

# Is gas-discharge plasma a new solution to the old problem of biofilm inactivation?

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Conventional disinfection and sterilization methods are often ineffective with biofilms, which are ubiquitous, hard-to-destroy microbial communities embedded in a matrix mostly composed of exopolysaccharides. The use of gas-discharge plasmas represents an alternative method, since plasmas contain a mixture of charged particles, chemically reactive species and UV radiation, whose decontamination potential for free-living, planktonic micro-organisms is well established. In this study, biofilms were produced using *Chromobacterium violaceum*, a Gram-negative bacterium present in soil and water and used in this study as a model organism. Biofilms were subjected to an atmospheric pressure plasma jet for different exposure times. Our results show that 99.6% of culturable cells are inactivated after a 5 min treatment. The survivor curve shows double-slope kinetics with a rapid initial decline in c.f.u. ml<sup>-1</sup> followed by a much slower decline with *D* values that are longer than those for the inactivation of planktonic organisms, suggesting a more complex inactivation mechanism for biofilms. DNA and ATP determinations together with atomic force microscopy and fluorescence microscopy show that non-culturable cells are still alive after short plasma exposure times. These results indicate the potential of plasma for biofilm inactivation and suggest that cells go through a sequential set of physiological and morphological changes before inactivation.

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## INTRODUCTION

Most studies dealing with the growth and physiology of bacteria have been carried out using planktonic cells in batch cultures. Although these studies have provided extensive information regarding the basic molecular mechanisms that control the growth of individual bacteria, most bacteria live primarily in communities referred to as biofilms (Stoodley *et al.*, 2002), where cooperative effects become important. Biofilms are microbial communities embedded in a matrix mostly composed of exopolysaccharides together with some proteins and nucleic acids. Biofilm formation can be considered as a developmental cycle that begins when planktonic bacteria attach to a surface, divide and recruit additional planktonic cells that attach to the cells already on the surface. In some cases, this process results in the development of a mature biofilm, in which cells cluster in pillar- and mushroom-like structures with water channels between them, forming a primitive

circulatory system (Kolter & Losick, 1998). In other cases, more compact, flat and homogeneous biofilm layers (Heydorn *et al.*, 2000; Picioreanu *et al.*, 2000), spherical clusters (Matsumoto *et al.*, 2007), discrete microcolony structures (Massol-Deyá *et al.*, 1995), ball-shaped microcolonies (Tolker-Nielsen *et al.*, 2000) and honeycomb-like structures (Marsh *et al.*, 2003; Russo *et al.*, 2006) have been reported. Although it is out of the scope of this paper to extensively review the different biofilm structures reported, it is generally accepted that biofilms are not a continuous monolayer surface deposit but instead a thin base film, ranging from a patchy monolayer of cells to a film several layers thick (Stoodley *et al.*, 1997). It has also been reported that a micro-organism such as *Pseudomonas aeruginosa* can produce biofilms with very different structures when growing under different sets of conditions (Davies *et al.*, 1998; Heydorn *et al.*, 2000; Murga *et al.*, 1995; Stewart *et al.*, 1993). Taken together, these results indicate that the wide variety of experimental biofilm model systems being used in different laboratories promote different kinds of biofilm structures (Heydorn *et al.*, 2000).

Abbreviations: AFM, atomic force microscopy; *D* value, decimal reduction time value.

Microbial biofilms on surfaces cost the USA billions of dollars per year in equipment damage, product contamination, energy losses and medical infections. Biofilm formation is a serious medical problem that leads to colonization of medical devices such as prosthetic implants (Costerton *et al.*, 1995; Elder *et al.*, 1995; Ell, 1996). Biofilms are responsible for diseases such as otitis media and play a role in cystic fibrosis and Legionnaire's disease, among others (Costerton *et al.*, 1999). Biofilms are also responsible for plaque formation on teeth and tooth decay. Many household surfaces, including toilets, sinks, countertops and cutting boards, are contaminated by biofilms. Biofilms impact adversely upon many industrial processes, leading to a decrease in process efficiency and end-product purity. Thus, biofilm control has become an area of intense study (De Kievit *et al.*, 2001).

Conventional methods of killing free-living bacteria through antimicrobial agents and disinfection are often ineffective against bacteria within a biofilm (Hoyle & Costerton, 1991; Stewart & Costerton, 2001; Stewart, 2002; Leriche *et al.*, 2003; Saravanan *et al.*, 2006). Therefore, the ability to destroy these living organisms is critical and the problem demands the development of alternative techniques. The use of gas-discharge plasmas potentially offers a good alternative to conventional sterilization methods, since plasmas contain a mixture of charged particles, chemically reactive species and UV radiation, which are effective in the destruction of individual micro-organisms (Laroussi, 1996; Moisan *et al.*, 2001, 2002; Purevdorj *et al.*, 2003). Gas-discharge plasmas are generated by supplying energy to a neutral gas, which causes the formation of charge carriers. Electrons, ions and free radicals are produced in the gas phase when electrons of sufficient energy collide with the neutral atoms and molecules in the feed gas. The most commonly used method of generating plasmas is by applying an electric field to a neutral gas (Conrads & Schmidt, 2000). Any volume of gas always contains a few electrons and ions that are formed, for example, as the result of interaction with cosmic rays or radioactive radiation. These free-charge carriers are accelerated by the electric field, and new charged particles are created when these carriers collide with atoms and molecules in the gas or with the surfaces of the electrodes. This process leads to an avalanche of charged particles that is eventually balanced by charged carrier losses, so that steady-state plasma is created.

Research on plasma-based sterilization and decontamination has been extensive for the past 15 years. Plasmas can be classified in terms of the pressure of the operating gas as low-pressure plasmas (1–10 mTorr range; 0.133–1.33 Pa), medium-pressure plasmas (0.1–10 Torr range; 13.3 Pa–1.33 kPa) and atmospheric pressure plasmas. The advantage of using atmospheric pressure plasmas is the possibility of obtaining the active agents mentioned above at relatively low temperatures ( $\leq 50$  °C) without the need for a vacuum system (Montie *et al.*, 2000). Furthermore, plasma sterilization is safe, both for the operator and the patient

(Moisan *et al.*, 2001). In addition, it is likely that synergistic effects among the active agents, such as charged particles, chemically reactive species and UV radiation, result in plasma being a more effective sterilization method (Lerouge *et al.*, 2001; Moisan *et al.*, 2001, 2002). These agents are well known to cause cell damage or cell death in micro-organisms exposed to them even for short periods. Park *et al.* (2003) reported bacterial disruption within 20 s of exposure to an argon plasma discharge. Purevdorj *et al.* (2003) reported similar results for spore-forming *Bacillus pumilus*. In this case, spore mortality varied depending on the composition of the gas feed and was higher with moisturized air plasma, suggesting that the inactivation may occur through hydroxyl free radicals generated from water molecules. Atmospheric pressure plasma applied to *Bacillus subtilis* reduced c.f.u. by four orders of magnitude for plasma exposure times of <10 min (Becker *et al.*, 2002; Panikov *et al.*, 2002). More recently, decimal reduction time (*D*) values of 2 s to 5.5 min have been reported for a wide variety of micro-organisms (Park *et al.*, 2007), including both bacteria and fungi (Halfmann *et al.*, 2007). The fact that sterilization by gas-discharge plasmas is at least as effective as, if not more effective than, sterilization by other methods which use a single killing agent suggests that there are important synergistic effects caused by the simultaneous and/or sequential effects of radiation, ions, electrons and reactive radicals on micro-organisms. These results clearly indicate that sterilization by plasmas has very attractive features in terms of effectiveness and practicality.

Therefore, numerous studies have demonstrated the effectiveness of gas-discharge plasmas for killing planktonic micro-organisms. However, to our knowledge, there are few reports about the use of plasma for biofilm disinfection or inactivation (Akishev *et al.*, 2005; Brelles-Mariño *et al.*, 2005; Abramzon *et al.*, 2006; Kamgang *et al.*, 2007). Traditionally, the effectiveness of plasma as a bacterial killing agent has been measured by counting the c.f.u. of a plasma-treated culture and calculating the number of surviving cells (Kelly-Wintenberg *et al.*, 2000; Laroussi, 2002; Akishev *et al.*, 2005; Brelles-Mariño *et al.*, 2005; Gallagher *et al.*, 2005; Abramzon *et al.*, 2006; Kamgang *et al.*, 2007). This approach relies on culturable cells alone, and does not take into account cells that might still be alive, but non-culturable, after plasma treatment. The objective of the present study was to achieve a better understanding of plasma-assisted biofilm inactivation by incorporating a variety of complementary techniques, including DNA and ATP determination together with atomic force microscopy (AFM) and fluorescence microscopy. These techniques allow us to determine the viability of biofilm-forming cells and their morphological changes after plasma treatment. In this paper we report the presence of living biofilm-forming bacterial cells after plasma treatment. Our results suggest that cells go through sequential physiological and morphological changes before becoming inactivated by plasma. Our study has important implications for the application of plasma to biofilms and indicates that longer

treatments are necessary to ensure complete inactivation/sterilization.

## METHODS

**Biofilm growth and plasma treatment.** Biofilms were produced in 96-well polystyrene microplates (Nunc) by adding 200  $\mu$ l of a bacterial suspension of *Chromobacterium violaceum* CV026 (McClellan *et al.*, 1997) with an OD<sub>650</sub> of 1.0. This strain was chosen based on preliminary experiments that showed that it produces a thick biofilm ring in agitated cultures. Bacteria were grown overnight in TY (tryptone–yeast extract) (Beringer, 1974) liquid broth at 28 °C. Microplates were incubated at 28 °C without shaking for 4 or 7 days. After incubation, unbound planktonic bacteria were removed by rinsing the wells twice with 100  $\mu$ l sterile distilled water. Plates were subjected to gas-discharge plasmas for various exposure times (5, 10, 15, 30 and 60 min, unless otherwise stated) under sterile conditions. A control without plasma treatment (0 min exposure time) was included. Bacteria were suspended in 200  $\mu$ l sterile distilled water, microplate lids were replaced and the cells were then disaggregated by a 5 min room temperature sonication treatment in a sonicator bath. Bacteria were then serially diluted and plated in duplicate on TY agar medium. Plates were incubated for 1 day at 28 °C and c.f.u. were counted.

**Plasma generation and conditions.** A gas-discharge plasma was produced by using an Atomflo 250 reactor that employs a capacitively coupled electrode design (Surfx Technologies). An atmospheric pressure plasma jet was generated by using a He flow of 20.4 l min<sup>-1</sup>, a secondary gas flow (N<sub>2</sub>) of 0.305 l min<sup>-1</sup> and an input power of approximately 4.8 W. Both gases were industrial grade. The plasma applicator was mounted such that the showerhead was only 0.7 cm away from the biofilm.

**DNA determination.** Four-day-old *C. violaceum* biofilms were produced in eight-well chambered coverglasses (Lab-Tec chambered coverglass, Nunc #15541), processed, and subjected to plasma treatment for 0, 5 and 20 min. A 750  $\mu$ l volume of 1 × Tris/acetate/EDTA (TAE) buffer (pH 8.0) was added to each well and after 15 min the content of each well was centrifuged at 10 000 r.p.m. for 5 min. A<sub>260</sub> and A<sub>280</sub> of the supernatants were determined using a Shimadzu BioSpec-mini spectrophotometer.

### Cell viability determination

**ATP estimation (BacTiter-Glo microbial cell viability assay).** Four-day-old *C. violaceum* CV026 biofilms were produced as described above and subjected to plasma treatment for 0 (control), 5, 60 and 240 min, followed by sonication and resuspension. A 100  $\mu$ l volume of the bacterial suspension was used to determine cell viability with the BacTiter-Glo microbial cell viability assay (Promega) according to the manufacturer's instructions. A control with no cells was included. Luminescence was measured with a Turner TD-20/20 luminometer.

**Fluorescence microscopy.** Four-day-old *C. violaceum* biofilms were produced in eight-well chambered coverglasses (Lab-Tec chambered coverglass, Nunc #15541), processed, and subjected to plasma treatment as described above. Biofilms were disaggregated by a 5 min room-temperature sonication treatment in a sonicator bath. Biofilm-forming bacterial cells were then resuspended and subjected to the LIVE/DEAD BacLight Bacterial Viability kit (Promega) according to the manufacturer's instructions. Stained cells were used to prepare smears on poly-L-lysine-coated glass slides. Samples were visualized with an Olympus BX61 fluorescence microscope.

**AFM.** Biofilms were grown in 96-well plates, treated with plasma for 0, 5 and 60 min, and processed as indicated above. Bacterial suspensions were used to prepare smears on glass slides. The smears were air-dried and atomic force microscope images were obtained in air in intermittent contact mode using a Quesant Instruments Universal Scanning Probe Microscope. Commercial silicon cantilevers from MikroMasch were employed with resonant frequencies between 150 and 190 kHz and spring constants from 25 to 60 N m<sup>-1</sup>. To identify appropriate areas for imaging, an optical microscope was first used to locate regions where the density of bacteria and other biomaterials was low enough to avoid multilayered clusters of organisms. The sample slide was then adjusted with a micromechanical stage manipulator to position the AFM tip over the area of interest. In this way, individual bacteria could be imaged on a flat glass background, allowing unambiguous identification. For each slide, at least seven widely separated regions were imaged to obtain a representative sample and ensure reproducibility.

## RESULTS AND DISCUSSION

Table 1 shows the percentage of *C. violaceum* culturable biofilm-forming cells removed at various plasma exposure times under the conditions indicated in Methods. The results show that four-day-old biofilms produced in polystyrene microtitre plates were almost completely inactivated by uniformly treating cells with atmospheric pressure plasma for 5 min. Ninety-six-well microtitre plates were chosen to grow biofilms because the internal size of each well is almost exactly the size of the plasma reactor head, which ensures a uniform exposure of cells. Experiments were also carried out with seven-day-old biofilms. Although we expected these biofilms to be more resilient than the four-day-old ones, similar results were obtained and there were no significant differences between the percentages of inactivated cells for the two sampling dates (Table 1).

To rule out effects of gas flow on biofilms, preliminary experiments were carried out to compare plasma-treated biofilm to biofilm exposed to a flow of gas in the absence of plasma (plasma source turned off). Although the flow of gas dried out cells and caused a decrease in the number of c.f.u. of about 5 to 10 %, this decrease was much smaller than the decrease produced by plasma (results not shown). Therefore, cell inactivation is due to plasma treatment and not due to excessive drying of cells by the gas flow.

**Table 1.** Percentage of *C. violaceum* CV026 biofilm-forming cells inactivated after various plasma exposure times

Results are the mean  $\pm$  SD of three independent experiments.

Exposure time (min)	Percentage inactivation	
	Four-day-old biofilm	Seven-day-old biofilm
5	99.61 $\pm$ 0.41	99.94 $\pm$ 0.08
10	99.41 $\pm$ 0.82	99.60 $\pm$ 0.69
15	99.70 $\pm$ 0.60	99.97 $\pm$ 0.06
30	99.76 $\pm$ 0.44	99.97 $\pm$ 0.06
60	99.98 $\pm$ 0.03	100 $\pm$ 0

We have previously reported the rotational temperatures of plasma species and calculated a gas temperature of 52 °C at the tip of the plasma applicator (Abramzon *et al.*, 2006). To determine the temperature that impacts the biofilm and to rule out the effect of temperature on biofilm inactivation, a K-type thermocouple was placed within an individual well of the 96-well plate, 0.7 cm away from the plasma showerhead. Equilibrium temperatures of 22–25 °C were reached within a few minutes, and remained constant over time, after applying the plasma to an empty well. Therefore, temperature is not responsible for biofilm inactivation.

The removal efficiency of a particular procedure can be assessed by determining the decimal reduction time ( $D$  value) (ISO, 1994, 2000); that is, the time required to reduce an original concentration of micro-organisms by 90 %. This parameter was originally defined for the thermal killing of micro-organisms by autoclaving.  $D$  values for plasma-treated planktonic cells and spores are in the range of 20 s to 10 min (Laroussi *et al.*, 2000; Laroussi, 2002; Moisan *et al.*, 2002). For *C. violaceum* biofilms, we have previously shown that the kinetics of biofilm inactivation have a double-slope behaviour (Abramzon *et al.*, 2006). In that study, the survivor curve [ $\log(\text{c.f.u. ml}^{-1})$  versus exposure time] showed a first phase consisting of a rapid initial decline in c.f.u. ml<sup>-1</sup> and a  $D$  value ( $D_1$ ) of  $2.3 \pm 0.34$  min followed by a much slower decline (second phase) with a  $D$  value ( $D_2$ ) of  $37.4 \pm 12.1$  min, which was longer than those reported for the inactivation of planktonic organisms, suggesting a more complex inactivation mechanism for biofilms. Bi- and triphasic behaviours for plasma-assisted killing of free-living micro-organisms and spores have been reported before. Two-slope behaviour with a smaller  $D$  value for shorter exposure times and a higher  $D$  value for longer exposure times has been reported for *Staphylococcus aureus*, *Escherichia coli* and *P. aeruginosa*. These biphasic curves have been explained as being due to damage to the cell membrane produced by the plasma reactive species in the first phase, followed by the penetration of the reactive species in the second phase, causing cell death (Kelly-Wintenberg *et al.*, 1998; Laroussi *et al.*, 2000; Critzer *et al.*, 2007; Kayes *et al.*, 2007). In the case of plasma-assisted spore killing, two- and three-slope survivor curves have also been reported (Moisan *et al.*, 2002). The process involves the action of UV radiation on isolated spores or on the first layer of stacked spores, followed by a further erosion of the various materials (coats, debris, dead spores) that cover still-living spores, therefore slowing down the process of DNA destruction of those spores by UV irradiation. We previously hypothesized that in our case, and in analogy to spores, the initial rapid decline of c.f.u. ml<sup>-1</sup> might have been due to the destruction of the upper layers of micro-organisms, easily available and more exposed to plasma. After this rapid removal, plasma had to penetrate layers of cell debris and dead cells before reaching the inner portion of the biofilm (Abramzon *et al.*, 2006).

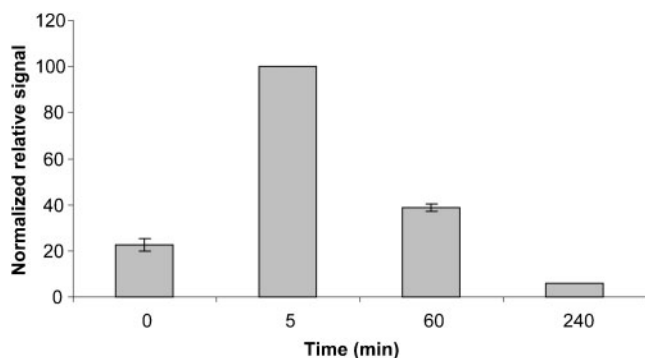
In a previous work we studied the chemistry of the generated plasma by spectrometry. We reported the presence of NO  $\gamma$ -bands at ~250 nm and an OH band at ~309 nm (Abramzon *et al.*, 2006). These reactive species have a direct impact on micro-organisms, especially on their outermost membranes (Laroussi *et al.*, 2000; Laroussi & Leipold, 2004; Laroussi, 2005). The OH radical is very reactive within the cell and will react with most biomolecules (Singh & Singh, 1982). Oxidative damage to membranes and cell walls is due to damage to either lipids or proteins. Oxidative stress has been shown to cause peroxidation of lipids, producing shortened fatty acids and therefore loss of the membrane structural integrity (Farr & Kogoma, 1991). A similar process may occur in the lipopolysaccharide of the outer membrane of Gram-negative cells. Interaction of oxygen radicals with proteins leads to the oxidation of several amino acids. Membrane proteins and amino acids from the peptidoglycan are susceptible to attack by OH radicals. It has been reported that NO adds to the lethality of the process (Laroussi, 2002). In summary, the presence of these radicals compromises the function and viability of the membrane and the cell wall. The plasma conditions chosen for our experiments were those that maximized OH and NO emissions and produced stable plasma (Abramzon *et al.*, 2006).

Laroussi and others (Laroussi, 2002; Laroussi *et al.*, 2001a, b) have observed cytoplasm leakage followed by total cell fragmentation when planktonic *E. coli* cells are subjected to plasma discharges for more than 30 s. The spectrophotometric measurement of bacterial extracellular media after plasma treatment has been reported by Kelly-Wintenberg *et al.* (1999) and Montie *et al.* (2000). An increase in  $A_{260}$  is considered to be evidence of leakage of UV-absorbing molecules such as nucleic acids and proteins from the cytoplasm into the extracellular fluid. In order to determine whether our decrease in the number of culturable cells was due to cell breakage and cytoplasmic leakage, we carried out experiments treating four-day-old bacterial biofilms with plasma for 0, 5 and 20 min and measuring  $A_{260}$  and  $A_{280}$ . The ratio of  $A_{260}$  and  $A_{280}$  was 1.42, 1.48 and 1.46 for 0, 5 and 20 min treatment, respectively (average of two independent experiments). These results do not indicate a release of DNA into the extracellular fluid. Therefore, in our experiments, there is no indication of a release of the cytoplasmic content into the medium after plasma treatment, suggesting that the cell membranes are still intact after 20 min of plasma treatment.

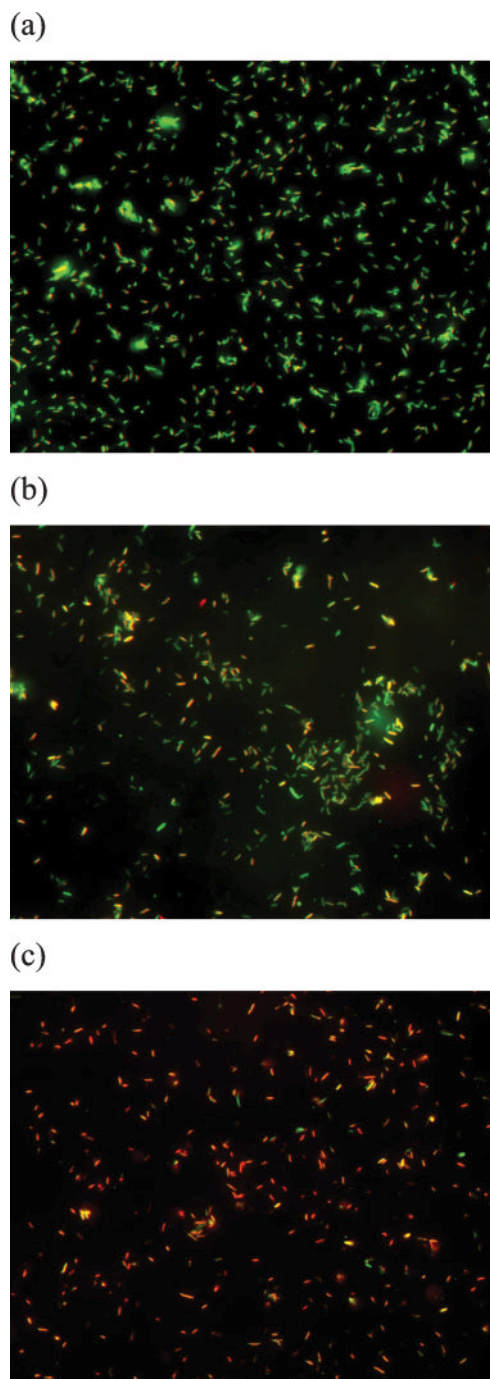
In order to measure viability of biofilm-forming cells after plasma treatment, two approaches were used: the indirect measurement of ATP production, and fluorescence microscopy using a combination of dyes that target dead and living cells. ATP production was indirectly measured by using the BacTiter-Glo microbial cell viability assay. This assay measures a luminescent signal that is proportional to the amount of ATP present, which is directly proportional to the number of cells in the culture. ATP is an indicator of metabolically active cells. Fig. 1 shows the relative

luminescent signal for biofilm-forming bacteria after 0, 5, 60 and 240 min of plasma treatment. As can be seen from the results, the signal, and therefore the amount of ATP, reached a peak at 5 min and decreased at higher exposure times. Although viable cells may still exist after 60 min of exposure, metabolic activity decreases further at longer exposure times, indicating loss of viability. These results indicate that biofilm-forming bacterial cells respond to the stress produced by the plasma treatment. At short exposure times, bacteria respond either by increasing respiration and thus ATP production, or by uncoupling ATP production from respiration as a way of coping with the stress. At longer plasma exposure times, bacteria succumb to oxidative stress and are no longer able to respond to it, resulting in loss of viability.

Another way of measuring the viability of cells after plasma treatment involves monitoring the membrane integrity of the cells by fluorescence microscopy. Fig. 2 shows the fluorescent images of biofilm-forming cells stained with the LIVE/DEAD BacLight Bacterial Viability test after 0, 5 and 60 min of plasma treatment. The assay utilizes a mixture of SYTO 9 green fluorescent nucleic acid stain and the red fluorescent nucleic acid stain propidium iodide. These stains differ in their ability to penetrate healthy bacterial cells. When used alone, SYTO 9 labels bacteria with both intact and damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, competing with SYTO 9 for nucleic acid binding sites when both dyes are present. When mixed in recommended proportions, SYTO 9 and propidium iodide produce green fluorescent staining of bacteria with intact cell membranes and red fluorescent staining of bacteria with damaged membranes. Therefore, the ratio of green to red fluorescence intensities provides a quantitative index of bacterial viability. As can be seen in the images, biofilm-forming



**Fig. 1.** Viability assay. Four-day-old *C. violaceum* CV026 biofilms were subjected to plasma treatment for 0 (control), 5, 60 and 240 min and suspended as indicated in Methods. Viability was determined with the BacTiter-Glo microbial cell viability assay and luminescence was measured. Results are the mean of four independent experiments. Error bars, SD; where no error bars are shown, the values were identical for independent experiments.



**Fig. 2.** Fluorescence microscopy images. Four-day-old biofilms were treated with plasma for 0, 5 or 60 min and cells detached from the biofilm were subjected to staining with the LIVE/DEAD BacLight Bacterial Viability kit, as indicated in Methods. (a) Control with no plasma treatment; (b) 5 min of plasma treatment; (c) 60 min of plasma treatment. Note that cells stained green are alive and cells stained red are dead.

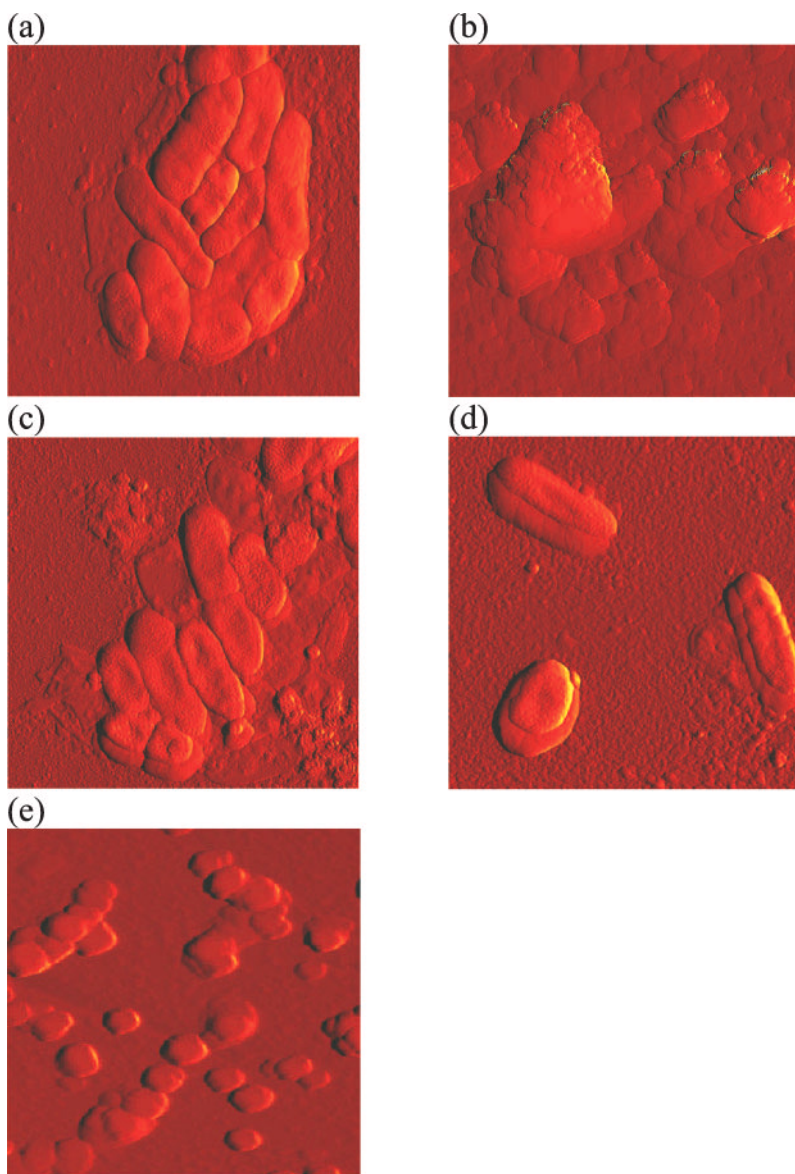
cells were predominantly green for the control and for the 5 min treatment, although in the latter there were some red cells as well. In the sample obtained after 60 min of plasma

treatment, most of the cells were stained red and were therefore dead.

Fig. 3 shows AFM images for *C. violaceum* biofilm-forming cells treated with plasma for different exposure times, as indicated in Methods. All images display a  $5 \times 5 \mu\text{m}^2$  scan of samples plasma-treated for 0 min (control, Fig. 3a), 5 min (Fig. 3c, d, e) and 60 min (Fig. 3b) and were obtained from widely separated regions of the sample. Fig. 3(a) shows aggregates with the typical rod-shaped morphology of *C. violaceum* cells of about  $2 \mu\text{m}$  in length. After 60 min of plasma treatment (Fig. 3b), bacterial cell images revealed broken or amorphous structures that were barely recognizable as bacterial remnants. These results for the 60 min plasma-treated sample were universal in that no recognizable intact bacterial cells were obtained in any of the 60 min images. This included images acquired over 10

widely separated regions of the sample. The 5 min plasma-treated samples showed different types of cell damage. Fig. 3(c) shows mostly intact cells in aggregates with only a few damaged and some undersized cells. Fig. 3(d) shows three two-layered cells, and an upper rough and a lower smooth layer (which was verified by cross sections of these images). Fig. 3(e) shows regular structures but distinctly different shapes and sizes from the normal bacterial cells. These rounded structures are approximately  $0.5 \mu\text{m}$  in diameter and resemble spheroplasts or protoplasts that might have arisen from cell wall/outer membrane removal due to plasma treatment.

The AFM results clearly indicated that biofilm-forming bacteria go through sequential morphological changes after plasma treatment. At shorter exposure times, bacterial cell walls may undergo modifications ranging from minimal



**Fig. 3.** Atomic force microscope images of *C. violaceum* cells after various plasma exposure times. The cells were detached from a biofilm treated with plasma for 0, 5 or 60 min. All images are  $5 \times 5 \mu\text{m}^2$  scans. (a) Biofilm-forming *C. violaceum* cells not subjected to plasma treatment (control); (b) 60 min of plasma treatment; (c–e) 5 min of plasma treatment.

changes (as evidenced by cells with 'rougher' surfaces, Fig. 3d) to putative loss of cell walls, leading to the production of spheroplasts as suggested by Fig. 3(e). In another experiment, we verified the relative 'roughness' of cells such as those in Fig. 3(d) by examining image cross sections and analysing the standard deviation of the surface height. These surface features were consistent with cells undergoing damage and were observed in a small percentage of the high-resolution 5 min plasma-exposure scans (Vandervoort *et al.*, 2008). After 60 min of plasma treatment, broken or amorphous structures were obtained. Cells that look damaged/lacerated under the AFM will be imaged as dead cells by fluorescence microscopy. Therefore, combining the results from fluorescence microscopy and AFM, it is clear that biofilm-forming cells undergo little change in cell morphology for the 5 min plasma treatment but incur major cell damage for 60 min exposure times.

The DNA spectrophotometric measurements, the colorimetric assays with vital dyes, and the viability tests show that although the c.f.u. count was very low, there were still living cells at short exposure times. It is possible that plasma-mediated biofilm inactivation proceeds through a first step in which bacterial cells enter a viable-but-nonculturable (VBNC) state, followed by a second step, characterized by a higher *D* value, in which cells are actually killed. This could explain the two-slope kinetics observed in our previous experiments. The VBNC state is a survival mechanism of bacteria facing environmental stress conditions, and has been reported for many Gram-negative organisms (Oliver, 1993; Colwell & Huq, 1994; Day & Oliver, 2004; Roszak & Colwell, 1987). Bacteria enter into this dormant state in response to one or more environmental stresses, which might otherwise ultimately be lethal to the cell. Plasma contains reactive agents including oxidative agents and radicals that are well known to cause environmental stress in bacteria. Changes in cell membrane composition have been reported for bacteria in the VBNC state (Oliver, 1993). Morphological changes have recently been reported for *Edwardsiella tarda* cells in the VBNC state; results show that when cells enter the VBNC state, they gradually change in shape from short rods to coccoid and decrease in size compared to the normal cells (Du *et al.*, 2007). These findings are consistent with our AFM findings. It has also been demonstrated that VBNC cells are active in metabolism (Zimmermann *et al.*, 1978), and our results with the cell viability assays show that luminescence, and therefore ATP production, reaches a peak at 5 min after plasma treatment, indicating that cells are metabolically active after that exposure time.

Our results are also compatible with the hypothesis that cell walls are damaged after plasma treatment, producing spheroplasts that are smaller in size. These spheroplasts are non-culturable but still alive, since they retain an intact cell membrane. Thus, no release of intracellular UV-absorbing products into the extracellular fluid is measured. Further research is required in order to validate this hypothesis.

Our results clearly show that bacterial biofilms can be inactivated by using gas-discharge plasma, and indicate the potential of plasma as an alternative sterilization method. Research is being carried out in our laboratories to determine the plasma conditions and chemistry necessary to achieve complete biofilm inactivation. However, these results also indicate that viability experiments should always be carried out before drawing the conclusion, based solely on measurement of culturable cells, that plasma is useful to kill cells.

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