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Article

Factors Compromising Antibiotic Activity Against Biofilms of *Staphylococcus epidermidis*

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Abstract Several factors associated with bacterial biofilms were studied for their role in phenotypic resistance to antibiotics. These factors included bacterial slime extracted from biofilms, reduced growth rates of biofilm-embedded bacteria and high bacterial inocula. Antibiotic activity against suspended bacteria in the presence of these factors, either alone or combined, was compared with activity against adherent biofilms. All MICs, determined by standard susceptibility tests, were below the sensitivity breakpoints for *Staphylococcus epidermidis* strain V2. The addition of bacterial slime to suspended bacteria reduced the bactericidal activity of glycopeptides but had less or no effect on the activity of the other antibiotics tested. High bacterial inocula affected the activity of flucloxacillin and quinolones only moderately or not at all, though a more pronounced effect on glycopeptides was observed. In contrast, the bactericidal activity of most antibiotics was severely compromised when adherent bacterial biofilms were used as inocula. In conclusion, the presence of slime, slow growth rates and high bacterial counts may explain the poor activity of glycopeptides against biofilm-embedded organisms, but these factors, either alone or in combination, do not explain the lack of bactericidal activity of other drugs against biofilms. Thus, additional factors need to be identified.

Introduction

In recent years, the growing use of indwelling medical devices such as intravascular catheters, artificial heart valves and orthopedic implants has resulted in a significant increase in the number of device-related infections. Coagulase-negative staphylococci are recognized as a major cause of these infections, with *Staphylococcus epidermidis* representing about 75% of all clinical isolates [1, 2].

Mechanisms that enable *Staphylococcus epidermidis* to cause serious health problems include its ability to adhere to surfaces and to grow as a biofilm. *Staphylococcus epidermidis* and other bacterial species produce an extracellular matrix called glycocalyx, or slime, a highly hydrated complex composed of exopolysaccharides, teichoic acids and proteins. Different components of *Staphylococcus epidermidis* slime have been identified that promote initial attachment [3, 4] or accumulative growth into multilayered cell clusters [5, 6]. These factors are frequently detected in clinical isolates but are rarely identified in saprophytic strains of *Staphylococcus epidermidis* [4, 7]. It has been postulated that these features represent a virulence factor as well as a factor for phenotypic resistance to antibiotic therapy [8, 9].

Once a biofilm has been established on a surface, the bacteria harbored inside are less exposed to the host's immune response and are, to some extent, protected against antibiotic action. It has been speculated that the glycocalyx may act as a penetration barrier to antibiotics [10, 11]. Besides the extracellular matrix material, the biofilm mode of growth encompasses numerous other factors that may render bacteria either more virulent or more resistant to the action of antibiotics. One such factor may be the high bacterial numbers concentrated in the confinement of a biofilm. Bacterial numbers in biofilms in vivo and in vitro may be orders

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of magnitude larger than the inoculum used in standard in vitro susceptibility tests. In an in vitro model of *Staphylococcus aureus* biofilms adhering to silicone catheter tubing, cell numbers of $>10^8$/cm (length) have been determined [12]. In animal models with biofilms of *Pseudomonas aeruginosa* adhering to peritoneal implants, cfu/cm^2 of $>10^7$ of were determined [13]. Another factor reducing bactericidal activity may be the slow growth rate of biofilm-embedded bacteria, which may even resemble that of stationary-phase bacteria [14, 15]. It has been shown that biofilm-embedded bacteria and nongrowing bacteria have similar antibiotic susceptibility [16, 17].

In this study the effects of (i) the presence of extracellular slime, (ii) reduced growth rates of biofilm-embedded bacteria and (iii) high bacterial densities on antimicrobial activity were evaluated and compared to the overall effect of the biofilm mode of growth, which encompasses a combination of factors mentioned above and of other properties not yet identified. The impact of a biofilm on antimicrobial activity was evaluated by using established biofilms of *Staphylococcus epidermidis* as inocula. The crude slime extract was prepared from biofilms of *Staphylococcus epidermidis* growing on the surface of a solid medium. The composition of extracts obtained from different strains and culture media was studied by gel electrophoresis.

Materials and Methods

Strains. Susceptibility tests were performed with the strains *Staphylococcus epidermidis* V2, KH11, B3972, RP62A and M7. Strain M7 is an accumulation-deficient but slime-producing mutant of RP62A generated by mitomycin mutagenesis [18].

Impact of Biofilm Factors on Antibiotic Activity Against Suspended Bacteria. Following a standard protocol [19], in vitro antibiotic activity was determined by a dilution broth procedure in tryptic soy broth (TSB) supplemented with 50 mg/l Ca^{2+} and 25 mg/l Mg^{2+}; a standard inoculum of circa 5×10^5 cfu/ml was used. Antibiotic solutions and serial twofold dilutions were prepared immediately before the experiment. The MIC was read after 18 h. After 24 h the MBC was determined for each tube without visible growth by subculturing serial tenfold dilutions on antibiotic-free TSB agar using a spiral plater (Spiral System Instruments, USA). For screening, MBCs were determined by subculturing volumes of 4 µl using a multipoint replicator. The MBC was defined as the lowest antibiotic concentration that reduced the inoculum by $\geq$99.9% [20].

To study the impact of high bacterial densities, MICs and MBCs were determined with higher inocula of circa 1×10^7 cfu/ml. To study the impact of extracellular matrix material on antibiotic activity, a crude slime extract (CSE) was added to the susceptibility assay together with the inoculum at a final concentration of 5 mg/ml. Determination of the generation time of *Staphylococcus epidermidis* V2 in TSB with and without CSE resulted in identical times of 35 min. The preparation of CSE is described below. To study a potential interaction or adsorption between CSE and vancomycin, a vancomycin solution (50 µg/ml) was incubated with and without 10 mg/ml CSE for 30 min at 35 °C. CSE was removed by centrifugation (10,000 × *g*, 5 min) and filtration (pore size 0.22 µm, Millex GV; Millipore, Switzerland). Vancomycin concentrations were determined in triplicate by a fluorescence polarization immunoassay (Cobas Integra 700; Roche Diagnostics, Switzerland).

To study the impact of reduced growth rates of biofilm-embedded strains, susceptibility testing was performed in a medium with a reduced glucose concentration. This slow-growth (SG) medium contained 2.38 g Na_2HPO_4, 0.19 g KH_2PO_4, 8 g NaCl, 0.9 g glucose and 1 g casamino acids per liter of distilled water. In a further modification, 2% pooled human plasma (serotype 0) was added to the SG medium. The generation time of *Staphylococcus epidermidis* V2 in SG medium with plasma (SG + P) and without plasma (SG-P) was 84 min and 221 min, respectively, as compared to 35 min in TSB. In growth controls of SG + P and SG-P, the average number of cells reached was 3×10^7 cfu/ml and 2.5×10^6 cfu/ml, respectively. These cell numbers did not cause turbidity of the samples. Therefore, only MBCs were determined in the two media.

To study the impact of adherence, a bacterial biofilm was established and used as an inoculum during susceptibility testing. Solid glass beads were submerged in 20 ml of TSB inoculated with *Staphylococcus epidermidis* V2. Within 20 h of incubation, a biofilm formed on the surface of the beads, with an average of 1.2×10^7 cfu per bead. These precolonized beads were gently rinsed in saline and used as inocula. All MIC/MBC determinations were performed in triplicate.

Preparation of Crude Slime Extract. A novel protocol was developed to isolate CSE from bacterial biofilms on solid media. CSE was extracted from the following *Staphylococcus epidermidis* strains: V2, a strong slime producer; RP62A; M7, an RP62A mutant; and KH11.

Biofilms were cultured in large agar plates (∅ 16 cm) on trypticase soy agar (TSA) and on SG + P solidified with 15 g of Bacto agar (SGA; Difco Lab, USA). The agar surface was covered with sterile moist cellophane sheets to ensure that no agar material was accidentally collected while harvesting the bacterial biofilm. For each strain, 3 ml aliquots of an overnight culture were plated onto ten agar plates and incubated for 24 h at 35 °C. The established biofilms were carefully harvested from the plates with a cell scraper. For each strain, the biofilm scrapings of all plates were pooled, vigorously vortexed for 5 min, subjected to 1 min of low-intensity sonication at 60 Hz (Labsonic 2000; B. Braun, Switzerland) and vortexed again for 3 min to homogenize the biofilm and disrupt the cohesion between bacteria and the exopolysaccharide matrix. The number of colony-forming units per milliliter was determined before sonication and after the second vortexing. Median numbers of colony-forming units before and after were unchanged (4.2×10^{10}). The volume of the mixture and the total number of colony-forming units were determined. An average total cell number of 2.1×10^{12} cfu was harvested per preparation and strain, with 7×10^{11} cells grown on SGA and 2.8×10^{12} cells on TSA.

The mixture of bacteria and exopolymers was centrifuged (30 min, 4,000 × *g*, 4 °C), and the supernatant containing the extracellular components was filter-sterilized (pore size 0.22 µm; Steritop; Millipore) to remove any remaining bacteria. Three volumes of 98% ethanol were added under constant stirring on ice to precipitate the extracellular substance. The precipitate was obtained by centrifugation (1 h, 16,000 × *g*, 4 °C), and the supernatant was discarded. The pellets of extracellular substance were air dried to remove traces of ethanol, dissolved in sterile distilled water, lyophilized, resuspended in water and dialyzed for 24 h at 4 °C (Spectrapor, molecular weight cutoff 12,000–14,000 kDa; Spectrum, USA). Following a second lyophilization step, a white cotton-like substance that was named “CSE” was obtained. An average of 28.4 mg of CSE could be harvested from a biofilm of 1×10^{12} cfu. Similar amounts of slime were harvested off TSA and SGA: 28.7 mg and 27.7 mg, respectively.

The composition of CSE derived from different strains and culture conditions was analyzed on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels following a standard protocol [21] and using Bio-Rad electrophoresis reagents, molecular markers and the Protean II xi vertical electrophoresis cell (Bio-Rad Laboratories, Switzerland). All samples were run on two gels. The wells of one gel were loaded with 400 μg of each sample and subsequently stained in 0.1% Coomassie Blue, 45% methanol and 10% acetic acid for 20 min at 37 °C and destained in the microwave in four to five volumes of fresh distilled water. The wells in the second gel were loaded with 40 μg of each sample and subsequently stained with Stains-all (Fluka Chemie, Switzerland) [22]. Proteins were stained red, glycoproteins purple and polysaccharides blue.

Results

Susceptibility of Staphylococcus epidermidis V2. Susceptibility testing with standard parameters showed that *Staphylococcus epidermidis* V2 was sensitive to all antibiotics tested according to NCCLS sensitivity breakpoints [23].

Effect of the Addition of Crude Slime Extract. Table 1 shows the standard MICs and the effect of the addition of CSE on antibacterial activity. While the activities of fleroxacin, rifampicin and flucloxacillin against *Staphylococcus epidermidis* V2 were not significantly influenced, the MICs of both vancomycin and teicoplanin increased eightfold, to 16 and 64 μg/ml, respectively, exceeding the sensitivity breakpoints. In TSB, the MBCs of vancomycin and teicoplanin increased 16- and six-fold, to 64 and 96 μg/ml, respectively. Vancomycin activity against *Staphylococcus epidermidis* RP62A, M7, KH11 and B3972 and teicoplanin activity against *Staphylococcus epidermidis* RP62A and M7 were similarly compromised in the presence of CSE (data not shown). Daptomycin activity against the V2 strain was less influenced, with MICs and MBCs increasing two- and four-fold, respectively. The inhibitory effect of CSE on the bactericidal activity of vancomycin was similar when the slime was isolated from biofilms of the strains *Staphylococcus epidermidis* RP62A, M7 and KH11 or when isolated from biofilms cultivated on either TSA or SGA (data not shown). Despite variations in slime composition, the compromising effect of slime was almost identical for all antibiotics. On average, vancomycin MICs and MBCs increased eight- and 16-fold, respectively, whether the slime was harvested from TSA or SGA. Teicoplanin MICs and MBCs increased eightfold with TSA-originated slime and four- to eight-fold with slime harvested from SGA. Concentrations of vancomycin in solutions that had been exposed to CSE 10 mg/ml for 30 min and in solutions without exposure to CSE (data not shown) were identical.

Slow Growth Rates. Reduction of the growth rate in the media SG + P and SG-P had different effects on the different groups of antibiotics (Table 1). Bactericidal activities of levofloxacin and flucloxacillin were barely affected by slow growth rates. MBCs of fleroxacin and rifampicin increased two- and four-fold in SG + P and SG-P, respectively, with rifampicin MBCs remaining well below the sensitivity breakpoint. The presence of CSE did not further increase the MBCs of these compounds. In contrast, bactericidal activities of vancomycin and teicoplanin were strongly influenced when the doubling time of *Staphylococcus epidermidis* V2 was increased from 35 min in TSB to 84 min in SG + P and to 220 min in SG-P. Teicoplanin MBCs in both media and vancomycin MBCs in SG-P were ≥ 64 mg/l in the absence and presence of CSE. Again, daptomycin activity was less compromised but showed a similar trend. While the median standard MBC of 2 μg/ml increased to only 3 and 4 μg/ml in SG + P and SG-P, respectively, a further increase to 16 and 32 μg/ml was observed when CSE was added.

High Bacterial Inocula. MICs and MBCs of most antibiotics increased moderately when a high inoculum of circa 1×10^7 cfu/ml was used (Table 2). The average increase of the MIC was twofold (range 1- to 4-fold, data not shown), while the increase of the MBC was more variable and more pronounced for some antibiotics. Activities of vancomycin, levofloxacin and flucloxacillin were affected only moderately or not at all, while MBCs of daptomycin, teicoplanin and fleroxacin

Table 1 Antimicrobial activities (μg/ml) in the absence and presence of 5 mg/ml crude slime extract (CSE) as determined by a microdilution broth procedure for *Staphylococcus epidermidis* V2. In tryptic soy broth (TSB), both MICs and MBCs were determined; in slow-growth media with and without human plasma (SG + P, SG-P), only the MBCs were measured

Antimicrobial agent	MIC in TSB		MBC in TSB		MBC in SG + P		MBC in SG-P	
	Without CSE	With CSE	Without CSE	With CSE	Without CSE	With CSE	Without CSE	With CSE
Vancomycin	2	16	4	64	8	>64	>64	>64
Teicoplanin	8	64	16	96	>64	≥64	>64	64
Daptomycin	2	4	2	8	3	16	4	32
Fleroxacin	0.5	1	1	1	2	2	4	2
Levofloxacin	0.125	n.d.	0.5	n.d.	0.5	0.25	1	0.5
Rifampicin	0.008	0.004	0.015	0.008	0.03	0.015	0.06	0.03
Flucloxacillin	0.125	0.06	0.25	0.125	0.5	0.25	0.25	0.5

n.d., not done

Table 2 Bactericidal activities (MBCs) determined by a macrodilution broth procedure in tryptic soy broth with different types of inocula of *Streptococcus epidermidis* V2

Antimicrobial agent	MBC (μg/ml)		
	10^5 cfu/ml, suspended	10^7 cfu/ml, suspended	10^7 cfu/ml, adherent
Vancomycin	4	8	>512
Teicoplanin	8	64	>512
Daptomycin	2	8	64
Fleroxacin	1	8	16
Levofloxacin	0.5	1	1
Rifampicin	0.008	16	>16
Flucloxacillin	0.25	0.25	>64

increased four- to eight-fold. In contrast, the antimicrobial activity of rifampicin was most severely compromised, with both the MIC and the MBC increasing more than 2000-fold. Surviving bacteria were subcultured on agar plates containing rifampicin 4 μg/ml, the NCCLS breakpoint of resistance. Bacterial growth on these plates was considered evidence of the presence of genotypic resistance.

Adhering Biofilms. The strongest impact on the activity of most antibiotics was observed when the bacterial inoculum was introduced in the form of established biofilms adhering to the surface of glass beads. Although the size of the adherent inoculum of circa 1×10^7 cfu/bead was similar to the increased suspended inoculum, a further loss in antibiotic activity was observed. The highest increases in the MBCs were detected for flucloxacillin (>256-fold) and vancomycin (>640-fold). The MBCs of teicoplanin and daptomycin increased by a factor of ≥8. In contrast, the MBCs of quinolones did not increase when adherent inocula were used.

Composition of Crude Slime Extract. Similar amounts of CSE were obtained from TSA and SGA, with an average of 28.7 and 27.7 mg per 1×10^{12} cfu, respectively. An average of 34.5, 23.3 and 26.7 mg CSE per 1×10^{12} cells could be isolated from biofilms of the strains *Staphylococcus epidermidis* V2, RP62A and M7, respectively.

Figure 1 shows the composition of extracellular proteins, glycoproteins and polysaccharides in the CSE of the strains *Staphylococcus epidermidis* V2, RP62A and M7 when grown on TSA and SGA. Each strain displayed a unique protein pattern (Figure 1A). In particular, the strains RP62A (lanes 4 and 5) and M7 (lanes 6 and 7) differed in the composition of extracellular proteins.

All three strains expressed different (glyco-) proteins and polysaccharides when grown on SGA compared to TSA (Figure 1B). All strains, V2 in particular, expressed a protein of approximately 25 kDa on SGA, identified by the red band, which was undetectable in the CSE of cells cultivated on TSA. The CSE of the strains RP62A (lanes 3 and 4) and M7 (lanes 5 and 6) contained large quantities of exopolysaccharides in the range of 21–25 kDa. These polymers stained red-purple when isolated from bacteria cultivated on TSA, indicating the presence of proteins or glycoproteins. In contrast, polymers in the CSE derived from SGA stained dark blue, indicating the presence of polysaccharides but an absence of a protein component. Despite the different composition of CSE isolated from different strains and culture media, the effect of CSE on bactericidal activity was nearly identical for all antibiotics.

Identical counts of colony-forming units determined before and after homogenization indicated that very few bacterial cells were disrupted in this process, during which only trace amounts of intracellular components would be released. It was therefore assumed that the polymers stained in the gels were of extracellular origin.

Discussion

The findings of this study suggest that numerous factors play a role in the resistance of biofilm-embedded bacteria. Any of the following, alone or in combination, may contribute to compromised antibacterial activity: interaction between constituents of the exopolymer matrices and the antimicrobial agents, suppression of growth rate due to a modified nutrient environment, and dense bacterial inocula. Schwank et al. [24] documented reduced bactericidal activity due to slime production in biofilms of *Staphylococcus epidermidis* RP62A and M7 and the additional impact of strong adherence and multilayered biofilm growth of RP62A. The latter strain was significantly harder to eradicate than its accumulation-deficient mutant, M7, which adhered weakly. Slime production and adhesion accumulation are two distinct processes and should be differentiated. CSE was added to suspended cultures to evaluate the contribution of a single factor.

Activities of different antibiotic groups varied in their response towards the factors tested. While glycopeptides were clearly affected by all factors tested, the quinolones, flucloxacillin and rifampicin were barely affected by a slow growth rate or the presence of CSE, indicating that further mechanisms present in biofilms may contribute to resistance of biofilm-embedded bacteria. Rifampicin bactericidal activity was severely reduced by an increased inoculum size due to rapid development of a genotypic resistance [25]. While the bactericidal activity of fleroxacin decreased with increased inocula and biofilm inocula, levofloxacin remained bactericidal under these conditions. Fluoro-

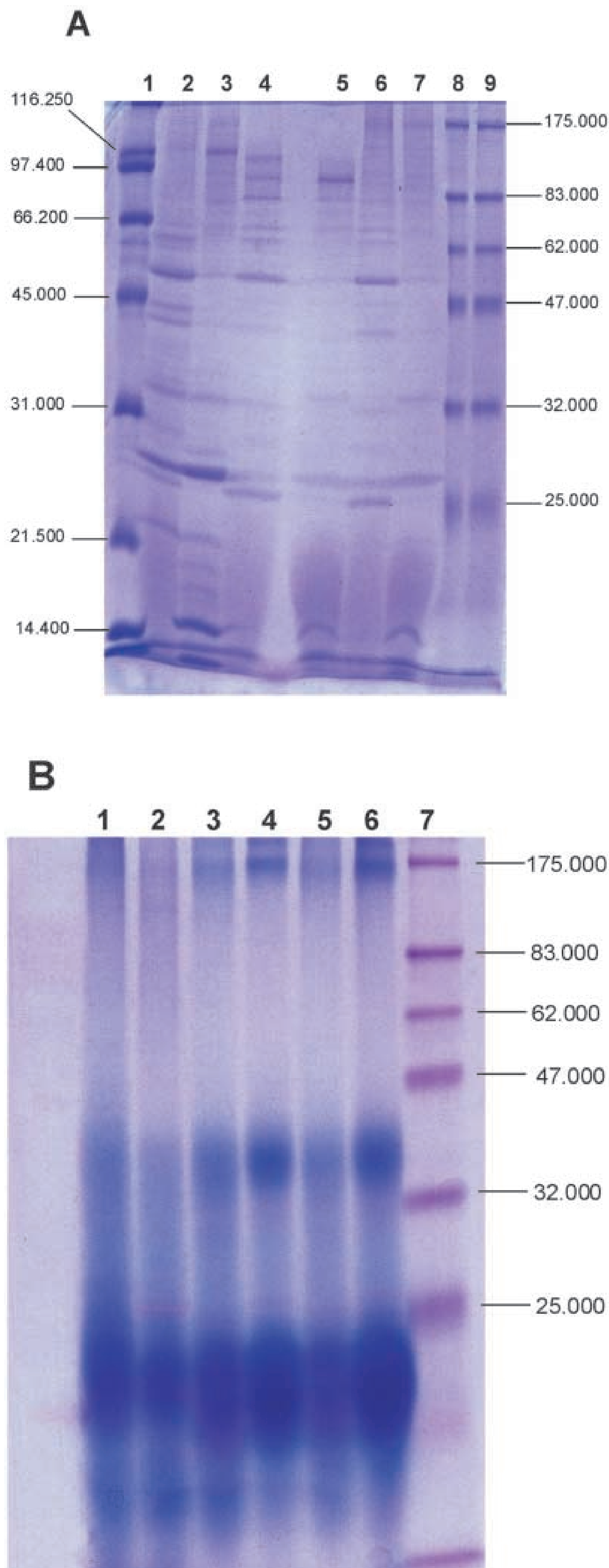

Figure 1A,B SDS-polyacrylamide gels (12%) showing the composition of crude slime extract isolated from the *Staphylococcus epidermidis* strains V2, RP62A and M7, cultured on tryptic soy agar (TSA) and slow-growth agar (SGA). **A** *lane 1*, marker; *lane 2*, V2 cultured on TSA; *lane 3*, V2 cultured on SGA; *lane 4*, RP62A cultured on TSA; *lane 5*, RP62A cultured on SGA; *lane 6*, M7 cultured on TSA; *lane 7*, M7 cultured on SGA; *lanes 8* and *9*, marker. Gel **A** is stained with Coomassie-Blue. Gel **B** *lane 1*, V2 cultured on TSA; *lane 2*, V2 cultured on SGA; *lane 3*, RP62A cultured on TSA; *lane 4*, RP62A cultured on SGA; *lane 5*, M7 cultured on TSA; *lane 6*, M7 cultured on SGA; *lane 7*, marker. Gel **B** is treated with Stains-all. Proteins are stained red, glycoproteins purple and polysaccharides blue

quinolones have exhibited strong bactericidal activity against biofilms of *Pseudomonas aeruginosa*, regardless of the growth rate [26, 27]. None of the factors tested, however, could explain the profound loss in the bactericidal activity of flucloxacillin against biofilm-producing bacteria.

Several hypotheses have been advanced to explain the strong reduction in antibiotic activity against strains in the presence of biofilm material. It has been shown that vancomycin reaches high concentrations in bacterial biofilms [28]. Reduced activities of some antibiotics in the presence of biofilm material has been suggested by the hypothesis that the glycocalyx acts as a penetration barrier by providing resistance to diffusion and delaying the equilibration of the antibiotic concentration between the external medium and the depth of the biofilm [10, 11, 29, 30]. Furthermore, the exopolysaccharide matrix of medically important bacteria is negatively charged or, less commonly, neutral. This may lead to an interaction with positively charged antibiotic molecules like vancomycin or various aminoglycosides, thereby reducing the free concentration of the antibiotic [10, 31]. In contrast, such an effect was not observed with neutral or negatively charged β-lactam antibiotics [32].

Since the activity of some antibiotics is growth-rate dependent, a possible effect of CSE on growth kinetics was also considered in this study. Given the possible influence of the variety of factors noted above, studies were conducted to minimize the effect of a physical barrier provided by a biofilm. Slime extract was resuspended with the inoculum and thoroughly mixed with the antibiotic, resulting in an even distribution without any diffusion barriers. A 30 min exposure of vancomycin to CSE did not reduce the concentration of the antibiotic, making the possibility of an adsorption process between CSE and vancomycin unlikely. Growth kinetics of *Staphylococcus epidermidis* V2 were almost identical in the absence and presence of CSE.

Collectively, these data show that CSE did not compromise glycopeptide activity by limited diffusion, adsorption or reduction of growth rate. Thus, CSE may influence antibiotic activity by mechanisms that directly

affect the bacterial cell other than changes in growth kinetics.

However, in established bacterial biofilms, reduced growth rates do represent another factor suspected to reduce antibiotic activity. The growth rate may be reduced because of limited diffusion of nutrients and/or oxygen and the accumulation of metabolic products. Acidic compounds produced by anaerobic metabolism may contribute to the development of a pH gradient. Acidic pH and hypoxia in the deeper regions of a biofilm may also reduce the activity of certain antibiotics. Quinolone and aminoglycoside activities are strongly affected by low pH values and reduced oxygen levels [33]. Little is known about a possible ion gradient that may influence antibiotic activity.

Bacteria respond to changes in their environment by profound phenotypic variations in enzymatic activity, cell wall composition and surface structures. Changes in the cell envelope may also include the expression of different penicillin-binding proteins, thereby reducing the bacterial susceptibility towards certain β-lactam antibiotics [34, 35]. Under conditions of nutrient limitation, different physiological pathways can be used. For example, alternative transport systems, more effective for the deficient nutrients, can be expressed to maintain growth [31]. Figure 1A documents different patterns of extracellular proteins expressed by three strains of *Staphylococcus epidermidis* when grown on either TSA or SGA.

One therapeutic approach to the eradication of biofilm-producing bacteria is the use of antimicrobial agents in combinations. One such combination included clarithromycin [36]. This macrolide had no bactericidal activity against the pathogen tested but instead caused a reduction in the glycocalyx material, thereby resulting in the increased diffusion or free concentration of the combination drug such as ofloxacin or gentamicin. Other antibiotic combinations, particularly those including rifampicin, also were quite effective against slow-growing and biofilm-embedded bacteria [17, 37].

The development of modified susceptibility tests that consider the complex situation of biofilm growth may be helpful in the identification of effective antibiotic regimens. Modified susceptibility tests using biofilms might be of greater clinical relevance in the context of foreign-body infections compared to routine susceptibility tests that follow highly standardized protocols. For such tests, a "Biofilm Eradication Concentration" could be determined as a more relevant endpoint compared to the conventional MIC and MBC values [38].

In conclusion, the presence of slime, slow growth rates and high bacterial counts may explain the poor activity of glycopeptides against biofilm-embedded bacteria. However, the impact of these factors, either alone or in combination, do not explain the lack of bactericidal activity of the other drugs against biofilm-embedded bacteria. Additional factors must be identified to fully explain the poor activity of these antibiotics against such bacteria.

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