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ORIGINAL ARTICLE

Biological responses of *Bacillus stratosphericus* to Floating Electrode-Dielectric Barrier Discharge Plasma Treatment

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Bacillus stratosphericus, DBD Plasma, disinfection, nonthermal plasma, oxidative stress, sterilization, VBNC, viable but nonculturable.

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Abstract

Aims: Dielectric barrier discharge (DBD) plasma is used for sterilization of contaminated inanimate surfaces but seldomly optimized and depends upon the type of organisms and the plasma treatment duration, (net energy deposited) this efficacy varies. The proposed study was designed to see biological responses of one of the robust organism, *Bacillus stratosphericus*.
Methods and Results: DBD plasma was applied over various durations to *B. stratosphericus* either surface-dried or suspension in de-ionized water, and viability, culturability, and viable but nonculturability (VBNC) were assayed using standard techniques. Depending upon the exposure of *B. stratosphericus* to DBD plasma resulted in three viability states, viable and culturable at low plasma doses and VBNC or disintegrated bacteria at higher plasma doses. Although organism's respiration levels at relatively low levels, immediately after plasma treatment, over the course of 24- h respiratory activity was increased *c.* eight times (and found still nonculturable during colony assays).
Conclusions: The loss of culturability is hypothesized to be induced as one of the responses to oxidative stress and it remains to be unclear if the response is temporary or indefinite. Appropriate plasma powers should be used to avoid VBNC-like status. 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay is a good alternative method to detect VBNC state.
Significance and Impact of the Study: *Bacillus stratosphericus* has the potential to turn into VBNC upon plasma application, and XTT assay can be an alternative method to detect VBNC state.

Introduction

Dielectric barrier discharge (DBD) plasma was initially introduced as a means of large-scale ozone production (Kogelschatz *et al.* 1999). With an evolution in electrical engineering technologies, voltage pulses can be generated at shorter rise-times and with less damage to the substrate being exposed (Cooper *et al.* 2007; Ayan *et al.* 2009). Compared to the effects of the more conventional thermal plasma, nonthermal plasma can be selective in its treatment because of the ability to avoid burn injury to healthy tissue. Most recently, our laboratories have successfully applied normal atmospheric nonthermal plasma using newly invented floating electrode dielectric barrier discharge (FE-DBD) plasma technique in the control of highly resistant methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* (Joshi *et al.* 2010). Living cells or tissues with water content and a relatively high dielectric constant have the required high capacity for charge storage (Fridman *et al.* 2006; Fridman 2008). In the case of FE-DBD, plasma is created in the gap between the living cells/tissues (which acts as the second

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electrode) and the other insulated electrode (primary electrode). While the current in the gaseous discharge gap is mainly because of motion of charge carriers (electrons and ions), it continues mostly in the form of displacement current through the cell/tissue. There are no special gaseous or air currents used in this technique, and the plasma is normal atmospheric (i.e. room air).

DBD plasma creates reactive oxygen and nitrogen (ROS) species to include superoxide, singlet oxygen, OH, hydrogen peroxide, NO and others, after applied (Laroussi and Leipold 2004; Laroussi 2005). ROS are known to cause bacterial inactivation and death. Therefore, one of the major reasons for its antimicrobial effect could be the generation of ROS, in addition to the flux of direct charges to the exposed surface, both of which are important in inactivation of microbes (Chirokov *et al.* 2004; Fridman *et al.* 2007; Laroussi 2009). And in turn, the latter too (i.e. flux of charged particles, UV photons, neutral species or electric charge) is known to generate ROS. It was reported previously by our laboratory that microbial exposure to DBD plasma when suspended in fluids yields an efficiency comparable to that of sterilization of surface-dried organisms in air and that the effect may be possibly in part owing to abundance of generation of ROS in fluids (Cooper *et al.* 2009; Dobrynin *et al.* 2009). In fluids, bacterial inactivation involves membrane lipid peroxidation through the production of superoxide anion and hydrogen peroxide in water (personal communication). In air, bacteria are inactivated by direct etching of the membrane which makes cells leaky, fenestrated and eventually complete disintegration upon exposed to plasma for long enough duration (Cooper 2009).

In this work, we show that *Bacillus stratosphericus* exhibits similar phenomena of etching and disintegration of the membrane, and damage to DNA with plasma treatment in dry air, and furthermore, when treated in liquid, the oxygenated species are able to inactivate bacteria to cause lethal and sublethal damages. Oxidative stress was previously shown to induce a viable but nonculturable (VBNC) state in bacteria (Arana *et al.* 1992; Gourmelon *et al.* 1994), and it remains consistent with bacteria whose membrane is not peroxidised beyond the repairable limit by plasma. *Bacillus stratosphericus* tolerates up to 17·4% NaCl and is resistant to UV as well as selected antibiotics such as penicillin, vancomycin and erythromycin (Shivaji *et al.* 2006). Its resistance characteristics make it an interesting choice for understanding the effect of plasma exposure. The organism is multi-resistant and originally isolated from high altitude where radiation, UV and other rays are likely affecting it. Our goal in this work is to understand the effects of FE-DBD exposure at doses which are previously reported to be lethal to bacteria and to show that bacteria are able to adapt and survive these treatments. For this reason, the established methods for comparing culturability, membrane integrity, bacterial morphology and respiration capabilities are used as a means to discern the viability state of *B. stratosphericus*.

Materials and methods

Bacterial isolate and growth

Bacillus stratosphericus samples originally isolated from cryogenic tubes when air samples were collected from the altitudes of 24, 28 and 41 km (Shivaji *et al.* 2006). The isolate was generously donated by the Biotechnology and Planetary Protection Group at the NASA Jet Propulsion Laboratory. An overnight culture of the isolate (MTCC 7305[T]) was re-inoculated in luria broth (LB) media to grow to mid-logarithmic phase and harvested by centrifugation. Cell pellets were rinsed twice and re-suspended in distilled water to final concentrations of a range of 10^7 to 10^9 cells ml^{-1} and used fresh preparations each time. The samples used were maintained at room temperature and diluted appropriately in LB medium post-plasma treatment to get isolated discrete colonies. Samples were plated on brain heart infusion agar and incubated at 37°C. Colony counts were performed in quadruplicate and observed daily for up to a week after plating to assess any latent growth.

DNA amplification

The method of genomic DNA amplification is widely used to demonstrate genes carried in conserved regions and to correlate the absence of such signature genes as evidence of absence of given species. Our experimental observations showed that plasma treatment of *B. stratosphericus* results in a rapid disintegration of DNA and the DNA fragments were not observed on agarose gel electrophoresis. Therefore, to confirm that DNA is completely destroyed during such plasma treatments, we used a recent method of identification of the presence of bacterial genomic DNA using a set of two commonly recommended primers for amplifying the DNA between positions 27 and 142 of bacterial 16S rRNA genes (originally numbered according to the *E. coli* rRNA). For the amplification of DNA, polymerase chain reaction (PCR) was carried out based on primers as recommended earlier (Frank *et al.* 2008). Primers used were 27F, forward primer (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (reverse: 5′-TACGGYTACCTTGTTACGACTT-3′). A 5 μl of bacterial suspension from the stock solution was placed on the scanning electron microscope (SEM) stub and allowed to dry for 30 min. Once the bacteria dried, they were treated for 60 s or left untreated

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(control). Water (5 μl) was added to the sample and pipetted off. A volume of 1 μl of each sample was used for PCR analysis. PCR master-mix contains per sample contained: 1 μl forward primer 27F, 1 μl reverse primer 1492R, 5 μl σ-buffer, 12·9 μl water, 0·1 μl Taq (Fermentas 5x Green GoTaq M791B) and 1 μl of the bacterial sample. The amplification reactions were performed with an initial denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, at 59°C for 60 s, at 72°C for 90 s and lastly a final extension at 72°C for 7 min. The 1% agarose gel was prepared with 1 μl of SYBR Safe DNA Gel Stain (Invitrogen Molecular Probes, Eugene, OR, USA) for visualization of DNA in gel. A 1- kb DNA Ladder (Promega, Madison, WI, USA) was used as a marker, and 1 μl of 6× Orange Loading Dye Solution (Fermentas Inc., Glen Burnie, MD, USA) was mixed with each sample for loading/visualization in the gel. The gel was run on 100 Volts using RunOne™ electrophoresis system (Embi Tec, San Diego, CA, USA) with constant voltage. After 15 min, it was removed from the power supply and imaged with the UVP EPI ChemDoc Imaging system at 254 nm.

Scanning electron microscopy

Characterization of the destruction of dry *B. stratosphericus* as a result of plasma treatment was performed at NASA laboratories, by bacterial cell surface scanning, collecting SEM (FEI/Philips XL30 Field Emission Environmental SEM) images with and without plasma treatment, essentially as per manufacturer's instructions. The samples were coated with Platinum/Palladium mixture at 40 mA for 40 s and processed as per the standard protocol from Manufacturer.

LIVE/DEAD assay

LIVE/DEAD BacLight Bacterial Viability kit was used to determine the membrane integrity as recommended by the manufacturer (Molecular Probes, Invitrogen, CA, USA). To prepare a fresh working solution of stain, 1·5 μl of solution A (SYTO9 dye) and 1·5 μl of solution B (Propidium iodide) were added to 997 μl of sterile de-ionized water, and after mixing thoroughly 100 μl was added to each tube containing homogeneous suspension of bacterial cells (either plasma treated or untreated) in the dark. The tubes were incubated in darkness at room temperature for 15 min. The stained samples were then viewed using a Leica DMRX fluorescence microscope with attached Leica DG300FX digital camera system, using fluorescein and Texas red band-pass filters (for corresponding SYTO9 green dye and Propidium iodide). The images were captured from five randomly selected areas for three different sets of experiments, saved as TIFF file and edited using Adobe Photoshop CS3 using the 'ImageJ' program to calculate mean number of green and red pixels in each area. In parallel, the fluorescence measurements were acquired using microplate assay, centered at 485 nm for live and dead bacteria at 530 and 630 nm, respectively. BioTek Synergy 4 Hybrid Multi-Mode Microplate reader and Gen5 (1.06) software were used to acquire data.

Quantification of viable cells by XTT assay

For each assay, fresh 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reagent solution was prepared as described previously (Peeters *et al.* 2008). From the aliquots, 0·5 mg XTT (Molecular Probe) and 1 umol l^{-1} Menadione (Sigma Chemical Co.) working solution was made up in de-ionized water. A 65 - μl sample of *B. stratosphericus* in a hanging-drop glass slide was either treated or not-treated with plasma, and 50 μl of the sample was collected and harvested in the microtubes by centrifugation at 8000 rev min^{-1}/6 min, and the supernatant discarded. The cells were resuspended in 200 μl of XTT reagent, mixed thoroughly, and tubes incubated in dark at 37°C/2 h. After centrifugation, the supernatant (100 μl) containing orange-coloured XTT metabolic product was measured by reading absorption at 492 nm using a microtiter plate reader [BioTek Synergy 4 Hybrid Multi-Mode Microplate reader and Gen5 (1.06) software]. The readings were normalized, and per cent surviving cells were calculated against untreated samples. In a parallel experiment, the pellet was then re-suspended in 100- μl sterile de-ionized water and either observed under fluorescence microscopy or a performed colony count was performed after appropriate dilutions for comparison.

Plasma treatments

Samples were directly treated with plasma by mounting a plasma generating primary electrode *c.* 2 mm above the coupon on which bacterial sample was deposited (Fig. 1). The surface power density of the discharge was kept at 1 W cm^{-2} at the applied voltage of *c.* 30 kV at the frequency of *c.* 10 kHz. Details of the power supply construction were reported by the authors previously (Cooper *et al.* 2007; Fridman *et al.* 2007).

Data analysis

All experiments were carried out a minimum of three times in duplicates. The data whenever necessary was analysed using GraphPad Ver. 3.0 (San Diego, CA, USA). Error bars indicate standard error mean unless

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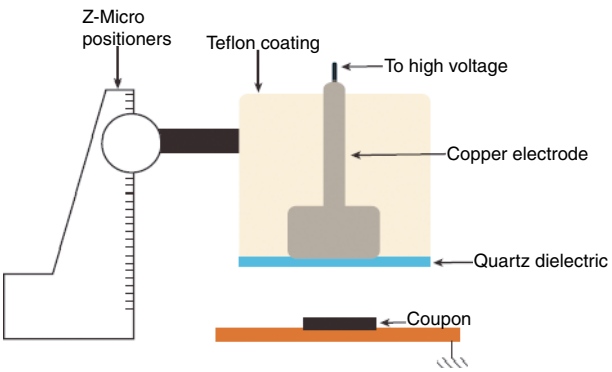


Figure 1 A schematic diagram of the experimental setup where primary electrode mounted above the coupon bearing bacterial sample is covered with quartz.

otherwise stated. Multiple comparisons were performed by one-way analysis of variance (ANOVA) and the value considered statistically significant if $P \leq 0{\cdot}05$, wherever applicable.

Results

To understand how DBD plasma influences the *B. stratosphericus* cell morphology, viability and culturability, we carried out series of experiments using various plasma exposure times, both under dry environment and wet environment (cell suspension). We looked for the cell wall and membrane-associated physical changes using SEM, genomic DNA-associated changes using PCR amplification of remnants of DNA (post-plasma treatment), viability of bacteria using standard colony count assays, cell membrane integrity using LIVE/DEAD fluorescence assays, culturability and dormancy using standard respiratory metabolic activity with XTT assays, morphological features peculiar to nonculturability under oxidative stress-induced cell elongation using SEM.

Figure 1 shows the schematic diagram of the plasma-generating device and the sample-holding mechanical stage. The distance between the plasma probe and biological samples can be adjusted with fine adjustment knob, and thus exact distance can be calculated. The function of quartz dielectric cover lowers the temperature generated across biological samples being exposed to plasma. The assembly works at normal atmospheric pressure and does not use any special gas, explains its simplicity and accessibility.

Destruction of *Bacillus stratosphericus* through etching phenomenon when present on dry surface

To prove the efficacy of our plasma device system, we selected a highly resistant phenotype of *B. stratosphericus*, originally isolated during astrobiological studies (Shivaji *et al.* 2006). This spore-bearing bacterium is multi-drug resistant and with extreme tolerance to salinity and ultraviolet rays, which makes it the ideal challenge organism. Existing reports at the beginning of our work were suggesting that plasma causes membrane damage (Joshi *et al.* 2010). Therefore, we started looking at the physical damage to the cell envelope. Figure 2A indicates a surprisingly clear evident of punch-out porosity to bacterial cells when the dried samples are treated with plasma on stainless steel surface. Figure 2A represents the picture of SEM images taken at three different time points, and the graded changes were observed over time. The images of untreated (2a), 60 s plasma-treated (2b) and 120 s plasma-treated (2c and 2d) specimens are shown for comparison and exhibited typical porosities through which cytoplasmic contents can freely leak out or conversely plasma effect can reach inside cell in fraction of seconds.

Similarly, the bacterial cells on dry surface were treated, and harvested cells were tested for their DNA amplifications as mentioned under materials and methods. Figure 2B is the findings of PCR products run on agarose gel electrophoresis. The gel shows amplified products of untreated samples in normal phosphate buffered-saline (150 mmol l^{-1} sodium chloride and 150 mmol l^{-1} sodium phosphate, pH 7·2 at 25°C) (positive control) and plasma-treated samples. A 60-second's plasma exposure was sufficient to disintegrate DNA in possibly minute fragments, affecting the given genetic regions of DNA, which the specified primers failed to amplify. The Fig. 2B therefore also suggests an extensive DNA damage. Concurrent colony count assay did not reveal any growth (zero colonies).

Inactivation of *Bacillus stratosphericus* when present in fluid (cell suspension)

Contrary to the plasma treatments of bacteria present on dry surfaces, wet treatment took a relatively longer time to inactivate bacilli but had shown a similar pattern. Figure 3a shows the responses of *B. stratosphericus* to plasma when present in fluid medium (cell suspension). It appears from Fig. 3a that the bacteria were viable from 60 s through 120 s of 24 h post-treatment, and about two magnitudes the organisms were live even after 60 s treatment. The relationship between plasma exposure time and decrease in number of viable cells was almost linear (Fig. 3a).

Plasma treatment compromises cell membrane integrity over time

Bacterial viability was also assessed by a fluorescent dye-labelling of the bacterial cells, wherein the cells

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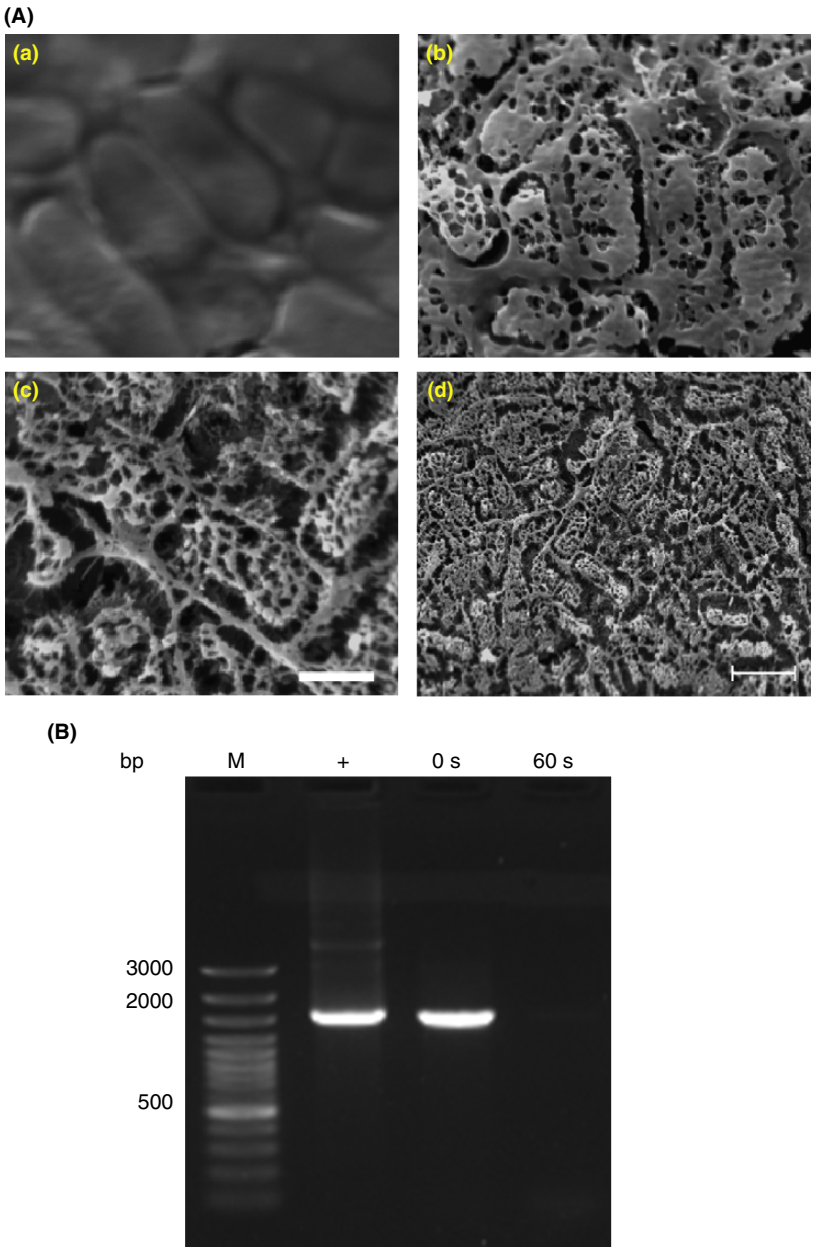


Figure 2 (A) The representative scanning electron microscopic images of plasma treatment of *Bacillus stratosphericus* on dry surface shows etching of bacterial cell envelopes. Such types of changes were visible from 60 s plasma treatment and onwards. (a) Untreated, (b) 60 s plasma-treated and (c, d) 120 s plasma-treated samples are seen. Bar in (c) 1 μm and (d) 2 μm. (B) Agarose gel showing PCR amplified products of DNA isolated from *B. stratosphericus*. A drastic reduction in the amount of DNA by plasma treatment is observed when surface dried *B. stratosphericus* exposed to dielectric barrier discharge plasma for 60 s onwards and compared with untreated (0 s) sample as mentioned under materials and methods. M, marker DNA ladder; + is the positive control; 0 and 60 s are the plasma treatment times in seconds.

whose membrane integrity is compromised their genomic DNA stained predominantly red by propidium iodide (otherwise impermeable to cells) and the healthy cells predominantly stained green upon taking up a cell permeable dye, SYTO9 (Molecular Probes, Invitrogen). Thus, a per cent of live *vs* dead cells can be calculated. Alternatively, a fluorescence microplate reader can be used to capture red and green fluorescence signals to generate real-time live/dead graphs. We did not see any significant difference in both of these methods, and findings were close to each other with a deviation of ±2–5% (data not shown). Figure 3b shows the findings of live/dead assay and essentially demonstrates that under wet environment (fluid) treatments, bacteria survives even up to 120 s when detected immediately post-treatment. The assay also demonstrates that the integrity of cell membrane of these organisms was compromised substantially and that at 24 h post-treatment these membrane-associated changes were significant ($P < 0{\cdot}05$) when compared with 0 h post-treatment. These types of changes indicate that the cells remained viable for longer time (e.g. 120 s), even after relatively prolonged plasma treatments, and on colony assay did not reveal any growth (Fig. 3a).

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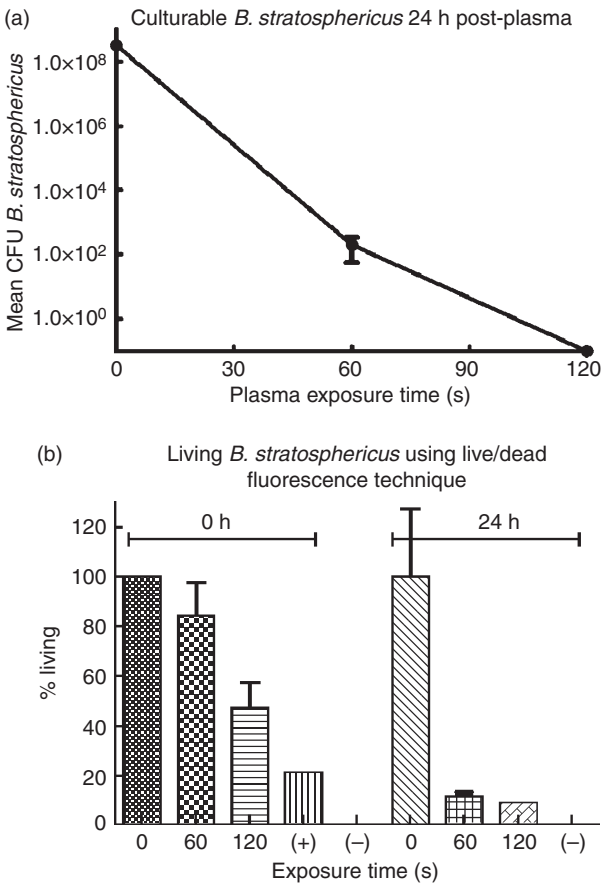


Figure 3 (a) A representative colony count assay showing viable and culturable *Bacillus stratosphericus* as colony form units (CFU) 60 s post-plasma treatment under wet environment (suspended cells), with *c.* 6 log reduction. No colonies were noted at 120 s post-plasma treatment. Bar, standard error mean. (b) A representative LIVE/DEAD fluorescence assay demonstrating a loss of cell membrane potential and compromise of membrane integrity upon application of plasma over time under wet environment (cell suspension treatments). Although viability of *B. stratosphericus* is reduced in plasma dose-dependent manner, there are undamaged (viable) cells seen even by the end of 120 s of treatment, which could not be detected by culture (colony assay, Fig. 4). Cells were either immediately assayed (0 h) or 24 h post-treatment. Bar, standard error mean.

Cells exist in VBNC state

A series of experiments were undertaken to determine whether the increasing amount of plasma-induced stress leads to the death of bacterial cells and subsequent sterilization or cells remain in VBNC state. Figure 4 shows the responses of *B. stratosphericus* over plasma exposure times. This XTT assay determines the respiring bacterial cells by measuring an orange-coloured metabolic product of XTT, and the amount is proportional to number of viable cells. From Fig. 4A, it appears that after 60 s of plasma treatment cells probably undergo latency stage (dormancy) as early as 2 h post-treatment through >24 h (variable per cent, depending on the amount of plasma energy applied). These small fractions of cells were viable, but upon colony assay could never be cultured and detected. This unusual property is possessed by very few organisms. This newly reported *B. stratosphericus* species has shown VBNC state upon plasma-induced stress. The 70% ethanol treatment of *B. stratosphericus* cells are seen as built-in control, for comparison. Table 1 shows the per cent surviving cells and an analysis of minimum three sets of experiments in triplicate. Looking at the extreme narrow range of standard error measurement, the data is convincing. Figure 4B shows the low-power fluorescence microscopic images, concurrently taken at 2- and 24-h holdings in NB medium, 120 s post-plasma treatment. The image of 24-h panel exhibited a relatively higher XTT activity, but colony assay did not show any growth of bacteria (plates were incubated and observed for 7 days).

Cell elongation phenomenon and plasma-induced stress

Various types of cellular stress and variable morphogenesis have been reported in other bacterial species. As we observed a shift in viable to VBNC status of *Bacillus* during plasma treatment, we thought of looking at the changes in morphologic features of these bacteria. After 60 s through 120 s plasma treatment under wet environment, we observed that *B. stratosphericus* cells undergo cellular elongation. Figure 4C is a representative SEM image of such finding, suggests that *Bacillus* too undergo cellular elongation like that of many other gram-positive and gram-negative organisms during VBNC stage. The internal controls (untreated samples) were run in parallel, and the findings were highly reproducible.

Discussion

DBD plasma is demonstrated as an effective antimicrobial technique, and it is important to understand the bacterial response to its exposure. We proposed to observe that the DBD normal atmospheric nonthermal plasma treatment of *B. stratosphericus* results in three viability states: viable and culturable at low plasma doses, VBNC bacteria, and disintegrated bacteria at higher plasma doses. Bacteria in VBNC state retain the ability to perform functions such as respiratory activity (Besnard *et al.* 2000; Laflamme *et al.* 2004), metabolism of incorporation of radio-labelled substrates (Rollins and Colwell 1986) and cellular elongation (Roszak and Colwell 1987). The detection of bacteria in this state requires assays which are independent of culturability. Therefore, the assessment is made employing

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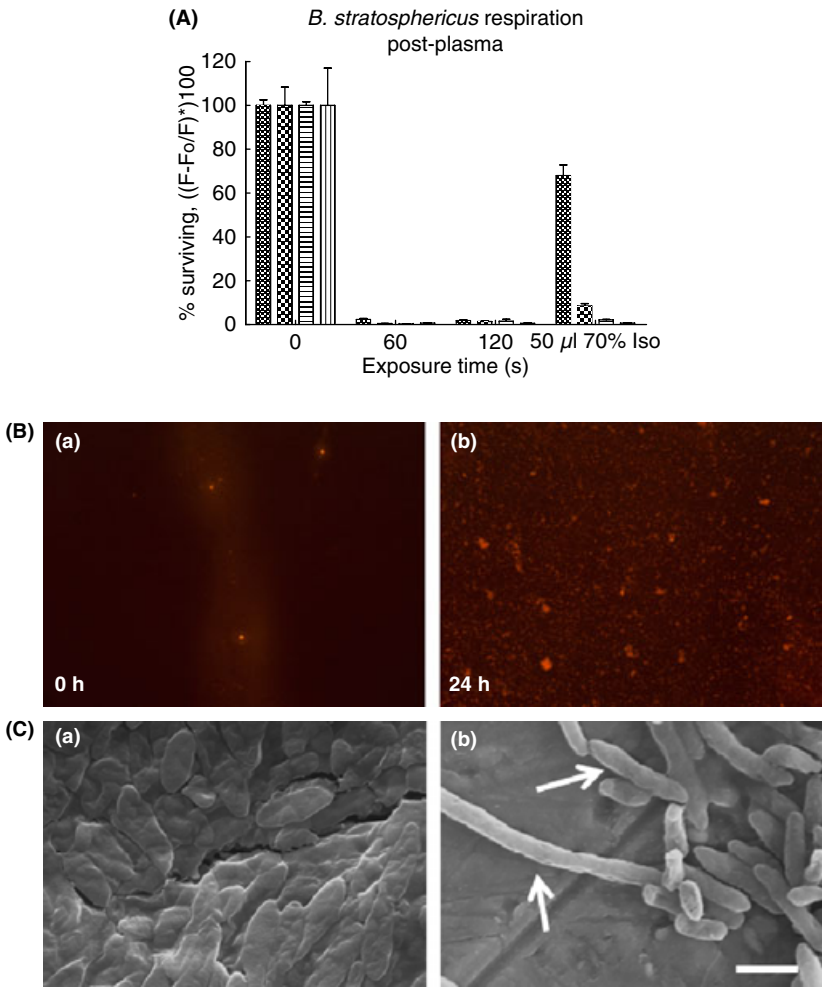


Figure 4 (A) XTT assay was used to detect all culturable as well as viable but nonculturable (VBNC) cells of *Bacillus stratosphericus* after holding the cells (2, 6, 18 and 24 h) in nutrient medium upon plasma treatment over time. Each set had negative control (reagents without cells) and positive control (70% ethanol treated cells) for comparison. The experiments were repeated for three times in triplicate. About 1×10^8 CFU ml^{-1} were starting cells (shown as 100%). Bar, standard error mean. (Table 1 demonstrates the per cent deviations and can be read with this figure). (B) The representative microphotographs of the smear of suspended cell pellet showing respiring *B. stratosphericus* from few initial survivors of plasma treatment for 120 s after 2 h (a) to increased respiration after 24 h (b; numerous cells) which were still remained nonculturable. (C) Scanning electron microscopic image showing heterogeneous morphology of *B. stratosphericus* after 120 s of plasma treatment under wet environment (b) as compared to untreated (a). Such cellular elongation is found associated with VBNC bacteria. Arrow points divided cells. Bar, 2 μm. XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide. (A) (▩) 0 h; (▩) 6 h; (▤) 18 h and (▥) 24 h.

Table 1 The XTT assay showing respiratory status of the cells of *Bacillus stratosphericus* post-plasma treatment

Observation time (post-plasma)	2 h	6 h	18 h	24 h
Treatment time (s)				
0	100·00 ± 3·19	100·00 ± 2·65	100·00 ± 11·04	100·00 ± 1·05
60	2·31 ± 0·50	0·45 ± 0·06	0·31 ± 0·04	0·65 ± 0·12
120	0·08 ± 0·13	0·69 ± 0·08	0·67 ± 0·19	0·65 ± 0·13

± SE, standard errors mean; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

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a comparison of the methods of culturability, membrane integrity, bacterial morphology and respiration in present studies on the responses of bacteria under plasma-induced stress.

It is known that plasma-mediated inactivation mechanisms differ based on the amount of fluid present in the biological sample (Dobrynin *et al.* 2009). When *B. stratosphericus* was dried on a stainless steel surface at room air prior and then exposed to DBD plasma, etching of cell membrane by charged particles observed. A flux of a cocktail of neutral or charged particles or photons within plasma interacts directly with the cell envelope and penetrates the bacteria at numerous locations, thus exposing internal components directly to plasma (Cooper *et al.* 2009). In 120 s of plasma treatment, the physical disintegration of bacteria can be clearly observed (Fig. 2a). Similar pore formation and disintegration of cell envelope have been observed on occasion, wherein the authors reported a release of cellular components including genomic DNA, resulting in loss of viability of *E. coli* and *Bacillus subtilis* (Hong *et al.* 2009). The resulting physicochemical reaction was so strong that DNA was completely destroyed. In 60 s of plasma treatment, nearly all DNA was disintegrated to the extent that PCR amplification of the remnants was not successful (Fig. 2b). Under dry environment, longer treatment of plasma probably led to severe ionic itching of the membrane of *B. stratosphericus*, which resulted in pore formation in it, and might be responsible for leakage of cytosol into the surroundings. Such type of observation has been made earlier (Hong *et al.* 2002, 2009). But in the present studies, we collected all biomaterial from defined area under treatment. Therefore, even if DNA is released from such fenestrated membrane, PCR amplification would not have missed it. Furthermore, when we ran agarose gel electrophoresis (not shown), did not see any DNA ladder of fragmented DNA (which was surprising) and therefore PCR amplification was set up. One possibility could be that the power applied to generate plasma was too high and therefore in fraction of seconds would be destroying DNA. Such type of finding has been reported (Hong *et al.* 2009). The lack of fragmentation may be explained as a result of DNA destruction to the extent where remaining pieces are too small to be amplified by the PCR technique. The lack of fragmentation may also be explained in part by a linearization of the chromosomal DNA by plasma treatment and the subsequent cleavage of polynucleotides by exonuclease enzymes, thus rapidly degrading the remaining DNA (Henikoff 1984). Nonetheless, the DNA digestion by either plasma and/or enzymes indicates that DNA is destroyed extensively where it drastically reduces the probability of bacterial viability. The subsequent colony count assay had shown no growth on TSA plates.

We observed that this novel multi-drug resistant, high salinity and temperature tolerating *B. stratosphericus* when treated under wet environment (cell suspension) it loses its culturability in <120 s of plasma treatment (Fig. 3a). At this point, it is not uncommon to assume complete sterilization has taken place; however with further analysis, it can be seen that viable *B. stratosphericus* remains. The ROS and other oxidants are generated by plasma, and their lethal effect on bacteria is known. Researchers (Hong *et al.* 2009) demonstrated that *E. coli* and *B. subtilis* spores are inactivated by ROS, and the effect was proportional to the oxygen radical species generated by the atmospheric plasma. The ROS generation has been shown to induce VBNC state via oxidative stress (Gourmelon *et al.* 1994). In our studies, the response to hydrogen peroxide treatment (a positive control (+) in Fig. 3b) and the generated ROS produced by DBD Plasma, *B. stratosphericus* has shown to inhibit its ability to culture while maintaining its membrane integrity in small per cent of cells (Fig. 3b) and a baseline respiration (Fig. 4A and Table 1). In earlier studies, inactivation kinetics is found plasma exposure time-dependent, as well as bacterial species specific, such as *Salmonella*, *Staph. aureus*, *E. coli*, *Bacillus atrophaeus*, *Clostridium botulinum*, etc (Muranyi *et al.* 2007; Venezia *et al.* 2008). Therefore, the bacterial species which require higher exposure of plasma treatment are likely to get exposed to sublethal doses, and may lead to a stage of incomplete inactivation. Such exposures may be responsible for genetic switch of viable to VBNC state. Recent studies show that this genetic mechanism of switching from classical viable stage to VBNC stage is not present in all micro-organisms, is largely a bacterial species specific (Ozcakir 2007). It is likely that *B. stratosphericus* is a highly resistant organism and therefore requires prolonged exposure to plasma treatment or also possible that this bacterium possess a genetic switch for VBNC stage under adverse conditions. A complete inactivation and destruction of *B. stratosphericus* cells was observed in 5 min of plasma treatment.

A reduction in respiration rate >98% is logical for bacteria which enter a dormant or quiescent lifestyle change (Binder and Anderson 1990). Transfer to growth media is typical to increase respiration in such micro-organisms (Binder and Anderson 1990) and is observed in our case as well. Further observations revealed that the bacteria producing the baseline signal (residual) after plasma treatment represent a small fraction of surviving bacteria when compared to 24 h post-treatment condition, which showed *c.* 8-times more bacteria which were respiring (Table 1). The later were viable but remained nonculturable. This increase in respiration gives rise to the question of whether this viability is reversible or irreversible. Upon

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literature searches, we assumed that this change in the level of respiration may have resulted from incubation in LB medium (i.e. in nutrients) for 24 h post-plasma treatment, as it is known to enhance respiration of bacteria that has experienced an external stressor.

Correlation of the culturability with membrane integrity and respiration activity is indicative of the VBNC state. Concurrently the observation in cellular morphology shows that the *B. stratosphericus* vegetative cells are also able to elongate (Fig. 4C) an additional indication of VBNC bacteria. *Bacillus stratosphericus* upon plasma treatment revealed heterogeneous population comprising of predominantly elongated larger cells and normal cells. The cells were overall more flattened with and negligible visible binary fission. Such features found associated with VBNC stage of both Gram-positive and Gram-negative bacteria (Besnard *et al.* 2000; Coutard *et al.* 2007). This may also suggest that a small portion of cells may be able to undergo cell division in later life (Coutard *et al.* 2007). It is evident that such VBNC bacteria are able to maintain their antibiotic resistance markers, and during restoration of classical progeny division, they continue to express the resistance trait, and therefore represent an additional risk to human health (Lleo *et al.* 2003). In either case, a complete inactivation of such resistant bacteria using appropriately higher and optimized plasma doses is advisable to minimize potential future threat.

Conclusions

An exposure to DBD plasma at otherwise 'lethal' doses induces a VBNC state in *B. stratosphericus* which are not completely inactivated/disintegrated by such treatment. It is possible that such doses might be leading to activation of genetic mechanisms of switch from viable to VBNC state. The presence of VBNC cells poses a major public health hazard. These cells cannot be detected by traditional culture methods, and the cells may remain potentially pathogenic upon favourable conditions. We hypothesized that the ROS produced by plasma may be inducing this state, and further studies are required in this direction. The mechanisms of inactivation in *B. stratosphericus* may be different under dry environment (directly ionic interactions with cell envelop) and wet environment (probably via ROS). Further studies on characterization of ROS are underway. To ensure the death of such bacteria, relatively longer plasma treatment time is advisable.

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