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Biofilm Formation by *Pseudomonas aeruginosa* and Disinfectant Susceptibility of Planktonic and Biofilm Cells

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Abstract

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Epifluorescence microscopy (EFM) was used to study the biofilm formation of *Pseudomonas aeruginosa* after 6, 24, 30, 48, 54, 72, 78, and 96 h growth in a chamber slide system. For this purpose, the biofilm was stained with the Live/Dead BacLight, wherein live and dead cells were visualised based on the cell membrane integrity. With the use of EFM we described 8- of 9-stage biofilm characteristics after 78 h of growth, since the majority of microscopic fields were fully covered with attached cells. However, the 96-h growth resulted in the cell detachment and revealed 30% of dead cells of all those cells that remained on the surface. The susceptibility testing of planktonic and biofilm cells to two disinfectants, chlorine-based and quaternary ammonium compound-based, revealed that biofilm cells were more tolerant to a chlorine-based sanitiser than planktonic counterparts. *P. aeruginosa* was inhibited by lower concentrations of the quaternary ammonium compound-based sanitiser than the chlorine-based sanitiser, which on the other hand was more effective in cell inactivation, as both the MIC/MBC (inhibitory/bactericidal) measurement and the CFDA/PI (carboxyfluorescein diacetate/propidium iodide) staining indicated.

Keywords: mode of existence; disinfection; fluorescence microscopy

Bacterial communities which colonise surfaces, forming a thin film-like structure, have been referred to as biofilms (Takenaka *et al.* 2001). It has been documented that biofilms are more resistant to antimicrobial agents, i.e. antibiotics, surfactants, disinfectants, than planktonic counterparts (Stewert & Costerton 2001; Kim *et al.* 2009). Also, their physiology significantly differs from that of planktonic cells (Simões *et al.* 2010). This is an emerging problem for the human medicine, because biofilms may be responsible for persistent infections, as well as for the food industry sectors such as brewing, dairy processing, fresh produce, poultry processing, and red meat processing which face many issues concerning biofilms, e.g. industrial biofouling, contamination of food production lines, microbial regrowth in distribution systems (Le Thi *et al.* 2001; Kim *et al.* 2008; Simões *et al.* 2010). The resistance of biofilms makes their elimination a big challenge since standardised methods for the biofilm control do not exist (Simões *et al.* 2009). Hence, the ongoing design of control strategies against biofilms on a case-by-case basis is crucially needed. The deepened understanding of how biocides interact with cells of a planktonic and biofilm mode is crucial, because many of them are effective against planktonic cells but not against biofilm cells (Kim *et al.* 2008). Therefore, a comparison of their susceptibilities to various antimicrobials has not been completed yet.

Diverse microscopic approaches are incorporated to explore biofilms. Scanning electron microscopy (SEM), epifluorescence microscopy (EFM), confocal laser scanning microscopy (CLSM) have been used to investigate the bacterial attachment and biofilm structure (Arnold & Bailey 2000; Takeuchi & Frank 2000, 2001; Takeuchi *et al.* 2000; Bagge *et*

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al. 2001; Kubota *et al.* 2008; Nosyk *et al.* 2008). EFM and CLSM make it possible to detect microorganisms and exopolysaccharides of biofilms by an appropriate staining method (Nosyk *et al.* 2008). What is more, the differentiation of live and dead cells within a biofilm can be achieved by the application of double staining, e.g. SYTO®9 and propidium iodine – PI (Takenaka *et al.* 2001). Suitable staining procedures combined with microscopic analysis were previously employed to evaluate the antimicrobial activities of biocides, e.g. disinfectants on *Pseudomonas* spp. biofilms (Wirtanen *et al.* 2001); examine the effect of detrimental conditions such as air-drying on the survival of biofilm cells (*Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*) (Fuster-Valls *et al.* 2008); assess the effect of antimicrobials such as chlorine, silver, tobramycin on *P. aeruginosa* biofilms (Kim *et al.* 2008), and on *P. aeruginosa* active and dormant cells which had been sorted from a biofilm (Kim *et al.* 2009).

The purpose of this study was to investigate the development of a *Pseudomonas aeruginosa* biofilm by EFM using Live/Dead BacLight viability kit, and determine the susceptibility of planktonic and biofilm cells to two disinfectants using the MIC/MBC measurement, and the difference in the physiological state of cells visualised by CFDA/PI (carboxyfluorescein diacetate/propidium iodide) staining.

MATERIAL AND METHODS

Preparation of planktonic and biofilm cultures. *Pseudomonas aeruginosa* ATCC 27853 was used in this study. The culture was stored at 6°C in a nutrient agar –NA slant and streaked on a NA (both Merck, Darmstadt, Germany) plate from which a single colony was selected for the preparation of a planktonic or a biofilm culture. A planktonic culture was grown at 35°C in nutrient broth – NB (Merck, Germany) for 24 hours. For biofilms, the overnight culture at 35°C in a NB (Merck, Germany) was first diluted to an optimal density of 0.10 at 600 nm and one 10 µl drop of the diluted solution was used to inoculate polycarbonate membranes (ø 13 mm, 0.22 µm pore size; Millipore, Billerica, USA) on NA plates. The plates were incubated at 35°C, and the membranes were transferred to fresh NA plates every 24 h and were grown for 48 hours. Membrane-supported colony biofilms were placed in sterile saline (0.85% NaCl) and disaggregated by sonication and vortexing for 1 minute. The cell suspension was further used for the MIC/MBC measurement.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). In this study, two commercially available surface cleaners and disinfectants were tested: Medicarine (Ecolab, Krakow, Poland), which is a chlorine-based sanitiser, and Pursept-AF (Merz, Frankfurt, Germany), which is a quaternary ammonium compound-based representative. For this purpose, the disinfectant dilutions in NB (Merck, Germany) containing tubes in the range between 4% and 0.0001% were prepared, which were further inoculated with planktonic or biofilm cultures. An initial inoculation for controls (NB) and the inhibition experiments (disinfectant containing NB) was ~ 10^6 CFU/ml. The minimum inhibitory concentration was followed until no additional growth was observed after 24 h incubation at 35°C. The MBC values were estimated based on the plating of 0.1 ml of disinfectant-containing probes onto NA (Merck, Germany) followed by the incubation at 35°C for 24 hours. The lowest disinfectant concentration which did not give any visible colonies on plates was assigned as MBC. The experiments were repeated twice.

Physiological state of cells. The EFM analysis was used for the evaluation of the physiological state of planktonic and biofilm cells. The controls, the disinfectant-containing probes below MIC and MIC probes were stained with CFDA/PI (5-[and-6]-carboxyfluorescein diacetate/propidium iodine; Biochemika Fluka, Buchs, Switzerland) dyes (the activity of intracellular enzymes and membrane integrity labels, respectively). The cell suspensions were first incubated with 50 µM CFDA (5-[and-6]-carboxyfluorescein diacetate; Biochemika Fluka, Switzerland) delivered in anhydrous dimethyl sulphoxide (Sigma-Aldrich, Poznan, Poland) at 37°C for 30 min and then with 30 µM PI (propidium iodine; Biochemika Fluka, Switzerland) delivered in double-distilled water for 10 min in dark. After incubation the samples were filtered using a vacuum pump (Millipore, Schwalbach, Germany) and black polycarbonate filters (ø 13 mm, 0.22 µm pore size; Millipore, Germany) and mounted on glass slides using immersion oil (Molecular Probes, Eugene, USA). Microscopic analysis was performed with an Olympus BX51 microscope equipped with a 100 W mercury lamp, XC digital camera (Olympus, Hamburg, Germany) and a set of appropriate filters: U-MNB2 470-490 nm for detection of green fluores-

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cence, U-MNG2 530-550 nm for red fluorescence. CellSens Dimension System 1.5 (Olympus, Germany) software was used for cell counting.

Growth of biofilm in a LabTek™ chamber slide system. The biofilms were grown based on Jurcisek *et al.* (2011) protocol. The bacterial colonies grown overnight on NA (Merck, Germany) were suspended in 1 ml phosphate-buffered saline (0.1 M PBS; Sigma-Aldrich, Poland) and diluted in NB to a final concentration of ~ 10^3 CFU/ml. 400 µl of the cell suspension was placed into each well of four-well chamber slides (Nunc™ Lab-Tek™; Thermo Fisher Scientific, Waltham, USA), and further incubated at 35°C for 96 hours. The medium was changed every 24 h to maintain biofilm viability. To visualise the cell adherence scale fluorescent staining was performed after 6, 24, 30, 48, 54, 72, 78, and 96 h of incubation. Chambers were washed gently with sterile saline and stained with Live/Dead BacLight™ viability kit (Molecular Probes, Life Technologies, Oregon, USA). The staining mixture was prepared by adding 3 µl of component A and 3 µl of component B per 1 ml of sterile saline. 400 µl of the freshly prepared staining mixture was added to each chamber and incubated at room temperature for 15 min in dark. After incubation, chambers were washed with sterile saline and removed from slides. Slides were closed with coverslips and with the use of saline for filling the biofilm growth area between a glass slide and a coverslip, and with BacLight™ Mounting Oil (Molecular Probes, Life Technologies, USA) for sealing the edges of the coverslip. The image analysis was conducted by the EFM as described above. Adherence characteristics of bacterial cells were performed for each sample based on 10 randomly selected fields which were analysed in order to classify the sample to the adherence scale (Table 1).

Table 1. 9-stage adherence designation of bacterial cells to a substratum based on Le Thi *et al.* (2001)

Stage No.	Stage characteristics
1	up to five bacterial cells in each field
2	only individual bacterial cells, no microcolonies
3	countable individual bacterial cells, small microcolonies
4	not confluent, large microcolonies – more than 100 bacterial cells
5	confluent microcolonies and individual bacterial cells
6	microcolonies occupying one-quarter of at least one field
7	at least one of the fields entirely covered by the biofilm
8	the majority of the fields fully covered, but portions of the substratum remain visible
9	all fields entirely covered by bacterial cells

Statistical analysis. Statistical analysis was conducted by the Statistica software version 9 (StatSoft Inc., Tulsa, USA). Samples were tested by a one-way ANOVA test. Differences were considered significant at the $P < 0.05$ level of probability.

RESULTS AND DISCUSSION

In order to analyse adherence characteristics of *P. aeruginosa* ATCC 27853, a biofilm growth in the LabTek™ chamber slide system was introduced. For this purpose, a microscopic analysis and fluorescent staining with Live/Dead® viability kit was adopted. The kit includes two nucleic acid stains, i.e. SYTO®9 and propidium iodine (PI). SYTO®9 has a low molecular weight and stains cells green. In turn, PI penetrates damaged cells staining them red. This gives an effect of live and dead cells to be distinguished within a population (Jurcisek *et al.* 2011). The cell adherence results are shown in Table 2 and Figure 1. Over a period of 78 h, stages from 2 to 8 were recognised (Figure 1) and the approximate share of live and dead cells was 80–20% (Table 2). In this study, the development of large microcolonies was observed for stage 6 and 8. The smaller percentage of live cells was noted after 96 h of growth (68.8%), indicating also the dispersal of a biofilm due to the lack of observable microcolonies (Figure 1). This study clearly established that a biofilm of *P. aeruginosa* could be easily produced in a chamber slide system. The usefulness of SYTO®9 and PI in biofilm staining of *P. aeruginosa* was also demonstrated. However, specifically for stage 8, a binding to components of a biofilm matrix by SYTO®9 was observed. It was previously reported by Whitchurch *et al.* (2002) that the extracellular DNA is required for the establishment of biofilms by *P. aeruginosa*, and it may influence their rheological properties and stability. In general, the composition of extracellular matrix comprises polysaccharides, proteins, nucleic acids, lipids, phospholipids, and humic substances (Simões *et al.* 2010). It is believed that major and fundamental structural components

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Table 2. BacLight live and dead biofilm cell shares and adherence stages of *P. aeruginosa* ATCC 27853 during 96-h growth in the LabTek™ chamber slide system

Time (h)	Percentage share (%)			Adherence stage (1–9)	
	live biofilm cells	dead biofilm cells	SD	mean	SD
6	100.0	0.0	0.0	2.4	0.1
24	81.2	18.8	0.4	3.6	0.1
30	93.9	6.1	2.5	6.0	0.4
48	91.9	8.2	6.7	6.0	1.5
54	80.6	19.5	21.7	4.8	0.6
72	95.1	4.9	3.5	5.2	0.7
78	81.6	18.5	26.0	8.0	0.6
96	68.8	31.2	7.8	2.5	0.4

The results shown are representatives of two replicate trials

of the biofilm matrix are polysaccharides and proteins (Tsuneda *et al.* 2003). It has frequently been observed that the function of extracellular substances is linked with protection from hostile conditions, e.g. high concentrations of biocides (Simões *et al.* 2005). Moreover, the biofilm matrix accumulates the molecules required for cell-cell communication and community behaviour (Simões *et al.* 2010). Besides extracellular substances, the presence of extracellular appendages produced by cells may also affect the process of binding cells to the surface (Simões *et al.* 2010). *Flagella, pili* or *fimbriae* are found on many Gram-negative bacteria, including *Pseudomonas*, and they are believed to overcome the repulsion barrier between the cell and the surface (Simões *et al.* 2010). Our results indicate the usefulness of fluorescent staining in bacterial community investigations and also suggest that more attention should be focused on studies related to particular EPS components and of a specific microorganism. Moreover, the biofilm growth in the chamber slide system combined with microscopy could be a suitable experimental model for many purposes. In fact, the chamber slide system was previously used by Takenaka *et al.* (2001) for reconstruction of a three-dimensional structure of the *P. aeruginosa* biofilm

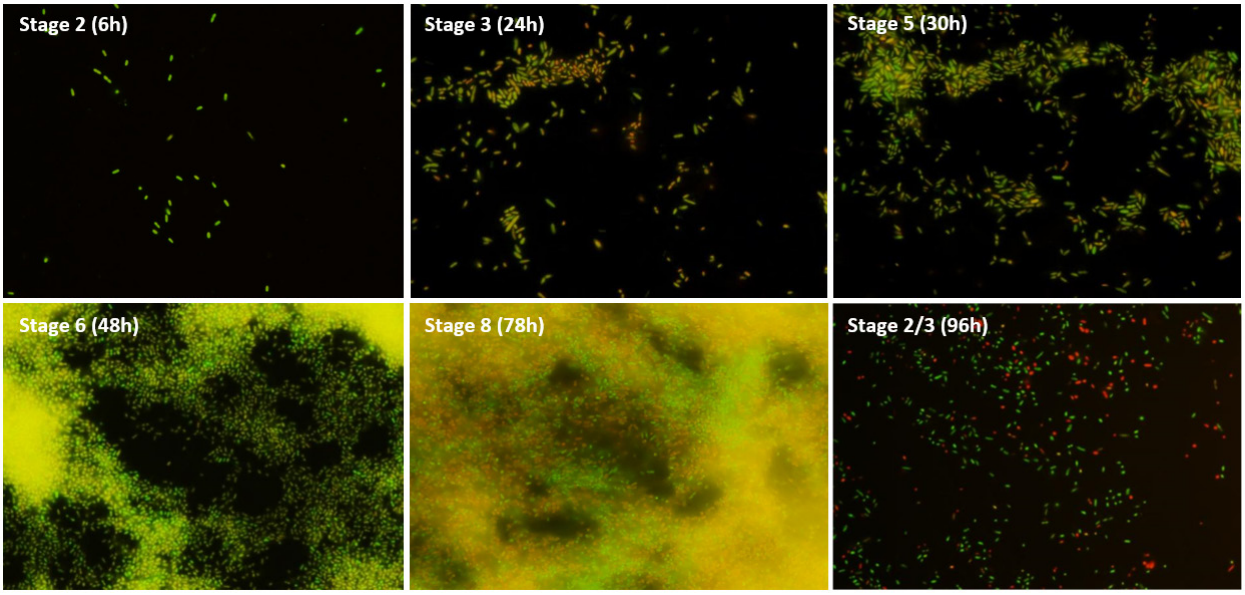

Figure 1. Stages of adherence of *P. aeruginosa* ATCC 27853 to glass during growth in the LabTek™ chamber slide system. Fluorescent staining with Live/Dead BacLight™ as described in Material and Methods. The micrographs shown are representatives of two replicate trials

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by CLSM. A different example shows that biofilm samples of *Pseudomonas fluorescens* were inspected by EFM with the aim of identifying the exopolymer layer characteristics after disinfectant exposure on stainless steel (Simões *et al.* 2003). Interestingly, Myszka *et al.* (2005) studied the biofilm formation of several microorganisms, including *P. aeruginosa* on different surfaces under nutrient-limited conditions. In the case of *P. aeruginosa*, they described 7 stages of biofilm formation on glass and Teflon surfaces, irrespective of the nutrient concentration. These different examples indicate that *P. aeruginosa* adhesion may be comprehensively studied in a wide variety of experimental conditions and the choice of a certain one is dictated by the goals of the experiment. Nevertheless, the fluorescence-based tools seem to be suitable approaches to evaluation of biofilm development and physiological properties of cells.

The susceptibility testing results showed that *P. aureginosa* ATCC 27853 exhibits some degree of resistance to the tested disinfectants. The MIC/MBC of Medicarine and Pursept are shown in Table 3.

The strain had the same MIC and MBC values for Medicarine, but the values differed between planktonic and biofilm cells (0.025 and 0.035%, respectively). Regarding Pursept, the difference was observed between MIC (0.002%) and MBC (0.005%) values, but not between planktonic and biofilm cultures. As a result, Pursept was not bactericidal at a MIC concentration for *P. aeruginosa,* although Pursept concentrations were about 10 times lower than those required to inhibit the growth of cells by Medicarine. Interestingly, Medicarine inactivated cells at MIC values, but in the case of this chlorine-based disinfectant the formation of persistence within a biofilm may be taken under consideration. The cell

Table 3. Antimicrobial efficiencies of Medicarine and Pursept for inhibition

		Disinfectant concentration (%)	
		planktonic cells	biofilm cells
Medicarine	MIC	0.025	0.035
	MBC	0.025	0.035
Pursept	MIC	0.002	0.002
	MBC	0.005	0.005

The results shown are representatives of two replicate trials; MIC – minimum inhibitory concentration and inactivation; MBC – minimum bactericidal concentration of planktonic and biofilm cells of *P. aeruginosa* ATCC 27853

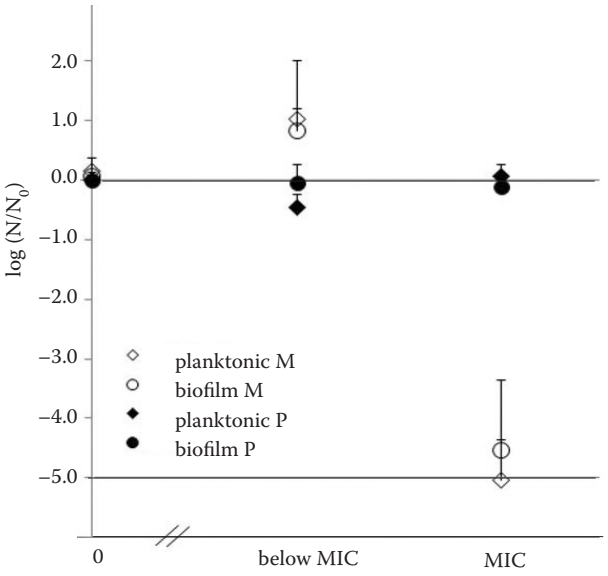

Figure 2. The effect of different concentrations of Medicarine (M) and Pursept (P) based on MIC test results on the recovery of planktonic and biofilm cells of *P. aeruginosa* ATCC 27853 as assessed by CFDA cell counts (mean ± SD for two replicate trails). The detection limit was –5.0 log (N/N_0)

count results shown in Figure 2 revealed the lack of cell inactivation by Pursept in a MIC concentration and confirmed an inactivation effect of Medicarine on cells. The cell staining used in this study refers directly to activity of intracellular enzymes (esterases) and membrane integrity of cells. This is due to carboxyfluorescein diacetate (CFDA), which enters the cells and is hydrolysed by the action of esterases. Consequently, a cleaved fluorescent, green product (carboxyfluorescein – CF) is deposited in the interior of cells with intact cytoplasmic membranes. Whereas propidium iodine enters cells with damaged cytoplasmic membranes, and intercalates with nucleic acids, therefore cells are stained red (Rault *et al.* 2007). The results based on CFDA cell counts did not reveal any significant differences for Pursept-containing probes up to the MIC concentration, regardless of the culture ($P > 0.05$). Unlike for Pursept, a significant reduction was observed for Medicarine ($P < 0.05$) (Figure 2). For Medicarine as well, a significant difference in PI cell counts was observed between below MIC- and MIC-probes, indicating an occurrence of cell lysis over the period of incubation with MIC concentrations (data not shown). Chlorine-based biocides act as oxidants which destroy cells by chlorinating the lipid protein substance in the cell wall to form toxic chloro-compounds and also by inducing the leakage

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of cell content outside the cell (Kim *et al.* 2008). A significant reduction and high efficacy of chlorinated sanitisers to control *Pseudomonas* sp. was previously reported (Greene *et al.* 1993). However, single cells that may remain viable in biofilms even though a bactericidal treatment was applied, are significant hazardous factors. Especially when other studies confirm that biofilm bacteria of *P. aeruginosa* are less susceptible to chlorine than planktonic counterparts (e.g. Eginton *et al.* 1998; Cochran *et al.* 2000; Kim *et al.* 2008). According to Cochran *et al.* (2000) the increase in resistance of *P. aeruginosa* biofilms could not be completely explained by reduced diffusion of biocides in biofilms. The increase in resistance of *Pseudomonas* biofilms to different disinfectants were reported by numerous papers, e.g. Simões *et al.* (2003), Surdeau *et al.* (2006), Bjarnsholt *et al.* (2007), Smith *et al.* (2008), Hendry *et al.* (2009), and Toté *et al.* (2010). Thus, there is an ongoing need for further research to elucidate the nature and physiology of cell forming communities on different materials, specifically the prevalence and composition of biofilms. This will inform new approaches to disinfection for a wide variety of medical and industrial sectors.

In conclusion, this study provides useful information for a better understanding of biofilm formation by *P. aeruginosa*. This study also contributes to understating the impact of planktonic and biofilm growth on the susceptibility of *P. aeruginosa* to commercially available surface disinfectants. The recognition of bacteria e.g. on food-contact surfaces as they form biofilms is an essential area of focus towards their elimination from food processing environments. Therefore, these results are expected to facilitate future investigations on the effectiveness of various treatments for biofilms for the purpose of their elimination and food safety assurance.

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