Sporulation-associated mother cell lysis in *Bacillus* displays markers of programmed cell death

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Sporulation in *Bacillus* sp. requires the death of spore mother cell that has been hypothesized to be genetically programmed. However, there is lack of conclusive evidence supporting this hypothesis. The present study provides evidence showing expression of programmed cell death (PCD)-specific markers such as activation of caspase-3, externalization of phosphatidylserine (PS) detected by annexin V-FITC binding through flow cytometry, and damage to DNA evaluated by TUNEL assay during sporulation in *B. subtilis* and *B. megaterium*. Addition of cell-permeable irreversible inhibitor of caspase-3 was found to inhibit the sporulation process as also the caspase-3 activity and PS externalization. These findings were further revalidated using sporulation-deficient-mutants of *B. subtilis*, created using chemical mutagenesis. These mutants were found to be deficient in caspase-3 activity as well as the extent of PS externalization. Wild-type *B. subtilis* cells were found to have extracellular metal-dependent DNAse activity, which decreased in sporulation-deficient mutants. These findings provide evidence for the existence and association of markers of PCD during sporulation-associated mother cell lysis in *Bacillus* sp.

Keywords: *Bacillus* species, mother cell lysis, programmed cell death, sporulation.

THOUGH the need and mechanism of apoptosis or programmed cell death (PCD) is well understood in eukaryotes, in bacteria it is still relatively poorly understood. Recently, a number of studies have reported the occurrence of PCD-like processes in bacterial cells in response to different stresses. Some of these include starvation-induced cell death in *Escherichia coli* regulated by various toxin–antitoxin gene pairs[1], metabolic stress-induced PCD in *Xanthomonas*[2–5], and antibiotic-induced cell death in *Staphylococcus aureus*[3]. Besides, certain developmental processes in the bacterial life cycle have also been postulated to involve PCD. These include lysis of spore mother cell during sporulation in *Bacillus* sp.[6,7], lysis of vegetative cells during myxobacterial fruiting body formation[8] and spontaneous autolysis in streptococci[9].

Sporulation in *Bacillus* species is a complex phenomenon, involving development of the growing vegetative cell into two-cell-chambered sporangium; within one an endospore is formed, and the other representing the mother cell dies. Initiation of sporulation is signalled by at least three types of input, including nutritional status, population density and cell cycle stage[10,11]. In *Bacillus* species the final stage of sporulation is the release of spore upon lysis (death) of the mother cell, which is actively lysed prior to release of the spore, by a set of enzymes called autolysins, which hydrolyse the peptidoglycan layer of the mother cell wall[6]. They are postulated to play a major role in many important cellular processes such as cell separation and differentiation, cell wall turnover, sporulation, mother cell lysis, competence and antibiotic-induced lysis[6]. The expression of autolysins has been reported to be well-regulated by the products of the gene-pairs comprising hoiln–antiholin module, which resembles the Bax/Bcl-2 family of proteins in eukaryotic cells. Thus it is speculated that the process of PCD is mechanistically conserved in bacteria by possessing molecules similar to eukaryotic systems[3].

During early stages of the sporulation process *Bacillus subtilis* produces several hydrolytic enzymes, e.g. serine proteinase, alkaline phosphatase, ribonuclease and several phosphodiesterases capable of hydrolysing DNA and RNA[12]. One such is Mn^{2+}-stimulated extracellular DNAse. This may have role in the process of PCD occurring during sporulation in *B. subtilis*. Production of this extracellular Mn^{2+}-stimulated DNAse has been shown to be associated with stage II of the sporulation process. In *B. subtilis* the production and release of DNAse occurs as a part of the developmental process of sporulation. NucB nuclease is a major extracellular DNAse in *B. subtilis*[13], whose transcription is regulated by the mother cell-specific sigma factor (σ^E). Recently, PCD has been well documented in many bacterial systems and existence of PCD-specific markers such as caspase-3 like enzyme activity, phosphatidylserine (PS) externalization has also

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been reported during stress conditions[14]. However, till date there are no reports showing involvement of these markers during sporulation in *B. subtilis* or other species. The present study was undertaken to evaluate the involvement of PCD-specific proteins like caspase-3 and poly ADP ribosyl polymerase (PARP), and other markers during sporulation in *Bacillus* species. To revalidate the observations, sporulation-deficient mutants of *B. subtilis* were created and the profile of PCD-related markers was evaluated in these mutants.

Materials and methods

Media and chemicals

Luria broth (LB) [casein enzymic hydrolysate (1%), yeast extract (0.5%), sodium chloride (0.5%)], sporulation broth (SB) [peptic digest of animal tissue (0.6%), casein enzymic hydrolysate (0.4%), yeast extract (0.3%), beef extract (0.15%), dextrose (0.1%), and manganous sulphate (0.03%)], and ethyl methanesulphonate (EMS) were procured from Himedia Laboratories Pvt Ltd, Mumbai, India. 3-Aminobenzamide, a PARP inhibitor, was procured from Sigma-Aldrich Inc. (St. Louis, USA) and Ac–DEVD–CMK (acetyl–Asp–Glu–Val–Asp–chloromethylketone), a cell-permeable caspase-3 inhibitor, was procured from BD Pharmingen (San Diego, CA, USA).

Bacterial strains and growth conditions

B. subtilis and *B. megaterium* strains were procured from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. For enumerating total viable cells, these organisms were grown in LB or SB media on a rotary shaker (150 rpm) at 30°C for 24 h, whereas for enumerating spores, 1 ml of the cell suspension was incubated in a water bath at 80°C for 10 min, serially diluted using saline (0.85%), and spread-plated on LB agar plates. The counting of vegetative cells and spores was carried out at 24 h interval till 72 h of incubation at 30°C. For examining the involvement of caspase-3 or PARP during sporulation process, the cells were grown in SB in the presence of cell-permeable caspase-3 inhibitor (Ac–DEVD–CMK, 100 μM) or PARP inhibitor (3-aminobenzamide, 200 μM), as described earlier[15].

Chemical mutagenesis to screen sporulation-deficient mutants

B. subtilis cells were grown overnight in 10 ml of LB in a rotary shaker (150 rpm) at 30°C. The next day, 0.5 ml of this culture was inoculated in 5 ml fresh sterile LB (1 : 100 dilution) and allowed to grow till mid log phase under similar conditions. Mutagenesis was induced by adding 70 μl of EMS into 5 ml of bacterial cell suspension (effective concentration of EMS being 133 mM) and incubating at 30°C in a rotary shaker (75 rpm) for 45 min. Subsequently, the cells were centrifuged and the pellet was washed twice with 5 ml of LB medium and resuspended in the same. A 100 μl of this cell suspension was inoculated in 5 ml of LB broth, in different replicates, and further grown overnight under shaking conditions (150 rpm) at 30°C. The following day the cultures were serially diluted using saline (0.85%) and spread-plated on LB–rifampicin (100 μg/ml) plates and incubated at 37°C for 24 h. The rifampicin-resistant mutants were further screened on skimmed milk agar plates for loss of extracellular proteolytic activity. The colonies which showed no or less proteolytic activity were further selected to screen sporulation-deficient mutants.

Assay of caspase-3 activity

An (1 ml) aliquot of 24 h SB-grown cells was withdrawn and centrifuged at 12,500 *g* for 2 min. The cell pellet was washed twice with phosphate buffer saline (PBS; 10 mM, pH 7.4), and finally resuspended in 1 ml cell lysis buffer containing Tris-HCl (10 mM), sodium phosphate buffer (10 mM, pH 7.5), NaCl (130 mM), triton X-100 (1%), sodium pyrophosphate (10 mM) and lysozyme (1 mg/ml) and later incubated at 4°C for 4 h. The cell lysate was then centrifuged at 12,500 *g* for 15 min and the supernatant was collected in a fresh microfuge tube. Protein concentration was normalized using Bradford's assay for all the samples before the assay was performed. Caspase-3 activity was analysed using 50 μl of the supernatant and 10 μl of Ac–DEVD–AMC (acetyl-Asp–Glu–Val–Asp 7-amido-4-methylcoumarin), a synthetic flurogenic substrate (BD Pharmingen, CA, USA) according to the method described earlier[2].

Analysis of phosphatidylserine externalization using annexinV-FITC labelling

Annexin V-FITC labelling was performed using annexin V-FITC apoptosis detection kit (BD Pharmingen, CA, USA) according to the manufacturer's guidelines. An aliquot (1 ml) of 24 h SB-grown cells was withdrawn and centrifuged at 12,500 *g* for 2 min. The cell pellet was washed twice with PBS (10 mM, pH 7.4) and the cell number per ml was normalized in PBS before the assay. Later, the cells were centrifuged at 12,500 *g* for 2 min and resuspended in 1 ml of annexin V-binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl and 2.5 mM $CaCl_2$) containing 5 μl of annexin V and incubated in the dark for 15 min. Later, propidium iodide (5 μl of 50 μg/ml) was added to the cell suspension, which was further incubated at ambient temperature in the dark for 15 min. About 0.1 million cells were assayed during each flow cytometry analysis (Partec CyFlow Space, Germany).

Determination of DNA damage by TUNEL assay

TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) was performed according to the manufacturer's guidelines using *in situ* cell death detection kit containing fluorescein (Roche, Basel, Switzerland). Briefly, 1 ml aliquot of 48 h-grown cells was centrifuged at 12,500 *g* for 2 min, the pellet was washed twice with PBS (10 mM, pH 7.4) and the cell number per ml was normalized in PBS before the assay. Subsequently, the cells were centrifuged at 12,500 *g* for 2 min and resuspended in 50 µl of TUNEL reaction mixture [TdT enzyme solution (5 µl), and labelling solution (45 µl)]. The cell suspension was incubated at 37°C for 60 min. Later the suspension was spun at 12,500 *g* for 2 min and the pellet was washed twice with PBS. Finally, the pellet was resuspended in 500 µl of PBS and analysed by FACS using flow cytometry system (Partec CyFlow Space, Germany). For each analysis, 0.1 million cells were used.

Evaluation of DNAse activity

B. subtilis wild type and mutant strains were grown in sporulation broth media and incubated at 30°C with shaking (150 rpm) till 24 h of growth. An aliquot (1 ml) of culture was removed at 24 h and centrifuged at 6000 *g* for 10 min. Protein content was normalized using Bradford's assay before the DNAse activity assay was performed. pBR322 plasmid DNA (200 ng) was incubated at 37°C for 1 h with 15 µl of cell-free supernatant of wild type as well as mutant strains of *B. subtilis*, and further analysed by agarose (1%) gel electrophoresis. DNA bands were visualized by staining with ethidium bromide and later quantified (Gene Genius, Syngene, UK).

Statistical analysis

The experiments were repeated at least thrice, each in triplicate. The mean and standard deviation (SD) were calculated for each set of experiments. The mean values were further compared using independent sample Student's *t* test at 95% confidence limit. In the case of flow cytometric analysis and agarose gel electrophoresis, the findings from one representative set have been displayed.

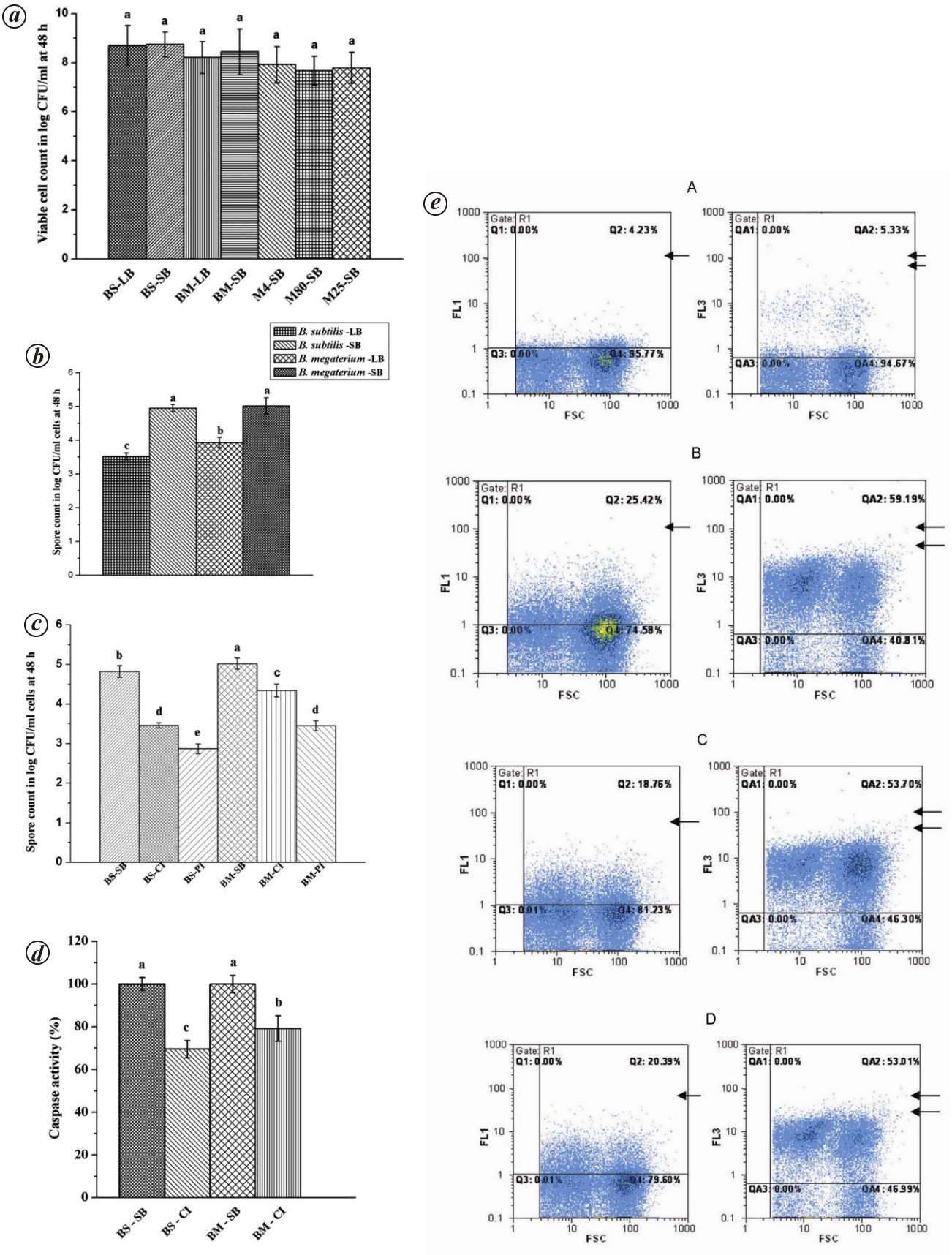
Results and discussion

Sporulation profile of Bacillus species

The cell count and spore count of both *B. megaterium* and *B. subtilis* were ascertained during growth phase after every 24 h intervals in SB and LB. The viable cell count of *B. megaterium*, *B. subtilis* as well as *B. subtilis* mutants (M4, M80 and M25) was about 8 log CFU/ml and there was no significant difference ($P \geq 0.05$) between the counts of all these bacterial strains (Figure 1 *a*). Sporulation efficiency of both *B. subtilis* and *B. megaterium* was found to be better in SB than in LB medium (Figure 1 *b*). In SB medium for both these *Bacillus* species the spore count was about 5 log CFU per ml of viable cells at 48 h. In LB medium spore count in case of *B. subtilis* was about 3.5 log CFU, while in *B. megaterium* it was about 4 log CFU per ml of viable cells (Figure 1 *b*). However, SB supported early sporulation in *B. megaterium* and the spores could be obtained after 24 h of growth, which was lacking in LB (data not shown). The spore count was variable for both the species in different media. The differential sporulation in these media could be attributed to the differences in their chemical composition. LB contained casein hydrolysate, yeast extract and sodium chloride, while SB contained peptic digest of animal tissue, beef extract, dextrose and manganous sulphate in addition to casein hydrolysate, and yeast extract as medium constituents. Peptone and beef extracts are sources of nitrogen, sulphur, amino acids and essential trace elements. Dextrose is an energy source for bacterial replication and manganous sulphate plays an important role in the process of sporulation[16]. Thus, the sporulation efficiency may vary depending upon the *Bacillus* strains and nutrient content of the medium. Thompson and Thames[17] also reported induction of sporulation in *B. stearothermophilus* using manganous sulphate (30 ppm) and higher amount of peptones (3%). Manganese is a co-factor for the enzymes such as phosphoglycerate phosphomutase that are involved in sporulation in *B. subtilis*[18]. In the absence of manganese, the cells generally do not sporulate. However, if the ratios of nutrient constituents like glucose or malate are altered, then the cells show sporulation[19]. A specific sporulation medium is required for producing spores from a specific group of spore-forming bacteria. For *B. megaterium*, it was observed that the spore production was maximum in tryptone yeast extract broth[20]. Though SB supported early sporulation in both *Bacillus* species, the sporulation efficiency attained saturation in both LB and SB media at later stages of incubation. For further studies on understanding the mechanism of cell death during sporulation, only SB was selected as a medium of choice for growth of both the *Bacillus* species.

Inhibition of sporulation

Since the presence of caspase-3-like proteases in *B. subtilis* has already been reported[21], the present study was carried out to evaluate the effect of caspase-3 inhibitor on sporulation process. In *B. subtilis*, addition of cell-permeable caspase-3 inhibitor resulted in about 90% reduction in the spore count, while with PARP inhibitor, reduction in the spore count was about 99% (Figure 1 *c*). In *B. megaterium*, with caspase-3 inhibitor the spore count reduced by about 80%, while with PARP inhibitor it reduced by about 98%. Similar trend in sporulation



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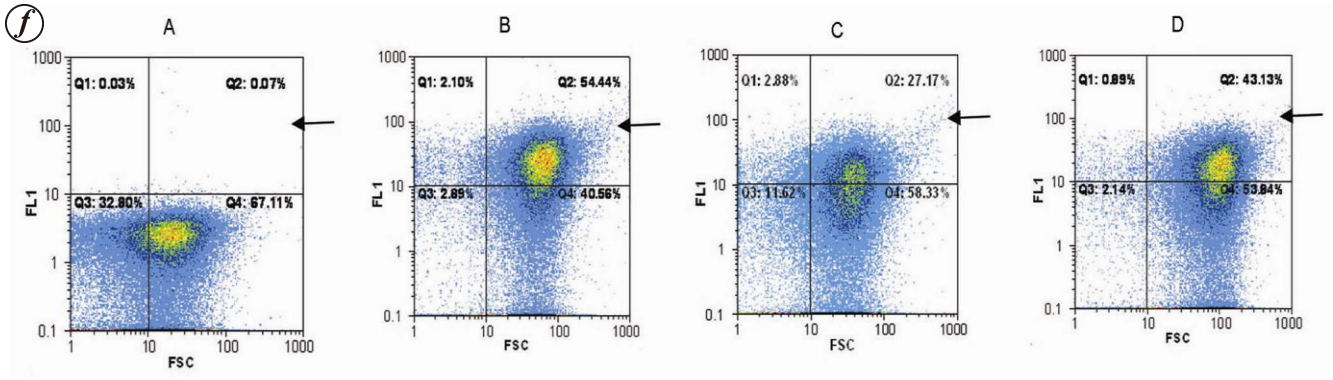

Figure 1. ***a***, Viable cell count of *Bacillus* species and *Bacillus subtilis* mutants in SB medium at 48 h of incubation. BS-LB, *B. subtilis* cells grown in LB medium; BS-SB, *B. subtilis* cells grown in SB medium; BM-LB, *B. megaterium* cells grown in LB medium; BM-SB, *B. megaterium* cells grown in SB medium; M4-SB, M4 cells grown in SB medium; M80-SB, M80 cells grown in SB medium, and M25-SB, M25, cells grown in SB medium. ***b***, Spore count of *Bacillus* species in SB and LB media at 48 h of incubation. ***c***, Spore count of *Bacillus* species at 48 h of growth having caspase-3 inhibitor (CI) and PARP inhibitor (PI). BS-SB, *B. subtilis* cells grown in SB medium; BS-CI, *B. subtilis* cells grown in SB medium in the presence of caspase-3 inhibitor; BS-PI, *B. subtilis* cells grown in SB medium in the presence of PARP inhibitor; BM-SB, *B. megaterium* cells grown in SB medium; BM-CI, *B. megaterium* cells grown in the presence of caspase-3 inhibitor and BM-PI, *B. megaterium* cells grown in the presence of PARP inhibitor. ***d***, Caspase-3 activity of *Bacillus* species during sporulation as well as in the presence of caspase-3 inhibitor. BM-SB, Activity of *B. megaterium* cells grown in SB medium; BM-CI, Activity of SB medium-grown *B. megaterium* cells in the presence of caspase inhibitor; BS-SB, activity of *B. subtilis* cells grown in SB medium and BS-CI, Activity of SB medium-grown *B. subtilis* cells in the presence of caspase inhibitor. $^{a–f}$Different letters indicate significant difference ($P \leq 0.05$) between the mean values compared as analysed by independent sample Student's *t* test. ***e***, Annexin V-FITC labelling indicating phosphatidyl serine (PS) externalization in *B. subtilis* undergoing sporulation. A, Unlabelled cells; B, Cells grown in SB medium; C, Cells grown in SB medium in the presence of caspase-3 inhibitor and D, Cells grown in SB medium in the presence of PARP inhibitor. Single arrow indicates the quadrant showing annexin V-FITC-labelled cells, while double arrow indicates the quadrant showing PI-positive cells. ***f***, TUNEL assay indicating extent of DNA damage in *B. subtilis* undergoing sporulation. A, Unlabelled cells; B, Labelled control; C, Cells treated with caspase-3 inhibitor and D, Cells treated with PARP inhibitor. Arrow indicates the quadrant showing TUNEL-positive cells.

profile was observed at 72 h of incubation. Inhibition of sporulation by treatment of cells with 3-aminobenzamide, a PARP inhibitor was unexpected. PARP is a protein involved in a number of cellular processes involving primarily DNA repair. Proteolytic cleavage of PARP by caspase-3 is a hallmark of apoptosis. Thus inhibition of PARP by 3-aminobenzamide should have resulted in more number of cells undergoing apoptosis and subsequent sporulation. But, surprisingly, reduced sporulation was observed. This contrary observation could be attributed to restoration of NAD^+ pool upon inactivation of PARP which requires NAD^+ for activity. Similar results showing suppression of apoptosis by 3-aminobenzamide have been reported earlier in human myeloid leukaemia cells[22]. They showed that intracellular NAD^+ concentration decreased due to stress and further decrease in NAD^+ was observed with the induction of apoptosis. Presence of 3-aminobenzamide during stress decreased the extent of apoptosis by preserving the intracellular NAD^+ content. Probably similar events could be occurring in sporulating *Bacillus* cells. These findings thus indicate the requirement of caspase-3 and PARP during sporulation in *Bacillus* species.

Inhibition of in vivo caspase-3 activity

Further studies were performed to analyse *in vivo* caspase activity in *Bacillus* strains under sporulating conditions. In *B. subtilis* cells treated with caspase-3 inhibitor Ac–DEVD–CMK, the enzyme activity decreased by about 31% compared to control cells (Figure 1 *d*). Similarly, in case of *B. megaterium* cells, about 21% decrease in caspase activity was observed. The reduction in caspase-3 activity can be thus linked with the reduction in spore count in the presence of caspase-3 inhibitor. Possibly, the caspase-3 inhibitor irreversibly binds to the enzyme and thus decreases the activity in inhibitor-treated population. Similar findings have been reported earlier in *Aspergillus nidulans*[23].

Phosphatidylserine externalization in sporulating cells

Major phospholipids present on membranes of bacteria include phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG), while other phospholipids include PS and phosphatidylcholine (PC). In *B. megaterium*, the relative percentage of PE, PG, DPG and PS is found to be 34, 49, 10 and 1 respectively[24]. Among the total phospholipids, PE and PS are present on the inner or cytoplasmic face of the cell membrane[25]. Dutt and Dowhan[26] have reported the presence of a CDP-diacylglycerol-dependent pathway for the formation of PS in *B. subtilis*. Externalization of PS occurs during later stages of apoptosis and is considered as one of its important markers. Annexin V is a protein that has high affinity for PS. Hence, annexin V tagged with fluorescein isothiocyanate (FITC) is routinely used to detect PS

externalization by flow cytometry[27]. In the case of *B. subtilis*, a significant decrease in PS externalization was observed in cells treated with caspase-3 inhibitor as well as PARP inhibitor at 24 h of incubation. In the total cell population, about 25% untreated *B. subtilis* cells were found to be annexin V-positive. Percentage of annexin V-positive cells decreased to about 19 in the case of caspase-3 inhibitor-treated cells, while it decreased to about 20 in PARP inhibitor-treated cells (Figure 1 *e*). A similar decrease was observed in the case of propidium iodide-positive cells, indicating dead cell population which was 59% in case of wild-type SB-grown cells and 53% in case of cells grown in the presence of caspase-3 inhibitor and PARP inhibitor. The small decrease in PS externalization could be due to the fact that a fraction of cells died due to nutrient deprivation. In case of *B. megaterium*, within the untreated population, about 27% cells were annexin V-FITC-positive. Cells treated with caspase-3 and PARP inhibitors showed significant reduction in the annexin V-FITC-positive population (Figure S1, see Supplementary Material online). These findings thus are additional supportive data for establishing a link between sporulation and PCD in *Bacillus* cells.

Evaluation of DNA nicking in sporulating cells

To further validate this observation, the extent of DNA damage in sporulating cells was measured using TUNEL assay[28]. This is based on the filling of DNA nicks by the enzyme terminal deoxynucleotidyl transferase. In doing so the enzyme adds dUTPs tagged with a fluorophore, FITC. The proportion of TUNEL-positive cells during sporulation in *B. subtilis* was found to be about 54%, which reduced significantly to 27% in cells treated with caspase-3 inhibitor, and 43% in the cells treated with PARP inhibitor (Figure 1 *f*). Similar results were obtained in *Bacillus megaterium*, where 21% of the control cell population was TUNEL-positive. After treating with caspase and PARP inhibitors, this population reduced significantly to about 5% (see Supplementary material online, Figure S2). The data thus indicate that extensive nicking of DNA takes place in *Bacillus* cells undergoing sporulation due to involvement of PCD-like processes.

Survival studies of B. subtilis sporulation-deficient mutant strains

To further confirm the role of PCD in sporulation of *Bacillus* species, sporulation-deficient (SD) mutants were created using EMS mutagenesis. The role of extracellular proteases during sporulation in *B. subtilis* has already been studied by Dancer and Mandelstam[29]. Based on this fact we hypothesized that mutant cells lacking extracellular proteases would be sporulation-deficient. As caspase gene has not yet been genetically characterized in bacteria, an approach of random chemical mutagenesis using ethyl methane sulphonate (EMS) was undertaken and mutants were screened for loss of extracellular protease activity. Simple primary screening for acquisition of rifampicin resistance due to *RpoB* mutagenesis and loss of extracellular proteolytic activity were used as markers for selection of putative mutants. One hundred and sixty mutants were obtained from which 40 were screened for sporulation efficiency in sporulation broth. Among these, three mutants (M-4, M-80, M-25) were found to have poor sporulation efficiency as sporulation reduced by about 98–99% (Figure 2 *a*).

Caspase-3 activity in B. subtilis SD mutant strains

The status of caspase-3 activity in wild type and mutant *B. subtilis* strains was assessed using fluorogenic substrate of caspase-3, Ac–DEVD–AMC[2]. The caspase-3 activity was found to decrease by about 19% for both M-80 and M-25 mutant strains in comparison to wild-type cells. In case of mutant M-4, the activity decreased by 30% with respect to wild type cells (Figure 2 *b*).

Phosphatidylserine externalization in B. subtilis SD mutant strains

A significant decrease in PS externalization was observed in SD mutant cells compared to wild-type *B. subtilis* cells. About 20% of wild-type SB-grown cells were annexin V-positive. Percentage of annexin V-positive cells decreased to about 4 for M-4 mutant, 5 for M-25 mutant and 8 for M-80 mutant (Figure 2 *c*). Thus, in comparison to *B. subtilis* wild-type strain, a significant decrease in PS externalization was observed in case of SD mutant cells. Similarly, PI-positive cell population in wild-type cells was ~9%, while in the case of all the mutants, the PI-positive cell population decreased to about 2% for M-4 mutant, 3% for M-25 mutant and 5% for M-80 mutant (Figure 2 *c*). This indicates the reduction in the number of cells undergoing death and sporulation in SD mutants.

Wild-type B. subtilis cells display extracellular DNAse activity

There is a report of a single gene encoding caspase-activated DNAse in mammals and *Drosophila*. Nuc-1 nuclease has been reported to be involved in DNA degradation in *Caenorhabditis elegans*[30]. In *B. subtilis* the production and release of DNAse occurs as a part of the sporulation process[12]. An extracellular DNAse (nucB) whose transcription is regulated by the cell-specific sigma factor σ^E in *B. subtilis* has been reported earlier[13]. NucB is known to be involved in DNA degradation. The expression of nucB is sporulation-specific and dependent

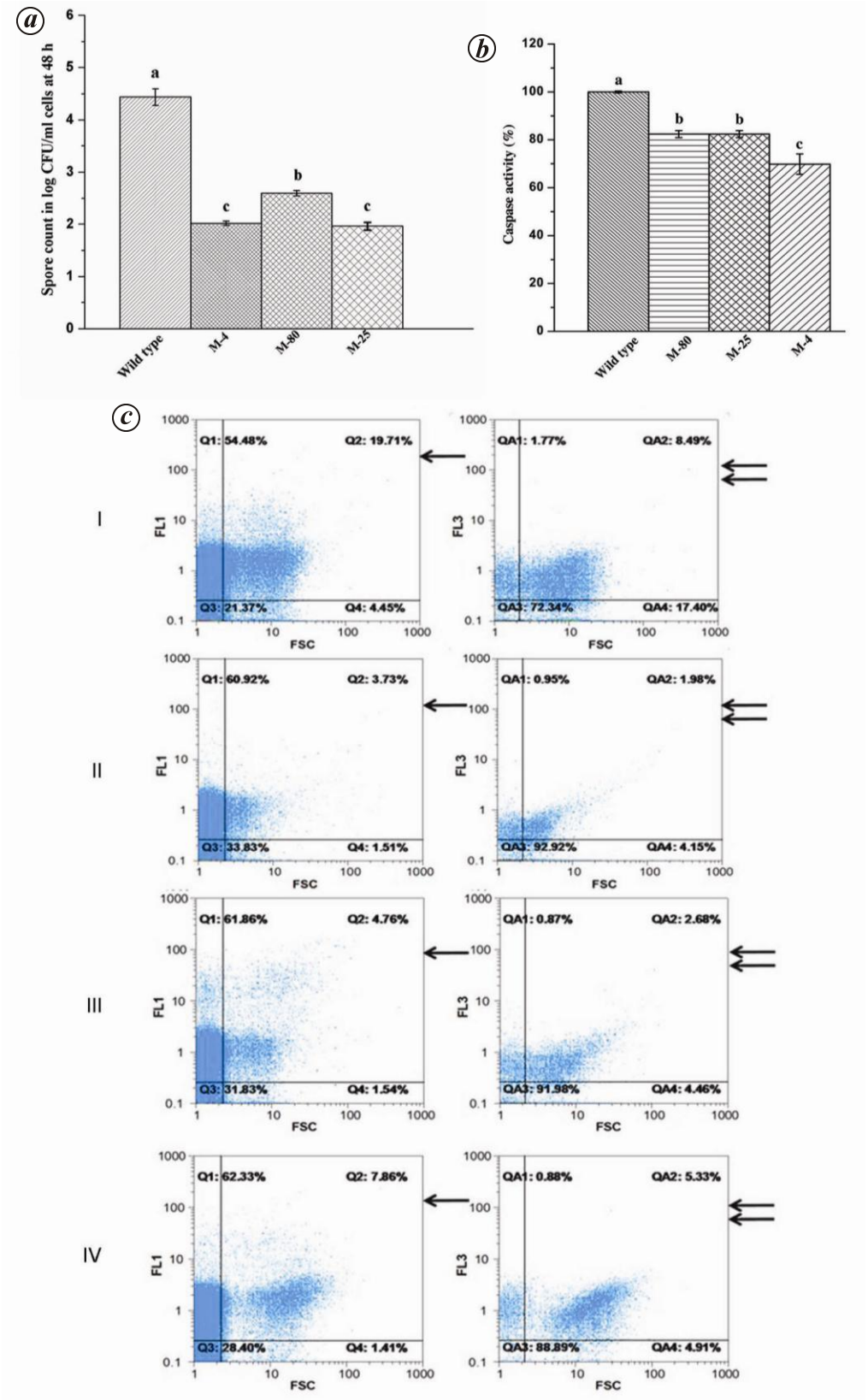

Figure 2. ***a***, Spore count of wild type and sporulation-deficient mutant strains of *B. subtilis* when grown in SB medium for 48 h. ***b***, Caspase-3 activity in wild type and sporulation-deficient mutant strains. [a–c]Different letters indicate significant difference ($P \leq 0.05$) between the mean values compared as analysed by independent sample Student's *t* test. ***c***, PS externalization in *B. subtilis* wild type and mutant strains undergoing sporulation in SB medium. I, Wild-type cells; II, M-4 cells; III, M-25 cells and IV, M-80 cells. Single arrow indicates the quadrant showing annexin V-FITC-labelled cells, while double arrow indicates the quadrant showing propidium iodide-positive cells.

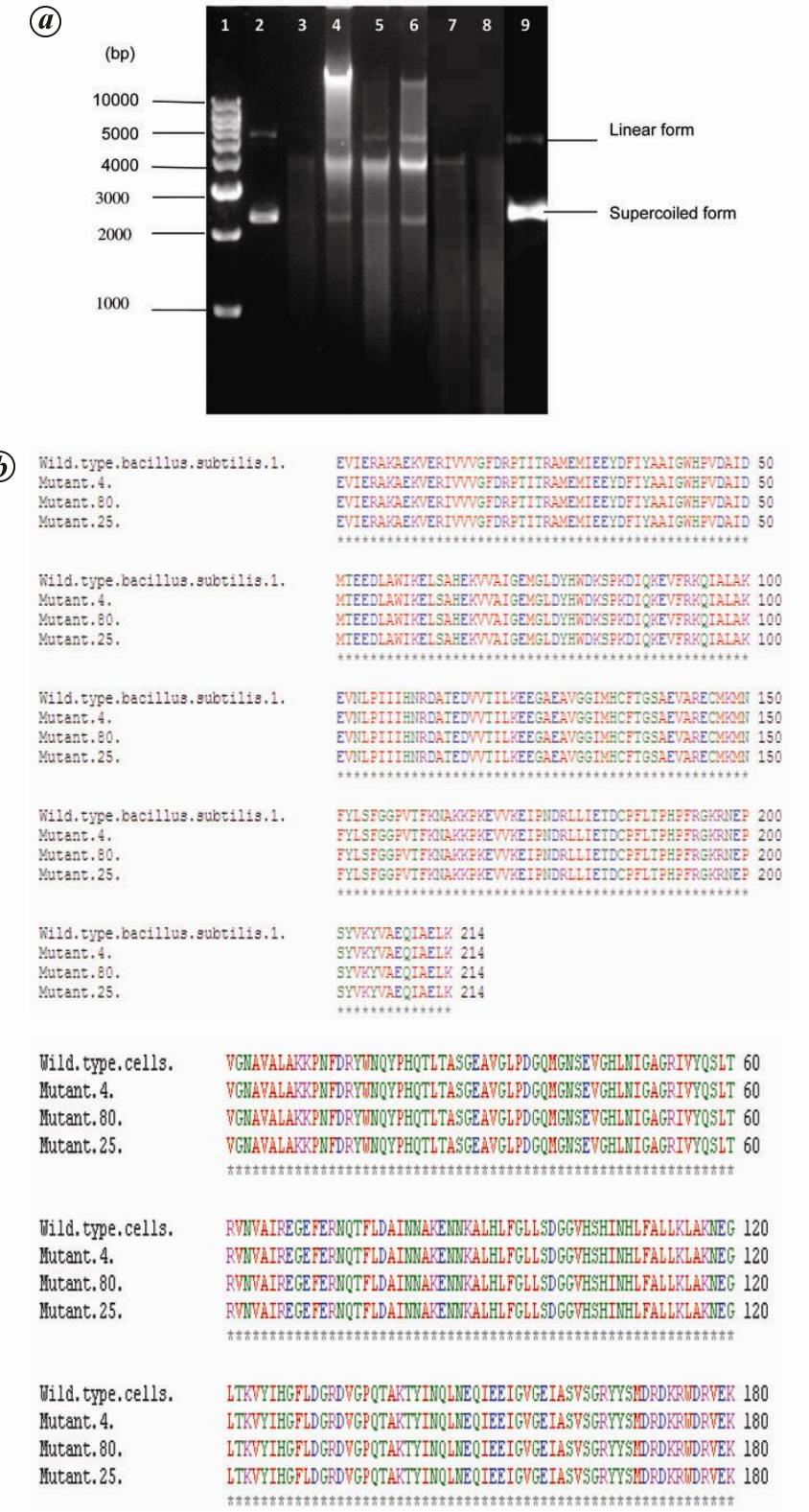

Figure 3. ***a***, DNAse activity as shown by the degradation pattern of pBR322 plasmid DNA (200 ng) by culture supernatant of 24 h, SB medium grown *B. subtilis*. Lane 1, 1 kb ladder; lane 2, pBR322; lane 3, pBR322 treated with culture supernatant of wild-type *B. subtilis* cells; lane 4, pBR322 treated with culture supernatant of M-80; lane 5, pBR322 treated with culture supernatant of M-4; lane 6, pBR322 treated with culture supernatant of M-25; Lane 7, pBR322 DNA treated with culture supernatant of wild-type cells grown in the presence of caspase-3 inhibitor; lane 8, pBR322 treated with culture supernatant of wild-type cells grown in the presence of PARP inhibitor and lane 9, Inhibition of pBR322 digestion by culture supernatant of wild-type *B. subtilis* cells in the presence of 0.05 M EDTA. ***b***, Alignment of the amino acid sequences of DNAse enzyme from mutant and wild type *B. subtilis* cells. Asterisks indicate identical amino acid residues. ***c***, Alignment of the amino acid sequences of phosphoglyceromutase enzyme from mutant and wild-type *B. subtilis* cells. Asterisks indicate identical amino acid residues.

on the gene products of Spo0A and SpoIIG in *Bacillus* species, which constitutes an operon responsible for the synthesis of the mother cell-specific sigma factor σ^E. It has been proposed to be involved in DNA degradation after mother cell lysis. The degradation of chromosomal DNA is a well-conserved apoptotic process. To examine the existence and role of any such DNAse during the present study, the culture supernatant of wild-type and PCD sporulation-deficient mutants was examined using pBR322 plasmid DNA as a model system. Cell-free supernatant of wild-type strain completely degraded the plasmid DNA pBR322. However, with culture supernatant of all the *B. subtilis* SD (M-4, M-25 and M-80) mutants, about 55% of the supercoiled form of plasmid DNA was found to be protected due to decreased sporulation in mutants leading to reduced mother cell lysis and release of DNAse enzyme. However, in the presence of caspase-3 inhibitor (CI) about 10% nicked form of plasmid DNA was protected, while in the case of PARP inhibitor (PI) the plasmid DNA was observed as smear (Figure 3 *a*). In case of wild-type *B. subtilis*, DNAse was released during sporulation. However, DNAse activity was drastically reduced in mutants. Reduced activity could be attributed to either getting regulated at the expression level or at post-translational activation level involving caspase-3 activity. This was further confirmed by alignment of the amino acid sequences of DNAse enzyme from wild type and mutant strains, which showed complete identity (Figure 3 *b*). Thus, it can be concluded that the activation of this DNAse is due to reduced caspase activity with no mutation in the DNAse gene.

Inhibition of DNAse activity

When the culture supernatant of wild-type *Bacillus* cells was added along with 0.05 M EDTA to pBR322 DNA, there was about 40% and 90% protection of linear form and supercoiled form of plasmid DNA respectively (Figure 3 *a*). Mn^{2+}-stimulated DNAse has been reported to be associated with sporulation in *B. subtilis*[12]. It indicates that Mn^{2+}, which is an essential cofactor for activation of DNAse enzyme, is inactivated by EDTA.

Mn^{2+} requirement for activation of phosphoglyceromutase

One more enzyme, phosphoglyceromutase, is important during sporulation in *B. subtilis*. This enzyme converts 3-phosphoglyceric acid to 2-phosphoglyceric acid and vice versa. Mn^{2+} is required for sporulation mainly to activate this enzyme, thereby preventing accumulation of the inhibitory 3-phosphoglyceric acid (3-PGA)[19]. Dormant spores contain a large depot of 3-PGA. The 3-PGA depot is stable in the forespore and dormant in the spore. However, during germination of the spore, 3PGA is utilized rapidly for generation of ATP. Hence if phosphoglyceromutase enzyme undergoes a mutation, 3-PGA will accumulate inside the cells and normal sporulation will be suppressed[31]. SB medium supported higher sporulation in comparison to LB medium, since SB contained Mn^{2+} ion which influenced spore composition, structure and germination. These two media mainly differ in Mn^{2+}, which is an essential cofactor for enzyme phosphoglycerate phosphomutase (E.C.5.4.2.1; PGA-mutase)[19]. To evaluate whether the phosphoglyceromutase gene is mutated, gene sequence of this enzyme from all the three mutants along with wild-type *Bacillus* cells was PCR-amplified and sequenced. Alignment of the amino acid sequences of phosphoglyceromutase enzyme from wild type and mutant strains showed complete homology (Figure 3 *c*). Thus it can be inferred that these mutants were not defective in the enzyme phosphoglyceromutase.

Thus, the present data reveal involvement of caspase-3-like enzyme during sporulation in *Bacillus* species. The data are supported by the experimental evidence showing reduction in sporulation in cells treated with cell-permeable caspase-3 as well as PARP inhibitors. The inhibitor-treated cells showed reduced PS externalization as well as DNA nicking, further confirming the role of PCD during sporulation. The hypothesis was further confirmed by generating sporulation-deficient mutants of *B. subtilis*. These mutants showed reduced caspase-3 enzyme activity as well as reduced PS externalization. The mutants showed significantly reduced DNAse activity but apparently no mutation in the DNAse gene, which also lacked PCD phenotype. To the best of our knowledge, there is no prior evidence of a direct link of sporulation in *Bacillus* with PCD. However, the association of these features of PCD in *Bacillus* and the regulation of mother cell lysis need to be further elucidated.

Conclusion

The present study provides evidence of involvement of PCD-specific markers, viz. caspase-3 and PARP activities in mother cell lysis during sporulation in *Bacillus* species. The process also displayed PS externalization and DNA nicking. The study complements the earlier reports where genetic evidence has indicated the occurrence of PCD during sporulation in *Bacillus* species. The results are further supported by creating the sporulation-deficient mutants of *Bacillus* which also lacked PCD phenotype. These mutants showed reduction in the sporulation efficiency as well as reduced caspase-3-like activity and PS externalization. SD mutants were also found to be lacking in extracellular metal-dependent DNAse activity reported to be involved in sporulation of *Bacillus* species. These findings thus provide direct evidence linking sporulation and caspase-dependent PCD process in *Bacillus* species.

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