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Tolerance of dormant and active cells in *Pseudomonas aeruginosa* PA01 biofilm to antimicrobial agents

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***Objectives*: The aim of the study was to determine the susceptibility of active and dormant cell populations from *Pseudomonas aeruginosa* biofilms to non-antibiotic antimicrobial agents such as chlorine, hydrogen peroxide and silver ions in comparison with antibiotics.**

***Methods*: Active cells in colony biofilm were differentially labelled by induction of a green fluorescent protein (GFP). Active and dormant cells were sorted in phosphate buffered solution by flow cytometry. Reductions in viability were determined with plate counts.**

***Results*: The spatial pattern of metabolic activity in colony biofilm was verified, and the active and dormant cells were successfully sorted according to the GFP intensity. Active cells had bigger cell size and higher intracellular density than dormant cells. While dormant cells were more tolerant to tobramycin and silver ions, active cells were more tolerant to chlorine. Metabolically active cells contain denser intracellular components that can react with highly reactive oxidants such as chlorine, thereby reducing the available concentrations of chlorine. In contrast, the concentrations of silver ions and hydrogen peroxide were constant during treatment. Aerobically grown stationary cells were significantly more tolerant to chlorine unlike other antimicrobial agents.**

***Conclusions*: Chlorine was more effective in inactivation of metabolically inactive dormant cells and also more effective under anaerobic conditions. The high oxidative reactivity and rapid decay of chlorine might influence the different antimicrobial actions of chlorine compared with antibiotics. This study contributes to understanding the effects of dormancy and the presence of oxygen on the susceptibility of *P. aeruginosa* biofilm to a wide range of antimicrobial agents.**

Keywords: antimicrobial susceptibility, chlorine, silver ion, antibiotics

Introduction

Biofilms are known to be more tolerant to antimicrobial agents than rapidly growing planktonic cells.[1,2] There are several hypotheses to explain the strong antimicrobial tolerance of biofilm cells such as the limitation of agent penetration,[3] the existence of dormant cells,[4–7] phenotypic variations,[8] a quorum sensing system[9–11] and multidrug efflux pumps.[12] Among these factors, the presence of dormant cells in biofilm has been reported as the major reason for its antimicrobial tolerance. The concentrations of nutrient and oxygen vary with the depth of biofilm and the altered microenvironment in biofilm causes the formation of dormant cells which have slow metabolic processes and no cell division.[4] The antibiotic tolerance of dormant cells in biofilm has been widely studied. It has been shown that most antibiotics are less effective for dormant cells[4,13,14] and oxygen limitation increases the antibiotic tolerance of biofilm.[14–16] Because dormant cells have little metabolic activity, including cell-wall synthesis and translation, the targets of antibiotics might be less susceptible in dormant cells compared with metabolically active cells. Although non-antibiotic antimicrobial agents such as chlorine are widely used for controlling biofilms, the susceptibility of dormant and active cells in biofilm to non-antibiotics is not well-understood. It is supposed that antibiotics and non-antibiotic antimicrobial agents might exert different effects on dormant and active cells depending on their antimicrobial actions. For example, the principally affected components of biofilm cells were related to the cell membrane integrity in the case of treatment with oxidizing agents and respiratory activity in the case of antibiotic treatment.[17] Therefore, in this study, we examined the

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antimicrobial tolerance of dormant and active cells in biofilm to non-antibiotic antimicrobial agents such as chlorine, hydrogen peroxide and silver ions compared with antibiotics. In addition, we investigated the influence of oxygen limitation on the susceptibility of cells to a broad range of antimicrobial agents.

Materials and methods

Bacteria and biofilm culture

Pseudomonas aeruginosa PA01(pAB1) colony biofilm was prepared as described previously.[14] PA01(pAB1), containing a plasmid with an isopropylthio-β-D-galactoside (IPTG) inducible green fluorescent protein (GFP) gene, was cultured overnight in Luria–Bertani (LB) broth with 100 mg/L carbenicillin at 37°C. The overnight culture was diluted to an optical density of 0.10 (at 600 nm) and one 5 μL drop of diluted solution was used to inoculate polycarbonate membranes (25 mm, 0.22 μm pore size; Fisher Scientific) on LB agar plates. The plates were inverted and incubated at 37°C, and the membranes were transferred to a fresh LB plate every 24 h and were grown for 48 h. In order to induce the GFP in PA01(pAB1) biofilm, the membranes were transferred to an LB plate containing 1 mM ITPG for an additional 4 h.

Cryoembedding and sectioning

In order to observe the protein synthesis activity in biofilm, colony biofilms were cryoembedded and cryosectioned. Membrane-supported biofilms were placed on a stainless steel coupon and the colony was covered with Tissue-Tek O.C.T. compound (VWR Scientific Products, USA). The coupon was placed in the cryo-chamber until the media froze and the edge of the membrane was trimmed. The embedded colony was sectioned into 5 μm sections using a cryotome (Microm HM505E, Germany). Sections were placed on glass slides and observed by fluorescence microscopy (Nikon Eclipse 80i, Japan).

Chemicals and analytic methods

Chlorine, hydrogen peroxide and silver ions were selected as non-antibiotic antimicrobials, and tobramycin and ciprofloxacin were chosen as general antibiotics for *P. aeruginosa*. Chlorine, hydrogen peroxide and silver ions were selected because they were the most widely used antimicrobials and have different antimicrobial mechanisms. It has been shown that chlorine is a strong oxidant;[18] and hydrogen peroxide has a relatively low oxidative reactivity;[18] and the major antimicrobial mechanism of silver is the interaction with cellular thiol groups rather than oxidation.[19] Chlorine and hydrogen peroxide (H_2O_2) were prepared by dilution of NaOCl (~10%) and 30% H_2O_2 solution, respectively. Silver nitrate salt was dissolved for a silver ion solution. Antibiotics were obtained by dissolving tobramycin sulphate and ciprofloxacin. Neutralizing reagents used were 100 mM sodium thiosulphate for chlorine, and 14.6% sodium thiosulphate and 10% sodium thioglycolate solution for silver ions.[17] All chemicals were obtained from Sigma-Aldrich and all solutions were prepared with Milli-Q water (Millipore, USA).

The chlorine concentration was measured by the DPD (*N*,*N*-diethyl-*p*-phenylenediamine) method using a portable spectrophotometer DR/2010 (HACH Co., USA). The hydrogen peroxide concentration was measured using the DMP (2,9-dimethy-1, 10-phenanthroline) method,[20] and the concentration of silver ions was determined as described elsewhere.[21] The organic carbon concentration of solutions was determined with a total organic carbon (TOC) analyser (Sievers Instrument, USA).

Flow cytometry

PA01(pAB1) colony biofilms were disaggregated and sorted into active and dormant cells by flow cytometry. Membranes with colony biofilm were placed in PBS (pH 7.2) and disaggregated by 1 min of sonication and 1 min of vortexing. Suspended cells were diluted to the range of 10^7 cfu/mL (0.01 optical density at 600 nm) and sorted by a Fluorescence Activated Cell Sorter (FACS Aria, Becton Dickinson, USA) with 15 000 events per second in the single cell mode. In order to maintain physiological states of cells, FACS was finished within 1 h. Cells were collected in PBS, and not LB, solutions in order to avoid the resuscitation of dormant cells to the active state. Solutions were kept at around 10°C to avoid the risk that metabolic activity of active cells might decrease at lower temperatures such as 4°C.

Determination of antimicrobial tolerance

Active and dormant cells sorted by FACS were diluted with PBS and treated with antimicrobial agents as soon as possible. An initial microbial population of PBS solution was ~10^6 cfu/mL. Cells were treated with chlorine (0.2 and 0.5 mg/L; 3 and 7 μM), hydrogen peroxide (3 and 30 mg/L; 88 and 882 μM), silver nitrate (10 mg/L; 169 μM) or antibiotics, including tobramycin sulphate (10 mg/L; 23 μM) and ciprofloxacin (1 mg/L; 3 μM). The selected concentration of each antimicrobial corresponded to the concentration required for 2 ± 1 log reduction of sorted biofilm cells for 30 min.

After treatment, 1 mL of sample was quenched with 20 μL of neutralizing reagents (100 mM sodium thiosulphate for chlorine and H_2O_2, 14.6% sodium thiosulphate and 10% sodium thioglycolate for silver ions, distilled water for antibiotics). Disinfection efficiencies were evaluated by plate counting. The level of inactivation was expressed as the $\log_{10}$ reduction calculated using the pre-treatment control as a reference. All antimicrobial experiments were repeated at least three times. Student's *t*-test (two-tailed) was performed to determine statistical significance. A P value of <0.05 was interpreted to indicate a significant difference.

Aerobically or anaerobically cultured cells

In order to examine the effect of oxygen concentration on antimicrobial efficiency, stationary phase cells were prepared under aerobic and anaerobic conditions. Aerobically cultured cells were prepared as follows. Cells were grown in LB overnight and diluted at 1:100 with fresh LB then grown for 24 h under aerobic conditions. In order to prepare anaerobically cultured cells, overnight cultured cells were diluted at 1:100 with LB broth bubbled with nitrogen gas for 10 min and sealed with mineral oil (Sigma, USA) then grown for 24 h. The optical density of aerobic or anaerobic culture solution was monitored in order to confirm the stationary phase of cells. Cells grown under aerobic conditions reached a stationary phase after 16 h (1.5 as optical density at 600 nm) and those grown under anaerobic conditions reached the stationary phase after 20 h (1.0 optical density at 600 nm). Anaerobically cultured cells were sampled using a syringe because of the upper layer of mineral oil. Cultured cells were centrifuged and washed with PBS twice and diluted to around 10^6 cfu/mL, and then used for antimicrobial experiments. The oxygen concentration was measured as 5 mg/L in fresh LB, 2.5 mg/L in 24 h aerobic culture LB and below 0.5 mg/L under nitrogen bubbling LB by dissolved oxygen meter (YSI Co., USA).

For antimicrobial experiments, cells were treated with chlorine (0.1 and 0.2 mg/L; 1.5 and 3 μM), hydrogen peroxide (3 and 30 mg/L; 88 and 882 μM), silver nitrate (2 mg/L; 34 μM) and tobramycin sulphate (2 mg/L; 4.6 μM). The concentration of each

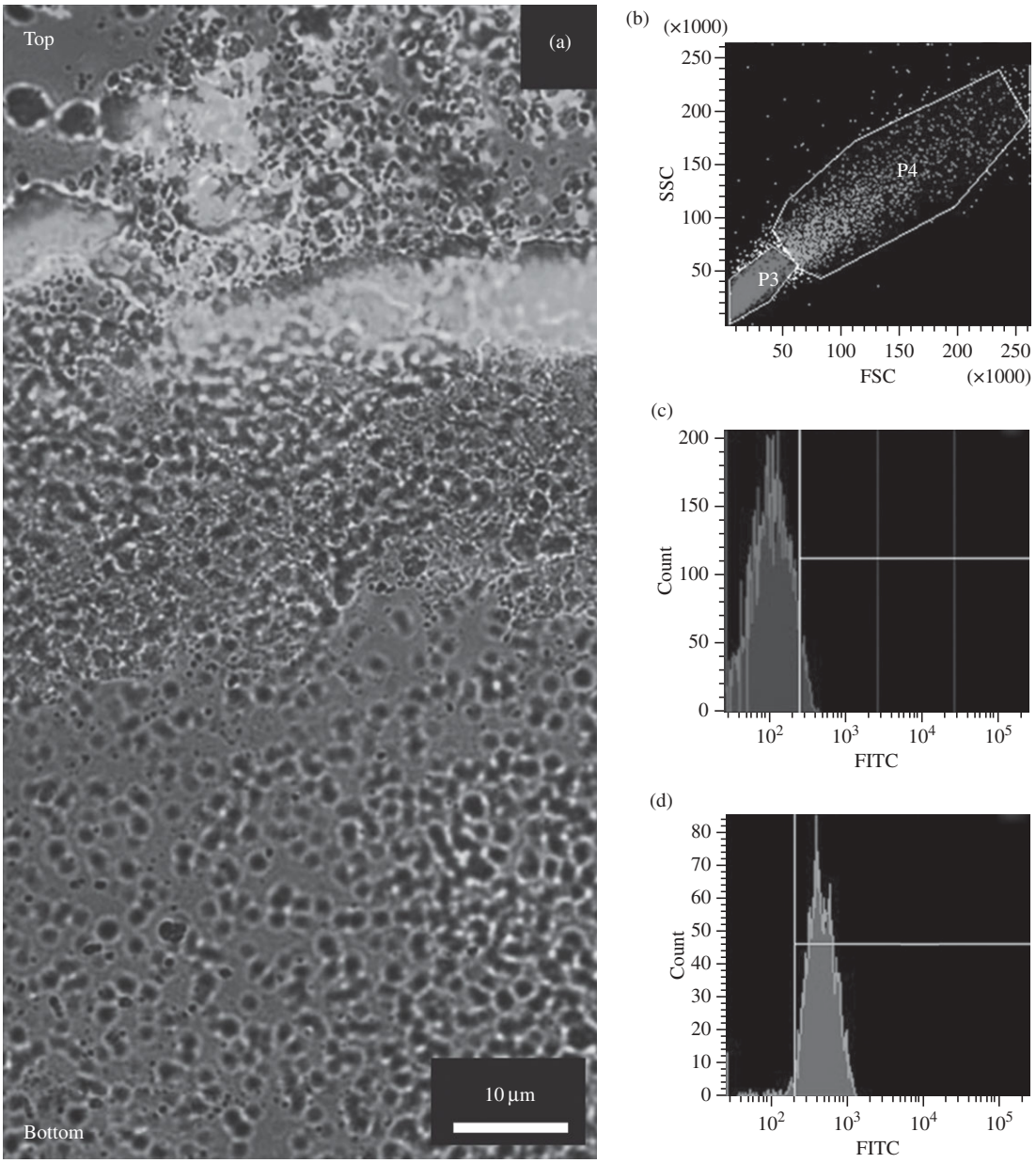

Figure 1. (a) Fluorescent and transparent merged image of cryosectioned PA01(pAB1) biofilm grown for 48 h, followed by GFP induction for 4 h by 1 mM IPTG. Cells in top area, but not the bottom area, of the colony biofilm expressed GFP. (b–d) FACS analysis diagrams of induced biofilm cells. (b) SSC–FSC diagram. FCS is related to cell size and SSC to cell metabolic activity. (c) GFP analysis of cells from the red region (P3) in (b). FITC indicates the GFP intensity. (d) GFP analysis of cells from the green region (P4) in (b). A colour version of this figure is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

antimicrobial was selected to correspond to the concentration required for 2 ± 1 log reduction of stationary planktonic cells for 30 min.

Results and discussion

Spatial patterns of metabolic activity in biofilm

PA01(pAB1) colony biofilms grown for 48 h were observed to be flat with a thickness of 130 ± 20 μm. After IPTG induction, active cells expressing GFP were observed in the upper layer of biofilm adjacent to the air interface as shown in Figure 1(a). This zone of activity was 30 ± 10 μm thick. Dormant cells lacking GFP expression were observed in the bottom layer. Figure 1(b–d) shows FACS results of disaggregated biofilm cells before sorting. Figure 1(b) is a typical plot of forward scatter (FSC) values and side scatter (SSC) values of cells, which are directly proportional to the relative cell size and the internal complexity of cells, respectively. Cells in the red region (P3), which have lower FSC and SSC values in Figure 1(b), had low GFP intensity (Figure 1c), whereas cells with higher FSC and SSC values in the green region (P4) expressed high GFP intensity (Figure 1d). This shows that cells with higher GFP intensity, which are relatively active, were bigger than cells with lower GFP intensity, the relatively inactive cells.

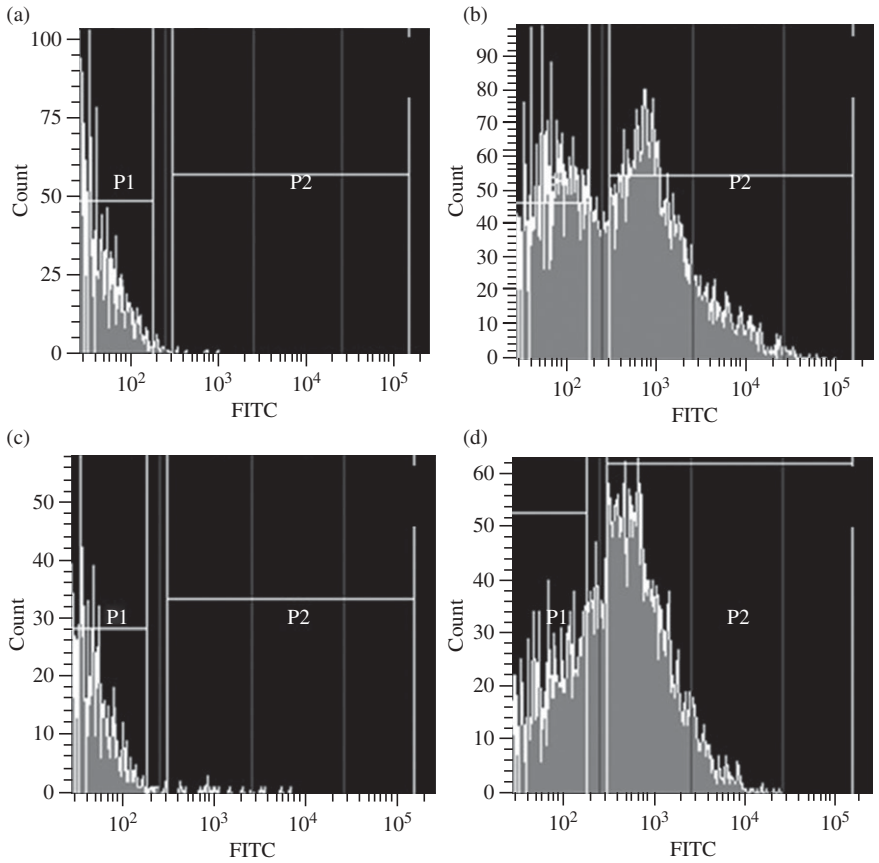

Figure 2. FACS diagram of (a) non-induced biofilm cells, (b) 4 h induced biofilm cells, (c) sorted cells with low GFP intensity, (d) sorted cells with high GFP intensity. FITC indicates the GFP intensity.

Next, the analysed samples were sorted into active and dormant cells according to the GFP intensity by FACS. GFP intensity diagrams of non-induced cells, 4 h induced cells, sorted dormant cells and sorted active cells are shown in Figure 2. The region exhibiting GFP intensity below 110 was defined as P1 and the region with GFP intensity over 120 was defined as P2 in Figure 2. Most of non-induced cells (>99%) were in P1 (Figure 2a) and 38% of cells in the induced sample were in P2 (Figure 2b). Cells in P1 were sorted as dormant cells and cells in P2 were sorted as active cells. Sorted dormant cells were almost all (>99%) in P1 (Figure 2c) and >50% of the sorted active cells were in P2 (Figure 2d).

There might be three possible reasons for the presence of almost 50% of the active cells in P1. First, the size of nozzle was suboptimal for sorting of PA01(pAB1) cells. Usually, a different nozzle size (70–200 μm diameter) is used for FACS according to the cell size. In our FACS, the 70 μm diameter nozzle was fitted because the average size of bacteria is around 2–6 μm. However, unfortunately, PA01(pAB1), which we used in this study, is 1 μm long so it is slightly out of the optimized range of nozzle. Second, through the sorting process, undesirable debris were usually generated, which were plotted in P1 area of diagram. The third reason could be the imperfection of cell dispersion, because *P. aeruginosa* has very sticky characteristics. However, this might not be a critical reason because we had almost 100% of dormant cells in P1. If a subset of cells were not separated, we should have observed dormant cells in the P2 area.

Antimicrobial susceptibility of active and dormant cells

Before the antimicrobial treatment, the viable cell concentration of sorted active or dormant cell fraction was measured. Viable cells in the active cell fraction were $100 \pm 10\%$ of the FACS recorded cell number. The deviation was observed due to an imperfect dispersion of biofilm cells. In the dormant cell fraction, the viable cell fraction was $60 \pm 10\%$ of the FACS recorded cell number. All antimicrobial experiments were conducted with adjustment to $\sim 10^6$ cfu/mL of viable microbial concentration. Active cell solution was prepared as 1.0×10^6 cells/mL and dormant cell solution was prepared as 1.7×10^6 cells/mL.

Log reductions of active and dormant cells after 30 min of antimicrobial treatments and P values are listed in Table 1. There was no reduction in untreated controls for 30 min. Active cells showed significantly higher tolerance against chlorine ($P = 0.005$). Dormant cells were more tolerant to silver ions and tobramycin, which is consistent with previous studies.[4,13] Dormant cells also showed higher tolerance to hydrogen peroxide and ciprofloxacin, but the differences were not significant ($P > 0.05$). In our preliminary experiments, the log reduction of the whole biofilm cells was almost the average value of the active and dormant cells. There was ~2.5 log reduction of unsorted cells after 30 min of 0.5 mg/L chlorine treatment, 2 log reduction after 30 mg/L hydrogen peroxide, 0.5 log reduction after 10 mg/L silver and 2 log reduction after 1 mg/L tobramycin.

Table 1. Effect of metabolic activity state on tolerance of dispersed biofilm cells to several antimicrobial agents

	Log reduction in cfu after 30 min treatment		
	active biofilm cells	dormant biofilm cells	*P* value
Chlorine (0.2 mg/L)	0.43 ± 0.28	1.53 ± 0.38	0.005
Chlorine (0.5 mg/L)	1.69 ± 0.10	4.24 ± 1.00	0.038
Hydrogen peroxide (3 mg/L)	0.46 ± 0.21	0.31 ± 0.09	0.093
Hydrogen peroxide (30 mg/L)	2.27 ± 0.78	1.73 ± 0.40	0.288
Silver ions (10 mg/L)	0.61 ± 0.19	0.35 ± 0.16	0.041
Tobramycin (10 mg/L)	2.37 ± 0.80	1.84 ± 0.53	0.028
Ciprofloxacin (1 mg/L)	2.12 ± 0.16	1.88 ± 0.06	0.37

In order to confirm these observations, the active and dormant cells were treated with antimicrobials for 60 min, and then antimicrobial tolerance was measured at each 10, 20, 40 and 60 min. During experiments, 1 mL of sample was neutralized at each sampling time. The inactivation curves of active and dormant cells treated with several antimicrobial agents are shown in Figure 3. There was no significant reduction in untreated controls over 60 min. Interestingly, active cells were more tolerant to 0.2 and 0.5 mg/L chlorine for 60 min (Figure 3a), whereas dormant cells were more tolerant to silver ions and tobramycin (Figure 3c). The difference in tolerance to each antimicrobial agent between active and dormant cells increased with treatment time. The difference between tolerance of active and dormant cells to hydrogen peroxide was not significant.

The cell-type-specific antimicrobial effects could be interpreted in terms of the different roles of metabolic activity in the antimicrobial mechanisms of each agent. Chlorine is a destructive, non-selective oxidant that reacts avidly with a variety of subcellular compounds.[18] Metabolic activity is less important for the activity of chlorine, whereas it is closely related to the antimicrobial mechanisms of antibiotics. Tobramycin is one of the aminoglycosides that inhibits protein synthesis, and ciprofloxacin is a fluoroquinolone compound that inhibits DNA replication.[22] Antimicrobial mechanisms of silver ions and hydrogen peroxide are slightly related to metabolic activity. Silver ions interact with thiol groups of proteins to inactivate their functions.[19] Hydrogen peroxide has a relatively low reactivity, so it can diffuse well into the cells and oxidize proteins and lipids, or it reacts with ferrous ion to produce very reactive hydroxyl radicals.[18,23]

Effect of oxygen growth conditions on antimicrobial susceptibility

In addition to metabolic activity, the difference in oxygen concentration could be one of the reasons for the differential susceptibility of dormant and active cells in biofilm to different antimicrobial agents. Anaerobic conditions have been reported at depths below 50 μm in biofilm colonies where the dormant cells

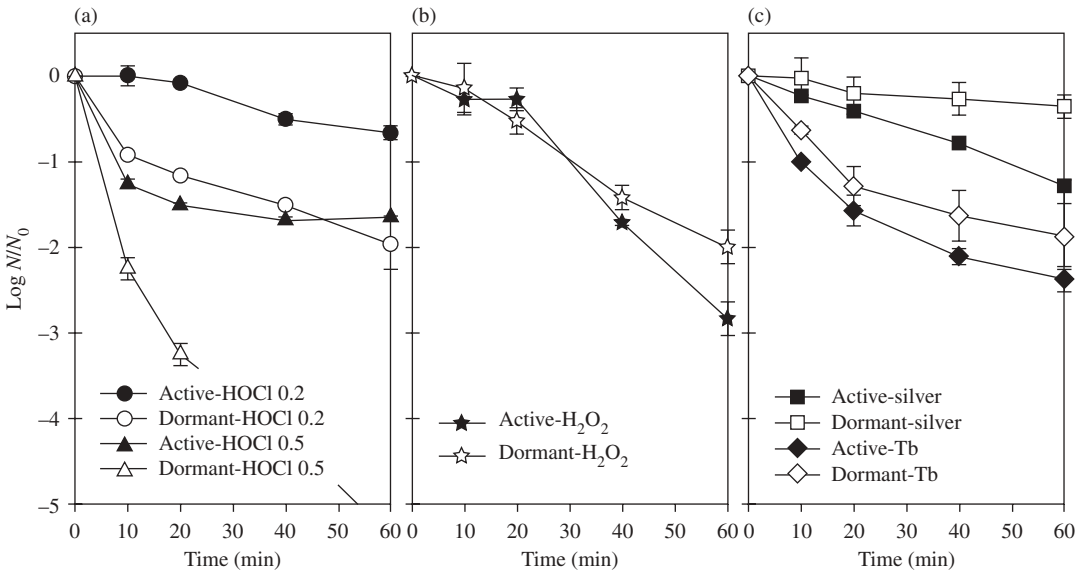

Figure 3. Log inactivation of active and dormant cells dispersed from biofilm treated with (a) 0.2 mg/L and 0.5 mg/L chlorine, (b) 30 mg/L hydrogen peroxide and (c) 10 mg/L silver ions and tobramycin. The values were averaged after three repetitions for each experiment.

Table 2. Effect of oxygen growth condition to tolerance of cells to several antimicrobial agents

	Log reduction in cfu after 30 min treatment		
	aerobically grown stationary cell	anaerobically grown stationary cell	*P* value
Chlorine (0.1 mg/L)	0.06 ± 0.03	0.14 ± 0.01	0.025
Chlorine (0.2 mg/L)	0.96 ± 0.18	3.49 ± 0.49	0.017
Hydrogen peroxide (3 mg/L)	0.36 ± 0.23	0.57 ± 0.20	0.019
Hydrogen peroxide (30 mg/L)	1.89 ± 0.42	3.80 ± 0.70	0.095
Silver ions (2 mg/L)	3.30 ± 0.20	1.79 ± 0.60	0.045
Tobramycin (2 mg/L)	2.87 ± 0.48	2.15 ± 0.32	0.164

exist.[24] In order to examine the effect of oxygen limitation on antimicrobial efficiency, stationary phase cells under aerobic and anaerobic conditions were prepared, and log reductions after 30 min of treatment were determined (Table 2). Aerobically grown stationary cells were significantly more tolerant to chlorine unlike other antimicrobial agents. Anaerobically grown cells were more tolerant to silver ions and the effect of oxygen growth conditions on antimicrobial tolerance to the other agents was not significant. In a previous study, chlorine was reported to be more effective for *Mycobacterium* species at low oxygen concentrations.[25] Similarly, in our experiments, chlorine more effectively inactivated anaerobically grown cells. It is supposed that the oxidative effect of chlorine on anaerobically grown cells might be greater than on aerobically grown cells. A similar result was observed for treatment with hydrogen peroxide, a mild oxidant, although 30 mg/L hydrogen peroxide did not exhibit significantly different tolerance between aerobically and anaerobically grown cells. For antibiotics, it has been reported that aerobic bacteria were more susceptible in aerobic conditions[14–16] and bacteria that can grow anaerobically were susceptible in anaerobic conditions,[15,16] which shows that antibiotic susceptibility depends on the metabolic activity.

Importance of available chlorine concentration

The different chlorine consumption rate between the active and dormant cells caused by the differences in cell sizes and the amount of cellular components could be one of the factors affecting chlorine sensitivity. Two factors have been suggested as causes of chlorine consumption in biofilm. One is the extracellular polymeric substance (EPS) in biofilm, the other is the bacterial cell itself. Metabolically active cells produce more EPS than dormant cells. However, in our experiment, whole biofilm cells were dispersed in the same bottle and EPS in biofilm then mixed thoroughly during FACS. Therefore, it is believed that the amount of EPS in the active and dormant cell fractions is not significantly different. Therefore, we have focused on the other factor. As addressed in Figure 1, active cells were bigger and had higher metabolic activity than dormant cells. Metabolically active cells contain denser intracellular components that can react with highly reactive oxidants such as chlorine, thereby reducing its available concentration. In order to confirm this hypothesis, we analysed the TOC content and concentrations of antimicrobial agents after 30 min of treatment. Active cell solution showed 230 mg/L TOC, which is higher than that of dormant cell solution (176 mg/L). In agreement with the high reactivity of chlorine, its concentration was decreased from 0.5 to 0.01 mg/L in active cells in 30 min. In dormant cells, however, 0.08 mg/L chlorine was detected after 30 min, which might reflect the lower TOC of dormant cells compared with active cells. In contrast, the concentrations of silver ions and hydrogen peroxide were constant during treatment. These results suggest that the available concentration of chlorine, but not the concentrations of the other antimicrobial agents, might be reduced more rapidly in active cells than dormant cells, resulting in a higher susceptibility of the dormant cells to chlorine.

Conclusions

In conclusion, the dormant cells in biofilm which grow slowly under anaerobic conditions showed decreased tolerance to chlorine, but not to other antimicrobials. The high oxidative reactivity and rapid degradation of available chlorine might influence the different antimicrobial actions of chlorine compared with antibiotics. This study contributes to our understanding of the antimicrobial tolerance of heterogeneous biofilm cells and hence to the choice of effective antimicrobial agents for specific conditions. In the environment of limited supplies of nutrients and oxygen, chlorine treatment might be an effective way to control microorganisms or biofilms.

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Transparency declarations

None to declare.

Supplementary data

A colour version of Figure 1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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