

The effects of a biocide and a surfactant on the detachment of *Pseudomonas fluorescens* from glass surfaces

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Abstract

Application of antimicrobial chemicals is a general procedure in the cleaning and disinfection of food-contacting surfaces. Adhesion to glass surfaces and chemically induced detachment of *Pseudomonas fluorescens* ATCC 13525^T were studied *in situ*, under flow conditions, in a well-controlled parallel plate flow chamber (PPFC). *Ortho*-phthalaldehyde (OPA) and cetyltrimethyl ammonium bromide (CTAB) were applied separately, at several concentrations, to attached bacteria and their subsequent detachment was monitored. Following treatments the remaining adhered bacteria were characterized in terms of viability and cell size. Simultaneously, the planktonic cell surface was characterized in order to correlate PPFC results with thermodynamic approaches for adhesion evaluation, and surface free energy of chemically treated cells with adhesion strength. About 2.8×10^6 cells/cm² adhered to the glass surface after 30 min of bacterial flow, although thermodynamic analyses evidenced unfavourable adhesion. The independent application of OPA and CTAB promoted bacterial detachment to a small extent (16% of total cells). The remaining adhering bacteria were totally non-viable for OPA ≥ 0.75 mM and CTAB ≥ 0.25 mM, showing a lack of correlation between bacterial viability and detachment. The cellular size decreased as attachment proceeded and with chemical treatment. Both chemicals altered the cell surface properties, increasing the cell-glass adhesion strength, and promoting the emergence of polar characteristics. The overall results emphasize that OPA and CTAB were markedly ineffective in removing glass-attached *P. fluorescens*, demonstrating that bacteria can be non-viable but remain strongly attached to the adhesion surface.

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1. Introduction

In the majority of ecosystems, microbial cells grow on surfaces, with the formation of highly structured sessile microbial communities, called biofilms (Bakker et al., 2002; Bremer et al., 2006). The development of a biofilm is believed to occur in a sequential process that involves movement of microorganisms towards surfaces, initial microbial attachment, formation of microcolonies, extracellular polymeric substances production, and biofilm maturation (Byers, 2000; Sauer and Camper, 2001). To inhibit biofilm formation, it is necessary to prevent the initial adhesion of microorganisms (Busscher et al., 1990).

In a food processing line, the formation of biological deposits on processing equipment must be controlled (Lecrigny-Nolf et

al., 2000; Rossoni and Gaylarde, 2000). Microbial growth can be limited by good sanitation practices (Maukonen et al., 2003; Simões et al., 2005b; Meyer, 2006). Control of microbial attachment can facilitate disinfection (Meyer, 2003). The mechanism by which a chemical agent interacts with adhered cells has not been elucidated. The velocity of fluids on surfaces where bacterial attachment occurs, and the conditions of application of the chemicals both may affect attachment (Simões et al., 2003). Bacteria can adapt rapidly to hydrodynamic and chemical stresses (Suci et al., 1998), so it is likely that cells undergo complex physiological changes during the process of attachment of planktonic cells (Sauer and Camper, 2001). Those changes significantly reduce the susceptibility of cells to measures for their control (Cloete et al., 1997; Gilbert et al., 2002).

There is a lack of information about the behavior of cells in the earlier stages of biofilm development. The initial adhesion of a bacterium to a surface is a complicated process that is

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affected by various physicochemical properties of both the cell and the surface (Gallardo-Moreno et al., 2002a,b). The cell surface hydrophobicity is considered to be the most important physicochemical parameter controlling adhesion and detachment from surfaces (Razatos et al., 1998; Gallardo-Moreno et al., 2002a).

In this study the adhesion to glass surfaces and detachment rate of *Pseudomonas fluorescens*, after the application of a non-oxidising aldehyde-based biocide and a cationic surfactant were monitored *in situ*, under flow conditions in a parallel plate flow chamber. The relevance of cell surface properties and viability in the detachment process were also studied.

2. Materials and methods

2.1. Microorganism and culture conditions

P. fluorescens ATCC 13525^T was used throughout this study. A continuous culture was grown in a 2 l glass chemostat, at 27 °C, with an air flow rate of 7.08×10^{-3} l/s. The chemostat was continuously fed with 40 ml/h of sterile medium containing glucose, 5 g/l, peptone, 2.5 g/l, and yeast extract, 1.25 g/l in 0.02 M phosphate buffer (KH₂PO₄; Na₂HPO₄) at pH 7.0±0.2.

To perform the attachment and detachment experiments, the bacterial suspensions were prepared by aseptically removing 130±5 ml of *P. fluorescens* culture from the chemostat, centrifuging the portion of culture at 3777 g for 5 min at 4 °C; washing the pelleted cells twice with sterile 0.05 M citrate buffer (pH 6), and resuspending the cells in citrate buffer to obtain a bacterial suspension at 6×10^6 cells/ml.

2.2. Chemicals tested

The chemical agents used were a non-oxidising aldehyde-based biocide, *ortho*-phthalaldehyde, (OPA; Sigma, Lisbon, Portugal), used at concentrations of 0.15, 0.375, 0.75 and 3.75 mM; and a cationic surfactant, cetyltrimethyl ammonium bromide (CTAB; Merck, Lisbon, Portugal) used at concentrations of 0.125, 0.25, 0.5 and 0.9 mM.

2.3. Attachment and detachment monitoring

A parallel plate flow chamber (PPFC), previously described by Sjollem et al. (1989) was used. The PPFC consisted of a nickel-coated frame measuring 16×8×1.8 cm. The bottom and top 5.5×3.8 cm plates were made of glass. These were cleaned by 50% maximum amplitude sonication using an ultrasonic processor (Cole-Parmer Instruments, Illinois, USA), for 3 min, in a solution of commercial detergent (Sonasol Pril, Henkel Ibérica S. A., Portugal). After the sonication treatment, plates were rinsed with ultrapure water. A Teflon spacer was placed between the plates, to separate them by 0.06 cm. The PPFC device was mounted in an inverted microscope (Diaphot 300; Nikon, Badhoevedorp, The Netherlands). The images were acquired in a CCD camera (AVC, D5CE; Sony, Tokio, Japan) connected to the microscope, and coupled to an image analyser (Image Proplus 3.0; Media Cybernetics, Bethesda, Maryland,

USA). The images were examined according to the procedure described by Azeredo et al. (1997).

To assess the rate of *P. fluorescens* attachment to glass, a cell suspension was circulated through the PPFC at 0.11 ml/s for 30 min. Then, citrate buffer was circulated through the equipment to remove unattached and weakly adhering cells. After 30 min, the buffer was replaced by chemicals solutions flowing at the same rate. Images were recorded every 20 s, using an image analyser (Image Proplus 3.0; Media Cybernetics), and the data obtained were expressed as number of cells per cm².

2.4. Epifluorescence microscopy analysis

After each PPFC experiment, the bacteria that remained adhering to the glass surfaces were stained with Live/Dead BacLight bacterial viability kit (Molecular Probes, Leiden, The Netherlands), according to the procedure described by Simões et al. (2005a). A microscope (AXIOSKOP; Zeiss, Göttingen, Germany), fitted with fluorescence illumination and a 100× oil immersion fluorescence objective, was used to visualise the stained cells. The optical filter combination consisted of a 480 to 500 nm excitation filter, in combination with a 485 nm emission filter. Bacterial observations were recorded as micrographs obtained using a microscope camera (AxioCam HRC; Zeiss). A program path (Scan Pro 5; Sigma), involving object measurement and data output, was used to quantify the number of cells and to measure the equivalent cell radius, as an estimate of cellular size (Walker et al., 2005). The average equivalent spherical radius of the adhered cells was determined from the cell length and width measurement assuming a uniform distribution of bacterial cell morphologies.

The mean number of cells was determined from counts of a minimum of 20 microscopic fields for each glass surface.

2.5. Contact angles measurements, hydrophobicity and free energy of adhesion

Bacterial hydrophobicity was evaluated from contact angle measurements, and by the method of van Oss et al. (1987, 1988, 1989). With this method, the degree of hydrophobicity of a given material is expressed as the free energy of interaction between two entities of that material immersed in water—(ΔG_{iwi} mJ/m²). If the interaction between the two entities is stronger than the interaction of each entity with water ($\Delta G_{\text{iwi}} < 0$), the material is considered hydrophobic. Conversely, if $\Delta G_{\text{iwi}} > 0$, the material is hydrophilic. ΔG_{iwi} can be calculated from the surface tension components of the interacting entities, according to the equation

$$\Delta G_{\text{iwi}} = -2 \left(\sqrt{\gamma_{\text{i}}^{\text{LW}}} - \sqrt{\gamma_{\text{w}}^{\text{LW}}} \right)^2 + 4 \left(\sqrt{\gamma_{\text{i}}^+ \gamma_{\text{w}}^-} + \sqrt{\gamma_{\text{i}}^- \gamma_{\text{w}}^+} - \sqrt{\gamma_{\text{i}}^+ \gamma_{\text{i}}^-} - \sqrt{\gamma_{\text{w}}^+ \gamma_{\text{w}}^-} \right); \quad (1)$$

where γ^{LW} accounts for the Lifshitz–van der Waals component of the surface free energy and γ^+ and γ^- are the electron

acceptor and electron donor parameters, respectively, of the Lewis acid-base component (γ^{AB}), with $\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$.

The surface tension components of a solid material are obtained by measuring the angles of contact with three pure liquids. The liquids used are α -bromonaphthalene, which is apolar, and two polar liquids, water and formamide. All these liquids have known surface tension components. The surface tension components are estimated by the simultaneous resolution of three equations of the type

$$(1 + \cos\theta)\gamma_i^{\text{Tot}} = 2\left(\sqrt{\gamma_s^{\text{LW}}\gamma_i^{\text{LW}}} + \sqrt{\gamma_s^+\gamma_i^-} + \sqrt{\gamma_s^-\gamma_i^+}\right); \quad (2)$$

where θ is the contact angle and $\gamma^{\text{Tot}} = \gamma^{\text{LW}} + \gamma^{\text{AB}}$.

When studying the interaction (free energy of adhesion) between substances i and I that are immersed or dissolved in water, the total interaction energy, $\Delta G_{\text{iwI}}^{\text{Tot}}$, can be expressed as

$$\begin{aligned} \Delta G_{\text{iwI}}^{\text{Tot}} = & \gamma_{\text{il}}^{\text{LW}} - \gamma_{\text{iw}}^{\text{LW}} - \gamma_{\text{Iw}}^{\text{LW}} \\ & + 2\left[\sqrt{\gamma_w^+}\left(\sqrt{\gamma_i^-} + \sqrt{\gamma_I^-} + \sqrt{\gamma_w^-}\right)\right. \\ & \left. + \sqrt{\gamma_w^-}\left(\sqrt{\gamma_i^+} + \sqrt{\gamma_I^+} - \sqrt{\gamma_w^+}\right) - \sqrt{\gamma_i^+\gamma_I^-} - \sqrt{\gamma_i^-\gamma_I^+}\right] \end{aligned} \quad (3)$$

Thermodynamically, if $\Delta G_{\text{iwI}}^{\text{Tot}} < 0$ adhesion is favoured, while adhesion is not expected to occur if $\Delta G_{\text{iwI}}^{\text{Tot}} > 0$ mJ/m².

P. fluorescens suspensions, prepared as described for the PPFC experiments, were exposed to either OPA or CTAB solutions. Afterwards, lawns of *P. fluorescens* were prepared as described by Busscher et al. (1984), to ascertain the bacterial surface properties. Untreated cells were always used as control experiments.

The glass plates were prepared for surface characterization by immersion in a solution of the commercial detergent in ultrapure water for 30 min. After rising with ultrapure water the glass plates were dried at 65 ± 5 °C for 3 h.

The contact angles and surface tension of the untreated and treated bacteria, and the glass plates, were determined by the sessile drop contact angle measurements, using an apparatus (model OCA 15 Plus; DATAPHYSICS, Filderstadt, Germany) that allowed image acquisition and data analysis. These measurements were carried out at 25 ± 2 °C using water, formamide and α -bromonaphthalene (Sigma), as reference liquids. The surface tension components of the reference liquids were taken from literature (Janczuk et al., 1993). Contact angle data were obtained from at least 25 determinations for each liquid and for each experiment.

2.6. Statistical analysis

The data were analysed using the statistical program Statistical Package for the Social Sciences (SPSS), version 14.0. The means and standard deviations within samples were calculated for all cases. Paired *t*-test analyses were performed for PPFC results. Hydrophobicity and free energy of adhesion data were analysed by the Wilcoxon test. Statistical calculations were based on confidence level equal or higher than 95%.

3. Results

3.1. Microbial adhesion

The analysis of *P. fluorescens* adhesion (Fig. 1) shows the existence of three distinct adhesion rates. During the first 300 s the rate was only 1 cell/s/cm² (linear correlation— $R^2=0.827$). Between 301 and 1000 s the rate was 600 cells/s/cm² (linear correlation— $R^2=0.978$). Between 1001 and 1800 s the rate was about 2950 cells/s/cm² (linear correlation— $R^2=0.960$). The final numbers of attached bacteria were 2.80×10^6 cells/cm². The duration of the experiments was found to be enough for cells to adhere strongly, as during the 30 min of buffer circulation, no significant decrease ($P>0.05$) in the number of attached cells was observed.

3.2. Effects of OPA and CTAB on detachment

The 30 min applications of various concentrations of OPA (Fig. 2a) and CTAB (Fig. 2b) to attached *P. fluorescens* resulted in small reductions in the numbers of attached cells. Circulation of citrate buffer instead of OPA or CTAB solutions resulted in detachment <1% of adhering cells. With OPA solutions, a maximum of 12.9% of the attached cells, were removed. OPA at concentrations of 0.15, 0.375, 0.75 and 3.75 mM gave detachment rates of 89, 117, 83 and 200 cells/s/cm², respectively. With CTAB solutions, a maximum of 15.8% of the cells was detached. CTAB at concentrations of 0.125, 0.25, 0.5 and 0.9 mM gave detachment rates of 206, 244, 161 and 150 cells/s/cm², respectively.

3.3. Characterization of the attached bacteria

The lengths of attached and planktonic cells were 0.840 ± 0.061 and 1.16 ± 0.11 μm , respectively. The difference in the mean lengths was significant ($P<0.05$). The application of OPA or CTAB to adhering *P. fluorescens* reduced both cell size and viability (Fig. 3). With 0.75 and 3.75 mM OPA or 0.5 and 0.9 mM CTAB cells were non-viable. With CTAB but not OPA, cell size reduction was concentration dependent ($P<0.05$).

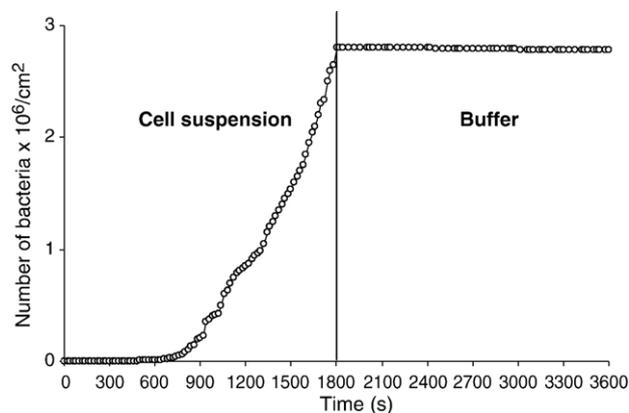


Fig. 1. *P. fluorescens* attached to glass during circulation of a suspension of the microorganism and subsequent circulation of citrate buffer.

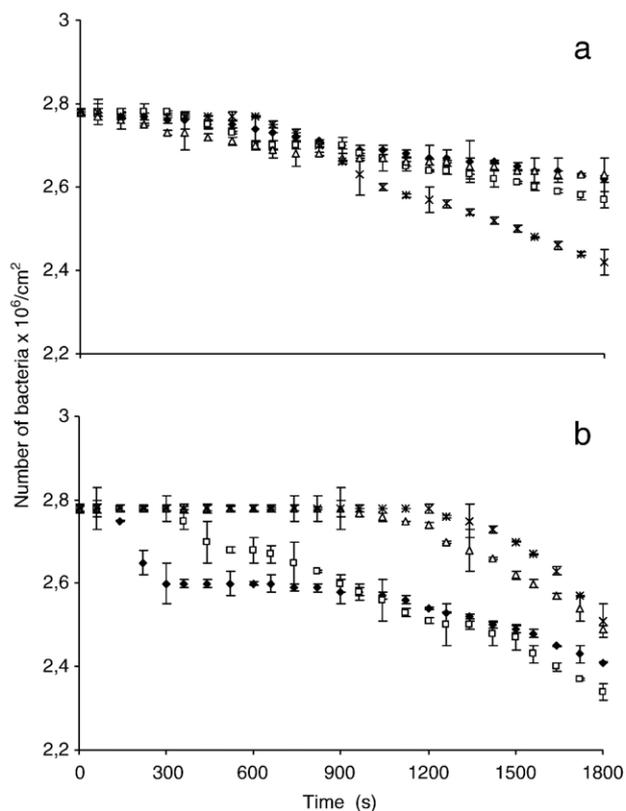


Fig. 2. *P. fluorescens* detachment from glass surface during treatment with (a) OPA at 0.150 mM (◆); 0.375 mM (□); 0.750 mM (△); 3.75 mM (✱) or (b) CTAB at 0.125 mM (◆); 0.250 mM (□); 0.500 mM (△); 0.900 mM (✱). Each symbol indicates the mean \pm SD of three independent experiments.

3.4. Bacterial surface properties and free energy of adhesion

P. fluorescens cells had hydrophilic properties ($\Delta G_{\text{bwb}}^{\text{Tot}} > 0 \text{ mJ/m}^2$). The free energy of adhesion of *P. fluorescens* to glass was about 29.8 mJ/m^2 , revealing that adhesion was not thermodynamically favoured. The application of OPA to *P. fluorescens* reduced the surface hydrophilic characteristics (Table 1), particularly at the lowest concentration tested. For other OPA concentrations the surface cellular changes were similar ($P > 0.05$). The values of the surface tension components demonstrated that bacteria developed polar properties after OPA treatment, as evidenced by the increases in γ^{AB} values. OPA decreased cellular electron donation (γ^-) ability and increased the electron accepting (γ^+) properties. This effect was not concentration dependent ($P > 0.05$). The apolar component (γ^{LW}) of bacteria was almost unaffected by OPA except at a concentration of 0.15 mM ($P > 0.05$). The analysis of the free energy of adhesion of OPA treated bacteria indicated an increase in the adhesion potential. The decreases in the free energy of adhesion for all the concentrations tested were not significantly different ($P > 0.05$).

Cell surface hydrophilic characteristics were progressively reduced with increasing CTAB concentrations (Table 1). With 0.9 mM CTAB, bacterial surface properties changed from hydrophilic to hydrophobic ($\Delta G_{\text{bwb}}^{\text{Tot}} < 0 \text{ mJ/m}^2$). The decreases of the bacterial apolar characteristics (γ^{LW}) for all CTAB

concentrations tested were not significantly different ($P > 0.05$). Polar characteristics (γ^{AB}), increased significantly ($P < 0.05$) with increasing CTAB concentration. Lower CTAB concentrations increased, but higher concentrations decreased γ^- values significantly ($P < 0.05$). The γ^+ values for cells increased significantly ($P < 0.05$) with increasing CTAB concentration. The thermodynamic evaluation of adhesion indicated that adhesion was not favourable when cells were treated with 0.125, 0.25 mM or 0.5 mM CTAB ($\Delta G_{\text{bwb}}^{\text{Tot}} > 0 \text{ mJ/m}^2$) even though the free energy of adhesion decreased with increasing CTAB concentration.

4. Discussion

Explanations of the adhesion of microorganisms to solid surfaces assume that the interfacial free-energy is the controlling force in cell adhesion. According to this concept, cell adhesion is favoured when the interfacial tension between the surface, the microorganism and the suspending liquid is reduced (Simões et al., 2007a). Moreover, the cell attachment has been shown to increase with increasing surface hydrophobicity (Oliveira et al., 2001). In a flowing system, microorganisms are transported to the surface by diffusion, convection, sedimentation and active movement (Gottenbos et al., 1999), and will tend to adsorb irreversibly to minimize the free energy of the system. In this study, adhesion data demonstrated that the rate of attachment

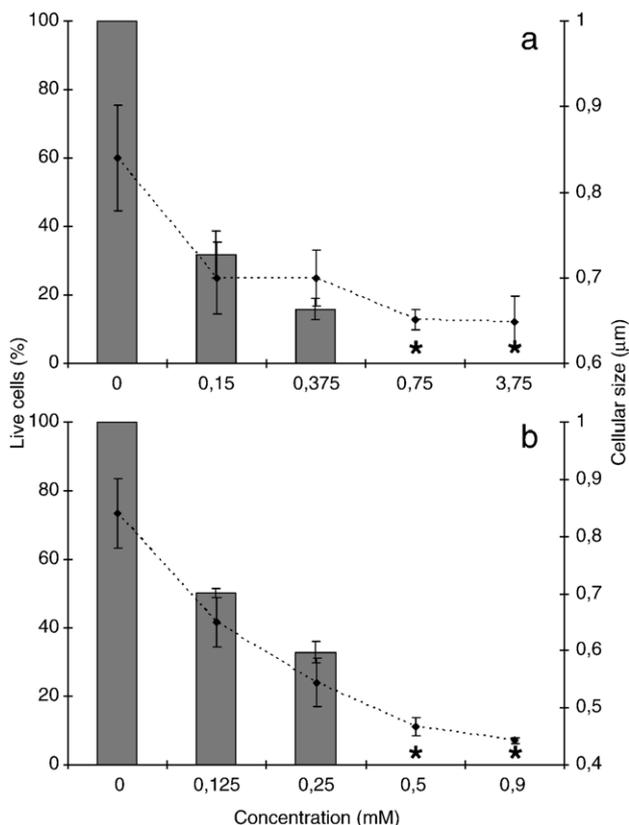


Fig. 3. Viability (■) and size (◆) of adhering cells treated with (a) OPA or (b) CTAB. * – indicates that all the cells were non-viable. Each symbol indicates the mean \pm SD of three independent experiments.

Table 1

Values for the means±standard deviation for the hydrophobicity ($\Delta G_{\text{bwb}}^{\text{Tot}}$), apolar (γ^{LW}) and polar ($\gamma^{\text{AB}}-\gamma^-$ electron donor and γ^+ electron acceptor of the γ^{AB} component) components of the surface tension of untreated bacteria and OPA or CTAB treated cells, and the free energy of adhesion ($\Delta G_{\text{bws}}^{\text{Tot}}$) between *P. fluorescens* (b) and glass (s) when immersed in water (w)

	Without treatment	[OPA] mM				[CTAB] Mm			
		0.150	0.375	0.750	3.75	0.125	0.250	0.500	0.900
$\Delta G_{\text{bwb}}^{\text{Tot}}$ (mJ/m ²)	65.7±4.8	6.08±1.3	19.8±2.1	23.4±0.79	24.4±2.9	71.4±3.3	57.9±0.88	7.90±1.1	-2.21±0.41
γ^{LW} (mJ/m ²)	24.1±1.1	11.7±1.2	23.4±1.7	24.4±1.1	26.2±2.1	13.2±0.88	13.0±0.93	15.4±1.3	17.4±0.87
γ^{AB} (mJ/m ²)	7.20±0.78	52.3±2.1	33.1±2.4	31.4±3.2	30.5±3.0	17.4±2.7	22.5±0.91	33.3±0.98	27.0±2.3
γ^- (mJ/m ²)	74.0±2.4	47.2±3.2	47.8±3.0	50.7±4.3	51.4±2.2	90.2±3.5	81.2±1.9	36.4±2.7	23.7±0.87
γ^+ (mJ/m ²)	0.200±0.01	14.5±0.30	5.70±0.85	4.90±0.09	4.50±1.0	0.80±0.03	1.60±0.21	7.60±1.3	7.70±0.99
$\Delta G_{\text{bws}}^{\text{Tot}}$ (mJ/m ²)	29.8±0.05	18.8±2.0	15.3±0.11	17.0±0.09	17.1±1.3	41.3±0.07	36.9±0.06	8.20±0.16	-3.89±0.08

was low during the initial 300 s. Thereafter the rate accelerated, probably because primary surface colonizers had formed a conditioning film (Gottenbos et al., 1999; Simões et al., 2007a) on which other cells could aggregate (Rickard et al., 2003). Free energy of adhesion results showed that *P. fluorescens* adhesion to glass is not favoured. However, it is known that other factors are involved in the adhesion process. Biological mechanisms, such as the presence of adhesive molecules on cell surfaces could be major factors in the process of microbial adhesion and further biofilm development (Doyle, 2000; Sauer and Camper, 2001). Moreover, microorganisms can adapt their attachment strategies to the nature of the surface (Tsibouklis et al., 1999).

The difficulty of removing *P. fluorescens* cells from glass using either CTAB or OPA seemed to be associated with changes to cell surface properties induced by the chemicals. According to Pasmore et al. (2002), the attraction between bacteria and the surface is expected to play an important role in the ability to remove biofilms from a surface. Moreover, decreased detachment could be influenced by electrostatic interactions, especially when cells were treated with CTAB. Azeredo et al. (2002) found that *P. fluorescens* treated with 0.5 mM CTAB become positively charged, suggesting that CTAB cements the cells to, rather than removing them from the negatively charged glass surface (Azeredo and Oliveira, 2000). Other study (Pereira and Vieira, 2001), performed with glutaraldehyde (GTA), an aldehyde-based biocide similarly to OPA, revealed that *P. fluorescens* treated with 1 mM of GTA generated unfavourable cell-glass electrostatic interactions. The application of CTAB or OPA allowed the existence of a remaining adhering pellicle that may be a source of problems, since bacterial recovery and development of resistant populations can occur (Bower and Daeschel, 1999). Furthermore, other microorganisms can adhere to the pellicle increasing the probability of pathogens survival in the process environment (Møretro and Langsrud, 2004; Lehner et al., 2005; Simões et al., 2005b; Lapidot et al., 2006).

Inactivated *P. fluorescens* resisted detachment. According to some authors (Barton et al., 1996; Gottenbos et al., 2000, 2001; Bruinsma et al., 2001; Liu et al., 2007), bacterial growth state and viability influences the ability of cells to adhere to abiotic surfaces. Strongly attached bacteria are unable to divide and grow, so attachment correlated with loss of viability (Gottenbos et al., 2000; Liu et al., 2007). According to Sauer and Camper (2001), a switch from planktonic and sessile growth involves

phenotypic changes. In this study, this phenomenon was evident even in the chemical-free assay, as cell size variation was enhanced by the stresses imposed by the chemicals.

The findings for OPA indicate that surface changes are one of the first effects of its antimicrobial action. OPA is a U.S. Food and Drug Administration (FDA) approved chemical, composed of an aromatic ring with two aldehyde groups, with broad antimicrobial activity. Its activity on Gram-negative bacteria is attributed to strong interactions with proteins which would alter cell surface properties, and with intercellular elements such as DNA (Simões et al., 2007b).

CTAB seems to act on cell functions before promoting significant surface changes, as surface properties were substantially altered by only higher concentrations. CTAB, a cationic surfactant/quaternary ammonium compound (QAC), causes cell membrane rupture by chemical reactions and electrostatic effects (Simões et al., 2006). The antimicrobial action of QAC's is attributed to their positive charge, which allows them to form electrostatic bonds with negatively charged sites on microbial cell walls (McDonnell and Russell, 1999). Those electrostatic bonds create stresses in the wall, leading to cell lysis and death. QAC's also cause cell death by protein denaturation, disruption of cell-wall permeability, and reduction of the uptake of essential nutrients (Cloete et al., 1997). It has been suggested that the primary site of action of CTAB is lipid components of the membrane with cell lysis being a secondary effect (Gilbert et al., 2002).

In conclusion, this study provides clear evidences of resistance to detachment of *P. fluorescens* attached to glass. Although both the antimicrobial chemicals tested interact strongly with bacteria, their application cannot be recommended for the induction of microbial detachment. The findings showed that OPA and CTAB interact with the cell surface to inactivate cells that remain strongly adhered to the surface. A layer of such dead cells may provide a surface to which other microorganisms can attach and so facilitate the process of biofilm formation.

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