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Effects of Growth in the Presence of Subinhibitory Concentrations of Dicloxacillin on *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* Biofilms

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Low concentrations of antibiotics can inhibit microbial adherence to medical device surfaces. However, little is known about the changes that occur in the physiology of bacteria within biofilms formed in the presence of subinhibitory (sub-MIC) concentrations of antibiotics. In this study, the densities and matrix compositions of biofilms formed by two coagulase-negative *Staphylococcus* species in the absence and in the presence of sub-MIC concentrations of dicloxacillin were evaluated. Biofilms formed in the presence of sub-MIC concentrations of dicloxacillin contained less biomass, and there were notable changes in the composition of the biofilm matrix. Changes in the spatial structure were also verified by confocal scanning laser microscopy, indicating that biofilms grown in the presence of sub-MIC concentrations of dicloxicilln had a lower cell density. Physiological alterations in the bacteria within biofilms grown in the presence of subinhibitory concentrations of the antibiotic were also evaluated. The results showed that there were differences in bacterial surface characteristics when cultures were grown in the presence of sub-MIC concentrations of dicloxacillin, including decreased hydrophobicity and decreased expression of the exopolysaccharide poly-*N*-acetylglucosamine. The elemental composition of the cell surface was also analyzed, and whereas in *Staphylococcus epidermidis* there were decreases in the oxygen and nitrogen contents, in *Staphylococcus haemolyticus* there were increases in these two parameters. Additionally, increases in resistance to several antibiotics were observed for the cells within biofilms formed in the presence of dicloxacillin.

Coagulase-negative staphylococci (CoNS), such as *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*, are now well established as major nosocomial pathogens associated with infections of indwelling medical devices (41). The major virulence factor associated with CoNS infections is the ability of the organisms to adhere to medical devices and subsequently form biofilms (9). Biofilms are a major clinical problem, mainly due to high levels of resistance to antibiotics (5, 23), and they often lead to persistent infections (10). In staphylococci, biofilm formation is associated with the production of the molecule poly-*N*-acetylglucosamine (PNAG) (28).

Many studies have demonstrated that low concentrations of antibiotics can inhibit, to some extent, biofilm formation on medical device surfaces (6, 33). One of the prophylactic strategies that are currently used is to provide a constant flow of a subinhibitory (sub-MIC) concentration of antibiotic (17). Another alternative is to use biomaterials impregnated with antibiotic (21, 32). However, besides the reduced abilities of bacteria to form biofilms, little is known about other changes implicated in the virulence of CoNS that might occur due to the presence of low concentrations of antibiotics. For example, a few studies have suggested that growth in the presence of sub-MIC concentrations of antibiotics can result in the development of antibiotic resistance (17, 37).

The aim of this work was to evaluate changes in the physiology of CoNS triggered by biofilm formation in the presence of a low concentration of dicloxacillin, a major antibiotic used to treat staphylococcal infections in Portugal. Factors such as biofilm-forming ability, biofilm spatial structure, cell surface properties, production of specific molecules responsible for biofilm formation (such as PNAG), and resistance to antibiotics were addressed in this work.

MATERIALS AND METHODS

Strains. In this work, two biofilm-forming CoNS clinical strains that have been characterized previously (7) were used; *S. epidermidis* M187 and *S. haemolyticus* M176 were both isolated from patients with peritonitis associated with renal dialysis.

Media and growth conditions. Trypic soy broth (TSB) and trypic soy agar (TSA) plates were prepared according to the manufacturer's instructions (Merck, Germany). All strains were grown for 24 ± 2 h at 37°C in a shaker rotating at 130 rpm in 15 ml of TSB, using bacteria grown on TSA plates not older than 2 days as inocula. After cells were harvested by centrifugation for 5 min at 10,500 × *g* and 4°C, they were washed twice and resuspended in saline (0.9% NaCl prepared in distilled water) at a concentration of approximately 1×10^9 cells/ml, as determined by the optical density at 640 nm. These cell suspensions were used in the subsequent biofilm assays.

Determination of growth parameters in the presence of a sub-MIC concentration of dicloxacillin. The concentration of dicloxacillin used in this study was 8 μg/ml, which is a sub-MIC concentration, as determined in a previous study (6). Bacterial growth parameters were determined by monitoring the increases in the optical densities at 640 nm of cell suspensions grown in TSB in the presence and absence of dicloxacillin. In the stationary growth phase the number of cells was

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determined by preparing 1:10 serial dilutions in TSB and plating 100 μl per dilution on TSA following 24 h of incubation at 37°C.

Biofilm formation. Biofilms were formed as described previously (8). Briefly, 50 μl of a cell suspension containing 1×10^9 cells/ml prepared in a 0.9% NaCl solution was added to 96- or 6-well polystyrene plates (Sarsted, Germany) containing TSB with 0.25% glucose. Biofilm formation was allowed to occur for 48 h at 37°C with rotation at 120 rpm. Every 12 h the TSB containing suspended cells was removed and fresh TSB with 0.25% glucose was added. The resulting biofilms were considered the controls. To evaluate the effect of sub-MIC concentrations of dicloxacillin on biofilm formation, biofilms were formed in culture medium (TSB with 0.25% glucose) supplemented with 8 μg/ml of dicloxacillin.

Biofilm quantification. Biofilm quantification was performed as described previously (20), with some modifications. Briefly, bacteria grown in 96-well polystyrene plates (Sarsted, Germany) were washed twice with a 0.9% NaCl solution, dried in an inverted position, and stained with 0.4% safranin for 10 min. The plates were washed with distilled water and dried overnight. To each well, 100 μl of a 0.9% NaCl solution was added, and the absorbance at 490 nm was determined with an enzyme-linked immunosorbent assay plate reader (Spectra Rainbow, Tecan, Austria). For each condition studied, three separate experiments were performed.

Confocal scanning laser microscopy (CSLM) analysis. Biofilm staining was performed as previously described (31), with some modifications. Briefly, biofilms that formed on six-well polystyrene plates were washed twice with 0.9% NaCl. Wheat germ agglutinin (WGA) conjugated to Oregon green (Molecular Probes, United States) at a concentration of 10 μg/ml was added to the biofilms, and the plates were incubated for 20 min at room temperature in the dark. After staining, the biofilms were gently rinsed with 0.9% NaCl.

The CSLM analysis was performed with an LSM 510 Meta (Zeiss, Germany) attached to an Axioplan II microscope (Zeiss, Germany), as previously described (25), with some modifications. Biofilms were observed using a 63× water immersion objective (Achroplan 63x/0.95W). The bacterial cells were detected by the refraction of light in the red spectrum, and WGA was detected by fluorescence in the green spectrum, using single-channel analysis. The excitation wavelengths were 633 nm, with an output power of 70%, for detection of bacterial cells and 488 nm, with an output power of 10%, for detection of WGA. The excitation beam splitter used was an HFT UV/488/543/633 beam splitter. The filter used to detect the light refracted by bacterial cells was an LP650 filter, and the filter used to detect the light emitted by WGA was a BP 505-530 filter. The beam splitter used was an NFT 545 beam splitter. For each condition, three independent biofilms were used, and in each biofilm four different regions of the surface were analyzed and the thickness of the biofilm was measured. Three-dimensional projections were made with the LSM 510 software (Zeiss, Germany).

Contact angle measurement. Measurement of contact angles for bacterial lawns was performed as previously described by Busscher et al. (4), with some modifications. Briefly, biofilms were scraped from the substrate, resuspended in TSB, and sonicated for 10 s at 20 W. Biofilm cells were then harvested by centrifugation (10,500 × *g*, 6 min, 4°C), washed twice with 0.9% NaCl, and resuspended to a concentration of approximately 1×10^9 cells · ml^{-1}. Twenty microliters of the suspension was filtered through a 0.45-μm cellulose filter that had previously been wetted with 10 ml of distilled water. The resultant lawn of cells deposited on the cellulose filter was then air dried for at least 3.5 h, until the so-called "dried plateau" was obtained (8). Water contact angles were measured by the sessile-drop contact angle technique, using an automated contact angle device (OCA 15 Plus; Dataphysics, Germany). All experiments were done in quadruplicate with three repeats.

XPS analysis of the bacteria surface. The elemental composition of bacterial cell surfaces was assessed by X-ray photoelectron spectroscopy (XPS) analysis as described by van der Mei et al. (38), with some modifications. Briefly, biofilms were scraped from the substrate, resuspended in TSB, and sonicated for 10 s at 20 W. Biofilm cells were then harvested by centrifugation (10,500 × *g*, 6 min, 4°C), washed twice in 0.9% NaCl, and resuspended to a concentration of approximately 1×10^9 cells · ml^{-1}. The cells were then filtered through a cellulose filter that had previously been wetted with 10 ml of distilled water. After filtering, the cellulose filter covered with bacteria was sliced into 1-cm^2 squares and quickly frozen in liquid nitrogen. Frozen filters were stored at −80°C for 1 to 2 h, and this was followed by 24 h of lyophilization (Christ Alpha2-4; B. Braun, Germany). The XPS analysis was performed using an ESCALAB 200A apparatus, with the VG5250 software and data analysis. The spectrometer used monochromatized Mg (Kα) X-ray radiation (15,000 eV). The constant pass energy of the analyzer was 20 eV, and it was calibrated with reference to Ag 3d5/2 (368.27 eV). The pressure during analysis was less than 1×10^{-6} Pa. The spectra were recorded following the sequence C1s, O1s, N1s, P2p. The elemental composition was defined as the ratio of oxygen to carbon (O/C), the ratio of nitrogen to carbon (N/C), or the ratio of phosphorus to carbon (P/C). Measurements were obtained for three independent cultures of each strain, and for each sample 20 XPS scans were performed for each of the four elements probed.

TABLE 1. Growth parameters for planktonic cells grown in the presence or absence of a sub-MIC concentration of dicloxacillin

Strain	Treatment[a]	Doubling time in log phase (min)	Stationary phase concn (10^9 CFU/ml)
M187	CT	60 ± 8[b]	8.2 ± 0.9
M187	DCX	66 ± 10	7.2 ± 1.4
M176	CT	59 ± 8	4.6 ± 0.8
M176	DCX	67 ± 5	5.1 ± 1.4

[a] CT, cells grown in the absence of dicloxacillin (control); DCX, cells grown in the presence of dicloxacillin.
[b] The values are means ± standard deviations.

Biofilm matrix composition. The biofilm matrix was extracted as previously described (2), with some modifications. Briefly, biofilms were scraped from the substratum surfaces, resuspended in 0.9% NaCl, and sonicated for 30 s at 20 W, and then the preparations were vortexed for 2 min. The resultant bacterial suspensions were adjusted so that the concentrations were approximately 1×10^9 cells · ml^{-1}, and they were centrifuged at 10,500 × *g* for 6 min at 4°C. The supernatants were filtered through a 0.2-μm nitrocellulose filter and stored at −20°C before they were used in the quantification assays. Proteins and polysaccharides of the biofilm matrix were determined by the methods of Lowry et al. (27) and Dubois et al. (15).

PNAG immunological detection. PNAG production by biofilms was detected as previously described (11), with some modifications. Briefly, biofilms were scraped from the substratum surfaces, resuspended in TSB, sonicated for 30 s at 20 W, and then vortexed for 2 min. The resultant bacterial suspensions were adjusted so that the concentrations were approximately 1×10^9 cells · ml^{-1}. The same volume of each suspension was resuspended in 300 μl of 0.5 M EDTA (pH 8.0) and incubated for 5 min at 100°C. Cells were harvested by centrifugation at 10,500 × *g* for 6 min, and 100 μl of the supernatant was incubated with 10 μl of proteinase K (20 mg/ml; QIAGEN, United States) for 60 min at 60°C. Then the proteinase K was heat inactivated by incubating the preparation for 30 min at 80°C. The solution was then diluted fourfold into 500 μl of Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl [pH 7.4]), and 100 μl of each dilution was immobilized on a nitrocellulose filter that was then blocked with 1% bovine serum albumin and incubated for 2 h with a rabbit antibody raised to *S. aureus* PNAG (kindly provided by T. Maira-Litran) (28) absorbed and diluted 1:5,000 as described by Gerke et al. (16). The secondary antibody used was a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody (Southern-Biotech, United States) that was diluted 1:6,000 and detected with the Amersham ECL (enhanced chemiluminescence) Western blotting system.

Resistance to antibiotics. The MICs of antibiotics for bacterial cells in biofilms formed in the presence and in the absence of a subinhibitory concentration of dicloxacillin were determined using the NCCLS protocol (30), with some modifications. Briefly, biofilms were scraped from the substratum surface, resuspended in TSB, and sonicated for 10 s at 20 W, and the preparations were adjusted to obtain a standard cell inoculum and incubated in 96-well microtiter plates with several twofold dilutions of dicloxacillin, tetracycline, and rifampin (Sigma, United States) for 24 h at 37°C in TSB.

Statistical analysis. Quantitative assays were compared using a one-way analysis of variance by applying Levene's test of homogeneity of variances and the Tukey multiple-comparison test, as well as paired sample *t* tests, using SPSS software (Statistical Package for the Social Sciences). All tests were performed with a confidence level of 95%.

RESULTS

Growth rate in the presence of a sub-MIC concentration of dicloxacillin. Table 1 summarizes the effect of dicloxacillin on the doubling time in the log phase and also the bacterial concentration reached in the stationary phase. When grown in the presence of dicloxacillin, both strains had a slightly longer doubling time in the log phase, which was statistically relevant ($P < 0.05$, as determined by a paired *t* test). However, in the

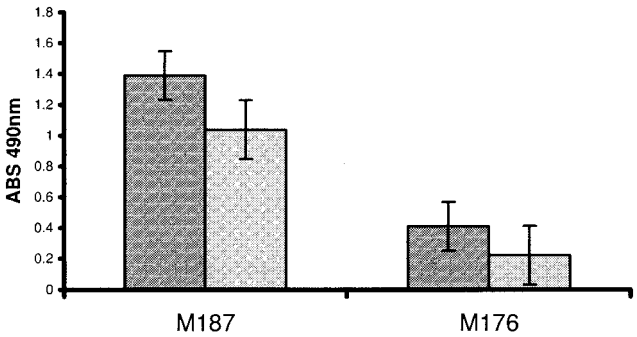

FIG. 1. Amounts of biofilm formed by *S. epidermidis* M187 and *S. haemolyticus* M176 in the absence of dicloxacillin (dark gray bars) and in the presence of a sub-MIC concentration of dicloxacillin (light gray bars), as evaluated by the safranin colorimetric assay. The error bars indicate standard deviations. ABS 490nm, absorbance at 490 nm.

stationary growth phase, there was no statistical difference between growth in the presence of dicloxacillin and growth in the absence of dicloxacillin ($P > 0.05$, as determined by a paired *t* test).

Amount of biofilm formed. The amount of biofilm formed by *S. haemolyticus* M176 in TSB supplemented with glucose was significantly lower ($P < 0.05$, as determined by analysis of variance and a Tukey multiple-comparison test) than the amount of biofilm formed by *S. epidermidis* M187 in the same conditions. The use of dicloxacillin at a low concentration (8 μg/ml [one-half the MIC]) significantly reduced ($P < 0.05$, as determined by a paired *t* test) the amount of biofilm formed by both strains (Fig. 1).

Biofilm structure. The CSLM observations clearly demonstrated that there were differences in the structure of biofilms formed in the presence of dicloxacillin (Fig. 2). First, biofilms formed in the presence of sub-MIC concentrations of dicloxacillin were thinner than control biofilms grown without the antibiotic. *S. epidermidis* M187 biofilms were 100 ± 28 μm thick when they formed without the antibiotic and 49 ± 14 μm thick when a sub-MIC concentration of dicloxacillin was present. For *S. haemolyticus* M176 biofilms, the thickness decreased from 30 ± 13 μm to 8 ± 3 μm. Both of these reductions were statistically significant ($P < 0.05$, as determined by a paired *t* test).

Besides the difference in biofilm thickness, a subinhibitory concentration of dicloxacillin also influenced the surface area covered. The CSLM top-view images of *S. epidermidis* M187 and *S. haemolyticus* M176 biofilms showed that control biofilms covered the entire abiotic surface (Fig. 2A2 and 2C), whereas biofilms formed in the presence of a sub-MIC concentration of dicloxacillin exhibited only partial surface coverage (Fig. 2B2 and 2D). The reduction was more obvious in *S. haemolyticus* biofilms.

The presence of PNAG as a major component of the biofilm matrix, as measured by wheat germ agglutinin binding, was detected only in *S. epidermidis* M187 biofilms, and PNAG was more abundant in the control biofilms than in the biofilms grown in the presence of a sub-MIC concentration of dicloxacillin.

Cell surface properties. Table 2 summarizes the effect of dicloxacillin on some bacterial cell surface properties. Cells entrapped in biofilms formed in the presence of subinhibitory concentrations of dicloxacillin had significantly lower ($P <$

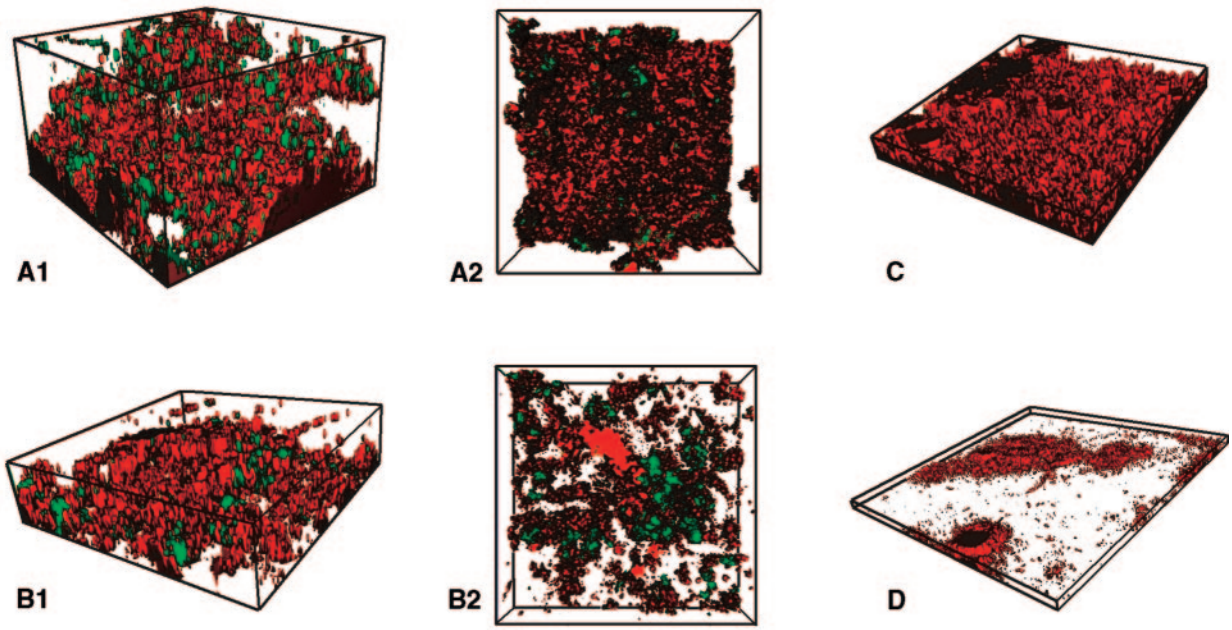

FIG. 2. Three-dimensional representations of the biofilms using confocal microscopy analysis. Green represents PNAG, which was asymmetrically distributed in the biofilm, and red represents the bacterial cells. (A1) M187 control, lateral view; (A2) M187 control, top view; (B1) M187 in the presence of dicloxacillin, lateral view; (B2) M187 in the presence of dicloxacillin, top view; (C) M176 control; (D) M176 in the presence of dicloxacillin. The images were adjusted with Paint Shop Pro 6.

TABLE 2. Bacterial cell surface properties of biofilms of *S. epidermidis* M187 and *S. haemolyticus* M176 formed in the presence and absence of a sub-MIC concentration of dicloxacillin

Strain	Treatment[a]	Contact angle (°)	Surface elemental composition		
			N/C ratio	O/C ratio	P/C ratio
M187	CT	28.0 ± 1.1[b]	0.192 ± 0.009	0.480 ± 0.037	0.032 ± 0.004
M187	DCX	22.9 ± 1.6	0.145 ± 0.025	0.399 ± 0.001	0.038 ± 0.005
M176	CT	36.4 ± 2.2	0.152 ± 0.045	0.397 ± 0.011	0.031 ± 0.004
M176	DCX	31.8 ± 1.7	0.191 ± 0.041	0.441 ± 0.052	0.027 ± 0.005

[a] CT, cells grown in the absence of dicloxacillin (control); DCX, cells grown in the presence of dicloxacillin.
[b] The values are means ± standard deviations.

0.05, as determined by a paired *t* test) water contact angles, reflecting a decrease in hydrophobicity (3). XPS analysis also revealed differences in the surface element compositions of the biofilm cells. The high O/C ratio observed is a common CoNS characteristic and can be related to the presence of a slime layer surrounding the cell wall (38), suggesting that *S. epidermidis* cells formed in the presence of a sub-MIC concentration of dicloxacillin may elaborate less slime than control biofilm cells. The opposite was found for *S. haemolyticus* biofilm cells, for which the O/C ratio was higher in the presence of dicloxacillin, suggesting that the control biofilms had less slime layer surrounding the cells.

Biofilm matrix. Figure 3 shows the detection and relative quantification of the PNAG present in the biofilm matrix. PNAG was detected only in *S. epidermidis* biofilms and was not present in *S. haemolyticus* biofilms. The amount of PNAG per cell produced in *S. epidermidis* biofilms was slightly lower in biofilms formed in the presence of a sub-MIC concentration of dicloxacillin.

Table 3 shows the composition of the matrix of biofilms formed in the presence and absence of a sub-MIC concentration of dicloxacillin. For both CoNS species, each biofilm matrix had a significantly lower ($P < 0.05$, as determined by a paired *t* test) protein content per number of cells in the biofilm when the biofilm was formed in the presence of dicloxacillin. When polysaccharide production was examined, *S. epidermidis* biofilms formed in the presence of a sub-MIC concentration of dicloxacillin produced significantly smaller amounts ($P < 0.05$, as determined by a paired *t* test) than the control. Conversely, the *S. haemolyticus* control biofilm matrix had a significantly higher ($P < 0.05$, as determined by a paired *t* test) polysaccharide content per cell in the biofilm than the biofilm matrix formed in the presence of a sub-MIC concentration of dicloxacillin.

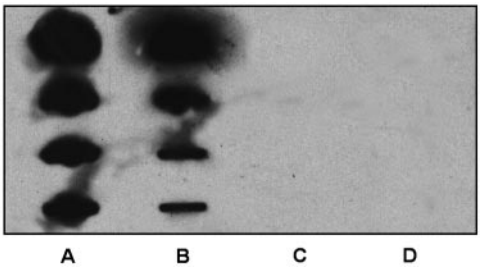

FIG. 3. Immunological detection and relative quantification of PNAG extracted from the biofilm matrix. Lane A, M187 control; lane B, M187 in the presence of dicloxacillin; lane C, M176 in the presence of dicloxacillin; lane D, M176 control. Rows from top to bottom represent serial fourfold dilutions of cell surface extract. The image was adjusted with Paint Shop Pro 6.

Antibiotic resistance. Growth in the presence of a sub-MIC concentration of dicloxacillin induced an increase in the resistance of the biofilm cells to dicloxacillin, as well as antibiotics with different mechanisms of action, including tetracycline and rifampin. The most pronounced increase in resistance was the increase in resistance to dicloxacillin (Fig. 4).

DISCUSSION

CoNS biofilm infections have become a serious problem in the last few years (39, 40). Several strategies to inhibit biofilm formation have been evaluated. Previous reports suggested that the use of sub-MIC concentrations of antibiotics could increase biofilm formation (35). However, more recent studies demonstrated that sub-MIC concentrations of some antibiotics are effective for reducing the amount of biofilm formed (6, 17, 34, 43), which may have some clinical interest. However, other changes that can occur in the physiology of biofilm cells might compromise the general use of this strategy to prevent biofilm formation. Several studies have reported increases in antibiotic resistance due to bacterial growth in the presence of sub-MIC concentrations of antibiotics (17, 37). Even so, the prophylactic use of antibiotics to prevent CoNS infections is widespread (18). In this study, changes in biofilm structure and matrix composition and also in antimicrobial resistance were observed when a sub-MIC concentration of dicloxacillin was used. It must be stressed that the concentration of dicloxacillin used was low enough not to inhibit the growth of bacteria (Table 1).

Bacterial adhesion to surfaces is the first step in biofilm formation (41). Hydrophobic interactions play an important

TABLE 3. Composition of the biofilm matrix of biofilms of *S. epidermidis* M187 and *S. haemolyticus* M176 formed in the presence and absence of a sub-MIC concentration of dicloxacillin

Strain	Treatment[a]	Protein concn (μg/10^8 cells)	Polysaccharide concn (μg/10^8 cells)
M187	CT	3.39 ± 0.20[b]	1.52 ± 0.32
M187	DCX	2.81 ± 0.74	0.99 ± 0.39
M176	CT	3.26 ± 0.35	0.98 ± 0.48
M176	DCX	1.37 ± 0.38	1.77 ± 0.59

[a] CT, cells grown in the absence of dicloxacillin; DCX, cells grown in the presence of dicloxacillin.
[b] The values are means ± standard deviations.

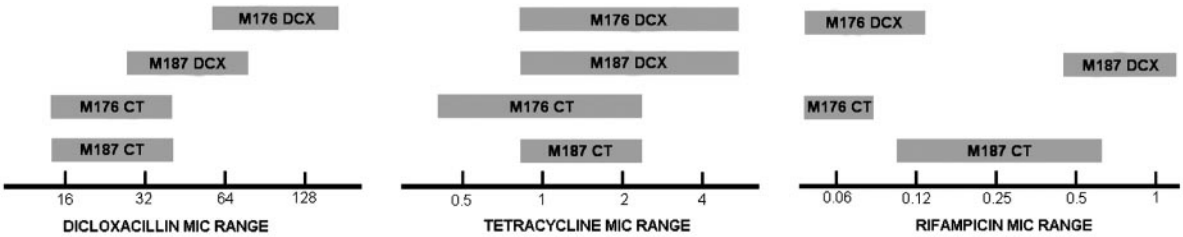

FIG. 4. Shifts in the MIC ranges for dicloxacillin, tetracycline, and rifampin for biofilm cells formed in the presence (DCX) or in the absence (CT) of a sub-MIC concentration of dicloxacillin. The image was created with Paint Shop Pro 6.

role in the initial adhesion to inert surfaces (14). Cells from both biofilms formed in the presence of a sub-MIC concentration of dicloxacillin had lower water contact angles, indicating that there was a decrease in hydrophobicity (3). The lower cell surface hydrophobicity could have had an impact on the initial adhesion to the surface. CSLM observations revealed reduced levels of surface coverage by the biofilms exposed to sub-MIC levels of dicloxacillin compared to the controls. While control biofilms formed in the absence of dicloxacillin covered the entire substratum, biofilms exposed to sub-MIC concentrations of dicloxacillin were not able to colonize the entire available surface. These data suggest that sub-MIC concentrations of dicloxacillin probably inhibit initial adhesion by diminishing cell surface hydrophobicity. Nonetheless, the reduction in cell surface hydrophobicity cannot fully account for the decrease in the amount and thickness of biofilms formed in the presence of sub-MIC concentrations of dicloxacillin. We demonstrated in previous work that initial adhesion and biofilm formation are not always directly related (9). Cell-to-cell adhesion, which is associated with the production of an intercellular adhesin (PNAG), plays a critical role in biofilm formation and maturation (41).

PNAG production was semiquantitatively evaluated by using an immunological detection assay and also by CSLM observations of biofilms treated with the lectin wheat germ agglutinin. CSLM has been used successfully in a wide range of assays as a tool to determine the composition and structure of biofilms (24, 26). In the present study, besides evaluating the spatial structure of biofilms, this technique also enabled detection of PNAG in the matrix of the biofilm. PNAG production was detected only in *S. epidermidis* biofilms. In these biofilms, the amount of PNAG produced per cell was slightly reduced in the biofilms formed in the presence of a sub-MIC concentration of dicloxacillin. Since PNAG production is related to biofilm formation in staphylococci (19, 29), these results can explain the reduction in biofilm formation. Interestingly, in *S. haemolyticus* biofilms no PNAG was detectable. This could mean either that (i) *S. haemolyticus* produces levels of PNAG that are below the level of detection of both methods, (ii) the exopolysaccharide of *S. haemolyticus* is structurally different and the probes used, which are very specific, were not able to detect it, or (iii) *S. haemolyticus* does not produce PNAG. To address these questions, we tried to detect the *icaC* gene in both species by PCR amplification (data not shown), and while *S. epidermidis* was positive for the *icaC* gene, *S. haemolyticus* was *icaC* negative. The absence of the *ica* locus seems to be common in CoNS species. de Silva et al. (13) probed for the presence of the *ica* locus in 180 CoNS strains and found that the prevalence of *ica* genes in *S. epidermidis* was around 50% and that the genes were absent in *S. haemolyticus* strains. In the present study, biofilm quantification assays clearly demonstrated that *S. haemolyticus* does not form a biofilm as thick as that formed by *S. epidermidis*. Since PNAG has been described as the molecule responsible for biofilm formation in many strains of staphylococci, our data seem to suggest that *S. haemolyticus* M176 does not produce PNAG and therefore cannot form a thick biofilm.

Interestingly, *S. haemolyticus* was very aggregative, especially in the presence of a sub-MIC concentration of dicloxacillin. However, the cell aggregates were not firmly attached to the surface. The results provided by the XPS analysis revealed that cells of *S. haemolyticus* biofilms formed in the presence of a sub-MIC concentration of dicloxacillin have a higher O/C ratio. This characteristic can be associated with an increase in the slime layer content of the outer cell surface (38) that can explain the more aggregative appearance of *S. haemolyticus* cells.

Biofilm formation in the presence of a sub-MIC concentration of dicloxacillin also influenced the composition of the biofilm matrix. It has been suggested that the matrix of biofilms can be responsible for the increased resistance to antibiotics by acting as a diffusion barrier (12, 36). Changes in the biofilm matrix can therefore influence the susceptibility of biofilm cells to antibiotics. For both species studied, the matrix compositions of the control biofilm and the biofilm formed in the presence of a sub-MIC concentration of dicloxacillin were clearly different. Biofilm cells, after growing for 1 day in the presence of a sub-MIC concentration of dicloxacillin, developed slightly increased resistance to several antibiotics with different mechanisms of action. The shift in the MIC range was more drastic when dicloxacillin was used in the MIC assay. Similar results have been reported by other authors (17).

The increase in antimicrobial resistance can compromise the use of the sub-MIC concentration approach to prevent biofilm formation. New approaches are being developed based on the same principle of sub-MIC alterations in growth but using other molecules, like nonsteroidal anti-inflammatory drugs (1) or specific enzymes that target biofilm formation (22, 42), which are not associated with antimicrobial resistance. However, new studies should also provide more information regarding other changes in biofilm physiology, like the results reported here, which should lead to a better understanding of biofilm formation and treatment processes, rather than just focusing on inhibition of biofilm formation.

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