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Dynamics of the Action of Biocides in *Pseudomonas aeruginosa* Biofilms^{▽†}

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The biocidal activity of peracetic acid (PAA) and benzalkonium chloride (BAC) on *Pseudomonas aeruginosa* biofilms was investigated by using a recently developed confocal laser scanning microscopy (CLSM) method that enables the direct and real-time visualization of cell inactivation within the structure. This technique is based on monitoring the loss of fluorescence that corresponds to the leakage of a fluorophore out of cells due to membrane permeabilization by the biocides. Although this approach has previously been used with success with various Gram-positive species, it is not directly applicable to the visualization of Gram-negative strains such as *P. aeruginosa*, particularly because of limitations regarding fluorescence staining. After adapting the staining procedure to *P. aeruginosa*, the action of PAA and BAC on the biofilm formed by strain ATCC 15442 was investigated. The results revealed specific inactivation patterns as a function of the mode of action of the biocides. While PAA treatment triggered a uniform loss of fluorescence in the structure, the action of BAC was first localized at the periphery of cell clusters and then gradually spread throughout the biofilm. Visualization of the action of BAC in biofilms formed by three clinical isolates then confirmed the presence of a delay in penetration, showing that diffusion-reaction limitations could provide a major explanation for the resistance of *P. aeruginosa* biofilms to this biocide. Biochemical analysis suggested a key role for extracellular matrix characteristics in these processes.

The control of microbial surface contamination is a major concern in terms of public health. *Pseudomonas aeruginosa* is a Gram-negative bacterium that is well known to be involved in a large number of human infections (14, 30). Numerous outbreaks have been linked directly to its presence on medical equipment (11, 15, 16, 25). The persistence of this bacterium in the environment can be attributed to its ability to form biofilms that increase its resistance to disinfection treatments. Numerous studies have indeed reported the high resistance of *P. aeruginosa* biofilms (compared to their planktonic counterparts) to numerous biocides, including chlorine, quaternary ammonium compounds, and aldehydes (5, 10, 13, 26). Although the precise mechanisms underlying this resistance remain unclear, it appears to be a multifactorial process that is primarily related to the physiological and structural characteristics of the biofilm. It is now generally accepted that biofilms constitute heterogeneous structures that group subpopulations with distinct physiological states and resistance phenotypes (28).

Data on biocide reactivity within these heterogeneous structures could provide a clearer understanding of the mechanisms involved in biofilm resistance and ultimately facilitate the development of new and more efficient treatments. Recently, a noninvasive technique based on confocal laser scanning microscopy (CLSM) was developed and used to investigate spatial and temporal patterns of antimicrobial action in biofilms

formed by Gram-positive strains (8, 29). This method enables the direct visualization of the patterns of loss of fluorescence in biofilms due to the leakage of unbound fluorophores (fluorescent calcein) out of cells after the bacterial membrane has been altered by antimicrobial agents. However, this method is not directly applicable to the study of *P. aeruginosa* because of limitations with respect to fluorescent staining. The principal limitation encountered with the fluorogenic esterase substrate is linked to active dye extrusion out of the cells by efflux pumps, resulting in weak fluorescent labeling (18). During the present study, we adapted the staining procedure to the time-lapse CLSM study of biofilms formed by the Gram-negative strain *P. aeruginosa*. The spatiotemporal action of peracetic acid and benzalkonium chloride was then visualized in the biofilms formed by the reference strain used for the testing of disinfectants (ATCC 15442). The observations were also extended to three *P. aeruginosa* clinical isolates for benzalkonium chloride, with characterization of the exopolymeric matrix and correlation to the kinetic profiles of inactivation obtained for the four strains, in order to shed light on the obstacles encountered by biocides in biofilms.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The results presented here were obtained using *Pseudomonas aeruginosa* ATCC 15442, the reference strain used for the testing disinfectants under the NF EN 1040 (1), and three *P. aeruginosa* clinical isolates provided by the Institute of Microbiology at Lausanne University Hospital (named Laus 3, Laus 16, and Laus 21). Bacterial stock cultures were kept at –20°C in tryptone soy broth (TSB; bioMérieux, France) containing 20% (vol/vol) glycerol. Prior to each experiment, frozen cells were subcultured twice in TSB at 30°C. The final overnight culture was used as an inoculum for the growth of biofilms.

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Antibacterial agents. An oxidizing agent and a quaternary ammonium compound were chosen, and both are widely used in medical environments: peracetic acid (PAA; molecular weight [MW], 76.05; 32% [by weight] in dilute acetic acid [Sigma-Aldrich, France]) and benzalkonium chloride C14 (BAC; MW, 368.04; puriss., anhydrous, $\geq 99.0\%$ [Fluka, France]). The disinfectants were diluted in sterile deionized water to the desired concentrations on the day of the experiment.

Determination of concentrations for biofilm and planktonic cell eradication. The biocide concentrations required to eradicate a biofilm of *P. aeruginosa* ATCC 15442 were evaluated by using a minimal biofilm eradication concentration (MBEC) assay (6) for PAA and BAC. This system consists in a standard, 96-well microtiter plate which has a lid with 96 pegs on which the biofilm can grow. Experimentally, the overnight culture was adjusted to an optical density at 600 nm (OD_{600}) of 0.01 ($\sim 10^6$ CFU/ml) in TSB, and 150 μ l of this culture was transferred into the wells of the microtiter plate before the lid with pegs was replaced (this level of inoculation would enable $\sim 10^8$ CFU/peg after biofilm development). The system was then incubated at 30°C for 24 h to allow biofilm development on the pegs. After incubation, the lid was removed, and biofilms on the pegs were rinsed in 150 mM NaCl. The biofilms were then transferred to microtiter plates containing increasing concentrations of PAA or BAC (200 μ l per well) for exposure to a biocide for 5 min at 20°C. After being rinsed in 150 mM NaCl, the biofilms on the pegs were then transferred to a neutralizing solution (3 g of L- α -phosphatidyl choline, 30 g of Tween 80, 5 g of sodium thiosulfate, 1 g of L-histidine, and 30 g of saponin liter $^{-1}$) in order to halt the action of the biocide (5 min at 20°C). Finally, the lid was transferred to a microtiter plate containing 200 μ l per well of TSB and was then incubated at 30°C for 24 h. After incubation, the MBEC corresponding to the concentration at which regrowth was not observed was determined for each biocide. In parallel, the biofilm population on pegs before the disinfectant challenge was determined by obtaining viable plate counts on tryptic soy agar (TSA; bioMérieux, France). After the biofilms were rinsed in 150 mM NaCl, the pegs were snapped off the lid and transferred to 150 mM NaCl before sonication for 10 min and vortexing for 30 s. The cells recovered were then enumerated on TSA after serial 10-fold dilutions, drop plating, and incubation at 30°C for 24 h.

Eradication concentrations were also evaluated for planktonic cells using a similar protocol in microtiter plates so that biofilm and planktonic susceptibilities to both biocides could be compared. Experimentally, 20 μ l of an adjusted overnight culture (to obtain a final concentration of 10^8 CFU/well in a microtiter plate) was transferred to the wells of a 96-well microtiter plate containing 180 μ l of increasing concentrations of the biocides and then left at 20°C for 5 min. After exposure to the biocide, 200 μ l was transferred to a 24-well microtiter plate containing 1.8 ml of neutralizing agent to stop the action of the biocide, and the plate was then left at 20°C for 5 min. A total of 2 ml of the neutralized suspension was then transferred to 18 ml of TSB and incubated at 30°C for 24 h. After incubation, biocide concentrations leading to the complete eradication of planktonic cells were determined, as previously described for biofilms. Each of these experiments was performed in triplicate.

Biofilm formation for CLSM analysis. Biofilms were grown in a polystyrene 96-well microtiter plates (Greiner Bio-One, France) with a μ Clear base (polystyrene; 190 ± 5 μ m thick), which enabled high-resolution imaging as previously described (4). Briefly, 250 μ l of the final overnight subculture adjusted in TSB to an OD_{600} of 0.01 (10^6 CFU ml $^{-1}$) was added to the wells of the microtiter plate. After 1 h of adhesion at 30°C, the wells were rinsed to eliminate any nonadherent bacteria before being refilled with 250 μ l of TSB. The plate was then incubated for 24 h at 30°C to allow for biofilm development.

Fluorescent labeling. The biofilms were stained with Chemchrome V6 (AES Chemunex, Ivry-sur-Seine, France). Chemchrome V6 is an esterase marker that can penetrate passively into a cell where it is cleaved by cytoplasmic esterases, leading to the intracellular release of fluorescent residues (green fluorescence). Experimentally, the biofilms were rinsed in 150 mM NaCl in order to eliminate any used medium and planktonic cells, and then the wells were refilled with 100 μ l of solution containing Chemchrome V6 (1:100 of commercial solution diluted in Chemsol B16 buffer [AES Chemunex, Ivry-sur-Seine, France]). The microtiter plate was then incubated in the dark at 20°C for 1 h in order to reach fluorescence equilibrium.

Time-lapse CLSM analysis. After fluorescent labeling of the biofilms, the microtiter plates were rinsed to eliminate any excess Chemchrome V6 and then refilled with 100 μ l of Chemsol B16 buffer. The microtiter plate was then mounted on the stage of a Leica SP2 AOBs confocal laser scanning microscope (Leica Microsystems, France) at the MIMA2 microscopy platform (<http://voxel.jouy.inra.fr/mima2>). The CLSM control software was set to take a series of time-lapse image scans (512×512 pixels corresponding to 140×140 μ m) at intervals of 15 s. The biofilms were scanned at 800 Hz using a $63\times$ objective lens

with a 1.4 numerical aperture and a 488-nm argon laser set at 10% of its maximum intensity. These settings had been shown to avoid photobleaching of the sample during preliminary scans performed using distilled water instead of biocides, and they were conserved for all of the time lapse experiments. Emitted fluorescence was recorded within a range of 500 to 600 nm in order to capture V6 Chemchrome green fluorescence. After the launch of the time series images, 100 μ l of PAA at 0.1 or 0.7% and BAC at 1%, respectively (in order to obtain final concentrations of 0.05 or 0.35% for PAA and 0.5% for BAC in the well), were gently added to the well just after completion of the first scan. The biofilms were then scanned every 15 s for 25 min, and all loss of fluorescence within the structure was recorded.

Image analysis. The intensity of green fluorescence was quantified by using the LCS Lite confocal software (Leica Microsystems). The fluorescence intensity was captured at the different time points (every 15 s) from three different square areas (areas 1, 2, and 3) of 100 μ m 2 in the cell clusters. Intensity values were normalized by dividing the fluorescence intensity recorded at the different measurement time points by the initial fluorescence intensity values obtained at the same location.

Application of bacterial destruction models to fluorescence intensity curves. GinaFIT, a freeware add-in for Microsoft Excel developed by Geeraerd et al. (12), was used to model inactivation kinetics. This tool can test nine different types of microbial survival models, and the choice of the best fit depends on five statistical measures (i.e., the sum of squared errors, the mean sum of squared errors and its root, R^2 , and adjusted R^2). During the present study, the “shoulder + log-linear + tail”, “log-linear + tail”, or “log-linear” inactivation models were fit to the fluorescence intensity curves obtained from the CLSM image series during biocide treatment. Two inactivation kinetic parameters were then extracted from this fitting: SL, the shoulder length (min) that corresponded to the length of the lag phase, and k_{max} , the inactivation rate (min $^{-1}$).

Resistance of cells recovered from biofilms or planktonic suspensions. The susceptibilities to PAA and BAC of *P. aeruginosa* ATCC 15442 biofilm cells immediately after biofilm disruption, and of planktonic cells, were evaluated and compared. Experimentally, 24-h-old biofilms were rinsed with distilled water, and attached cells were recovered from the microtiter plate by scraping the bottoms of the wells with tips and aspirating and expelling the suspension at least 10 times. The cells recovered were vortex mixed using glass beads before being washed in 150 mM NaCl after centrifugation (7,000 rpm, 10 min, 20°C) and adjusted to 10^8 CFU ml $^{-1}$ in 150 mM NaCl for the disinfection step. Planktonic cells were harvested from a 24-h-old culture in TSB at 30°C by centrifugation (7,000 rpm, 10 min, 20°C), washed in 150 mM NaCl, and then also adjusted to 10^8 CFU/ml. Biocide susceptibility was then tested according to the protocol of the European standard NF EN 1040 (1). Each experiment was performed in triplicate.

Determination of the sugar and protein contents of the biofilm matrix. The protein and sugar levels of the biofilm matrix were determined for the four strains of *P. aeruginosa*. After development, the biofilms were rinsed in distilled water and recovered from the microtiter plate by scraping the bottoms of the wells with tips and aspirating and expelling the suspension at least 10 times. The biofilm suspension thus recovered was then vortexed for 30 s, sonicated for 5 min to disperse aggregates, vortex mixed again for 30 s, and then centrifuged at 10,000 rpm for 10 min. The supernatants were then filtered at 0.45- μ m-pore-size to remove any remaining bacteria, and the solutions were kept at -20°C until biochemical assays were performed. Protein levels were determined by using the Bradford assay (3) with bovine serum albumin as the standard. Sugar levels were evaluated by using the phenol-sulfuric assay procedure with glucose as the standard (9). Each experiment was performed in triplicate on three separate biofilm extractions.

RESULTS

Resistance of biofilms and planktonic cells to biocides. The PAA and BAC concentrations required to completely eradicate *P. aeruginosa* ATCC 15442 biofilm cells in 5 min were determined by using an MBEC assay. A density of 7.98 ± 0.52 log (CFU/peg) was attained by *P. aeruginosa* ATCC 15442 after 24 h of development. The cell suspension density was adjusted to the same population level in order to determine planktonic cell resistance so that the eradication concentrations could be compared in both states (biofilm and planktonic). The eradication concentrations for planktonic and bio-

TABLE 1. Biocide concentrations required to eradicate planktonic bacteria and biofilms of *P. aeruginosa* ATCC 15442 after 5 min of contact^a

Biocide	Expt	Concn (%)	
		$C_{\text{planktonic}}$	C_{biofilm}
PAA	1	0.01	0.15
	2	0.01	0.15
	3	0.01	0.20
BAC	1	0.05	5
	2	0.05	5
	3	0.05	5

^a The biocide concentrations required to eradicate planktonic bacteria ($C_{\text{planktonic}}$) and biofilms (C_{biofilm}) of *P. aeruginosa* ATCC 15442 after 5 min of contact are presented. The results of three independent experiments are presented for both biocides.

film cells are presented in Table 1 (the three replicates are shown). The results demonstrated a higher resistance of biofilms to biocide treatments compared to planktonic cells. The PAA concentrations required to totally eradicate biofilm cells were 15- to 20-fold higher than those necessary to kill the same amount of planktonic cells. With BAC, total eradication of the biofilm was attained using a biocide concentration that was 100-fold higher than that used for planktonic cells.

Visualization and modeling of biocide action in *P. aeruginosa* biofilms. The action of PAA and BAC in *P. aeruginosa* ATCC 15442 biofilms was visualized by using time-lapse CLSM. During control experiments (treatment with distilled water), we observed a loss of fluorescence of less than 4% $\pm$ 3% of initial fluorescence, after 25 min of treatment. Illustrative experiments showing the spatial and temporal patterns of fluorescence loss in cell clusters treated with 0.5% BAC and 0.05% PAA are presented (Fig. 1 and 2; see also Videos S1 and S2 in the supplemental material). These images represent horizontal sections of the biofilms 0, 5, 10, 15, 20, and 25 min after addition of the biocide. The fluorescence intensity curves presented in Fig. 2 correspond to the intensity recorded at the different areas (areas 1, 2, and 3) indicated in Fig. 1 during

biocide treatments. GInaFIT inactivation models were applied to these experimental data. The “shoulder + log-linear + tail” inactivation model was applied to the fluorescence intensity curves for areas 1 and 2 (R^2 of 0.983 and 0.992, respectively), and the “log-linear + tail” inactivation model was applied to the curve for area 3 ($R^2 = 0.992$) under BAC treatment. The “log-linear” model was applied to the curves for the three areas under PAA treatment ($R^2 > 0.971$). Different patterns of fluorescence loss were observed as a function of the biocide used (Fig. 1 and 2). PAA treatments caused a homogeneous loss of fluorescence within the cell clusters. Indeed, the application of 0.05% PAA caused a simultaneous reduction in fluorescence in all layers of the cell cluster as from the beginning of treatment (SI = 0 min) (Fig. 2A). The inactivation rates ranged from 0.06 to 0.09 min^{−1}. Treatment with 0.35% PAA led to an immediate and uniform loss of fluorescence in the cell cluster. The mean inactivation rate in the center of cluster was thus very high (mean $k_{\text{max}} = 14.9$ min^{−1}), as shown in Table 2.

We found that the application of BAC led to a nonhomogeneous loss of fluorescence within the structure. Cells at the cluster periphery (area 3 in the white square) started to be inactivated immediately after application of the biocide (SI = 0 min), whereas cells located in the intermediate area (area 2 in the gray square) and in the center of the cluster (area 1 in the black square) were steadily inactivated during treatment (SI of 7.6 and 12.0 min, respectively) (Fig. 2B). Inactivation rate k_{max} values were between 0.37 min^{−1} in the intermediate region and 0.51 min^{−1} at the periphery of the cluster. It should be noted that few cells remained fluorescent throughout the structure after 25 min of treatment (Fig. 1 and Video S1 in the supplemental material).

These results showed that, depending on the biocides used, the spatiotemporal patterns of biofilm inactivation differed. We then investigated the action of BAC (the biocide with which we had observed a nonuniform activity pattern in the structure of *P. aeruginosa* ATCC 15442 biofilm) in different biofilm structures formed by the clinical *P. aeruginosa* isolates Laus 3, Laus 16, and Laus 21. The results of illustrative exper-

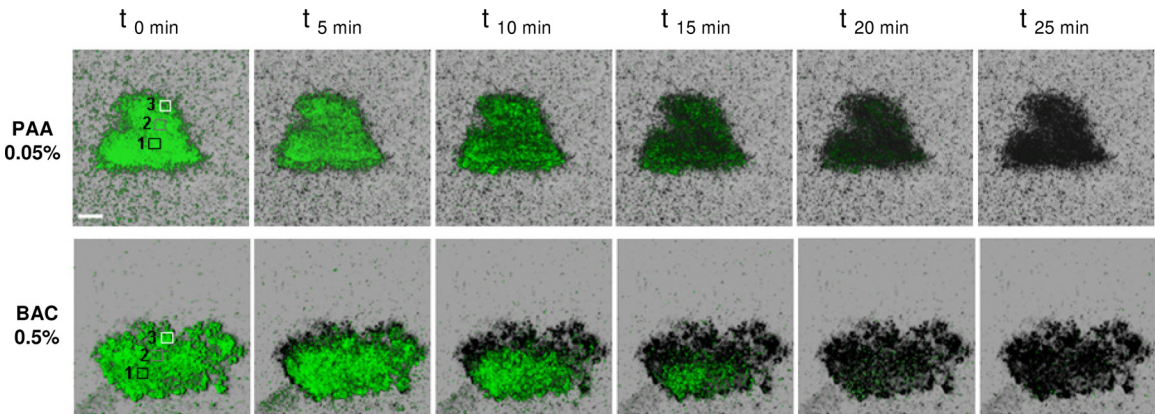


FIG. 1. Visualization of Chemchrome V6 fluorescence loss (cell membrane permeabilization) in *P. aeruginosa* ATCC 15442 biofilms during treatments with PAA and BAC biocides after 0, 5, 10, 15, 20, and 25 min of application. Each image corresponds to the superimposition of green fluorescence images on grayscale images of the initial fluorescent at the same location. Images were recorded ~5 μm above the bottom of the well. Three squares are indicated to represent area 1 (black square in the center of the cluster), area 2 (gray square in the intermediate region), and area 3 (white square at the periphery). Scale bar, 20 μm .

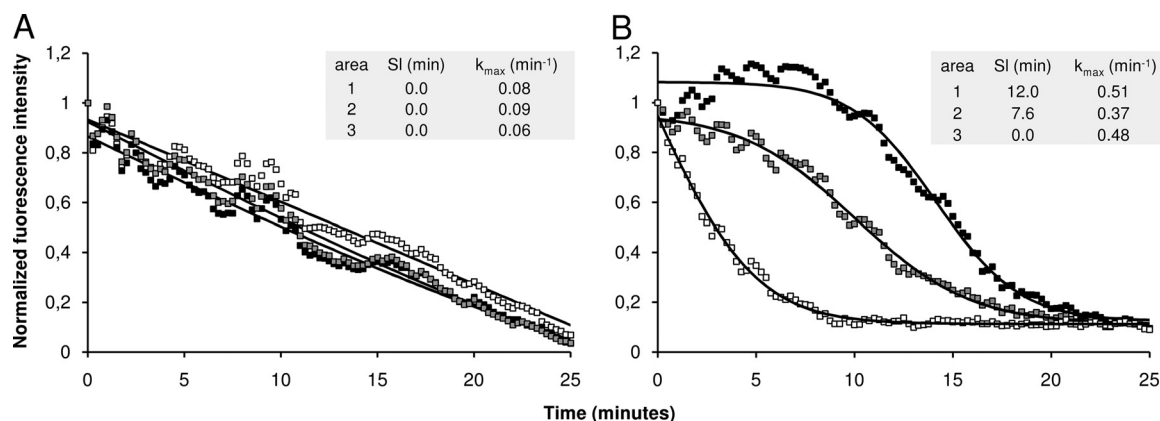


FIG. 2. Quantification of fluorescence intensity during biocide treatments. The values shown represent the loss of fluorescence at three different areas: 1 (■), 2 (□), and 3 (○) in the biofilm cluster under treatment with 0.05% PAA (A) and 0.5% BAC (B). Two inactivation parameters, SI (shoulder length) and $k_{\max}$ (inactivation rate), were obtained after fitting GInaFIT inactivation models (solid lines) to experimental data and are represented for each area.

iments are presented in Fig. 3, and videos are also available (see Videos S3, S4, and S5 in the supplemental material). The mean inactivation parameters, SI and $k_{\max}$, at the center of cell clusters were also determined by fitting GInaFIT inactivation models to experimental data and are shown in Table 2 for the different strains. The results revealed a variety of spatial and temporal inactivation patterns, depending on the strain. BAC activity was first localized at the periphery of the cluster of Laus 3 and Laus 21 strains and then gradually migrated toward the inner layers, as previously observed with *P. aeruginosa* ATCC 15442. However, the inactivation parameters differed between strains. With Laus 3, antimicrobial activity migrated rapidly to the center of the cluster (mean SI of 3.2 min), and the mean inactivation rate $k_{\max}$ of 4.0 min^{-1} was relatively high compared to that obtained with strain ATCC 15442 (mean $k_{\max} = 1.6 \text{ min}^{-1}$). Antimicrobial activity more slowly attained the center of Laus 21 cell clusters, there being a noticeable delay of 11.6 min after biocide application, and the inactivation rate was very low (mean $k_{\max} = 0.2 \text{ min}^{-1}$). A different pattern of fluorescence loss was observed with the Laus 16 strain. Treatment with BAC led to a stretching of the biofilm and a uniform loss of fluorescence from all parts of the biofilm, from the start of treatment (mean SI of 0.8 min^{-1}). After approximately 8 min of treatment, the loss of fluorescence became more rapid at the periphery of the cluster and then steadily reached the center of the cell cluster (Fig. 3 and see Video S5

in the supplemental material). The mean $k_{\max}$ value was similar to that obtained with Laus 3 (Table 2).

Involvement of the biofilm matrix in resistance to biocides.

In order to determine the role of the matrix in biofilm resistance to biocides, the susceptibilities of *P. aeruginosa* ATCC 15442 cells recovered from a biofilm immediately after washing or from a planktonic suspension were compared. Log reductions of 2.7 ± 0.2 and 2.8 ± 0.3 were obtained for planktonic and biofilm cells, respectively, when they were exposed for 5 min to 5 ppm of PAA. Exposure to 5 ppm of BAC for 5 min led to log reductions of 3.8 ± 0.2 and 3.9 ± 0.1 for planktonic and recovered biofilm cells, respectively. These cells did not therefore display any significant differences in terms of their resistance to PAA and BAC ($P > 0.05$), suggesting a major role for the three-dimensional structure and exopolymeric matrix in the resistance of *P. aeruginosa* biofilms to these biocides.

The sugar and protein contents of the biofilm exopolymeric matrix of *P. aeruginosa* ATCC 15442 and the three clinical isolates Laus 3, Laus 16, and Laus 21 were then determined by using biochemical assays. The results presented in Fig. 4 show that the biofilm of the Laus 21 clinical isolate was clearly characterized by a higher protein content ($88 \mu\text{g}/\text{well}$) than in the three other strains (ranging from 52 to $55 \mu\text{g}/\text{well}$) ($P < 0.05$). We also found that the biofilms of strains ATCC 15442 and Laus 21 displayed higher sugar contents than the Laus 3 and Laus 16 strains ($P < 0.05$).

TABLE 2. Inactivation parameters for biocides in the internal areas of cell clusters of the four *P. aeruginosa* strains^a

Strain	Biocide	C_{biocide} (%)	No. of expts	Mean $\pm$ SEM		
				SI (min)	$k_{\max}$ (min^{-1})	R^2
ATCC 15442	PAA	0.05	4	0.3 ± 0.6	0.4 ± 0.5	0.973 ± 0.028
	PAA	0.35	2	0.0 ± 0.0	14.9 ± 1.1	0.983 ± 0.015
	BAC	0.5	4	7.3 ± 3.7	1.6 ± 0.9	0.990 ± 0.004
Laus 3	BAC	0.5	3	3.2 ± 1.7	4.0 ± 4.3	0.961 ± 0.005
Laus 16	BAC	0.5	3	0.8 ± 1.3	4.6 ± 4.0	0.981 ± 0.014
Laus 21	BAC	0.5	3	11.6 ± 4.3	0.2 ± 0.1	0.970 ± 0.004

^a SI (shoulder length) and $k_{\max}$ (inactivation rate) values were obtained after fitting GInaFIT inactivation models to experimental fluorescence intensity data. C_{biocide} , biocide concentration.

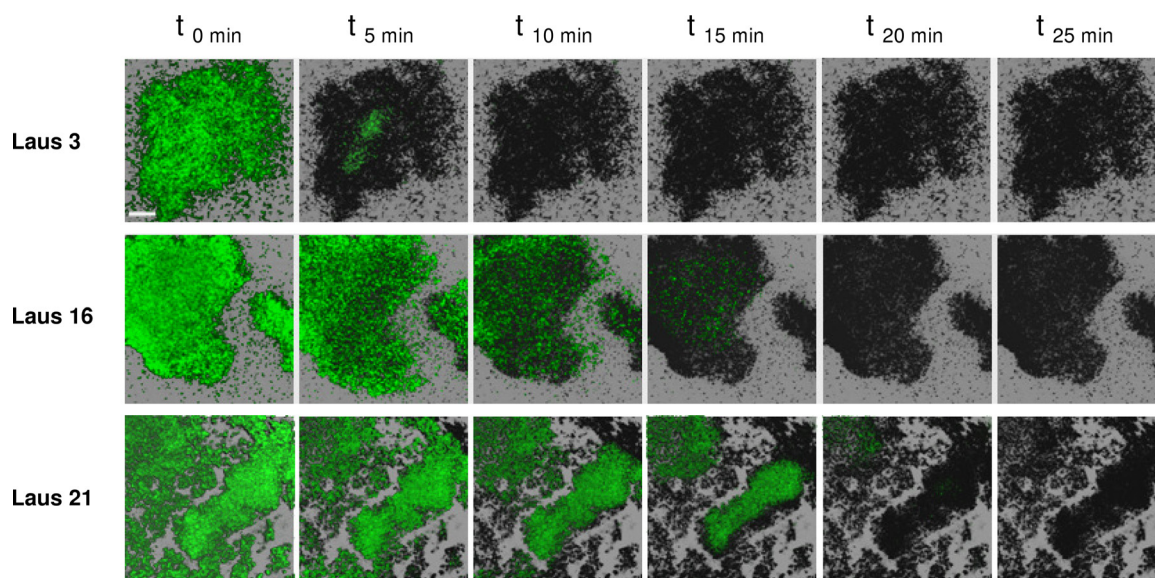


FIG. 3. Visualization of Chemchrome V6 fluorescence loss (cell membrane permeabilization) in *P. aeruginosa* clinical isolate biofilms during BAC treatments after 0, 5, 10, 15, 20, and 25 min of application. Each image corresponds to the superimposition of green fluorescence images on grayscale images of the initial fluorescence at the same location. Scale bar, 20 μ m.

DISCUSSION

Biofilms are well known to display a high degree of resistance to antibiotic and biocide treatments (17, 21, 32). In agreement with previous studies (5, 10, 13, 26), we confirm here that *P. aeruginosa* biofilm cells displayed resistance to PAA (an oxidizing agent) and more markedly BAC (a quaternary ammonium compounds) that was greater than that of their planktonic counterparts. It is now generally recognized that biofilms are heterogeneous structures (23, 28) and that the appearance of specific biofilm functions such as resistance to antimicrobial agents is intimately related to the inherent three-dimensional organization of cells and exopolymeric matrix and

results from multifactorial processes. The development of tools for the *in situ* investigation of antimicrobial activity within biofilms at a single cell level while taking account of local heterogeneity is thus essential to gain an understanding of the limitations of these treatments to develop new and more efficient strategies. A time-lapse CLSM method was recently developed and used to investigate the spatial and temporal patterns of antimicrobial activity in a biofilm formed by *Staphylococcus epidermidis* alone and a mixed biofilm of *Streptococcus oralis*, *Streptococcus gordonii*, and *Actinomyces naeslundii* (8, 29). During these studies, the bacteria were stained first by incubating the cells with calcein-AM. This fluorogenic esterase substrate penetrates passively into cells, where it is cleaved by cytoplasmic esterases, causing the release of fluorescent residues and thus triggering cell fluorescence. The inactivation of cells in the biofilm was then visualized by monitoring the fluorescence loss that corresponded to the leakage of fluorophores outside the cells once the biocide had permeabilized the membrane. Although this technique had been shown to be well suited to the study of some Gram-positive species, it is not directly applicable to studying other species, mainly because of limitations to fluorescent labeling. Indeed, one of the first requirements of this technique is that fluorescent residues must remain trapped in the cells if the membrane is not compromised. However, in Gram-negative strains, and particularly *Pseudomonas* sp., intense efflux pump activity can lead to the release of fluorescent residues from the cells, so that a stable and intense level of intracellular fluorescence cannot be achieved (18).

During the present study, we used the Chemchrome V6 esterase marker/Chemsol B16 staining buffer kit, which can block efflux pump activity and thus maintain fluorophores inside the cells (18). This staining proved to be stable for several hours with *P. aeruginosa* and was successful with other Gram-negative species such as *Salmonella enterica*. In addition, the levels of biofilm inactivation achieved by CLSM agreed well

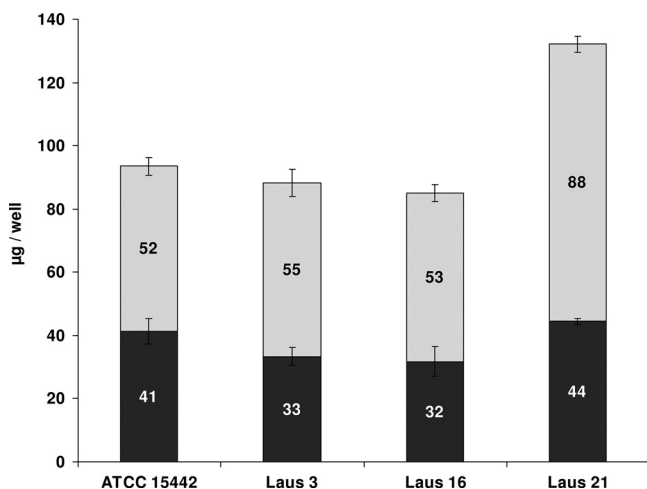


FIG. 4. Sugar (black bars) and protein (gray bars) levels in the biofilm of the four *P. aeruginosa* strains. Values (μ g/well) correspond to the mean of three independent experiments and are shown inside the bars. Error bars represent the standard deviations.

with those obtained using the plate count method (data not shown).

Using time-lapse CLSM combined with Chemchrome V6/Chemsol B16 staining, the action of PAA and BAC in *P. aeruginosa* biofilms was thus investigated. Different patterns of fluorescence loss were observed as a function of the biocides used, thus illustrating the specificity of action and limitations of each compound. PAA caused a uniform and linear loss of fluorescence in cell clusters of *P. aeruginosa* ATCC 15442, suggesting that the greater resistance of the biofilm compared to planktonic cells observed here could not be due to limitations affecting penetration of the biocide into the biofilm, as previously reported in the case of *P. aeruginosa* with other oxidizing agents such as chlorine or hydrogen peroxide (7, 27, 31). Nevertheless, even though PAA was able to diffuse inside the clusters, the biocidal compounds may partly have been consumed through quenching reactions with exopolymeric substances, leading to the greater biofilm resistance observed. In line with this, we observed that disruption of the biofilm and the washing of cells enabled the recovery of the same susceptibility as that observed for planktonic cells; this finding was consistent with the fact that biofilm resistance appeared mainly to be due to the presence of the exopolymeric matrix. The efficacy of oxidizing agents is indeed well known to be profoundly affected by the presence of organic materials such as the constituents of the biofilm matrix (polysaccharides, proteins, and nucleic acids) (2, 19, 22). In addition, the presence of protective enzymes such as catalases in the extracellular matrix has also been reported to be involved in the resistance of *P. aeruginosa* biofilms to oxidizing agents (27).

In contrast, BAC treatment caused a nonuniform loss of fluorescence in *P. aeruginosa* ATCC 15442 biofilms. Cells in peripheral layers were inactivated first, and then the action of the biocide spread steadily into the cluster structure. This gradual inactivation of the structure, together with the fact that disruption of the three-dimensional biofilm structure and elimination of the matrix led to a recovery of biocide efficiency, suggests that BAC encountered obstacles to penetration within the cluster, probably caused by interactions with biofilm components. In a recent study, Davison et al. (8) estimated that the time required for diffusive access in the absence of a reaction or sorption was 24 s for quaternary ammonium compounds (MW, 357) in a cell cluster with a diameter of $\sim 150\ \mu\text{m}$. Under our experimental conditions, the cell cluster diameters were smaller (80 to $120\ \mu\text{m}$), and the mean time before fluorescence decreased within the clusters (SI) under treatment with 0.5% BAC (MW, 368.02) was more than 7 min for *P. aeruginosa* ATCC 15442 (Table 2). The involvement of hydrophobic and/or charge interactions in barriers to the penetration of quaternary ammonium compounds has indeed already been proposed with respect to the biofilms formed by different strains, including *P. aeruginosa* (5, 8, 24). Another explanation for the resistance of an ATCC 15442 biofilm to BAC is that few cells remained alive at different areas in the cluster, despite the apparent penetration of the biocide after 25 min of treatment (Fig. 1 and see the videos in the supplemental material). These cells may have been located in areas difficult for the biocides to attain; for example, the cells may have been located in areas protected by a large quantity of matrix and other cells. In addition, it cannot be excluded that these few cells expressed

highly resistant phenotypes throughout physiological adaptations, e.g., persisters (20), or throughout genetic mutations.

Interestingly, visualization of the action of BAC in biofilms formed by clinical *P. aeruginosa* isolates also revealed patterns of inactivation that confirmed the existence of transport limitations and suggested that the restricted penetration of BAC into biofilms might be one of the key processes explaining the resistance of *P. aeruginosa* biofilms to this biocide. The characterization and comparison of the sugar and protein contents in the biofilms of the four *P. aeruginosa* strains supported the idea that the exopolymeric matrix plays a key role in these transport limitations. We observed that the biofilm of the Laus 21 clinical isolate, in which a high delay of BAC penetration was recorded, was characterized by a larger quantity of matrix than that of other strains, mainly due to a high protein content. Moreover, biofilms of Laus 3 and Laus 16 were characterized by the lowest sugar levels, which were associated with a more rapid penetration of BAC into biofilms compared to the ATCC 15442 and Laus 21 biofilms (see the kinetic inactivation parameters in Table 2). It should also be noted that the speed of penetration did not seem to be directly related to the size of cell clusters (Fig. 3). The diversity of the composition and density of biofilm matrix are thus more likely to explain the differences in BAC inactivation dynamics between the strains analyzed.

In conclusion, we adapted the time-lapse CLSM visualization and modeling of biocide action to biofilms formed by the Gram-negative pathogen *P. aeruginosa*. The dynamics of biocidal action thus recorded made it possible to identify mechanisms involved in biofilm resistance, such as spatial diffusion and/or reaction limitations. These local molecular processes need to be taken into account in the development of innovative and efficient strategies for biofilm control.

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