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Spatial and Temporal Patterns of Biocide Action against *Staphylococcus epidermidis* Biofilms▽†

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The dynamic antimicrobial action of chlorine, a quaternary ammonium compound, glutaraldehyde, and nisin within biofilm cell clusters of *Staphylococcus epidermidis* was investigated using time-lapse confocal scanning laser microscopy. The technique allowed for the simultaneous imaging of changes in biofilm structure and disruption of cellular membrane integrity through the loss of an unbound fluorophore loaded into bacterial cells prior to antimicrobial challenge. Each of the four antimicrobial agents produced distinct spatial and temporal patterns of fluorescence loss. The antimicrobial action of chlorine was localized around the periphery of biofilm cell clusters. Chlorine was the only antimicrobial agent that caused any biofilm removal. Treatment with the quaternary ammonium compound caused membrane permeabilization that started at the periphery of cell clusters, then migrated steadily inward. A secondary pattern superimposed on the penetration dynamic suggested a subpopulation of less-susceptible cells. These bacteria lost fluorescence much more slowly than the majority of the population. Nisin caused a rapid and uniform loss of green fluorescence from all parts of the biofilm without any removal of biofilm. Glutaraldehyde caused no biofilm removal and also no loss of membrane integrity. Measurements of biocide penetration and action time at the center of cell clusters yielded 46 min for 10 mg liter^{-1} chlorine, 21 min for 50 mg liter^{-1} chlorine, 25 min for the quaternary ammonium compound, and 4 min for nisin. These results underscore the distinction between biofilm removal and killing and reinforce the critical role of biocide reactivity in determining the rate of biofilm penetration.

The action of a biocide or antibiotic against microorganisms in biofilms varies in time and space. There is insight to be gained into the phenomena important in this process by watching, through a microscope, the antimicrobial attack. Here we describe the application of a recently developed technique for visualizing antimicrobial action (29) to biofilms formed by *Staphylococcus epidermidis*. We describe distinct behaviors for the four antimicrobial agents examined, which were chlorine, glutaraldehyde, a quaternary ammonium compound (QAC), and an antimicrobial peptide, nisin.

S. epidermidis, a commensal resident of the skin and an opportunistic pathogen, is a common culprit in nosocomial infections (13, 15, 31). In particular, this microorganism is known to form biofilms on indwelling devices such as catheters, prosthetic joints, and contact lenses. There is therefore interest in understanding the efficacy of biocides against biofilms formed by this organism in such applications as control of contamination on hospital countertops and in catheter lock solutions, skin disinfectants, and contact lens storage case disinfection.

Numerous evaluations of biocide activity against *S. epidermidis* biofilms have been reported for such agents as chlorhexidine, hydrogen peroxide, povidone-iodine, alcohols, and chlorine (4, 6, 10, 17, 19, 21, 32). These data support the accepted paradigm that bacteria in biofilms are more difficult to kill than are the same microorganisms when in free aqueous suspension. Analytical techniques such as colony formation assays (plating), regrowth assays, crystal violet staining for total biomass, tetrazolium salt reduction, and ATP content measurement have been employed. These measurements provide an indication of overall efficacy, in terms of either viability reduction or biofilm removal, but do not shed light on fundamental phenomena important in the interaction of the biocide with the biofilm. In other words, there is little or no mechanistic insight that can be gleaned from these conventional testing data.

The technique described in this article affords information about the time scale for penetration of biologically active concentrations of the antimicrobial agent into the biofilm interior, the failure of most of the antimicrobials to induce any release of biomass from the biofilm, the important role of hydrodynamics outside the biofilm in removing biofilm weakened by reaction with chlorine, the lack of correlation of antimicrobial molecular weight with observed penetration time, and the presence of a subpopulation of cells that are less susceptible to an antimicrobial.

MATERIALS AND METHODS

Bacterium and media. *Staphylococcus epidermidis* strain RP62A (ATCC no. 35984) was grown overnight in full-strength tryptic soy broth (TSB) at 37°C in a shaking incubator. This culture was used as an inoculum for the growth of biofilms in capillary flow cells. For planktonic experiments, the overnight culture was subcultured 1:10 into fresh TSB, reincubated for 2 h, and then diluted 1:10 in sterile phosphate-buffered saline (PBS) for staining and further analysis. PBS was an aqueous solution containing 2 μM $MgCl_2$ and 0.31 μM KH_2PO_4, with a pH of 7.2 ± 0.2.

Antimicrobial agents. A balance was used to measure 625 mg of the quaternary ammonium compound (QAC) *n*-alkyl (C_{14}, 50%; C_{12}, 40%; C_{16}, 10%)

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TABLE 1. Antimicrobial agents and their properties

Agent	MW	D_{aq}[a] ($\mu m^2 s^{-1}$)	t_{50} (s)
Hypochlorous acid	50	1,900	3
Glutaraldehyde	100	930	11
QAC	357	427	24
Nisin	3,354	190	55

[a] D_{aq}, diffusion coefficient in water.

dimethylbenzylammonium chloride (Barquat MB-80; Lonza, Fair Lawn, NJ) into a vial. The QAC was diluted by adding 100 ml of sterile PBS to make a stock solution of 5 g liter^{-1}. This stock solution was further diluted 1:100 in sterile PBS to reach the final working concentration of 50 mg liter^{-1} (0.140 mM) QAC.

Solutions of chlorine were prepared from a concentrated, reagent grade sodium hypochlorite (NaOCl) solution containing 10 to 13% available chlorine (Sigma, St. Louis, MO) that was diluted into filter-sterilized PBS to attain a working concentration of 10 mg liter^{-1} (0.28 mM) chlorine or 50 mg liter^{-1} (1.41 mM) chlorine, depending upon the treatment (high or low concentration, respectively). The pH of the treatment solution was 7.2 $\pm$ 0.3.

A reagent grade solution of 50% (wt/wt) glutaraldehyde (Fisher Scientific, Pittsburgh, PA) was diluted by adding 885 μl to 100 ml sterile PBS to make a stock solution of 5 g liter^{-1}. This stock solution was further diluted 1:100 to a working concentration of 50 mg liter^{-1} (0.50 mM) glutaraldehyde.

A powder containing 2.5% (wt/wt) nisin (Sigma-Aldrich, St. Louis, MO) was diluted by adding 0.5 g powder to 5 ml sterile PBS. This stock solution was diluted further into sterile PBS for a final working concentration of 50 mg liter^{-1} (14.9 μM) nisin.

Aqueous diffusion coefficients for hypochlorous acid, glutaraldehyde, and QAC were published estimates (26). The aqueous diffusion coefficient of nisin was taken as the average of the estimates using the Wilke-Chang correlation (16) and the method of Polson (18). The effective diffusion coefficient (D_e) in the biofilm was taken as 50% of the value in water for hypochlorous acid (24) and 30% of the value in water for the other agents (20, 25). The biofilm diffusive penetration time in the absence of reaction or sorption (t_{50}) was calculated from the equation $t_{50} = 0.139R^2/D_e$, where the typical cluster radius (R) was taken as 150 μm.

Properties of the four biocides are summarized in Table 1.

Flow cytometry. Planktonic bacteria were analyzed by flow cytometry. An overnight culture of *S. epidermidis* bacteria was grown in TSB, subcultured 1:10 into fresh, 37°C TSB, and then incubated for an additional 2 to 3 h, which allowed the culture to reach exponential phase (optical density at 600 nm [OD_{600}] = 0.050 $\pm$ 0.005). The exponential-phase bacteria were diluted 1:10 into filter-sterilized PBS for staining with a fluorogenic esterase substrate and subsequent analysis. Calcein-AM (CAM; Invitrogen, Grand Island, NY) in individual vials containing 50 μg was dissolved in 100 μl of dimethyl sulfoxide (DMSO) to make a stock solution. Cells were stained for 1 h by adding 20 μl of this stock solution into 1 ml of cell suspension to give a working concentration of 9.85 μM CAM. Samples were further diluted 1:10 in filter-sterilized PBS and added to special test tubes via filter caps. These test tubes were placed on the flow cytometer for analysis. Measurements were collected after 0, 20, 40, and 60 min of CAM staining. Staining progress and rates were measured using a BD FACSAria cell sorter flow cytometer.

Following CAM staining, the planktonic cells were centrifuged at 4,600 $\times$ *g* for 4 min, the supernatant was removed, and the cells were resuspended in sterile PBS in order to remove any CAM in the bulk fluid. This was analogous to the PBS wash period for biofilm experiments. Samples were taken during this PBS "wash" phase of the experiment (at 0, 20, 40, and 60 min) in order to determine whether cells retain the fluorescent calcein. An antimicrobial agent was then added to the cell suspension, and fluorescence loss was tracked with the flow cytometer in order to determine changes in cellular permeability. During the antimicrobial treatment phase, samples were analyzed at 0, 5, 10, 15, 20, 40, and 60 min. The action time of an antimicrobial agent against planktonic cells, $T_{50\text{-P}}$, was determined as the time for the median fluorescence intensity of the population to fall to one-half of its initial value.

Planktonic viability. Planktonic cell viability was assayed in conjunction with the flow cytometric analysis. Serial dilutions were performed on the planktonic cultures for both an untreated control sample and a biocide-treated sample. These serial dilutions were taken immediately before and after the 1-h treatment period, and the dilutions were enumerated using the drop-plate method on tryptic soy agar (TSA) plates (7). In order to eliminate the presence of residual biocide after the treatment period, 1-ml samples of both the untreated control and biocide-treated cell suspensions were filtered onto polycarbonate membranes which had a 0.22-μm pore size. The filtered samples were rinsed with 5 ml of deionized (DI) water, and the cells were resuspended in 1 ml PBS by vortexing for 1 min. The cells which were removed from the filters were serially diluted and enumerated using the drop-plate method. Efficacy of the 1-h biocide treatment was reported as a log reduction (LR).

Chlorine residual. The concentrations of residual total and free chlorine were measured in triplicate to determine the chlorine demand associated with planktonic bacterial cells. The planktonic cultures were prepared as if they were to be analyzed using flow cytometry by diluting the cells from an exponential-phase culture 1:10 in sterile PBS. This diluted culture was centrifuged at 4,600 $\times$ *g* for 4 min, the supernatant was removed, and the cells were resuspended in sterile PBS. Concentrations of 10 and 50 mg/liter chlorine were added to the cell suspension, and chlorine residuals were measured at several time points. Total and free chlorine measurements were performed spectrophotometrically using *N*,*N*-diethyl-1,4-phenylenediamine sulfate (DPD) chemistry (Hach, Loveland, CO).

Biofilm analysis. The glass capillary biofilm reactor was used for growing *S. epidermidis* biofilms under continuous flow using techniques similar to those previously published (20, 25, 29). This reactor is a glass tube with a square cross section (inside diameter = 0.9 mm) and wall thickness of 0.17 mm, allowing for easy observation under a microscope when attached to the microscope stage with a capillary holder (Biosurface Technologies, Bozeman, MT). A turbid overnight culture of *S. epidermidis* grown in TSB served as the inoculum for the capillary biofilm experiments. The inoculum was statically incubated in the reactor for 4 h at 37°C, allowing time for the bacteria to attach to the capillary walls. After the initial attachment period, 1/10 strength TSB was pumped through the capillary at a rate of 1 ml min^{-1} for 24 h at 37°C. At this flow rate and temperature the Reynolds number was 27.

There were three subsequent phases of staining and observation: stain, rinse, and treat. These steps were performed in the microscope room at an ambient temperature of 23°C. At this temperature the Reynolds number was 20 and the wall shear stress was 0.171 N m^{-2}.

During each of these phases, spatiotemporal patterns of fluorescence in isolated biofilm clusters were observed using a Leica TCS-SP2 AOBS confocal scanning laser microscope (CSLM) and an NPLAN L 20×, 0.40-numerical-aperture (NA) objective. A 488-nm laser was used for excitation, and the detector bandwidth was set to collect CAM emission fluorescence between 497 and 550 nm.

In the first phase, a 24-h biofilm was statically stained with CAM for 1 h by injecting 9.85 μM CAM in PBS to fill the glass tube. After the staining period, the biofilm was rinsed by pumping sterile PBS through the capillary at a rate of 1 ml min^{-1}. The purpose of the rinse phase was to remove excess CAM substrate and allow the intracellular fluorescence to stabilize. In the third phase, an antimicrobial agent was pumped through the capillary at the same flow rate and the spatiotemporal patterns of loss of fluorescence were observed with the CSLM. Images were recorded every 30 s for a total of 1 h for each phase: CAM staining, PBS wash, and antimicrobial treatment. $T_{50\text{-B}}$, was determined as the time required for the fluorescence intensity at the center of a cell cluster to fall to one-half of its initial value.

Image analysis. Digital images captured using the CSLM were analyzed using MetaMorph (MDS Analytical Technologies, Downingtown, PA), a quantitative image analysis program. The average green fluorescence intensity was measured at different locations within each selected biofilm cluster. Average intensity measurements were collected from the periphery, the center, and an intermediate location within biofilm clusters termed the medial region. The regions at the periphery and medial areas of biofilm clusters were square regions with dimensions 8.79 by 8.79 μm, while the single center region had dimensions 17.58 by 17.58 μm. Therefore, the total areas analyzed at the periphery, medial, and center of each cluster were equal. Changes in intensity were normalized by dividing by the initial intensity at the beginning of each 1-h time series of antimicrobial treatment. This normalized intensity (I/I_0) was used to compare values of relative loss of fluorescence during 1-h biocide treatment periods.

Changes in cluster size during antimicrobial treatment were also analyzed using this software. A freeform polygon was traced around the edge of the transmission mode image of the cluster prior to antimicrobial treatment. A threshold was applied to the images, which designated how much biofilm (dark pixels) covered the area enclosed by the freeform polygon region. For each image in the 1-h time series and for each antimicrobial treatment, the cluster area normalized to initial cluster area (A/A_0) was recorded.

TABLE 2. Summary of effects of antimicrobial agents on *S. epidermidis* biofilms

Agent	C_o[a] (mg liter^{-1})	$T_{50\text{-B}}$ (min)	A_{60}/A_0	I_{60}/I_0	n[b]
Control	NA[c]	ND[d]	1.003 ± 0.005	0.954 ± 0.031	5
Hypochlorous acid	10	46 ± 33	0.574 ± 0.348	0.281 ± 0.473	3
Hypochlorous acid	50	21 ± 16	0.096 ± 0.142	0.006 ± 0.015	6
Glutaraldehyde	50	ND	1.009 ± 0.020	0.886 ± 0.103	5
QAC	50	25 ± 15	0.908 ± 0.096	0.030 ± 0.014	4
Nisin	50	4 ± 1	0.919 ± 0.092	0.006 ± 0.001	2

[a] C_o, applied concentration.
[b] n, number of experiments.
[c] NA, not applicable.
[d] ND, not determined, as fluorescence intensity did not reach 50% of initial intensity within the 60-min experimental test.

RESULTS

A method for visualizing the spatial and temporal patterns of antimicrobial action in biofilms, based on loss of an unbound fluorophore loaded into the cytoplasm of bacterial cells, was applied to investigate the behavior of four antimicrobial agents against *S. epidermidis* biofilms. The method detects loss of membrane integrity and is compatible with antimicrobial treatments that cause at least some membrane permeabilization within a 60-min period. This was the case for a QAC biocide, chlorine, and the antimicrobial peptide nisin. The method was not suitable for analyzing the action of glutaraldehyde. Biofilms treated with glutaraldehyde retained intracellular fluorescence, probably because this agent did not permeabilize cell membranes.

In untreated controls, CAM-stained biofilms retained 95% of their initial fluorescence over the 60-min experimental test (Table 2). All biocides except glutaraldehyde caused a loss of fluorescence compared to the untreated control (Table 2). None of the biocides caused non-CAM-stained bacteria to become fluorescent (data not shown).

Distinct spatiotemporal patterns of fluorescence loss were observed for each of the agents investigated (Fig. 1 and 2; see videos in the supplemental material). The antimicrobial action of chlorine was localized around the periphery of biofilm cell clusters. Green fluorescence was lost in a highly stratified zone, approximately 10 to 30 μm deep, at the interface between the biofilm and the bulk fluid (Fig. 1). Chlorine also appeared to weaken the mechanical cohesiveness of the biofilm, causing it to be vulnerable to removal by shear forces from the flowing fluid. This removal phenomenon is discussed in greater depth below. Treatment with QAC resulted in a complex pattern of fluorescence loss. Fluorescence was lost first at the periphery of the cell cluster, and then the antimicrobial action migrated steadily inward. The action of QAC was not as sharply stratified as in the case of chlorine. A secondary pattern superimposed on the penetration dynamic suggested a subpopulation of cells that lost fluorescence much more slowly than the majority of the population. Nisin caused a relatively rapid and uniform loss of green fluorescence from all parts of the biofilm.

Chlorine was the only antimicrobial agent that caused some removal of biomass from the biofilm (Fig. 3). Even with an aggressive chlorine treatment (50 mg liter^{-1}), removal occurred as a slow erosion. The video of this process (see the supplemental material) makes it apparent that a surface layer of fluidized biomass behaves as a viscous fluid, flowing in a slow-moving film over the mechanically intact core of the biofilm. Velocities of this biomass flow were of the order of magnitude of 1 μm per second in comparison to the average bulk fluid flow velocity of 2 cm per second. When the liquefied biomass reached the downstream edge of a cell cluster, it could be stripped from the biofilm and carried away by the flow. Treating biofilms for 60 min with 10 mg liter^{-1} chlorine reduced the area of isolated cell clusters to 57% of their initial area on average (Table 2; Fig. 3). Treatment with 50 mg liter^{-1} for 60 min completely removed some biofilm cell clusters. On average, cell clusters were reduced to approximately 10% of their initial area.

Treatment with glutaraldehyde, QAC, or nisin caused little or no removal from biofilms. In biofilms treated with glutaraldehyde or in the untreated control, the area of cell clusters did not change at all (Table 2). The possible reductions in biofilm cluster area recorded following QAC and nisin treatments (to 91 and 92% of original cluster area, respectively), though not demonstrable with statistical confidence, may have been due to slight contractions of the biofilm. Again, these slight reductions did not appear to involve any release of biomass from the biofilm.

To aid in the interpretation of these results, measurements of viability loss and fluorescence loss in planktonic cells exposed to antimicrobial agents were made. All four of the antimicrobial agents achieved at least a 3 log reduction in viable cell numbers when applied to planktonic bacteria at the same concentration as was used against biofilms (Table 3). The exposure time was 60 min for this challenge. We observed the loss of CAM staining fluorescence in antimicrobial-challenged planktonic cells with flow cytometric analysis. QAC and nisin both caused rapid loss of fluorescence from bacterial cells; the time scale of fluorescence loss was quantified by the parameter $T_{50\text{-P}}$, the time for the median fluorescence intensity of the population to fall to one-half of its initial value (Table 3). In contrast, neither glutaraldehyde nor chlorine treatments induced enough loss of fluorescence to allow determination of $T_{50\text{-P}}$. It appears that QAC and nisin cause a concomitant loss of membrane integrity and viability, whereas glutaraldehyde and chlorine bring about a rapid loss of viability without concurrent permeabilization of the membrane. Prolonged chlorine treatment must eventually result in loss of membrane integrity, because green fluorescence is lost from the perimeter of biofilm clusters.

An additional consideration with chlorine is the transformation of this highly reactive agent. When planktonic *S. epidermidis* cells were incubated with 10 mg liter^{-1} chlorine, the

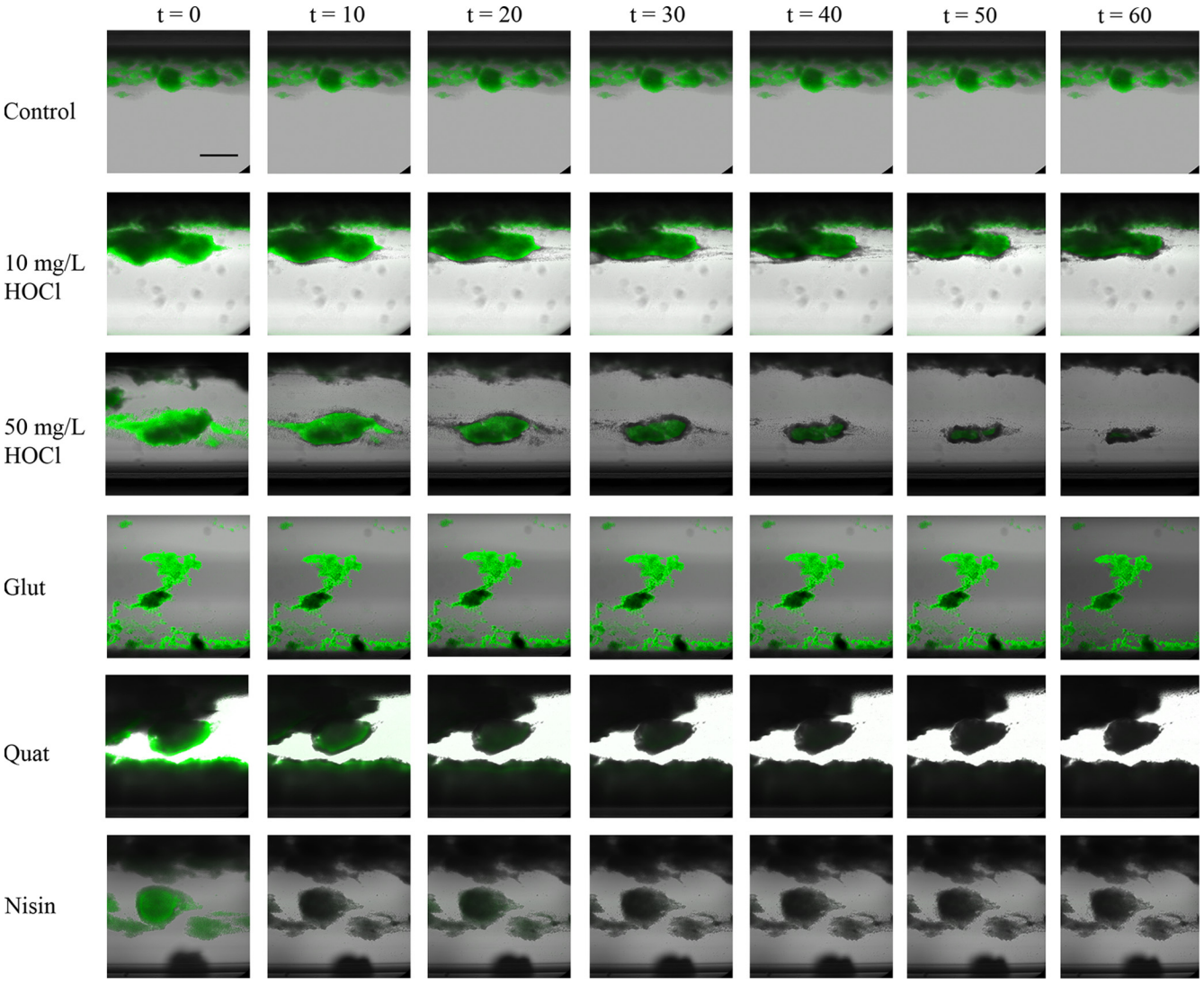

FIG. 1. Visualization of antimicrobial action against *S. epidermidis* biofilms. Biofilm structure and removal are apparent in the transmission images (gray), and disruption of membrane integrity is revealed via loss of CAM staining (green). In each series, the image at far left is the transmission image of biofilm immediately prior to treatment and the image at far right is the transmission image after 60 min of treatment under continuous flow. The intervening panels are overlays of the transmission and green fluorescent channels. Quat, QAC; glut, glutaraldehyde. Scale bar, 200 μm.

concentration of chlorine decreased. At the first time point measured, 15 min, the concentration of total chlorine was 3.3 mg liter^{-1} and the concentration of free chlorine was 0.3 mg liter^{-1}.

The penetration of antimicrobial agents can be inferred from the loss of green fluorescence in the interior of biofilm cell clusters during treatment. We quantified a combined penetration and action time, denoted by $T_{50\text{-B}}$, as the time required, after the onset of antimicrobial exposure, for the fluorescence intensity at the center of a cell cluster to fall to one-half of its initial value. This time was only a few minutes for nisin but was between 21 and 46 min for chlorine and QAC (Table 2). For nisin, the values of T_{50} for biofilm and planktonic cells were similar (Table 3), suggesting that biofilm and planktonic cells have similar intrinsic susceptibilities to this agent. In contrast, QAC permeabilized planktonic cells much faster ($T_{50\text{-P}}$ = 0.6 min) than it did biofilm cells ($T_{50\text{-B}}$ = 25 min). This discrepancy could be due to lower susceptibility of biofilm cells or to retarded transport of the QAC into the biofilm interior. For both QAC and chlorine, the estimated time for penetration by diffusion in the absence of reaction (t_{50} in Table 1) was much shorter than the actual $T_{50\text{-B}}$ measured in biofilms. This large discrepancy, along with the obvious spatial patterns apparent in Fig. 1, suggests that sorption or reaction of the antimicrobial agent in the biofilm retards its access.

Nisin (molecular weight [MW], 3,354) accessed the interior of biofilm cell clusters faster than the smaller QAC (MW, 357) and chlorine (MW, 50) (Fig. 4A). Quicker penetration for the larger agent is exactly opposite of what intuition suggests. This result underscores the importance of the reactivity of the antimicrobial in the biofilm in controlling its penetration. The biofilm penetration time of nisin indicates that this agent is not

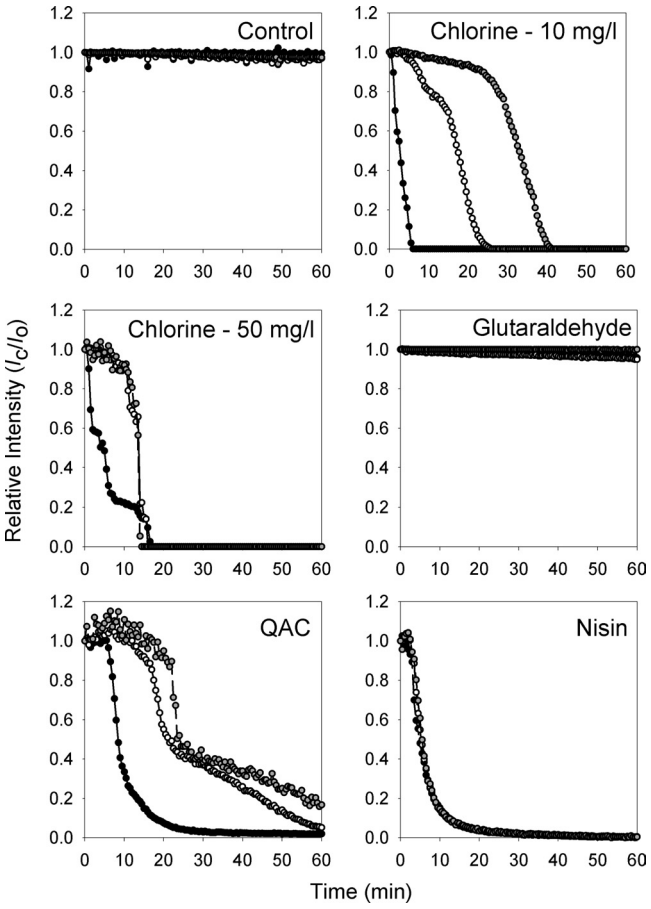

FIG. 2. Image analysis of fluorescence loss in CAM-stained *S. epidermidis* biofilms treated under continuous flow as indicated in each panel. The relative intensity plotted on the *y* axis is the fluorescence intensity at a particular region of the biofilm divided by the initial intensity in that same region. Shown are measurements made near the periphery of the cell cluster (black symbols), a medial location of the cell cluster (open symbols), and the center of the cell cluster (gray symbols).

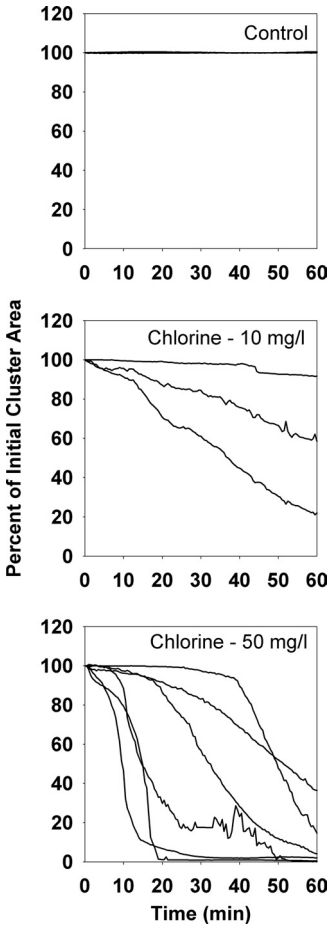

FIG. 3. Erosion of biofilm clusters, as indicated by reduction in cluster area, during treatment with PBS (control), 10 mg liter^{-1} chlorine, or 50 mg liter^{-1} chlorine.

neutralized or bound by the cells or matrix of the biofilm. In contrast, both QAC and chlorine must react with or be sorbed into the biofilm, interactions that retard penetration.

Biofilm penetration time ($T_{50\text{-B}}$) failed to correlate with the size of biofilm cell clusters (Fig. 4B). We noticed, rather, that $T_{50\text{-B}}$ could be correlated with measures of the speed of biocide action at the periphery of cell clusters. For the QAC, there was a delay in antimicrobial action at the periphery of cell clusters that correlated well with the penetration and action time measured at the center for cell clusters (Fig. 5A). We speculate that this delay represents sorption of the QAC by biomass in the capillary and tubing upstream of the imaged cluster. In other words, the amount of upstream biomass was an important factor in determining the time-dependent concentration of QAC at a particular location in the system. For chlorine, the delay in action was not evident, but the rate of fluorescence loss at the periphery was related to $T_{50\text{-B}}$ (Fig. 5B). The rate of the fluorescence loss at the edge of a cell cluster is likely proportional to the local chlorine concentration in the fluid. Here again, the reaction of the antimicrobial with upstream biomass was an important determinant of the local behavior, which also likely contributes to the variation in the erosion rates observed for clusters exposed to chlorine (Fig. 3).

DISCUSSION

The antimicrobial action of chlorine, a QAC, and nisin was visualized within *S. epidermidis* biofilm cell clusters under continuous-flow conditions. The confocal scanning laser micros-

TABLE 3. Summary of effects of antimicrobial agents on planktonic *S. epidermidis*

Agent	C_o[a] (mg liter^{-1})	LR	$T_{50\text{-P}}$ (min)
Control	NA[b]	0.40 ± 0.21	ND[c]
Hypochlorous acid	10	6.26 ± 0.41	ND
Hypochlorous acid	50	6.21 ± 0.55	ND
Glutaraldehyde	50	3.83 ± 0.25	ND
QAC	50	3.61 ± 0.60	0.6 ± 0.0
Nisin	50	5.67 ± 0.86	5.6 ± 8.2

[a] C_o, applied concentration.
[b] NA, not applicable.
[c] ND, not determined as fluorescence intensity did not reach 50% of initial intensity within the 60-min experimental test.

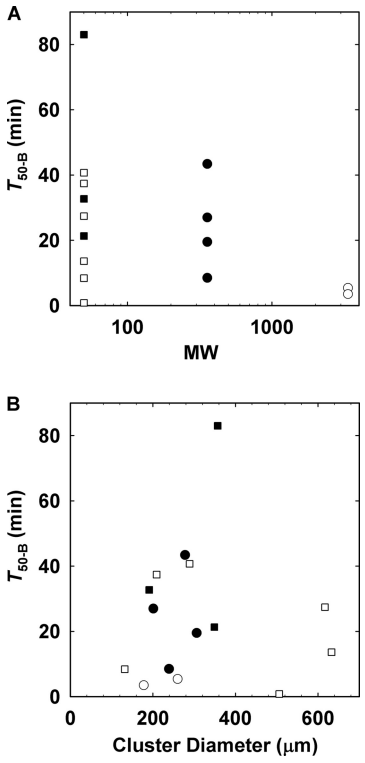

FIG. 4. Biofilm penetration and action time, $T_{50\text{-}B}$, dependence on molecular weight of the antimicrobial agent (A) and biofilm cluster diameter (B). The treatments include 10 mg liter^{-1} chlorine (filled squares), 50 mg liter^{-1} chlorine (open squares), QAC (filled circles), and nisin (open circles). M, molecular size.

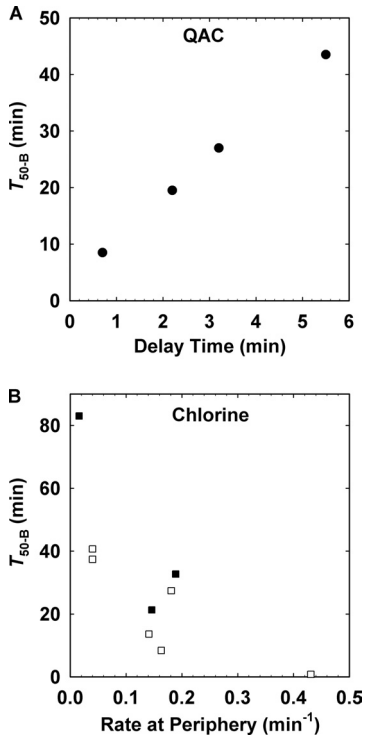

FIG. 5. Biofilm penetration and action time, $T_{50\text{-}B}$, dependence on delay in evidence of antimicrobial action at the periphery of clusters exposed to QAC (A) and dependence on observed rate of antimicrobial action at the periphery for clusters exposed to chlorine (B). Symbols: 10 mg liter^{-1} chlorine, filled squares; 50 mg liter^{-1} chlorine, open squares; QAC, filled circles.

copy technique used allows for the simultaneous imaging of biofilm structure and of loss of cellular membrane integrity. Results from this investigation support the general observation that staphylococci in biofilms are difficult to kill or remove with a wide variety of antimicrobial agents, including those used in this investigation (1, 2, 6, 8, 11, 12, 14, 17, 22, 23, 30). Distinct spatial and temporal patterns of antimicrobial action and biomass removal were observed for these three agents (summarized in Table 4), suggesting that the physical, chemical, and biological phenomena that are important differ from one agent to another. In the following paragraphs, we summarize our interpretation of the behavior of each of the antimicrobial agents when applied to the *S. epidermidis* biofilm.

Chlorine is highly reactive, and its attack on the biofilm was localized around the periphery of the cell cluster. The reaction-diffusion-limited penetration of chlorine into biofilms has been previously described (3, 5, 27, 33) and is known to be important for other reactive oxidizing biocides (9, 28). Cells and exopolysaccharide (EPS) in the surface layer of the biofilm consumed chlorine in neutralizing reactions faster than it could diffuse into the biofilm interior. The time required to achieve antimicrobial action at the center of cell clusters was about 30 min (Table 2), much longer than the 3-s time estimated for diffusive penetration of chlorine in the absence of reaction (Table 1). This represents a retardation factor of about 600, which is consistent with previous analysis of the reaction-diffusion interaction of chlorine in biofilms (3). Cells near the biofilm-fluid interface were killed and permeabilized by the reaction with chlorine. The biofilm matrix around the outside of the cluster was also affected: it was weakened and became fluid. The flow of water around the biofilm caused the weakened biomass to ooze downstream and to be detached. The rate of this process depends on the local chlorine concentration and mass transfer characteristics and is affected by the amount of biomass in the tube and tubing upstream.

Though we did not gain insight into the antimicrobial action of glutaraldehyde against the biofilm, we can conclusively state that glutaraldehyde failed to remove biofilm. This is consistent with the known cross-linking properties of this dialdehyde, which is commonly used as a fixative for biological specimens.

The QAC antimicrobial acted first on cells near the biofilm-fluid interface. The agent then progressively penetrated and acted on more deeply embedded cells. As with chlorine, the penetration was radially symmetric. That is, QAC penetrated on the upstream and downstream edges of the cluster similarly. This observation shows that convective transport within the cell cluster was negligible and that diffusion is responsible for biocide transport within the biofilm. The penetration of QAC was retarded, probably by sorption of the positively charged and hydrophobic biocide to the biofilm. The measured time to penetration to the center of cell clusters of 25 min (Table 2) was much longer than the estimated time for diffusive access in the absence of sorption of 24 s (Table 1). This difference corresponds to a retardation factor of about 60. The biphasic

TABLE 4. Summary of effects of antimicrobial agents on *S. epidermidis* planktonic cells and biofilms

Agent	Effect on planktonic cells	Biofilm penetration	Biofilm removal
Chlorine	Kills rapidly without loss of membrane integrity, slower permeabilization upon continued exposure	Very slow penetration limits antimicrobial action	Progressive erosive removal from biofilm surface
Glutaraldehyde	Kills rapidly, no loss of membrane integrity	Unable to diagnose	None
QAC	Kills rapidly with concurrent loss of membrane integrity	Retarded penetration	None, perhaps slight contraction
Nisin	Kills rapidly with concurrent loss of membrane integrity	Rapid penetration; antimicrobial action not penetration limited	None, perhaps slight contraction

shape of the loss of CAM fluorescence in time, especially noticeable at the centers of cell clusters (Fig. 2), suggests the presence of a tolerant subpopulation. This less-susceptible population constituted approximately 28 to 73% of the population at the cluster centers. No biofilm was removed by QAC treatment.

Nisin rapidly penetrated and permeabilized cells throughout biofilm cell clusters. The fact that the biofilm and planktonic action times were similar (4 min and 6 min, respectively) suggests that nisin action was limited by the intrinsic kinetics of killing and that biofilm and planktonic cells had similar susceptibilities. The estimated penetration time of about 1 min (Table 1) was fast enough to not interfere with the biological action. This interpretation is consistent with the lack of spatial pattern in fluorescence loss during nisin treatment (Fig. 1). No evidence of a nisin-tolerant subpopulation was seen. Nisin caused no removal of biofilm.

The results of this investigation reinforce the distinction between biofilm removal and killing of cells in the biofilm. These processes can occur in parallel, but they are fundamentally different. Nisin was perhaps the most effective antimicrobial in terms of its rapid bactericidal action, but it caused no biofilm removal at all. In contrast, much of the effectiveness of chlorine was due its ability to erode biofilm. Many antimicrobial treatments used to control biofilms may not induce much removal. Even if these treatments produce a drop in viable cell numbers, they may leave most of the dead cells on the surface. With chlorine treatment, biomass removal appeared to depend on fluid shearing of weakened biomass at the periphery of the cell cluster. Increasing the fluid shear during treatment with chlorine could both improve chlorine transport to the biofilm and increase the rate of biomass detachment.

This technique did not afford insight into the antimicrobial action of glutaraldehyde against the biofilm because glutaraldehyde did not permeabilize cells. The CAM loading technique is not compatible with biocides that do not disrupt membrane integrity and may not be suitable for agents that act relatively slowly, such as most antibiotics. Another limitation of this method is that it is not possible to recover bacteria quantitatively from the biofilm in the flow cell. This precludes determination of log reductions in viable cells by plating for the biofilm in this system.

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