional planktonic breakpoints were to be applied ($\text{MIC} \leq 0.5 \mu\text{g/ml}$).

The biofilms of a few isolates were more susceptible to a larger number of drugs than were other isolates. IDRL-5204 had low BBCs for all agents tested, except daptomycin, rifampin, and trimethoprim-sulfamethoxazole, the last to which it was planktonically resistant. IDRL-2554 BBCs were below the CLSI planktonic susceptibility breakpoints for moxifloxacin, quinupristin-dalfopristin, rifampin, trimethoprim-sulfamethoxazole, and vancomycin. Only two isolates, IDRL-2640 and IDRL-5256, formed biofilms that exhibited resistance (as defined according to CLSI planktonic susceptibility breakpoints) to all agents tested.

Effect of subinhibitory concentrations of antimicrobial agents on *S. lugdunensis* biofilm formation. Using the microtiter plate biofilm assay, we incubated isolates with antimicrobial concentrations below the MIC₅₀ under conditions that support

staphylococcal biofilm formation (26). The growth of some isolates was hindered by certain drugs. In order to directly compare the relative production of stained biofilm material at the various antimicrobial concentrations tested for each isolate, negative growth effects caused by the presence of subinhibitory concentrations of antimicrobial agents during biofilm formation were taken into account. Using an approach similar to those previously described for CNS (9, 12, 39) and *Enterococcus faecalis* (28), the amount of stained biofilm (reported as OD₄₉₂) under each condition was normalized by the cell and biofilm optical density (OD₆₀₀). This allowed us to directly assess whether biofilm production was positively or negatively affected by various antimicrobial agent concentrations under conditions in which cell growth was also affected. In all cases, increases or decreases in biofilm production were defined by comparing the OD₄₉₂/OD₆₀₀ ratio of bacteria grown in the presence of drug to the OD₄₉₂/OD₆₀₀ ratio for the uninhibited growth control.

Results of three independent experiments are summarized in Table 4, and representative results of single experiments for select antimicrobial agents are shown in Fig. 1. The most pronounced and pervasive effect occurred with nafcillin, which significantly increased biofilm formation, relative to the change in cell density, in 93% (14 of 15) of *S. lugdunensis* isolates (Table 4 and Fig. 1). IDRL-2622 biofilm increased 14-fold at $0.5\times$ the MIC₅₀. Notably, biofilm formation of the two reference strains, *S. aureus* SA113 and *S. epidermidis* RP62A, was significantly decreased in the presence of subinhibitory concentrations of nafcillin (Fig. 1). No other agents increased biofilm in more than 27% of the *S. lugdunensis* isolates (Table 4).

Linezolid and tetracycline decreased biofilm formation, relative to their effects on cell growth, in 80% (12 of 15) and 93% (14 of 15) of *S. lugdunensis* isolates, respectively (Table 4). One subinhibitory concentration of tetracycline stimulated biofilm

TABLE 4. Effects of subinhibitory antimicrobial concentrations on *S. lugdunensis* biofilm formation^a

Antimicrobial agent	% (no. of isolates/total no. of isolates)		
	Increase	Decrease	No effect
Cefazolin	13 (2/15)	7 (1/15)	80 (12/15)
Daptomycin	13 (2/15)	0 (0/15)	87 (13/15)
Linezolid	0 (0/15)	80 (12/15)	20 (3/15)
Moxifloxacin	27 (4/15)	40 (6/15)	33 (5/15)
Nafcillin	93 (14/15)	0 (0/15)	7 (1/15)
Quinupristin-dalfopristin	20 (3/15)	33 (5/15)	47 (7/15)
Rifampin	7 (1/15)	0 (0/15)	93 (14/15)
Tetracycline	7 (1/15)	93 (14/15)	0 (0/15)
Trimethoprim-sulfamethoxazole	20 (3/15)	40 (6/15)	40 (6/15)
Vancomycin	27 (4/15)	27 (4/15)	47 (7/15)

^a Biofilms were formed in the presence or absence of three subinhibitory concentrations of each antimicrobial agent as described in Materials and Methods. The effects on biofilm formation in the presence of at least one subinhibitory antimicrobial concentration that significantly differed from biofilm formation in the absence of the antimicrobial agent ($P < 0.05$ [Student *t* test]) in two or more independent experiments are reported.

formation in *S. lugdunensis* IDRL-5256 (Fig. 1), whereas linezolid did not increase biofilm formation of any isolates. Further, the effect of linezolid on the reference strains was exactly opposite that observed for *S. lugdunensis*, having greatly increased biofilm formation at $0.25\times$ the MIC₅₀ for both *S. aureus* and *S. epidermidis*. Tetracycline caused a small, but significant, drop in *S. epidermidis* RP62A biofilm at one concentration and had no effect on *S. aureus*. The effects of subinhibitory concentrations of the remaining antimicrobial agents were more varied among isolates than for the drugs shown in Fig. 1 (Table 4). Cefazolin, daptomycin, and rifampin had no substantial effect on the majority of *S. lugdunensis* isolates.

DISCUSSION

The necessity to develop methods to treat and prevent biofilm infections is becoming of increased importance (14). The results of conventional antimicrobial susceptibility testing are difficult to apply to biofilm-associated infections since traditional antimicrobial treatments fail to eradicate surface-attached bacteria (37, 50). Techniques for in vitro antimicrobial susceptibility testing of biofilm bacteria have just recently become available for gathering biofilm susceptibility data on collections of clinical strains (5, 32). These studies, which have been conducted on pathogenic isolates of several species (42, 44, 46), facilitate the identification of potentially efficacious antibiofilm agents. The planktonic and biofilm susceptibilities we report here provide a comprehensive evaluation of clinically useful drugs against biofilm-forming *S. lugdunensis* isolates.

Compared to other CNS, *S. lugdunensis* is an atypically virulent pathogen associated with both nosocomial and community-acquired infections; nevertheless, it has remained susceptible to most antimicrobial agents. Not surprisingly, the MIC₉₀s of the isolates tested here were susceptible to 10 antimicrobial agents with activity against staphylococci (Table 2). However, despite the apparent in vitro susceptibility of planktonic bacteria to most antimicrobial agents, surgery or removal of infected medical devices is often needed for treatment of deep-

seated *S. lugdunensis* infections (1, 13). Two of the few known virulence factors characterized to date in *S. lugdunensis* are fibrinogen-binding and von Willebrand factor-binding proteins (31, 35, 36). The functions of these proteins suggest that a mechanism for primary attachment to host tissues exists in *S. lugdunensis*, thus enabling in vivo biofilm formation. In vitro *S. lugdunensis* biofilms were not susceptible to drug concentrations more than 10 times greater than the planktonic susceptibility breakpoints for 80% of the antimicrobial agents we evaluated for biofilm bactericidal activity (Table 3). We found that 90% of the trimethoprim-sulfamethoxazole BBCs were fourfold higher than the published breakpoint for planktonic cells (7). Likewise, planktonic trimethoprim-sulfamethoxazole MBCs, which tested a maximum concentration one doubling dilution higher than the BBC assay, displayed bacteriostatic results (Table 2).

As with biofilms formed by most other species (37), our results verify that *S. lugdunensis* biofilms, as a whole, confer antimicrobial resistance through as-yet-uncharacterized mechanisms. It is interesting that biofilm resistance profiles were not uniform throughout our collection of isolates. Although two isolates (IDRL-2554 and IDRL-5204) formed biofilms that were killed by low drug concentrations of most antimicrobial agents tested, the few drugs to which they were resistant were dissimilar. The antimicrobial agents we tested represent many drug classes, leading us to speculate that different modes of antimicrobial resistance are used by individual strains of a single species during biofilm formation.

Moxifloxacin, the only antimicrobial agent with activity against *S. lugdunensis* biofilms in the present study, eradicated biofilm growth of 73% isolates at clinically achievable concentrations. Moxifloxacin is a relatively new antimicrobial agent with activity against staphylococci (23). In a rat model of implant-associated *S. aureus* osteomyelitis, moxifloxacin monotherapy significantly reduced the number of organisms recovered from bone, soft tissue, and the surface of the implanted device (25). In vitro biofilms of *S. epidermidis* clinical isolates have been shown to be decreased upon exposure to the quinolones pefloxacin, ciprofloxacin, norfloxacin, and ofloxacin (52). However, a recent in vitro study of moxifloxacin suggested that concentrations of up to 100 times the MIC were required to reduce viable cell counts of CNS biofilms (39). Nonetheless, our results merit further investigation of the antibiofilm activity of moxifloxacin against *S. lugdunensis* (and other staphylococcal) biofilms.

Based on the previously reported finding that subinhibitory concentrations of tetracycline and quinupristin-dalfopristin enhance elaboration of biofilm matrix by increasing *S. epidermidis* *ica* transcription (41), we questioned whether subinhibitory levels of the 10 antimicrobial agents studied here would have similar effects, since increased biofilm production in response to inadequate drug dosing would be an undesirable consequence. Only 20 and 7% of isolates produced more biofilm, relative to cell density, when grown with low quinupristin-dalfopristin or tetracycline concentrations (Table 4 and Fig. 1), respectively. Intriguingly, tetracycline substantially lessened the amount of biofilm formed by 93% of the isolates. Linezolid exposure caused a similar drop in *S. lugdunensis* biofilm formation. However, the β -lactam nafcillin stimulated biofilm formation in most isolates (Table 4, Fig. 1). We did not observe

FIG. 1. Biofilm response of *S. aureus* SA113, *S. epidermidis* RP62A, and 15 *S. lugdunensis* isolates to subinhibitory concentrations of antimicrobial agents. Biofilms were formed in the wells of polystyrene microtiter plates in the presence or absence of antimicrobial agents at the indicated sub-MIC levels. Asterisks indicate statistically significant increases or decreases in biofilm formation compared to the level of biofilm formation without the antimicrobial agent for each isolate ($P < 0.05$ [Student *t* test]). Assays were repeated three times with similar results. Representative data are shown for selected drugs.

similar effects with any of these agents on the *S. aureus* and *S. epidermidis* reference strain biofilms. Further, other researchers have reported that penicillin G and oxacillin had no effect on *S. epidermidis* and that dicloxacillin actually limited CNS biofilm formation (4, 41). Our results suggest that alternate mechanisms of gene regulation, compared to *S. epidermidis*, govern biofilm formation in *S. lugdunensis* when cells are grown in the presence of subinhibitory concentrations of antimicrobial agents. Further, this work suggests that nafcillin may not be ideal for the treatment of *S. lugdunensis* biofilm-associated infections, although in vivo studies are needed to definitively address this issue.

Several antimicrobial agents we assayed produced varied responses in biofilm formation among *S. lugdunensis* isolates (Table 4), which partially agreed with previously published work on other CNS. *S. epidermidis* biofilms were found to increase or decrease in a strain-dependent manner in response to subinhibitory doses of cefamandole, as well as of vancomycin (12). Whereas we observed a similar effect with vancomycin, cefazolin had no effect on 80% of *S. lugdunensis* isolates. Subinhibitory concentrations of moxifloxacin were previously shown to have no effect on CNS biofilms (39). In contrast, *S. lugdunensis* biofilm production was either elevated or reduced in the presence of subinhibitory concentrations of moxifloxacin in two-thirds of our isolates. The heterogeneity of the biofilm response displayed by individual *S. lugdunensis* isolates may result from differential gene regulation patterns utilized among strains living in biofilms.

An unexpected outcome in the present study was the lack of bactericidal activity of vancomycin against 93% of the isolates. Vancomycin, a glycopeptide, is one of few available antimicrobial agents active against and considered capable of killing staphylococci, the latter of which is particularly important for the treatment of infections that require bactericidal therapy, such as endocarditis (10). *S. lugdunensis* native valve endocarditis is recognized as a particularly aggressive infection associated with severe valve destruction that often requires surgical intervention, in addition to antimicrobial therapy (1, 48). The use of vancomycin, either alone or in combination with another antimicrobial agent (e.g., aminoglycosides or rifampin), for the treatment of *S. lugdunensis* infections has been reported (1, 45, 48). Vancomycin tolerance, a phenomenon in which bacteria with susceptible MICs are refractory to killing in MBC assays, has been studied in *S. aureus* endocarditis isolates, and it has been suggested that tolerance should be considered when treating endocarditis (30, 40). Although none of 10 CNS included in one study were found to be vancomycin tolerant (40), several *S. epidermidis* strains from implant-associated infections described in a recent study had reported vancomycin MBC/MIC ratios of ≥ 64 (42). Our findings are consistent with the recent work of Bourgeois et al. (2), who reported tolerance to vancomycin or teicoplanin in 6 of 13 *S. lugdunensis* isolates. Regardless of the frequency of occurrence of vancomycin tolerance among non-*S. lugdunensis* CNS, the high rate of vancomycin tolerance among *S. lugdunensis* isolates is an observation that justifies in vivo studies of vancomycin against *S. lugdunensis* biofilm infections. This point may bear consideration in the management of severe *S. lugdunensis* infections, especially endocarditis.

In conclusion, we have used in vitro susceptibility tests and

biofilm formation assays to examine how antimicrobial agents affect *S. lugdunensis* clinical isolates collected from a multitude of sources. Our results identified several conditions that warrant further studies to delineate whether the observed effects are of clinical relevance. In particular, our data suggest that moxifloxacin holds promise as a useful treatment for *S. lugdunensis* biofilm infections. We also observed that nafcillin increases *S. lugdunensis* biofilm formation and that vancomycin lacks bactericidal activity against most *S. lugdunensis* isolates. The existence of antimicrobial agent-specific effects may be an important factor in the selection of antimicrobial therapy for this virulent organism.

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