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ORIGINAL ARTICLE

Characterization of *Bacillus subtilis* spore inactivation in low-pressure, low-temperature gas plasma sterilization processes

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Abstract

Aims: To identify structural components of *Bacillus subtilis* spores serving as targets for sterilization with microwave induced low-pressure, low-temperature nitrogen-oxygen plasma.

Methods and Results: The inactivation of spores followed a biphasic kinetics consisting of a log-linear phase with rapid inactivation followed by a slow inactivation phase. In the course of plasma treatment, damage to DNA, proteins and spore membranes were observed by monitoring the occurrence of auxotrophic mutants, inactivation of catalase (KatX) activity and the leakage of dipicolinic acid, respectively. Spores of the wild-type strain showed the highest resistance to plasma treatment. Spores of mutants defective in nucleotide excision repair (*uvrA*) and small acid-soluble proteins (*ΔsspA ΔsspB*) were more sensitive than those defective in the coat protein CotE or spore photoproduct repair (*splB*). Exclusion of reactive particles and spectral fractions of UV radiation from access to the spores revealed that UV-C radiation is the most effective inactivation agent in the plasma, whereby the *splB* and *ΔcotE* mutant spores were equally and slightly less sensitive, respectively, than the wild-type spores. Finally, the extent of damages in the spore DNA determined by quantitative PCR correlated with the spore inactivation.

Conclusions: Spore inactivation was efficiently mediated by a combination of DNA damage and protein inactivation. DNA was identified to be the primary target for spore inactivation by UV radiation emitted by the plasma. Coat proteins were found to constitute a protective layer against the action of the plasma.

Significance and Impact of the Study: The results provide new evidence to the understanding of plasma sterilization processes. This knowledge supports the identification of useful parameters for novel plasma sterilization equipment to control process safety.

Introduction

Resulting from the introduction of specialized high-tech polymer materials for medical instruments and novel polymeric packaging materials for liquid pharmaceuticals and foods, there is a need for low-temperature sterilization processes that are safe to operate and that produce reliable sterilization even of complex shaped objects. A novel process with a potential to fulfil these requirements works with low-temperature gas plasmas. Although the temperature of the objects to be sterilized is maintained at material compatible levels, it has been shown that plasma treatment can effectively inactivate a wide range of micro-organisms including spores (Kelly-Wintenberg

et al. 1999; Feichtinger *et al.* 2003; Lee *et al.* 2006). Currently, plasma treatment of packaging (Deilmann *et al.* 2008) and of foods to improve their microbiological safety is the subject of research (Vleugels *et al.* 2005; Basaran *et al.* 2008; Selcuk *et al.* 2008). Other applications for plasma sterilization include surface decontamination after bioterrorism attacks (Herrmann *et al.* 1999) and planetary protection from carry over of biological material in space missions (Bol'shakov *et al.* 2004; Schuerger *et al.* 2008). More than producing sterility, plasma treatment even has the potential of depyrogenation and inactivation of prions (Rossi *et al.* 2006).

Plasma sterilization methods are characterized by the use of gas or gas mixture that is partially excited through introduction of an electric or electromagnetic field. The resulting nonequilibrium gas discharge contains UV photons, as well as potentially reactive particles such as radicals, ions and free electrons. These plasma agents may contribute to the sterilization by interacting with the biological material. The concentrations in which the agents occur in plasma depend greatly on the device set-up, operating conditions (gas pressure, type and power of plasma excitation) and gas composition. In addition, it is of importance whether the substrate to be sterilized is in direct contact with the plasma or located remote from it (reviewed in Moisan *et al.* 2001; Laroussi 2005; Boudam *et al.* 2006).

The mechanisms by which plasma agents interact with biological materials and finally lead to the inactivation of micro-organisms are currently under investigation. For sterilization with low-pressure plasmas, Moisan *et al.* (2001) proposed a general model, which is derived from the observation of at least two sequential phases of rapid and slow inactivation of micro-organisms and from properties of the plasmas used. The corresponding inactivation rates are limited by the action of UV radiation (fast inactivation) and reactive particle mediated erosion of the biological material (slow). Mogul *et al.* (2003) showed that low-pressure oxygen plasma can degrade lipids, proteins and DNA of *Deinococcus radiodurans* cells. Kim and Kim (2006) reported the inactivation of enzyme activity and accumulation of reactive oxygen species inside *Escherichia coli* cells after treatment with atmospheric pressure helium-oxygen plasma. Exposure of *E. coli* cells and spores of *Bacillus globigii* to atmospheric pressure plasmas was shown to cause mutations in surviving cells leading to metabolic changes (Laroussi *et al.* 2002, 2006). Nevertheless, still little is known about the details of interaction of the different plasma agents with the different components of bacterial cells or spores. Especially, the question arises which components of a cell or spore are the primary targets, and which of the agents are most effective in the inactivation process.

In this study, we investigated the effect of low-pressure, low-temperature nitrogen-oxygen plasma treatment on *Bacillus subtilis* spores with respect to inactivation and changes in proteins, DNA, and membranes. To identify the primary targets of plasma sterilization, spores of *B. subtilis* mutants defective in two independent DNA repair systems (*splB1* and *uvrA42*), synthesis of DNA protecting small acid-soluble proteins (Δ*sspA Δ*sspB*), and protein coat structure (Δ*cotE*) were included in our study.

Materials and methods

Micro-organisms, growth conditions and preparation of spore suspensions

The strains of *B. subtilis* used in this study are listed in Table 1. Culturing was routinely performed on modified dextrose tryptone agar (DTA; glucose (unless stated otherwise, reagents were obtained from Merck, Germany) 5 g l^{-1}; tryptone 10 g l^{-1}; yeast extract 1 g l^{-1}; Bacto-agar (Difco) 15 g l^{-1}). For strain PS3328, tetracycline (Sigma–Aldrich) was added to a final concentration of 20 μg l^{-1}. For preparation of purified spore suspensions, strains were grown on Schaeffer's sporulation medium agar plates (Harwood and Cutting 1990) without antibiotics by incubating at 37°C until abundant sporulation

Table 1 *Bacillus subtilis* strains used in this study

Strain	Relevant characteristic	Reference
168	*trpC2*	(Burkholder and Giles 1947)
1A757*	Wild-type (Trp$^+$)	
PS3328†	Derivative of 168, Δ*cotE*	(Paidhungat *et al.* 2001)
1S111*	Derivative of 1A757, Δ*sspA* Δ*sspB* (referred to as $\alpha^-\beta^-$)	(Mason and Setlow 1986)
1A488*	Derivative of 168, *splB1*	(Munakata and Rupert 1974)
1A345*	Derivative of 168, *uvrA42*	(Munakata and Ikeda 1969)

*Strains were obtained from the Bacillus Genetic Stock Center (BGSC).
†Strain was provided by P. Setlow, University of Connecticut Health Center, Farmington, CT, USA.

occurred (up to 5 days) as monitored by phase contrast microscopy. From the agar plates, the biomass was harvested with a sterile spreader using 5 ml of ice-cold-deionized water and subjected to centrifugation (10 min, 5000 ***g***, 4°C). The spore suspension was washed five times by resuspension in ice-cold water followed by centrifugation and finally stored at 4°C in deionized water. Spore counts were determined by plating onto DTA. The spore preparations contained >99% phase bright spores and were used no longer than 2 weeks. Sporulation conditions are known to influence resistance properties of the spores (reviewed in Nicholson *et al.* 2000). To minimize this, spores of all *B. subtilis* strains were produced under the same conditions.

Preparation of spore-coated substrates for plasma exposure experiments

Microscopic slides were used as carriers for the spores. The surface of the slides was flamed briefly to sterilize them and make them evenly wettable with water. To delimit the area to be contaminated, a circle of 20 mm diameter was drawn onto the glass surface using a lacquer paint marker. Stock suspensions of spores were diluted with cold-deionized water and aliquots of 50 μl containing $1{\cdot}5 \times 10^8$ colony-forming units (CFU) were transferred onto the slides. The particles were allowed to sediment for 1 h at 4°C before freezing for 30 min at −25°C. The slides were freeze-dried in a SpeedVac vacuum concentrator (Savant, Farmingdale, NY, USA) producing a dense and uniform layer of partially stacked spores on the glass carriers, which appeared as an opaque area.

Plasma exposure

As depicted in Fig. 1, the vacuum plasma system consisted of an evacuable vessel, a plasma source inside the vessel and a substrate mount that allowed positioning of the substrates in variable distances relative to the plasma source. The substrate mount provided the possibility to cover the glass slide substrates with optical filter plates; thus, shielding the plasma-generated particles and parts of the plasma's radiation emission spectrum. The plasma source consisted of eight Duo-Plasmalines® (Muegge Electronic, Reichelsheim, Germany) arranged in parallel and was driven by two 2·45 GHz magnetron microwave (MW) generators, each with a maximum power output of 2 kW. A defined atmosphere was maintained by the controlled flow of a mixture of oxygen and nitrogen through the vessel. Details on the properties of the plasma generated by the Duo-Plasmaline® source are given in Kaiser *et al.* (1999). Substrates were placed onto the mount and

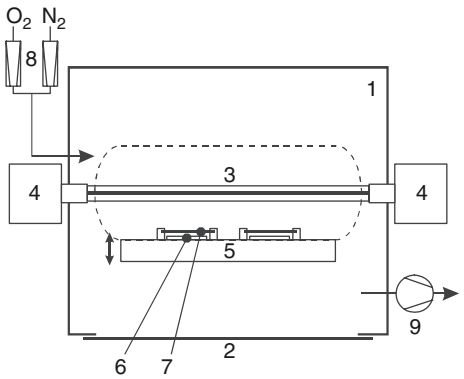

Figure 1 Scheme of the vacuum plasma system: (1) evacuable stainless steel vessel; (2) vessel door; (3) Duo Plasmaline® plasma source (the approximate extension area of the plasma is shown as broken line); (4) magnetron heads; (5) substrate mount allowing the distance between substrates and plasma source to be adjusted; (6) glass slide substrate coated with spores; (7) optical filter plate (optional); (8) gas flow controllers; and (9) vacuum pump.

the vessel was evacuated to a pressure below 10 Pa. The gas flow was started and the plasma was ignited by switching on the MW power when the vessel pressure had equilibrated. After plasma treatment, the vessel was flooded to atmospheric pressure and the substrates were removed. Plasma treatments were performed at a pressure of 70 Pa, MW power of 4 kW, gas mixture of 80% (v/v) N_2 and 20% (v/v) O_2 unless stated otherwise. For exposure longer than 10 s, pauses of 50 s where the plasma was switched off were introduced after every 10 s to prevent excessive heating of the substrates. Plasma emission spectra were recorded using an Avantes AVS-SD 2000 spectrometer equipped with an optical fibre.

Determination of surviving cell counts

After plasma exposure, the glass slide substrates were placed evenly on the bottom of sterile beakers containing magnetic stir bars. Cold sterile deionized water with a volume of 2 ml was added, and the spores were resuspended using a magnetic stirrer at 300 min^{-1} for 2 min. Colony-forming units (CFU) were determined by plating on DTA using an automatic spiral plater (Don Whitley Scientific, Shipley, UK). When only a small number of survivors were expected, the pour-plating method was employed. The plates were incubated at 30°C for 18 h. Based on the arithmetic mean of the colony counts of at least four replicates of nonplasma exposed substrates N_0 and that of plasma exposed substrates N, survival was calculated as $N/(N_0)$. Corresponding errors were estimated through error propagation and indicated in the graphs as error bars.

Modelling of spore inactivation

Mean values of the inactivation kinetics data were fitted to a biphasic inactivation model (Cerf 1977) with the GInaFiT macro (Geeraerd *et al.* 2005). In this model, the relation between survival and exposure time is given by the equation

$$N/(N_0)^{=} f \cdot e^{-k_1 \cdot t} + (1-f) \cdot e^{-k_2 \cdot t},$$

where in N and N_0 are the numbers of survivors and the initial population, respectively, f is a constant designating the transition from the first phase to the second, k_1 and k_2 are the inactivation rates for the two distinct phases, and t is the exposure time.

For comparison of kinetic data with other plasma inactivation studies, decimal reduction values (D values) expressing the exposure time required for a reduction of surviving cell counts by 90% of the initial value were estimated for the first and the second phase by $D_1 = \ln 10/(k_1)$ and $D_2 = \ln 10/(k_2)$, respectively. Survival at the transition from the first to the second inactivation phase was estimated by $N_{Tr}/(N_0) = 1 - f$, and the corresponding plasma exposure time was determined by numerical solving. The root mean sum of squared error (RMSE) values calculated by GInaFiT for each fit (not shown) were in the ranges of the coefficients of variations of the spore counts, indicating that the biphasic model was well suited to represent the experimental data (Geeraerd *et al.* 2005).

Screening for auxotrophic mutants

The suspensions from four replicate substrates were pooled, diluted and spread onto DTA plates to yield about 200 colonies per plate upon incubation at 30°C for 24 h. Colonies of auxotrophic mutants were identified by replica plating onto Spizizen's minimal medium supplemented with 50 μg ml^{-1} L-tryptophan (Harwood and Cutting 1990).

Determination of catalase activity and dipicolinic acid release from spores

An aliquot (4 ml) of an ice-cold aqueous suspension of plasma-treated spores was centrifuged (5 min, 10 000 ***g*** at 4°C). The supernatant was filter sterilized and stored at −25°C. The pellet was suspended in 200 μl of 10 mmol l^{-1} phosphate buffer (pH 7·0) containing Complete Mini protease inhibitor cocktail (Roche, Mannheim, Germany). Zirconia beads (0·3 g, 0·1 mm diameter) were added and the spores were disrupted by shaking in a Mini Bead Beater (Biospec, Bartlesville, OK, USA) for 15 min. After centrifugation (5 min at 10 000 ***g***), the supernatant was used to determine the catalase activity photometrically (Goldblith and Proctor 1950). Catalase activity was expressed as μmol H_2O_2 converted per minute using an external calibration standard of bovine liver catalase (Sigma–Aldrich). To determine the release of dipicolinic acid (DPA) from plasma injured spores, the supernatants were thawed and fourfold concentrated under vacuum. The DPA content was assayed according to Scott and Ellar (1978). Results are reported in nanograms of DPA per substrate.

Determination of DNA damage

Aliquots (1 ml) of the spore suspensions from each of four replicate substrates were pooled and collected by centrifugation (10 min; 10 000 ***g***; 4°C). For chemical decoating, the sediment was suspended in 200 μl of 50 mmol l^{-1} Tris–HCl (pH 8·0) containing 8 mol l^{-1} urea, 1% sodium dodecyl sulfate, 10 mmol l^{-1} EDTA, and 50 mmol l^{-1} dithiothreitol and incubated for 90 min at 37°C (Fairhead *et al.* 1993). The decoated spores were washed three times by repeated centrifugation with cold water. Disruption was accomplished by suspending the spores in 200 μl STE (sodium chloride-Tris-EDTA) buffer (10 mmol l^{-1} Tris–HCl, pH 8·0; 10 mmol l^{-1} EDTA; 150 mmol l^{-1} NaCl) containing 2 mg ml^{-1} lysozyme and incubated 60 min at 37°C. Chromosomal DNA was purified from the disrupted spores using the High Pure PCR Template Preparation Kit (Roche, Penzberg, Germany). Concentrations of DNA were determined by fluorometry using the QuantiT HS DNA Kit (Invitrogen). To determine the degree of DNA damage semi-quantitatively, a real-time PCR-based ratio detection system Bauer *et al.* was employed (Bauer *et al.* 2004). This method assumes a random distribution of defects along the DNA double strands, and therefore the probability of detecting such defects is increasing with the length of the DNA fragment in examination. Based on the genome sequence of *B. subtilis* 168 (accession no. NC_000964) PCR primer pairs Bs_dnaK855f (5′-CACAATGGGTCCTGTCCGTC-3′)/Bs_dnaK1254r (5′-AGACATTGGGCGCTCACCT-3′) and Bs_dnaK1154f (5′-ACACGACGATCCCAACAAGC-3′)/Bs_dnaK1254r were constructed to amplify a 400 bp fragment and an internal 101 bp fragment (used as an internal standard) from the *dnaK* locus, respectively. Both fragments were amplified on an ABI Prism 7000 SDS real-time PCR system (Applied Biosystems, Foster City, CA, USA) in separate 25 μl reaction volumes containing Quantitect SYBR Green reagents (Qiagen, Hilden, Germany), 0·2 μmol l^{-1} of each primer and 0·25 ng template DNA. Absolute copy numbers were determined using serial dilutions of genomic DNA from *B. subtilis* 168 as an external standard. The degree of DNA damage was expressed as

ratio of the detectable copy numbers between the 400 bp fragment and the 101 bp fragment. This method allows the detection of damages like thymidine dimers (Sikorsky *et al.* 2004) and double or single strand breaks.

Results

Effects of plasma treatment on DNA, membranes and proteins of spores

To investigate the effect of plasma treatment on membranes, DNA and proteins of spores, the leakage of DPA, generation of auxotrophic mutants and spore specific catalase (KatX) activity, respectively, were determined from spores of *B. subtilis* 168, which have been subjected to plasma treatment under moderate conditions (5 cm distance from the plasma source, exposure up to 30 s). The results are compiled in Table 2. Viable spore counts decreased over plasma exposure time and after 30 s of treatment a reduction in three orders of magnitude was obtained. A biphasic model (Cerf 1977) using the GinaFiT tool was applied to the inactivation data and resulted in a good fit (parameters $f = 0{\cdot}988846$, $k_1 = 1{\cdot}4\ s^{-1}$, $k_2 = 0{\cdot}1\ s^{-1}$, $N_0 = 1{\cdot}2 \times 10^8$; RMSE = 0.0977). This suggests an inactivation kinetics consisting of two distinguishable log-linear phases: a rapid inactivation phase ($D_1 = 1{\cdot}7$ s) at the first 4·2 s of plasma exposure followed by a slow one ($D_2 = 26$ s). When spores were subjected to one evacuation and gas flow cycle without ignition of the plasma, no inactivation was observed (data not shown).

Plasma treatment of the spores caused release of DPA, generation of auxotrophic mutants and reduction in KatX activity. Monitoring of these effects was possible for the first 15 s plasma exposure only. Thereafter, the continued plasma exposure resulted in a degradation of organic material into volatile products. Although 97% of the spores were inactivated during the first 3 s of plasma exposure, only a minor release of DPA to the suspension medium was observed (Table 2). In the subsequent phase up to 9 s of exposure, an enhanced DPA release was determined, while the inactivation rate of the spores was decreased. After 3 s, first auxotrophic mutants occurred and the mutation frequency increased to a maximum of 0·82% at 15 s exposure. Further exposure revealed a remarkable drop in the mutation frequency, likely caused by the low-surviving fraction of the spores and the resulting lack of sublethally injured cells. In contrast, the activity of KatX the catalase present in *B. subtilis* spores (Casillas-Martinez and Setlow 1997) decreased almost linearly with exposure time and after 15 s of plasma exposure fell under the limit of detection.

Inactivation kinetics of *Bacillus subtilis* mutant spores

To further investigate the mechanism of action of plasma treatment, spores of the *B. subtilis* mutants PS3328, 1S111, 1A488 and 1A345 as well as of the wild-type strain 1A757 were generated and used for preparation of substrates. These substrates were treated at a distance of 2 cm to the plasma source, leading to faster spore inactivation compared to the experiment described earlier. As the substrates were placed well inside the plasma, the spore containing area normally appearing opaque became progressively clear during exposure for longer than 5 s, indicating a proceeding erosion of the biologic material. As shown in Fig. 2, the response of survival to plasma exposure time suggested a biphasic inactivation for all strains with a first phase (first 2·5–5·9 s of plasma exposure) of rapid inactivation followed by a second phase of slow inactivation. Various models were applied using GInaFiT and the most appropriate fits were actually produced by the biphasic model of Cerf (1977). The parameters obtained for the inactivation kinetics of spores of the five *B. subtilis* strains are shown in Table 3. The use of the biphasic model allowed a more consistent estimation of D_1-values for the first inactivation phase because the transition between the two phases is mathematically defined and all available experimental data points are included in its calculation.

Table 2 Effects of plasma treatment on viability, dipicolinic acid (DPA) release, catalase activity and mutation of *Bacillus subtilis* 168 endospores. Purified spores were lyophilized on glass slides and exposed to nitrogen-oxygen plasma at a distance of 5 cm to the plasma source

Plasma exposure time (s)	Viable spore count (CFU per substrate)*	DPA released to the suspension medium (μg)	Catalase activity (μmol min^{-1})	Auxotrophic mutation frequency (%)
0	$1{\cdot}2 \times 10^8$ ($1{\cdot}1 \times 10^7$)	0·57	4·5	<0·10
3	$3{\cdot}0 \times 10^6$ ($2{\cdot}2 \times 10^6$)	0·89	2·7	<0·22
6	$1{\cdot}0 \times 10^6$ ($2{\cdot}8 \times 10^5$)	6·63	1·6	0·28
9	$4{\cdot}8 \times 10^5$ ($1{\cdot}2 \times 10^5$)	8·50	0·8	0·58
15	$3{\cdot}9 \times 10^5$ ($3{\cdot}1 \times 10^5$)	<0·33†	<0·4†	0·82
30	$1{\cdot}0 \times 10^5$ ($4{\cdot}8 \times 10^4$)	<0·33†	<0·4†	0·12

*Standard errors of the four replicates in parenthesis.
†Below the limit of detection.

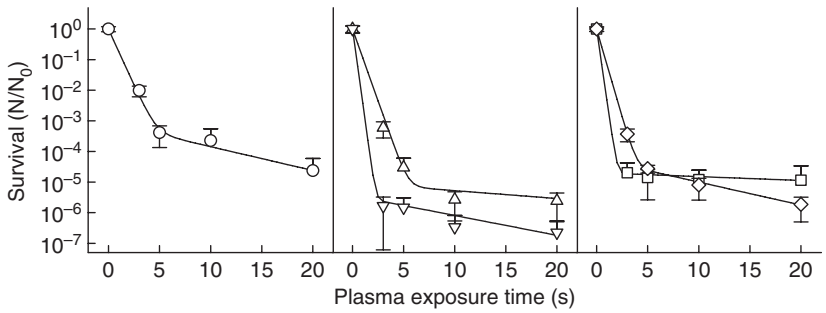

Figure 2 Inactivation kinetics of spores of *Bacillus subtilis* strains 1A757 (○), PS3328 (△), 1S111 (▽), 1A488 (◇), and 1A345 (□) on glass slide substrates obtained by direct plasma exposure. The distance from plasma source to the substrates was 2 cm. Initial viable spore counts (N_0) before exposure were $1{\cdot}5 \pm 0{\cdot}1 \times 10^8$ CFU per substrate for strains 1A757, PS3328, 1S111, 1A488 and $1{\cdot}4 \pm 0{\cdot}2 \times 10^7$ CFU per substrate for strain 1A345. The line represents the biphasic model of inactivation fitted to the data points. Error bars indicate ± one standard error of at least four replicates.

Strain	f	t_{Tr}* (s)	N_{Tr} $(N_0)^{-1}$ †	k_1 (s^{-1})	D_1 (s)	k_2 (s^{-1})	D_2 (s)
1A757	0·999143	4·5	$8{\cdot}6 \times 10^{-4}$	1·7	1·4	0·2	12·9
PS3328	0·999991	5·9	$9{\cdot}5 \times 10^{-6}$	2·2	1·1	0·1	‡
1S111	0·999996	2·5	$3{\cdot}6 \times 10^{-6}$	5·6	0·4	0·1	‡
1A488	0·999944	4·0	$5{\cdot}6 \times 10^{-5}$	2·6	0·9	0·2	‡
1A345	0·999980	2·5	$2{\cdot}0 \times 10^{-5}$	5·3	0·4	<0·1	‡

Table 3 Parameter values f, k_1, and k_2 obtained by applying a biphasic model to the inactivation kinetics data of spores of *Bacillus subtilis* wild-type and mutant strains

*Plasma exposure time at the transition from the first to the second inactivation phase as calculated from the model.

†Spore survival at the exposure time t_{Tr} as calculated from the model parameters.

‡D_2 values were not calculated because of the higher error of spore counts near the limit of detection.

Based on the characteristics of the inactivation kinetics (Fig. 2, Table 3), differences in the resistance to plasma between the spores of the wild-type and mutant strain can be observed. Spores of the wild-type strain 1A757 showed the highest resistance resulting in $D_1 = 1{\cdot}4$ s and $D_2 = 12{\cdot}9$ s as determined from the biphasic model fit for the first and second inactivation phases, respectively. Spores of the *cotE* (PS3328) and *splB* (1A488) mutants exhibited an intermediate resistance in the first phase with D_1 values of 1·1 s and 0·9 s, respectively, while spores of the *uvrA* mutant (1A345) and the $\alpha^-\beta^-$ mutant (1S111) were highly sensitive. Because of their extremely fast inactivation, there is no data point in the middle of the first phase. Therefore, the estimation of the D_1 values to 0·4 s by the model is accompanied with a greater uncertainty. Because of low spore counts at the end of the second inactivation phase, determination of useful D_2 values was impeded by the limit of detection.

Spore inactivation by fractions of the plasma UV emission spectrum

To investigate the role of radiation involved into spore inactivation in more detail, various optical long-pass filter plates were introduced 2 mm in front of the substrates (Fig. 1). This prevented access of short-wavelength-photons and plasma-generated reactive particles. In this setup, spores of the wild-type strain 1A757 and mutant strains PS3328, 1S111, 1A488 and 1A345 were treated in equal plasma processes (10 s exposure at 2 cm distance to the plasma source), but filters with different 50% transmission cut-off wavelengths ranging from 125 nm to 305 nm were used. The plasma used in our experiments emitted UV radiation in the spectral ranges of UV-C (200–290 nm) as well as of UV-A+B (290 nm to 320 nm + 320 nm to 400 nm) (Fig. 3). As depicted in Fig. 4a, the wild-type spores were inactivated less than one log when radiation of wavelengths smaller than 280 nm was excluded. Radiation between 215 and 280 nm caused inactivation of more than 3 logs. No difference in spore inactivation was observed when filters with cut-off wavelengths below 215 nm were used, as well as when direct plasma access (without filter) was allowed.

Spores of the *cotE* mutant strain PS3328 exhibited a spectral inactivation response comparable to that of the wild-type spores. However, direct plasma exposure resulted in a one log higher inactivation of PS3328 spores

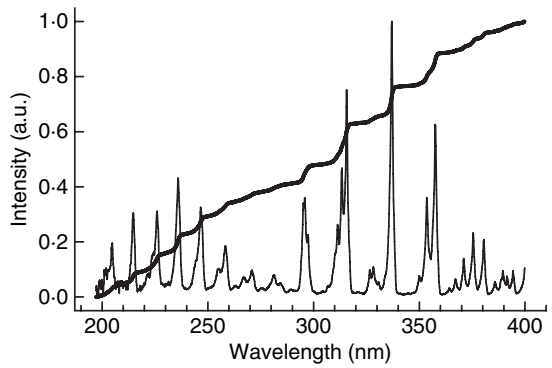

Figure 3 Emission spectrum (thin line) in the UV-A/B/C range normalized to the highest peak at 337 nm and integrated intensity (thick line) normalized to its maximum of the plasma used for the spore inactivation experiments. (a.u., arbitrary units).

than of wild-type spores. On the contrary, PS3328 spores tended to slightly higher survival than wild-type spores when filters with cut-off wavelengths lower than 280 nm were used. This observation has also been reported for UV-inactivation of *cotE* mutant spores (Riesenman and Nicholson 2000). Strongest inactivation was observed with spores of the $\alpha^-\beta^-$ mutant strain 1S111 when filters with cut-off wavelengths of 280 nm and lower were used. In contrast to the observations with spores of the other strains, 1S111 spores still showed a 3·7 log inactivation when covered with 305 nm long pass filters. Survival of spores of the *splB* mutant strain 1A488 carrying a defect in the spore photoproduct repair pathway was comparable to that of the wild-type and *cotE* mutant spores, when filters were applied. However, the spores of strain 1A488 were still more susceptible to the direct exposure to plasma than the wild-type spores. Spores of the nucleotide excision DNA repair defective mutant 1A345 exhibited the most pronounced difference in inactivation between plasma treatments using the 280 and 164 nm long-pass filters.

To investigate the effect of filters on spore inactivation in the different phases of inactivation, an inactivation curve was determined using the SiO_2 optical filter and spores of the wild-type strain *B. subtilis* 1A757. The biphasic model was also applicable to these data with good fitting (Fig. 4b, parameters not shown). Use of the filter caused a prolongation of the initial inactivation phase (from 4·5 to 7·3 s) resulting in an increase in D_1 to 2·1 s. In the second inactivation phase, spore survival as well as the D_2 value was not affected significantly.

Impact of plasma exposure on spore DNA

Because DNA is likely to be a target for spore inactivation, the effect of plasma treatment on the damage of DNA was investigated by monitoring the destruction of a particular chromosomal DNA fragment. After exposure of spores of *B. subtilis* 1A757 to plasma (10 s) and extraction of DNA, the concentration of the 400 bp target DNA fragment was determined using quantitative real-time PCR. Plasma exposure may affect the quality and efficiency of DNA extraction from spores (e.g. by generating cross-links between DNA and other spore core components), which may in turn unpredictably influence the detection of target DNA fragments by PCR. To account for this, a ratio detection system (Bauer *et al.* 2004) was

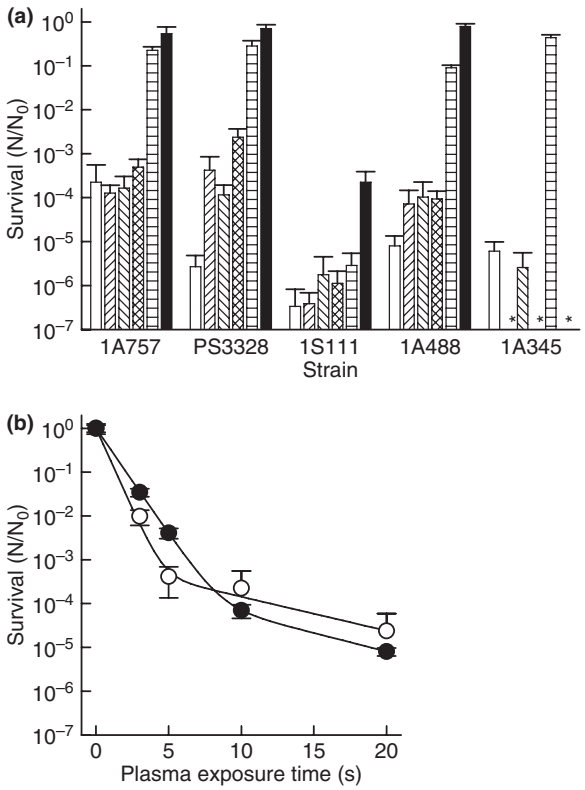

Figure 4 (a) Inactivation of spores of wild-type *Bacillus subtilis* 1A757 and mutant strains by plasma exposure without shielding (□) and with shielding of the substrates from the plasma-generated activated particles and parts of the emission spectrum by optical long pass filters MgF_2 (▨) SiO_2 (▧), BG24A (▩), WG280 (▤), and WG305 (■). The 50% transmission cut-off wavelengths of these filters were 125, 164, 215, 280 and 305 nm, respectively. All substrates were exposed for 10 s in a distance from the plasma source of 2 cm. (b) Comparison of inactivation kinetics of the *B. subtilis* 1A757 spores without shielding (○) and with shielding (●) of the substrates by the SiO_2 optical filter during plasma exposure at 2 cm distance from the plasma source. The line represents the biphasic model of inactivation fitted to the data points. Initial viable spore counts (N_0) before exposure were $1{\cdot}2 \pm 0{\cdot}6 \times 10^8$ CFU per substrate. (*, not determined for filters MgF_2, BG24A, and WG305). Error bars indicate one standard error of at least four replicates.

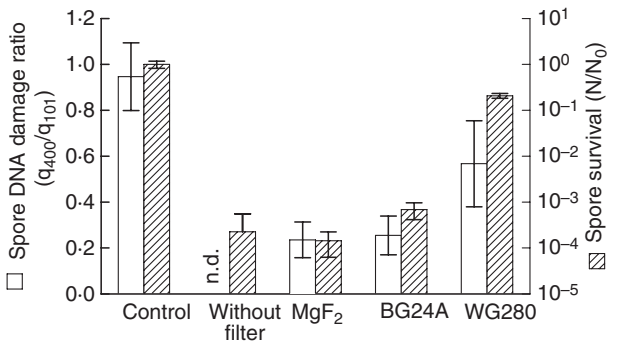

Figure 5 Damage in DNA extracted from *Bacillus subtilis* 1A757 spores after plasma exposure expressed as ratio of the copy numbers of a 400 bp target fragment (q_{400}) and an internal control fragment of 101 bp length (q_{101}) detected by quantitative real-time PCR (white bars). For comparison, spore survival of the plasma treatments is shown (hatched bars). The spores were plasma treated on glass-slide substrates with and without shielding of the substrates using the optical filters indicated (for details on the filters and exposure conditions see legend of Fig. 4). In the treatment without filters, the spore biomass was eroded so heavily that no detectable traces of DNA could be purified from the samples and therefore the q_{400}/q_{101} ratio could not be determined (n.d.). Error bars indicate ± one standard error of four replicates.

established, wherein the target fragment copy numbers q_{400} were normalized to those of an internal subfragment of 101 bp (q_{101}). The resulting ratio is equal to 1 for non-degraded DNA and attains lower values for increasing degrees of degradation. Because the correlation between the actual degree of DNA damage (in lesions per base pair) and the ratios is unknown, we used this method for qualitative evaluation only. As shown in Fig. 5, when the substrates were shielded with the WG280 long-pass optical filters (50% cut-off wavelength 280 nm), the DNA damage was moderate corresponding to the weak spore inactivation. Plasma treatment using filters BG24A and MgF_2 (cut-off below 215 and 164 nm, respectively) resulted in a high degree of DNA degradation. Comparing the effects of these two filters, although spore survival decreased when using the lower cut-off wavelength filters, the DNA degradation remained constant (q_{400}/q_{101} = 0·25 and 0·24 for the treatment using filters BG24A and MgF_2, respectively).

Discussion

In this study, we showed that in low-pressure, low-temperature nitrogen-oxygen plasma *B. subtilis* spores are inactivated efficiently by a combination of protein inactivation and DNA damage. The plasma produced high radiation output in the UV-A + B + C range but also caused high erosion of the biomaterial. The following results support the dominating role of UV radiation in the spore inactivation process. First, plasma-generated radiation between 215 and 280 nm exhibited strongest inactivation of spores (Fig. 4a). This behaviour was also reported for plasma treatment of *B. atrophaeus* spores (Halfmann *et al.* 2007) and *B. subtilis* spores (Feichtinger *et al.* 2003). Second, spore inactivation was correlated with detectable damages in the DNA (Fig. 5). Third, spores of the following mutants were found to be the most sensitive (Fig. 2, Table 3, Fig. 4a). In mutant 1A345, the nucleotide excision repair (NER) pathway is defective, prohibiting efficient repair of various DNA damages after germination including UV-induced ones (Munakata and Ikeda 1969). In spores of mutant 1S111, the α/β-type DNA-binding small acid-soluble proteins are absent, leading to an increased sensitivity against harsh environmental conditions including UV radiation (Setlow 2006). On the other hand, we found that spores of strain 1A488 deficient in spore photoproduct lyase (SPL) DNA repair pathway were less sensitive than spores of the NER deficient strain. The SPL pathway specifically repairs the spore photoproduct (SP) 5-thyminyl-5,6-dihydrothymine (Slieman *et al.* 2000), which accounts for >90% of the bipyrimidine photolesions induced by UV-C irradiation (Moeller *et al.* 2007). The low sensitivity of spores of the mutant 1A488 indicated that SP is not the dominating type of DNA damage induced by plasma and that additional DNA-damaging mechanisms exist. Although the strains used in this study were not isogenic and were derived from different ancestors, the phenotypes of their spores are well characterized in various inactivation studies (summarized in Setlow 2006) and, thus, these interpretations of the results can be considered valid.

Our results indicate that proteins also may be targets for the plasma-generated agents, as shown by the linear decrease in KatX activity in the first phase of spore inactivation (Table 2). This is supported by the findings of other workgroups (Mogul *et al.* 2003; Rossi *et al.* 2006), which demonstrated a destruction of isolated protein and enzyme inactivation by low-pressure, low-temperature plasma. On the other hand, the results of our studies with Δ*cotE* mutant strain PS3328 (Figs 2 and 4a) clearly showed that coat proteins function as protectors against plasma-generated reactive agents. Spores of strain PS3328 are lacking the outer coat protein layer and possess a loosely bound inner coat only (Driks 2002). Consistently, these spores exhibited a significantly lower survival than wild-type spores when directly exposed to plasma, whereas when shielded from plasma-generated reactive particles their inactivation was comparable to that of the wild-type spores (Fig. 4a). Because we used an oxygen-containing plasma, it is very likely that in our study these particles are reactive oxygen species (ROS) such as atomic

oxygen, peroxide, superoxide, hydroxyl radicals and ozone, which were shown to be involved in spore inactivation by reacting with proteins (Gaunt *et al.* 2006). The assumption that the coat serves as a 'reactive shield' to prevent access to reactive agents like ROS to the spore core is supported by the work of Riesenman and Nicholson (2000) and others (reviewed by Setlow 2006) showing that the spore coat is part of the resistance mechanism against reactive chemicals.

With increasing exposure time, we observed a progressive erosion of the spore material off the carriers. This erosion is caused by two processes (Moisan *et al.* 2001): first, photodesorption defined as cleavage of chemical bonds by high-energy photons resulting in formation of volatile compounds and second, etching defined as chemical reaction of plasma-generated reactive particles (like ROS) with biomolecules resulting in slow combustion into volatile products. According to the model of Moisan *et al.* (2001), the spores accessible to UV radiation are rapidly inactivated. Before spores unreachable to UV radiation, e.g. stacked or embedded in a biofilm, can be inactivated, the erosion of the covering layers is required. This is a slow process limiting the inactivation rate, resulting in a second phase of inactivation, as observed in other plasma sterilization studies (Hury *et al.* 1998; Moreau *et al.* 2000; Feichtinger *et al.* 2003). In our study, we also observed a biphasic inactivation of spores of all *B. subtilis* strains. Remarkably, this was still the case when the spores were covered with UV-A + B + C transparent optical filters (Fig. 4b). Because the inactivation rate observed in the second phase was identical to that without filters, it is indicated that among the erosion processes only photodesorption but not etching contributes to the spore inactivation in the second phase. Given the very short exposure times in our experiments, the process of photodesorption might greatly be enhanced by simultaneous action of other agents in the plasma. However, further investigations are needed to prove this speculation. Nevertheless, this finding further extends the model of Moisan *et al.* (2001) for spore inactivation by low-pressure plasma.

For industrial application of plasma sterilization, the biphasic nature of spore inactivation is a critical aspect with regard to the sterilization safety. This is because natural contamination mainly occurs in biofilms or aggregates of germs associated with dust particles, both limiting the access of UV radiation to its targets. Our finding that UV radiation is the most effective inactivation agent in plasma suggests to control low-pressure, oxygen-nitrogen plasma processes in such a way that highest UV output is obtained. To enhance the efficiency of sterilization, a two-step process with high erosion rates in the second step seems to be more practicable. The applicability of such processes however depends on the material and object to be sterilized. Plasmas as voluminous sources of UV radiation and reactive particles offer the potential to sterilize even complex shaped objects such as artificial joint implants. In addition, the possibility to remove pyrogens or prions and to maintain the integrity of high-tech materials as well as the absence of residuals makes plasma processes ideal for medical or pharmaceutical applications. For high-volume applications like food packaging sterilization, the plasma technology is also of advantage because of its energy efficiency and very short processing times.

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