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Properties of *Bacillus anthracis* spores prepared under various environmental conditions

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Abstract *Bacillus anthracis* makes highly stable, heat-resistant spores which remain viable for decades. Effect of various stress conditions on sporulation in *B. anthracis* was studied in nutrient-deprived and sporulation medium adjusted to various pH and temperatures. The results revealed that sporulation efficiency was dependent on conditions prevailing during sporulation. Sporulation occurred earlier in culture sporulating at alkaline pH or in PBS than control. Spores formed in PBS were highly sensitive towards spore denaturants whereas, those formed at 45°C were highly resistant. The decimal reduction time (D-10 time) of the spores formed at 45°C by wet heat, 2 M HCl, 2 M NaOH and 2 M H_2O_2 was higher than the respective D-10 time for the spores formed in PBS. The dipicolinic acid (DPA) content and germination efficiency was highest in spores formed at 45°C. Since DPA is related to spore sensitivity towards heat and chemicals, the increased DPA content of spores prepared at 45°C may be responsible for increased resistance to wet heat and other denaturants. The size of spores formed at 45°C was smallest amongst all. The study reveals that temperature, pH and nutrient availability during sporulation affect properties of *B. anthracis* spores.

Keywords *Bacillus anthracis* · Spore sensitivity · Sporulation · Germination · Dipicolinic acid

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Introduction

In the natural environment, bacteria acclimatize to a variety of adverse conditions, such as fluctuating temperatures, pH and nutrient limitation. Members of the genus *Bacillus* respond to such hostile conditions by adopting an alternative developmental pathway leading to sporulation. *Bacillus anthracis*, a mammalian pathogen, is the etiological agent of anthrax. The infection occurs from intradermal inoculation, ingestion or inhalation of the spores (Klein et al. 1966). Bioterrorist attacks with *B. anthracis* spores have sparked renewed interest in studying methods of bacterial spore inactivation. In earlier studies, electron beam irradiation and chlorination have been used for inactivating *Bacillus anthracis* spores (Helfinstine et al. 2005; Rice et al. 2005). The sensitivity of *B. subtilis* spores, another member of the genus *Bacillus*, towards different organic solvents, heat, hydrogen peroxide, hydrochloric acid and sodium hydroxide has also been studied (Melly et al. 2002). These inactivation studies were performed with *Bacillus*

spores prepared in routinely used sporulation medium. It has been shown that variations in temperature during sporulation affects spore properties of *B. subtilis*, including sensitivity towards various chemical agents (Melly et al. 2002). In another study, it has been shown that heat shock applied to *B. subtilis* and *B. megaterium* during sporulation increases the heat resistance of the resultant spores (Movahedi and Waites 2000; Sedlak et al. 1993). Various physico-chemical properties of endospores, such as moisture and dipicolinic acid content, cortex layer thickness, and external conditions (media composition, pH, salinity and temperature) are thought to contribute towards their heat resistance (Gerhardt and Marquis 1989). Bacillus species are the best-studied endospore-formers, however, the studies assessing efficiency of endosporulation have been performed with other endospore-formers as well.

Strains of *Moorella thermoacetica* (JW/DB-2 and JW/DB-4) isolated from autoclaved media were highly heat resistant (Byrer et al. 2000). The spores obtained from these strains under various conditions were exposed to 121°C and D-10 time was determined. Chemolithoautotrophically grown spores obtained at 25°C from JW/DB-2 and JW/DB-4 had D-10 times of 43 and 23 min, whereas, spores obtained at 60°C showed D-10 times of 83 and 111 min, respectively. However, the spores formed at 60°C from cells grown chemo-organoheterotrophically had D-10-times of 44 and 38 min. These observations suggested that growth conditions and sporulation at higher temperature contribute to heat resistance in *M. thermoacetica* (Byrer et al. 2000). Another study revealed that the endospores of *Clostridium botulinum* TMW 2.357 and *Bacillus amyloliquefaciens* TMW 2.479 are inactivated by combination of pressure and heat. It was shown that increase in pressure and temperature accelerates the inactivation of spores, however, a pressure mediated spore protection was observed (Margosch et al. 2006).

In the present study, we undertook a comprehensive approach to analyze the effect of various stress conditions on sporulation of *B. anthracis*. The *B. anthracis* spores were prepared at different pH, temperatures and in nutrient deprieved medium (PBS), and further analysis was carried out to study the chemical/physical properties of the spores produced in response to the stress applied to the culture. In addition, the growth and sporulation efficiency of *B. anthracis* were also studied under these stress conditions. The sensitivity of the spores formed under various stress conditions towards UV radiations, wet heat, hydrochloric acid, sodium hydroxide, hydrogen peroxide and formaldehyde was determined. The spores were further characterized by estimating their size, DPA content and germination efficiency. The spores formed in PBS were found to be highly sensitive towards physio-chemical agents. Therefore, proteomic analysis of these spores was carried out by two-dimensional gel-electrophoresis (2-D gel electrophoresis). In summary, the results suggest that conditions during sporulation play a significant role in determining the time required for spore formation and also affect the properties of spores formed subsequently. Apart from providing the effect of environmental conditions on the sporulation, this study may help in preparing surrogate spores of *B. anthracis* to evaluate inactivation procedures.

Materials and methods

Bacterial strain and growth kinetics

Bacillus anthracis Sterne, an attenuated strain used extensively as an animal vaccine, was employed during this study. The growth and sporulation was carried out in modified G (mG) medium, as described previously (Liu et al. 2004). Different stress conditions were applied at an early stationary phase. The cells from a 300 ml culture were harvested and washed with PBS and divided into six aliquots to prepare the spores. The pellet was suspended in 50 ml mG medium, pH 7.0 to prepare control spores. For starvation stress, the pellet was resuspended in 50 ml PBS. The effect of pH stress was studied by growing the bacteria in 50 ml of mG medium adjusted to pH 5 or pH 9. The cultures were incubated at 37°C with continuous shaking at 250 rpm for sporulation, unless indicated otherwise. Temperature stress was given by resuspending the pellet in 50 ml mG medium (pH 7.0) and maintaining the growth temperature at 25 or 45°C. The sporulation efficiency was measured at various time intervals after applying the stress. Sporulation efficiencies were calculated by plating the culture before and after heat treatment (65°C, 30 min) on 2× SG agar followed by incubation for 24 h at 37°C. The initial population of untreated cells was expressed as N_0 and percentage survival as number of CFU/ml $(N_t/N_0) \times 100$, where N_t is number of colony forming units (CFU) of treated culture.

Purification of spores

The cells were grown in mG sporulation medium under various conditions with continuous shaking at 250 rpm. Cultures with >98% refractile spores, as determined by phase contrast microscopy, were then harvested, washed repeatedly, and stored as described previously (Ireland and Hanna 2002).

Determination of spore sensitivity

Sensitivity of spores (10^8 colony-forming units per sample) towards various chemical denaturants was assessed by

treating them individually with, 2 M HCl, 2 M NaOH, 0.2 M H_2O_2 and 3.5% formaldehyde for specified periods at room temperature. For wet heat, spore suspensions were incubated at 95°C for different time intervals. UV irradiation was performed in a crosslinker (UV Stratalinker 2400) producing 254-nm-wavelength UV radiation. The spore suspension (10^8 spores in 5 ml PBS) was aliquoted into the 6-cm-diameter petri dish and exposed to various doses of UV: 173, 346, 519, 692 and 785 J/m^2. The dose was measured using radiometry (model IL 1400A, international light, Newburyport, MA, USA). After treatment, spores were washed with sterile distilled water and recovered by centrifugation (10,000×*g*, 10 min, 4°C). All the spore suspensions were serially diluted in sterile distilled water and number of CFU of spores was determined by plating on 2× SG agar. The percentage survival was calculated as mentioned above.

Dipicolinic acid content and germination assay

The dipicolinic acid (DPA) content of various spore preparations was assayed according to the method described by Amiteye et al. (2003). In brief, purified spore suspensions were pelleted by centrifuging 1.5 ml of culture (10,000×*g* for 5 min), prior to the assay. Spore's dry weight was determined by completely drying the spore suspension in a dry bath and then determining their weight. DPA was assayed by suspending equal weight of various spore pellets in 1 ml of sterile distilled water. Subsequently, the suspension was incubated at 121°C, 15 lb/sq in. for 20 min, cooled on ice for 15 min and then centrifuged at 6,000×*g* for 5 min. A portion of supernatant (600 µl) was treated with 200 µl of solution containing 25 mg L-cysteine, 0.31 g of $FeSO_4.H_2O$, 80 mg of $(NH_4)_2SO_4$, and 25 ml of 50 mM sodium acetate (pH 4.6). A standard curve of DPA was used to calculate the amount of DPA in supernatants obtained from various spore samples.

To determine the rate of germination of spores formed under various stress conditions, spores were radiolabeled by adding ^{45}Ca (specific activity = 21.8 mCi/gm) in the mG medium during sporulation. Equal number of radiolabeled spores were added to 2× YT germination media containing 4 mM alanine as a germinant, and the ^{45}Ca released (percentage germination) in the medium was monitored at specified time intervals using liquid scintillation counter. The percentage of calcium released (percentage germination) was then calculated as described previously (Weiner and Hanna 2003).

Atomic force microscopy (AFM)

For AFM studies, purified spore suspensions were diluted in sterile distilled water. The suspension (2 µl) was loaded onto a mica plate and incubated for 5 min at room temperature as described earlier (Zaman et al. 2005). AFM imaging was carried out in air using PicoSPM (MI, AZ, USA). Imaging was done in the tapping mode using AAC cantilevers (Mikromasch), having a spring constant of 0.2–0.4 N/m and a resonance frequency of ~28 kHz. The spore size was determined by measuring spore length and width from two independent sets of ~200 spores for all the conditions. Dimensional measurements were carried out using standard Image Processing Software, Scanning Probe Processor (SPIP, version 4.1).

Two-dimensional polyacrylamide gel electrophoresis

Crude lysates of *B. anthracis* spores were prepared for 2D-gel electrophoresis as described previously (Movahedi and Waites 2000). Two hundred and fifty micrograms of crude lysate in 125 µl of carrier ampholyte [0.5% (v/v), pH 3–10) was loaded on a 7 cm immobilized pH gradient (IPG) pH 4–7 strips and separated by isoelectric focusing electrophoresis (IEF) in the first dimension. IEF was carried out at following conditions: (1) 0–250 V, 25 min, (2) 400 V for 2 h and 30 min, (3) 4,000 V applied until it reaches up to 10,000 VH. The strip was then subjected to a two-step equilibration in DTT and iodoacetamide buffers before proceeding to SDS-PAGE at a constant voltage of 100 V. Separated proteins were silver stained using silver stain plus (BIORAD). The 2D-gels were scanned and analyzed. The protein spots of interest were manually excised from gel for subsequent MALDI analysis. The peptide fingerprints obtained from mass spectrometer were searched using Mascot (Perkins et al. 1999), and ProFound (Zhang and Chait 2000) databases.

Results and discussion

Effect of temperature, pH and nutrient deprivation on sporulation efficiency

Nutrient deprivation is one of the major contributing factors for the commitment of bacteria for sporulation. In the present study, to assess the effect of various environmental conditions, particularly pH, temperature and nutrient deprivation, on the sporulation process of *B. anthracis*, cultures were subjected to individual stress conditions before the onset of sporulation as described in the material and methods. Whereas, the cultures maintained at alkaline pH and in nutrient-deprived medium showed 5 and 10% sporulation, respectively, after 6 h of stress (Fig. 1), no sporulation was observed in control cultures (pH 7.0) after the initial 6 h of incubation. After 11 h of incubation under various stress conditions, a sporulation efficiency of >80% was

observed in the culture grown at acidic pH, and >95% in the culture maintained at alkaline pH and in nutrient deprived medium (Fig. 1). These results revealed that sporulation in alkaline and nutrient deprivation condition takes place earlier than in the control. Yazdany and Lashkari (1975) reported that pH of the growth medium significantly affects the sporulation efficiency in *B. stearothermophilus*. They observed that at a given time, >80% sporulation was obtained in the medium with pH 7.7–8.7, which declined sharply when the pH of medium was altered to a greater extent, either acidic or basic. In another report, Nakata studied the effect of pH (6.4, 7.0 and 7.4) on growth, sporulation and the intermediates secreted in the medium during sporulation of *B. cereus* and reported that only slight differences were noted in the final number of thermo-resistant spores produced by each of the cultures, indicating that sporulation processes are not significantly impaired within these pH range (Nakata 1963). In the present study, we used a relatively wider pH range (pH 5.0–9.0) to study the effect of pH on sporulation efficiency of *B. anthracis* and observed significant variations in sporulation efficiencies in response to pH alterations (Fig. 1).

The sporulation efficiency of the culture maintained at 45°C was lower than control at all the time points studied and an efficiency of >80% was attained only after 26–30 h (Fig. 1). In the case of culture grown at 25°C, only 1–2% sporulation was observed after 36 h, and at the end of 48 h only 70% bacteria survived which eventually formed spores as determined by plating the culture before and after heat treatment (data not shown). These results indicate that the changes in temperature during sporulation significantly delay the spore formation which may be a consequence of the time required for adaptation of microorganisms to variation in temperature.

We also studied *B. anthracis* sporulation at pH 3.0 and 11.0 and temperatures below 25°C and above 45°C. In all these conditions, significant cell death was observed within 2 h of stress (data not shown) and therefore, sporulation, which requires at least 6–8 h from onset to completion could not be observed. The results thus define pH and temperature extremes that inhibit growth and sporulation in *B. anthracis*.

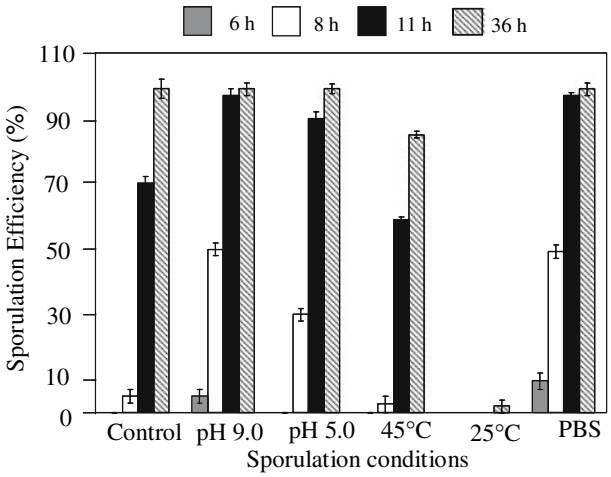

Fig. 1 Sporulation efficiency of *B. anthracis* under different conditions. Sporulation efficiency was calculated by plating the culture before and after heat treatment (65°C, 30 min) on 2× SG agar. Control cells were grown in mG medium at pH 7.0 for various time periods as indicated. Sporulation in control cultures and those maintained at pH 5.0 and at 45°C was observed only after 8 h of incubation. Sporulation was not observed in cultures grown at 25°C. The data shown are mean ± SE of three separate values obtained from two independent experiments

Spore sensitivity towards physical and chemical denaturants

Chemical and physical denaturants are widely used for the inactivation of *Bacillus* spores (Setlow et al. 2002). Wet heat resistance is one of the most important properties of the *Bacillus* spores. In the present study, spores formed under various stress conditions showed varied sensitivity towards wet heat (Table 1). Considering the D-10 time for wet heat, spores formed at a higher temperature (45°C) were more resistant than those formed at a lower temperature (25°C) (Table 1). Spores formed at acidic pH were also found to be more resistant to wet heat compared to spores formed at alkaline pH and control spores. Notably, the spores purified from PBS were most sensitive to wet heat (Table 1). The spores prepared from nutrient-deficient media have been shown to possess low mineral content and are generally more sensitive to denaturants (Marquis and Shin 1994; Marquis et al. 1994). Further, the mineralization of bacterial spores with Ca^{2+} and other mineral cations has been shown to enhance resistance to heat damage and it was partly associated with increased dehydration of the mineralized protoplast or spore core (Marquis and Shin 1994). In view of these facts spores formed in PBS are expected to have a low mineral content, a plausible cause for the observed sensitivity to wet heat (Table 1).

Irradiation is yet another routinely employed method for killing the *Bacillus* spores. The UV sensitivity profiles indicated that spores prepared under different conditions exhibit similar sensitivity at low doses of UV irradiation whereas, the spores prepared at 25°C and PBS showed increased killing at the higher dose of UV exposure (Fig. 2). In an earlier report, Nicholson and Galeano (2003) showed that UV dose that were effective against *B. subtilis* spores were also sufficient to inactivate *B. anthracis* spores. The spores of *B. anthracis* Sterne exhibited LD_{90} (lethal dose to kill 90% spores) at 275 J/m^2 (Nicholson and Galeano 2003) which was approximately three fold lower than the dose required for LD_{50} (lethal dose to kill 50% spores) in the present

Table 1 Spore sensitivity towards physical and chemical denaturants

Spore denaturants	Sporulation conditions					
	Control	pH 5.0	pH 9.0	45°C	25°C	PBS
Decimal reduction time (D-10 time) (min)						
Wet heat	37 ± 1.5	50 ± 3.0	40 ± 2.5	57 ± 2.0	33 ± 2.0	29 ± 1.0
2.0 M HCl	45 ± 2.0	59 ± 1.0	59 ± 1.0	71 ± 3.0	42 ± 1.0	15 ± 1.6
2.0 M NaOH	19 ± 1.5	22 ± 3.0	49 ± 2.5	49 ± 2.0	18 ± 1.5	15 ± 1.0
2.0 M H_2O_2	27 ± 1.5	14 ± 1.5	28 ± 2.0	52 ± 1.1	24 ± 2.0	12 ± 1.0

For wet heat resistance, various spore suspensions were incubated at 95°C and for resistance towards chemical denaturants, spores were incubated with 2.0 M HCl, 2.0 M NaOH and 0.2 M H_2O_2 for specified time. After treatment, spores were recovered and their sensitivity towards various denaturants was determined. The decimal reduction time (D-10 time) is the time of exposure which reduces spore viability by a factor of 10 (reduction in viable spore counts by 90%). The data shown are mean ± SE of three separate values obtained from two independent experiments

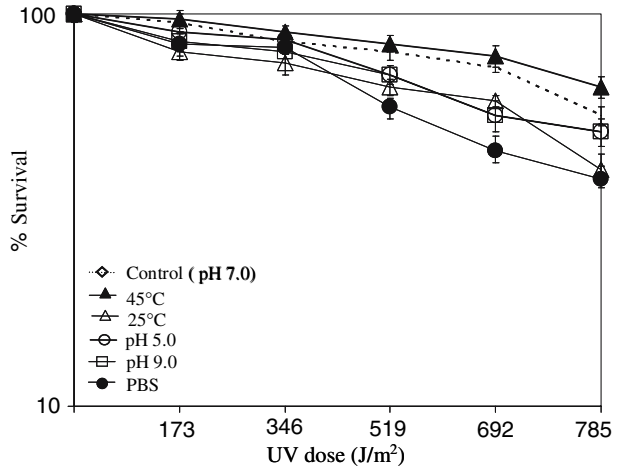

Fig. 2 Survival of spores exposed to UV radiations. The spores were treated with UV-radiation and the dose was measured using radiometer. The spores were recovered by centrifugation and suspended in sterile distill water. The number of CFU was determined by plating the spore suspension on 2× SG agar. The data shown are mean ± SE of three separate values obtained from two independent experiments

study. The observed variation in the UV dose requirement could be due to the higher spore density or the different sporulating conditions used in the present study. The results obtained in the present study suggest that spores prepared in PBS or at 25°C are more sensitive to UV radiation.

Earlier reports have identified several factors that play important role in determining the spore resistance. In addition to genetic makeup these factors include sporulating conditions such as temperature, impermeability of the spore core, water content and level of minerals in the spore core, saturation of spore DNA with a/b-type small, acid-soluble proteins, and repair of damage to macromolecules during spore germination and outgrowth (Nicholson et al. 2000). The spores of *Bacillus subtilis* exhibit higher degree of resistance to a variety of spore denaturants such as acid, alkali, oxidizing agents and formaldehyde as compared to the vegetative cells (Nicholson et al. 2000).

In the present study, we further explored the sensitivity of spores formed under different conditions towards various denaturants including acid (HCl), alkali (NaOH), hydrogen peroxide (H_2O_2) and formaldehyde (HCHO). Purified spores were treated with aqueous solutions of various denaturants for different time periods and the viability was assessed by number of CFU. It was observed that the spore survival decreased with the increase in time for which cells were exposed to various denaturants (data not shown). In spite of this common trend, response of individual spore preparations varied towards different denaturants. Spores formed at 45°C were more resistant to acid, alkali and hydrogen peroxide compared to the control spores (Table 1). In consensus with our results, Melly et al. (2002) have reported that *B. subtilis* spores formed at higher temperature were more resistant towards various chemical decontaminants, as compared to spores prepared at lower temperature. The sensitivity of the spores formed either at acidic pH or at alkaline pH was similar towards HCl (Table 1). However, the spores formed at acidic pH were more sensitive towards H_2O_2 as compared to the control spores and spores formed at alkaline pH (Table 1). Spores formed in PBS were generally much more sensitive towards various chemical denaturants (Table 1). A plausible explanation is that during growth in PBS, sporulating cells are deprived of key growth elements since media is completely depleted in carbon, nitrogen source and divalent metal ions. It is well established that spore's mineral content is an important factor to confer resistance to spores. Marquis et al. have analyzed the properties of mineralized and demineralized spores of *B. megaterium*, *B. subtilis* and *B. stearothermophilus*, and observed that divalent cation-loaded spores are more resistant towards wet-heat (Marquis and Shin 1994; Marquis et al. 1994) and oxidizing agents (Marquis and Shin 1994) than the monovalent cation-loaded demineralized spores.

Spores formed under various stress conditions were also treated with 3.5% aqueous solution of formaldehyde for

various time periods. However, no significant difference was observed in the decimal reduction time (data not shown). Therefore, while spores prepared under different conditions exhibited variations in sensitivity towards HCl, NaOH and H_2O_2, their sensitivity towards UV and formaldehyde remained unaltered. The variation in sensitivity of spores prepared under different conditions may be due to the difference in relative amount of proteins that are assembled into several distinct layers under the direction of specific assembly proteins (Driks 1999). The spore coat structure protects the peptidoglycan cortex from attack by lytic enzyme and could be one of the factors responsible for the spore resistance to a variety of chemicals (Russell 1982). Melly et al. (2002) analyzed the coat proteins from spores of *B. subtilis*, prepared at different temperatures and revealed a number of differences responsible for their differential susceptibility towards hydrogen peroxide, butadiene, formaldehyde, gltaraldehyde and sterilox. In our studies, an effort was made to analyze the proteome of spores prepared in PBS.

Dipicolinic acid content and germination efficiency of spores

The spores formed under different conditions were purified and further characterized by assessing specific parameters including DPA content of the cell. Dipicolinic acid (DPA) is a major determinant of spore's unique properties including dormancy and wet-heat resistance (Paidhunghat et al. 2000). The compound is exclusively present in the core of bacterial spores in form of a chelated complex with calcium (Powell 1953). We observed that the amount of DPA in *B. anthracis* spores formed at an elevated temperature (45°C) was two-fold higher as compared to the control spores (Fig. 3a). The mechanism by which DPA increases in spores formed at 45°C is not clear. One function of DPA in spore resistance is to lower the core water content, probably by replacing some core water. This process can elevate spore resistance to wet heat by protecting core proteins from inactivation or denaturation. In agreement with the increased DPA content of spores formed at 45°C we observed a reduced size of these spore which probably is a direct consequence of reduced core water content. Since DPA content correlates well with spore sensitivity towards wet heat and chemical decontaminants like H_2O_2 (Paidhunghat et al. 2000), it appears likely that the increased DPA content of spores prepared at 45°C contributes towards their increased resistance against wet heat and chemicals.

It has been shown that DPA photosensitizes spore DNA to damage and favors the formation of 5-thyminyl-5,6-dihydrothymine when exposed to UVC (254 nm) radiation (Douki et al. 2005). DPA has also been reported to maintain spore dormancy and DPA-less spores were unstable and germinated spontaneously (Paidhunghat et al. 2000). Three genetic loci (gerA, gerB, and gerK) have been shown to be responsible for nutrient-induced germination of *B. subtilis* spores. Spores of *B. subtilis* lacking *gerA*, *gerB* and *gerK* operon have been reported to be more sensitive to UVC radiation than wild type spores (Slieman and Nicholson 2001). However, a difference in the pattern of UV resistance was noted among spores of strains lacking germination operon and inactivated DPA synthase, when irradiated as air-dried films or in aqueous solution. The spores lacking germination operon and DPA synthase were more resistant to UVC radiation whereas, sensitivity of spores lacking germination operon but intact DPA synthase were similar in aqueous suspension or as air dried film (Slieman and Nicholson 2001). These results indicated that role of DPA in UV resistance is

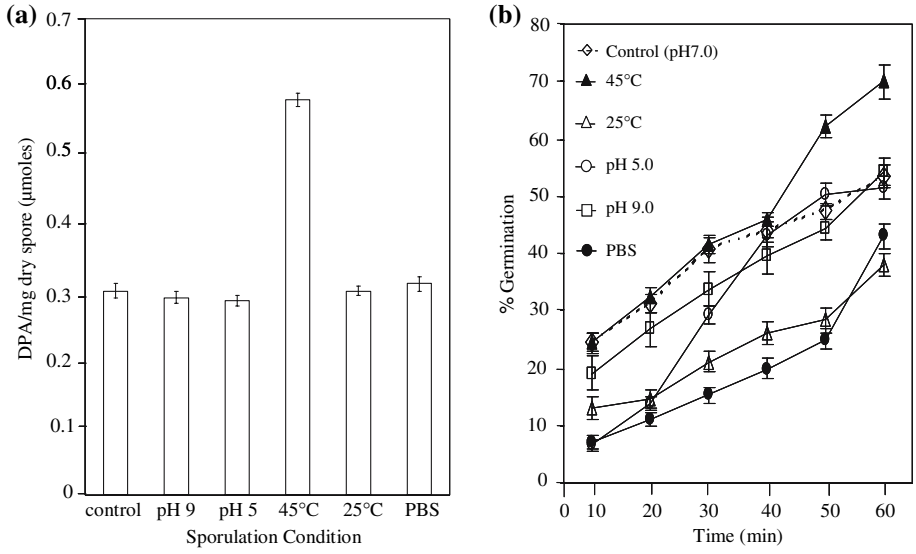

Fig. 3 Dipicolinic acid content and germination efficiency of various spores. **a** The spores were incubated at 121°C, 15 lb/sq in. for 20 min and DPA content in the supernatant was determined. **b** Germination of spores was studied using radiolabeled spores and percentage germination was calculated. The data shown are mean ± SE of three separate values obtained from two independent experiments

complex and is associated with DPA-dependent germination pathway of spores. In the present study, the sensitivity of spores containing higher amount of DPA was similar to that of control (Fig. 2). It appears that increase in DPA content above the normal level present in control spores may not have any impact on sensitivity of spores towards UV radiation.

Release of calcium ions from the spore into the environment is an early post-commitment stage of germination and is directly proportional to the number of actively outgrowing bacilli in a rich media containing germinant (Suzuki et al. 1971). The spores formed at 45°C germinated much earlier (50% efficiency at 43 min) compared to the control spores with 50% efficiency at 60 min (Fig. 3b). The spores formed at alkaline and acidic pH showed no significant difference in the time required for 50% germination as compared to control spores. The spores formed at low temperature (25°C) and in PBS germinated at a relatively low rate where 50% germination was not achieved even after 60 min as compared to control spores. Therefore, it can be concluded that temperature during sporulation and composition of sporulation media affects the relative composition of the spore, especially DPA and calcium as observed in this study.

Atomic force microscopy of various spores

The size of the spores formed under different conditions has not been analyzed so far for any of the *Bacillus* species. In the present study, we determined the dimensions of various spore preparations (~200 each) using atomic force microscopy, in hydrated environments (Table 2). Significant variations were observed in the sizes of spores formed at 45°C (0.6 × 0.44 μm) and at 25°C (1.06 × 0.35 μm) as compared to control spores (0.76 × 0.50 μm). However, the size of spores remained unaltered in case of spores formed under acidic or alkaline conditions or in PBS in comparison to the control spores. All the spore populations were viable. As discussed above, the smaller size of the spores formed at 45°C may be due to the increased DPA and lower core water content in these spores as compared to the control ones. However, the reason for the change in size of the spores formed at 25°C was not clearly understood. In a recent study, it has been reported that the spore is capable of relatively rapid expansion and contraction without breaking its dormancy (Driks 2003). Hence the coat, and/or cortex are sufficiently flexible to accommodate these volume changes without impairing spore integrity. Since, the state of hydration of spores is governed by the temperature during sporulation (Gerhardt and Marquis 1989), it is probable that the spores formed at 25°C will have higher water content making them more dynamic with respect to changes in dimensions and hence they appear to be elongated.

2D-gel analysis of spore proteins prepared PBS

The spores prepared under different stress conditions showed varied susceptibility towards physiochemical agents. The spores formed in PBS were found to be highly susceptible to these agents. Therefore, in order to identify proteins involved in spore resistance/sensitivity, the proteome of these spores was analysed by 2D-gel electrophoresis. The comparison of spots on 2D-gels between spores prepared in PBS and mG medium showed 32 spots that were differentially expressed. Out of the 32 protein spots identified, 8 yielded significant Mascot scores (≥60) (Table 3). The analysis showed upregulation of three stress related proteins namely catalase, peroxiredoxin and universal stress protein A (Table 3). Increased levels of catalase and peroxiredoxin have been reported in *Bacillus stearothermophilus* in response to oxidative stress (Topanurak et al. 2005). Universal stress protein A (UspA) belongs to the orthologous group of proteins called UspA superfamily and the levels of UspA have been shown to be elevated in response to a variety of stress conditions including starvation (Gustavsson et al. 2002). It has been reported that adaptation during starvation is important for cells to initiate long-term survival under conditions of not only nutrient depletion but to develop resistance to other stresses (McDougald et al. 2002). Moreover, there is an overlap in the cell's use of global regulators to deal with both starvation and oxidative stress (McDougald et al. 2002). Hence, the upregulation of both catalase and peroxiredoxin in

Table 2 Size of *B. anthracis* spores formed under different conditions as determined by atomic force microscopy

Sporulation conditions	No. of spores	L_{av} (μm)	D_L (μm)	D_L (%)	W_{av} (μm)	D_W (μm)	D_W (%)
pH 7.2	180	0.76	0.07	9.1	0.50	0.09	21
pH 5.0	202	0.75	0.137	13.4	0.48	0.103	21
pH 9.0	190	0.747	0.137	18.6	0.46	0.086	21
45°C	200	0.6	0.1	18.0	0.44	0.08	21
25°C	220	1.058	0.1	10.0	0.346	0.055	15.9
PBS	202	0.72	0.129	12.9	0.50	0.103	21

L_{av} average length, W_{av} average width, D_L absolute deviation in length, D_w absolute deviation in length width

Table 3 Differentially expressed proteins of *B. anthracis* spores formed in PBS

S. no.	Protein description	NCBI accession number	Coverage of peak matched (%)	Mascot score[a]	Trends on 2D-gel of PBS spores	Function
1	Catalase	gi: 49185946	32	96	Increased	Reduces peroxides
2	Peroxiredoxin	gi: 65317955	50	60	Increased	Reduces peroxides
3	Universal stress protein A	gi: 49183647	40	68	Increased	Stress protein
4	Malonyl CoA acyl carrier protein transacylase	gi: 49186702	39	89	Increased	Fatty acid biosynthesis
5	Ribonuclease Rne/Rng family	gi: 49187338	25	65	Present only in PBS	RNA hydrolysis
6	Glutamate dehydrogenase	gi: 49184418	26	70	Decreased	Glutamate synthesis
7	Hypothetical protein	gi: 49187188	23	60	Present only in PBS	–
8	Hypothetical protein	gi: 49184277	30	60	Increased	–

[a] Mascot score is based on absolute probability (P) that the observed match between the experimental data and the database sequence is a random event. The reported score is $-10 \log (P)$

spores formed in PBS could be due to the nutrient stress encountered by the *B. anthracis* culture when they were allowed to sporulate in PBS.

Fatty acids are essential components of membrane that determines sensitivity of spores towards denaturants. The levels of Malonyl CoA ACP transacylase were increased in spores formed in nutrient-deprived medium. Inspite of increased levels of Malonyl CoA ACP transacylase, the spores formed in nutrient deprived medium may have altered membrane composition due to lack of precursors for fatty acid synthesis.

Therefore, the spores prepared in PBS may become more sensitive to chemical denaturants. The reason for decreased levels of glutamate dehydrogenase and presence of ribonuclease in spores formed in PBS is not clear.

Conclusions

In conclusion, the results of the present study indicate that spores formed at higher temperature were highly resistant whereas, spores formed in PBS were sensitive to various denaturants. *B. anthracis* is a natural soil-inhabiting bacterium and changes in the microenvironment of the spore can cause structural alterations that may influence characteristics of the spore including infectivity. It has been reported that soil of alkaline pH with moisture, high concentration of organic matter and ambient temperature in excess of 15°C provides microenvironment which promotes cycling of *B. anthracis* spores (Van Ness 1971). This observation is supported by our results wherein we also observed an early sporulation at alkaline pH. The spores thus formed were found to be more resistant to both acidic and basic pH conditions that may help in survival of a higher percentage of viable spores in such a microenvironment.

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