World Journal of Microbiology & Biotechnology (2005) 21: 1077–1081
DOI 10.1007/s11274-004-8288-1

Enhancement of glutaraldehyde biocidal efficacy by the application of an electric field. Effect on sessile cells and on cells released by the biofilm

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Received 9 September 2004; accepted 28 December 2004

Keywords: Biocide, biofilm, *Pseudomonas fluorescens*, released cells, sessile cells

Summary

The aim of this paper was to evaluate the possible enhancement of the biocidal efficacy of glutaraldehyde against *Pseudomonas fluorescens* biofilms by the application of an electric field. The behaviour of sessile cells and cells released by the biofilms was assed. Biofilms were formed on thin stainless steel coupons immersed in culture media inoculated with *Pseudomonas fluorescens*. Treatments using glutaraldehyde (TGA) and both glutaraldehyde and electric field application (TGAEF) were carried out with the samples with biofilms. TGA: samples with biofilms were immersed in glass cells containing a buffer solution with different glutaraldehyde concentrations in the 25–500 ppm range. TGAEF: samples with biofilms were immersed in an electrochemical cell containing glutaraldehyde solution where a direct electric current (4×10^{-4} A cm^{-2}) was delivered to the chamber. The evolution of biofilms was observed through optical microscopy at real time. Results show that the electric field enhanced glutaraldehyde efficacy reducing the number of surviving cells in the range of one to four orders with respect to those with TGA treatment. The sensitivity of the cells to the treatments decreased in the following order: planktonic cells > cells released by the biofilm > sessile cells.

Introduction

Biofilm development can be divided into several stages (Stewart *et al*. 2000a; Stoodley *et al*. 2001): (a) transport of the planktonic cell towards the substratum surface,(b) attachment of pioneer planktonic cells to the surface in a reversible form, (c) production of extracellular polymeric substances which allow the cells to achieve irreversible attachment becoming sessile cells, (d) maturation of the biofilm, (e) release of some cells from the biofilm which could attach in another site completing a cycle (Singleton *et al*. 1997; Xu *et al*. 1998; Rice *et al*. 2002; Cortizo & Fernández L. de Mele 2003). It has been reported (Brözel *et al*. 1995; Sauer & Camper 2001; Sauer *et al*. 2002; Donlan & Costerton 2002) that *Pseudomonas* cells display multiple phenotypes during biofilm development. It was observed that pioneer planktonic cells that approached to the substratum changed regulation of motility, alginate production, and quorum sensing. These altered behaviours could affect the sensitivity of the microorganisms to antimicrobial agents (Lewandowski 1998; Geesey & Bryers 2000).

Glutaraldehyde (GA) is employed as a biocidal agent in different environments such as oilfields, industrial water treatment and sterilisation of medical and odontological instruments. Although GA is highly effective against planktonic cells, sessile cells are not easy to eradicate by conventional GA treatments (Videla *et al*. 1991; Grobe & Stewart 2000; Grobe *et al*. 2002). Consequently, it is a challenge to develop new techniques to enhance the efficacy of this biocide.

It has been reported that the efficacy of antibiotics can be improved in the presence of an electric field. Although the electric field alone only causes minor effects on Pseudomonas cells, the combined treatment, i.e. antimicrobial plus electric field, has been proved as effective. This effect has been called *bioelectric* and its mechanism remains elusive (Costerton *et al*. 1995; Stoodley *et al*. 1997, 1999; Wattanakaroon & Stewart 2000). Today, most of the research into the bioelectric effect has been focused on medical applications. However, it may be possible to exploit this phenomenon to control biofilms of industrial systems (Stoodley *et al*. 1997).

The aim of this work was to assess the possible enhancement of the biocidal efficacy of GA against *Pseudomonas fluorescens* (*P. fluorescens*) (planktonic cells, released cells and sessile cells) by the application of an electric field.

Materials and methods

Inoculum

Pure cultures of *P. fluorescens* isolated from an industrial environment were used in the experiments. *P. fluorescens* was maintained in Cetrimide agar (*selective* agar for *Pseudomonas*) at 28 °C. *P. fluorescens* inoculum was prepared by suspending a Cetrimide agar slant (24 h old) in 2 ml of sterile nutrient medium. Afterwards, the inoculum was poured into an Erlenmeyer flask containing 300 ml of the nutrient broth medium and kept on a rotary shaker for 3 h at 28 °C.

Planktonic cells

Aliquots (3 ml) of the nutrient broth culture were centrifuged (2500 rev min^{-1}) and the supernatant broth decanted. The bacterial pellets were then resuspended and challenged with GA at concentrations ranging from 25 to 500 mg l^{-1} (ppm) for 6 h. The disinfection solutions were made from a stock solution of GA (500 ppm) and phosphate buffer solution (NaCl: 8 g l^{-1}, KH_2PO_4: 0.34 g l^{-1}, K_2HPO_4: 1.21 g l^{-1}, pH = 7.2, which is described as phosphate buffer throughout the text) so that the final concentration was the desired. The liquid media were centrifuged. The bacterial pellets were resuspended in the above phosphate buffer solution. The number of surviving cells was enumerated using a drop plate method employing nutrient agar, after 48 h incubation time. A control assay was conducted in the same manner, with a buffer solution without GA addition.

Biocidal treatments against sessile cells and cells released from the biofilm

Thin stainless steel coupons (area: 3 cm^{-2}) were immersed for 3 h in an Erlenmeyer flask with *P. fluorescens* pre-growth culture during the stationary phase of growth, under static conditions. Biofilms were formed on the surface of the coupons. Experiments under laminar flow conditions ($5 < Re < 50$) were also made. In this case, *P. fluorescens* pre-grown culture at a stationary phase of growth was pumped to the glass cell containing the coupons. The number of planktonic bacteria at the beginning of the experiments was chosen to be between 10^7 and 10^{10} cells ml^{-1}.

The samples with biofilms were treated in two different ways (Treatments TGA, TGAEF). TGA: two samples with biofilms formed on their surfaces were removed from the batch culture and afterwards they were immersed in a specially designed 30 ml glass cell containing a buffer solution with different GA concentrations (25, 50, 100, 200 and 500 ppm) and a control buffer solution without GA. To maintain a constant biocide concentration, the solution exposed to the biofilms was changed every 1–2 h. Released cells were enumerated using standard plating method (spm). Sessile cells were enumerated by spm after scraping the sessile cells off the metal samples and suspending the cells in NaCl solution (9 g $l^{-1)}$. Then the suspension was centrifuged at 2500 rev min^{-1} for 15 min, the bacterial pellets were rinsed with a sterile buffer and the sequence was repeated twice to eliminate the rest of GA. Subsequently, the solutions were sonicated for 10 s three times, to disperse the cells.

TGAEF: The samples with biofilms formed on their surfaces were put into an electrochemical cell containing a buffer solution and 100 ppm GA. A direct electric current was delivered to the chamber by means of a circuit including a current controller and platinum (anode) and stainless steel (cathode) electrodes. Electric current flowed at a current density of c.a. 4×10^{-4} A cm^{-2} (Voltage: −1.1 V between the cathode and the reference electrode (saturated calomel electrode)). The samples with biofilms formed on their surfaces were placed within the electrolyte volume, confined between the electrodes during a 4 h period.

The early stages of the biofilm growth were observed through a simple non-invasive optical microscopy method previously developed (Cortizo & Fernández L. de Mele 2000) using a glass flow cell and a thin substratum.

Epifluorescence microscopy of the biofilms stained with acridine orange (McFeters *et al.* 1999) and difluorescein acetate were also made. Enumerations of bacteria during the initial period of biofilm growth (bacterial densities $< 10^6$ cells cm^{-2}) were made. Qualitative information about the covered area for mature biofilms was also obtained.

At least three separate experiments were performed for each experimental condition. Data were expressed as the mean ± SD. Statistical differences were analysed using a Student's *t*-test.

Results

Figure 1 shows that the effectiveness of GA against the sessile cells was significantly ($P < 0.05$) lower than those observed against planktonic cells and released cells. The eradication of *P. fluorescens* planktonic cells could be achieved after TGA and TGAEF using low GA concentrations (25 ppm, exposure period 4 h). The number of sessile cells enumerated after scraping the stainless steel coupon decreased only in two or three orders using 100 ppm GA in the absence or in the presence of the electric field respectively. However, initial concentrations of up to 500 ppm GA were unable to eliminate *P. fluorescens* sessile cells completely, even under the electric field treatment. The efficacy of GA treatments against biofilms formed in cultures with lower concentrations of cells ($<10^8$ cell cm^{-2}) was higher. In this case, the number of sessile cells decreased more than 3 orders of magnitude after treatments, reaching values lower than 10^4 cells cm^{-2}.

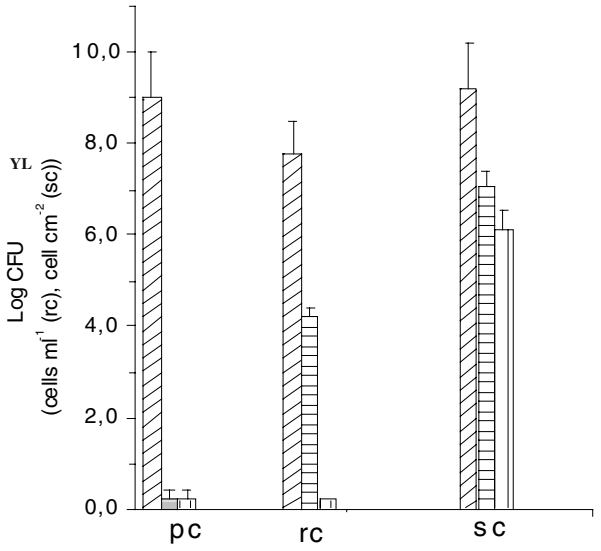

Figure 1. Log of the colony forming units (CFU) from planktonic cells (pc) the cells released by the biofilm (rc) and sessile cells (sc) obtained after the immersion of the biofilm in a sterile buffer solution (/) (control), in the buffer solution + 100 ppm GA (Treatment TGA) (—) and in the buffer solution + 100 ppm GA under an electric field (Treatment TGAEF) (|). Exposure period 4 h, batch culture.

After TGA and TGAEF the surviving released cells vs. time relationships attained a maximum while a stationary value was reached by the control curve (Figure 2 a). After the application of TGAEF an almost complete elimination of released cells was reached after 4 h. Figure 2b shows at higher magnification the curves of Figure 2a during the first 2 h. It can be observed that the slope of the control curve is higher than those of the other curves. Particularly, the number of living cells in suspension is zero after the first hour when the electric field was applied and when the concentration of glutarldehyde was increased up to 200 ppm.

Microscopic observations made *in situ* showed that in the presence of GA the biofilm matrix constricted (up to 50% the original thickness). The biofilm layer could be seen as more compact, thin, darker and less transparent.

Biofilm detachment could also be observed after TGA and TGAEF (Figure 3) in agreement with previous results (Videla *et al.* 1991; Viera *et al.* 1999a, b). However, the detachment was more evident when an electric field was applied.

Discussion

It was reported (Eagar *et al.* 1988) that, the sensitivity to GA of the sessile cells scraped from the biofilm, dispersed in solution and then treated with GA is between that of planktonic cells and sessile cells. The sessile phenotypic expression which could be related to the resistance to the biocidal action of antimicrobial agents (Cochran *et al.* 2000; Sauer & Camper 2001; Sauer *et al.* 2002) seems to be progressively lost by the sessile cells dispersed in a liquid medium (released

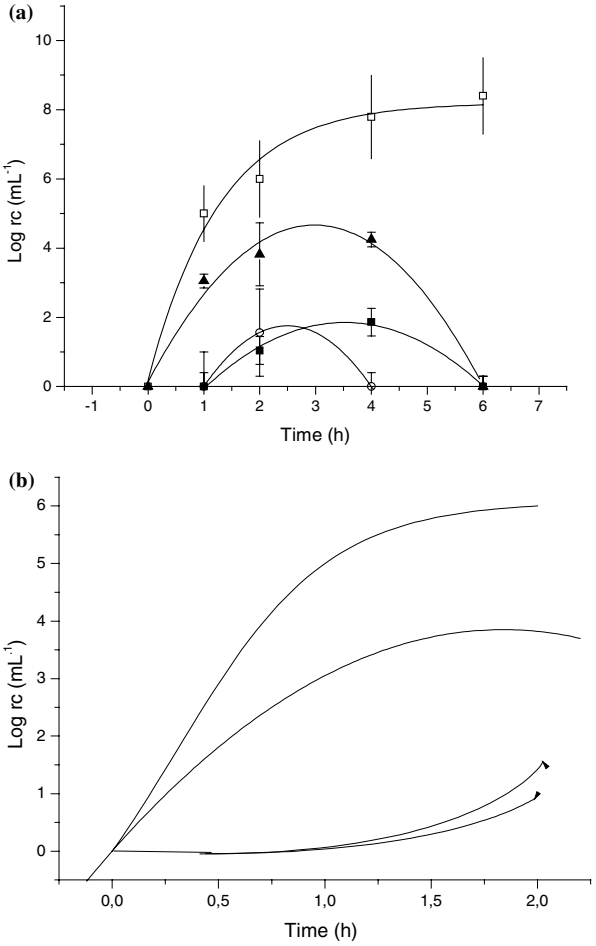

Figure 2. Log of the colony forming units (CFU) *vs* time (1, 2, 4 and 6 h) corresponding to the cells released by the biofilm (rc) after being immersed in a sterile buffer solution (□) (control), in the buffer solution + 100 ppm GA (●), or 200 ppm GA (△) (Treatment TGA) and in the buffer solution + 100 ppm GA under an electric field (Treatment TGAEF) (▼). Batch culture experiments.

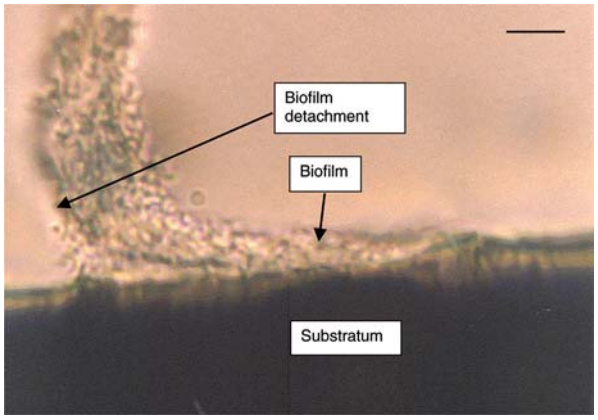

Figure 3. Microphotograph obtained through optical microscopy *in situ* and at real time of a *P. fluorescens* biofilm after treatment TGA (100 ppm GA). On the left hand, the detachment of a biofilm formed on the lateral border of a thin stainless steel sheet substratum (40 × objetive). The black bar on the right corresponds to 9.1 μm.

cells). Although frequently disregarded, the release of cells by the biofilms has practical importance because it reveals a survival mechanism that allows the spread of the cells and the initiation of new biofilm formation in another place (Rice *et al.* 2002; Stoodley *et al.* 2002).

Present results show that the efficacy of GA in TGA and TGAEF treatments decreases when the activity of the cells decreases. Thus, GA effectiveness diminishes in the following order: against planktonic cells > against released cells > against sessile cells.

Results of Figure 2a and b corresponding to released cells, are very different from those reported by Grobe & Stewart (2000) related to planktonic cells. The maximum value of released cells in TGA and TGAEF assays (Figure 2a) reveals the competition between at least two processes with positive and negative contributions respectively. On the contrary, the experiments previously described by Grobe & Stewart showed a logarithmic decay in the number of planktonic cells during treatments (without a maximum), which was the evident consequence of the action of the killing process alone.

Regarding the present results, it is interesting to investigate if a synergistic mechanism of GA plus electric field could take place, involving both the inhibition of the release process and/or the enhancement of the killing process when GA and the electric field are applied simultaneously. When the beginning of the curves of Figure 2a is observed at higher magnification (Figure 2b) it can be observed that the slopes of the curves, in the presence of GA, are lower than the slope of the control. In order to interpret these results and those of Figure 1, it must be taken into account that the surviving released cells after treatment ($\mathrm{Trc}(t)$) that were enumerated at a certain time t (released cells with TGA and TGAEF in Figure 2) is the consequence of the following balance: (under the assumption that the growth is negligible during treatments, for simplicity):

$$\mathrm{Trc}(t) = \mathrm{rc}(t)_{\mathrm{t}} - \mathrm{rc}(t)_{\mathrm{k}} \quad (1)$$

where $\mathrm{rc}(t)_{\mathrm{t}}$ stands for the total released cells and $\mathrm{rc}(t)_{\mathrm{k}}$ for the released cells that were killed at time t.

Additionally, the surviving cells of the biofilm after treatment at certain time t ($\mathrm{Tsc}(t)$) that were enumerated (sessile cells with TGA or TGAEF in Figure 1), are the result of the following balance:

$$\mathrm{Tsc}(t) = \mathrm{sc_i} - \mathrm{rc}(t)_{\mathrm{t}} - \mathrm{sc}(t)_{\mathrm{k}} - \mathrm{sc}(t)_{\mathrm{kd}} \quad (2)$$

Where $\mathrm{sc_i}$ indicates initial number of sessile cells of the biofilm before the treatment (close to the value of the sc control in Figure 1), $\mathrm{sc_{kd}}$ biofilm cells that were killed and detached and $\mathrm{sc_k}$ those that were not detached. According to (2) $\mathrm{sc_i}$ before treatment (initial) is the sum of the surviving sessile cells plus released cells after treatments ($\mathrm{Tsc}(t) + \mathrm{rc}(t)_{\mathrm{t}}$) plus the sessile cells that were killed (detached and non detached ($\mathrm{sc}(t)_{\mathrm{kd}} + \mathrm{sc}(t)_{\mathrm{k}}$)).

Because of the complexity of the balances, it is not simple to elucidate if the reason for the decrease of the surviving cells is the reduction of the release rate, the enhancement of the killing action or both reasons simultaneously. However, there are interesting features of Figure 2b that are worth investigating. During the first hour of treatment, the number of surviving released cells, when 200 GA were added and when the electric field was applied with 100 ppm GA, was zero. This indicates that either the release process was delayed or that the few released cells were killed under these conditions (Equation (1)). Besides, under the same GA concentration, the slope of the curve was lower when the electric field was applied, showing the reduction of the release process and/or the enhancement of the killing process (Equation (1)). Additionally, the number of sessile cells decreased one order during TGAEF with respect to TGA (Figure 1, Equation (2)), and the release of cells is probably dependent on this number. Consequently, it could be deduced that the release process possibly decreased during TGAEF because of the reduction of surviving sessile cells. Besides, the reduction of the release process could be caused by changes in the activity of the sessile cells during the treatments. It must also be considered that the released cells lose the resistant characteristics of the sessile cells with time (Cochran *et al.* 2000; Sauer & Camper 2001; Sauer *et al.* 2002), and this probably increases the rate of the killing processes in the presence of the electric field. Coincidently, Figure 2 shows that the maximum value of the released cells is lower and occurred earlier in the presence of the electric field, which maybe attributed, according to Equation (1), to both (*a*) the reduction of the release process of cells and (*b*) the enhancement of the killing process. According to this analysis, the reduction of the surviving released cells cannot be unequivocally attributed to only one reason, it is most likely the result of the contributions of both (*a*) and/or (*b*) which are enhanced in the presence of the electric field. The analysis is even more complex if the growth of bacteria during treatments is considered.

The mechanism of the bioelectric effect is still not understood. It has been reported that the electric field *per se* is not responsible for the effect because it causes only a log reduction of 0.65 ± 0.42 in viable *Pseudomonas* cell numbers, compared to the number of the untreated control. It has been hypothesised that the electric field may cause structural changes to the biofilm, variations in the interfacial pH, production of H_2O_2, high local concentrations of O_2, etc. However, all of these causes partially explain the bioelectric effect (Stoodley *et al.* 1997; Stewart *et al.* 2000b).

It could be concluded that the electric field was able to improve the GA treatment by increasing the detachment process of biofilm patches, by increasing the killing action and by decreasing the release of cells. The application of the combined method (TGAEF = GA + electric field) may be useful to improve the control of biofilms in industrial or health centre environments.

Acknowledgements

Authors wish to acknowledge the financial support received from UNLP, CONICET (PIP 02359/00) the Agencia Nacional de Promoción Científica y Tecnológica (PICT 06-12508) and the Commission of Scientific Investigation of Buenos Aires Province (CICPBA art. 14, 4686/68). S.G. Gómez de Saravia and M.F. Lorenzo de Mele are researchers of CICPBA and CONICET respectively.

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