Reinforcement of Bacillus subtilis spores by cross-linking of outer coat proteins during maturation

Abhyankar, W.; Pandey, R.; ter Beek, A.; Brul, S.; de Koning, L.J.; de Koster, C.G.

Citation for published version (APA):

Abhyankar, W., Pandey, R., ter Beek, A., Brul, S., de Koning, L. J., & de Koster, C. G. (2015). Reinforcement of Bacillus subtilis spores by cross-linking of outer coat proteins during maturation. *Food Microbiology*, *45*(Part A), 54-62. https://doi.org/10.1016/j.fm.2014.03.007

Reinforcement of *Bacillus subtilis* spores by cross-linking of outer coat proteins during maturation

Wishwas Abhyankar [a,b], Rachna Pandey [b], Alexander Ter Beek [b], Stanley Brul [b], Leo J. de Koning [a], Chris G. de Koster [a,*]

[a] *Mass Spectrometry of Biomacromolecules, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands*
[b] *Molecular Biology and Microbial Food Safety, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands*

A R T I C L E   I N F O

A B S T R A C T

Resistance characteristics of bacterial endospores towards various environmental stresses such as chemicals and heat are in part attributed to their coat proteins. Heat resistance is developed in a late stage of sporulation and during maturation of released spores. Using our gel-free proteomic approach and LC-FT-ICR-MS/MS analysis we have monitored the efficiency of the tryptic digestion of proteins in the coat during spore maturation over a period of eight days, using metabolically ^{15}N labeled mature spores as reference. The results showed that during spore maturation the loss of digestion efficiency of outer coat and crust proteins synchronized with the increase in heat resistance. This implicates that spore maturation involves chemical cross-linking of outer coat and crust layer proteins leaving the inner coat layer proteins unmodified. It appears that digestion efficiencies of spore surface proteins can be linked to their location within the coat and crust layers. We also attempted to study a possible link between spore maturation and the observed heterogeneity in spore germination.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Bacterial endospores are dormant, multilayered and highly resistant cellular structures formed in response to stress by certain Gram-positive bacteria belonging to the genera *Bacillus*, *Clostridium* and other related organisms. On return of more favorable conditions and in presence of nutrients spores germinate and grow out as normal vegetative cells via the process of germination and outgrowth (Henriques and Moran, 2007; Setlow, 2007). Two properties that make spores unique and incomparable are their resistance characteristics and the heterogeneity amongst them especially with regards to their germination.

Spore inactivation can be effectively achieved by wet-heat treatment but spores are generally resistant to high temperatures when compared to vegetative cells. Basal heat resistance of spores has been attributed to small acid soluble proteins (SASPs) protecting the spore DNA, the structure of the cortex, the Ca^{2+}-DPA levels of the core as well as spore core water content (Nicholson et al., 2000; Setlow, 2006, 2007). In a study by Sanchez-Salas et al. (2011), it was concluded that after being released from the

mother cells, a further maturation of spores, is necessary for the acquisition of full thermal resistance. In the same study it was suggested that changes in coat structure especially with regards to inter-protein cross-linking may be one of the factors that determine the generation of full thermal resistance in maturing spores. Importantly, it has been shown that there is a significant heterogeneity in the heat resistance of spores of a genetically homogeneous population (Xu et al., 2009). Since certain species of spore forming *Bacilli* and *Clostridia* are pathogenic, toxigenic as well as potential food spoilers the detailed study of spore resistance mechanisms is of high relevance.

It is reported that ∼30% of the protein fraction from the spore coat is characterized by extensive inter-protein cross-linking (Henriques and Moran, 2007). Three types of cross-links, namely, the dityrosine links, the ε-(γ)-glutamyl-lysine isopeptide linkages and the disulfide bonds — are predicted to render this fraction insoluble (Henriques and Moran, 2007). Also tyrosine and cysteine rich proteins as well as the transglutaminase enzyme Tgl have been identified from the coat thus providing a strong evidence for such chemical cross-linking occurring in the spore coat. Although the spore coat protein composition in the released spores is presumably constant, there can be differences in the level of cross-linking amongst the proteins. We hypothesized that the higher the level of protein cross-linking is, the higher the thermal stress resistance is of spores. As a consequence,

---

* Corresponding author. Room C2.263, Science Park 904, 1098 XH Amsterdam, The Netherlands. Tel.: +31 (0)20 525 5457.
E-mail address: C.G.deKoster@uva.nl (C.G. de Koster).

from a proteomics point of view, the higher the coat proteins are cross-linked the lower would be the efficiency with which they are digested by a protease. To investigate this hypothesis, we used a batch fermenter setup that allowed for the growth and sporulation of *Bacillus subtilis*. Using ^{15}N-labeled eight-day mature spores as a reference, we monitored the loss of digestion efficiency of spore coat proteins in spores prepared in ^{14}N-labeled medium. For the first time, the effect of spore maturation on the spore coat protein digestion was monitored in *B. subtilis* and was seen to be coupled to protein cross-linking indicated by the loss of digestion efficiency (the tryptic peptide ^{14}N/^{15}N isotopic ratio) of certain proteins and increased heat-resistance of spores. Compared to the matured spores, the younger spores showed enhanced protein digestion efficiency from the inner to the outer coat to the crust proteins reflecting the extent of cross-linking in these layers. This study, for the first time, identified the spore coat proteins that could prove critical in the spore maturation process. Finally, with spore live-imaging method (Pandey et al., 2013), we monitored the germination time (speed) of young and mature spores to map a possible linkage between spore coat cross-linking and germination speed in spores.

2. Materials and methods

2.1. Bacterial strain and sporulation conditions

B. subtilis wild-type strain PY79 was obtained from Dr. Eichenberger's lab (New York University, USA) and was used for preparing ^{14}N (Light) and ^{15}N (Heavy)-labeled spores. Bacteria were pre-cultured and sporulated as described previously (Abhyankar et al., 2011). For sporulation a defined minimal medium, buffered with 3-(N-morpholino)propane sulfonic acid (MOPS) to pH 7.4, was used (Kort et al., 2005). The in-house bench fermenter setup consisted of four autoclavable 0.5 l glass bioreactors, equipped with Tamson T1000 waterbath (Gemini BV, Apeldoorn, The Netherlands). Each bioreactor contained a double-layered glass jacket through which water was continuously flown to maintain the growth temperature at 37 °C. Sterile air was continuously plunged through at a constant rate (0.5 L/hr. at 200 rpm). Exponentially growing *B. subtilis* cells from the same pre-culture (20 ml, OD$_{600}$ 0.4) were inoculated into all the bioreactors and allowed to sporulate and mature to a maximum of eight days post inoculation. The query cultures were grown and sporulated in presence of ^{14}NH$_4$Cl while the reference cultures in ^{15}NH$_4$Cl as the sole nitrogen source. The final stock of reference spores consisted of spores pooled from three independent biological replicates while three independent biological replicates were separately analyzed for the query culture.

2.2. Spore harvesting

The ^{14}N-query spores were harvested as described elsewhere (Abhyankar et al., 2011) on day 2, day 4, day 6 and day 8 post-inoculation whereas the ^{15}N-labeled reference spores were only harvested on day 8 post inoculation. For two-day old spores the harvesting procedure also involved a final step of Histodenz gradient (Sigma Chemical Co., St. Louis, MO) centrifugation to get rid of the non-sporulated cells. For these spores the washed and harvested spore pellet fraction was suspended in a small volume of 20% Histodenz and then layered on 50% Histodenz medium. The tubes were centrifuged at 15 °C for 45 min at 15,000 × *g*. By this procedure the free spores were pelleted down which were used for further work.

2.3. Measurements of thermal resistance

Thermal resistance of spores to wet heat was assessed using the previously used screw-cap tube method (Kort et al., 2005). In short,

a 1 ml (heat-activated (70 °C, 30 min); OD$_{600}$ ∼2.0) spore suspension in sterile MQ water, for each time point, was injected with a syringe into a preheated metal screw-cap tube containing 9.0 ml of sterile MQ water. Any remaining vegetative cells in the sample were inactivated by the heat activation step. Next a mild thermal stress was applied by heating the tube through a 10 min complete immersion in a glycerol bath pre-warmed to 85 °C. After 10 min the tube was transferred to ice-water. Dilution series of spore suspension were prepared in sterile MQ water and 100 µl of sample was spread on Tryptic Soy agar plates. The number of colonies was counted after 24 h of incubation at 37 °C. As a control a same dilution series for non-heat stressed spores ware plated. In this way the % of mild heat stress survival was determined for each of the three spore samples at different stage of maturity. The significance of the data was assessed by a one-way ANOVA test.

2.4. Mixing of ^{14}N and ^{15}N-labeled spores, spore coat isolation and protein extraction

The harvested ^{14}N-spores were immediately mixed in 1:1 ratio with ^{15}N-reference spores based on OD$_{600}$. After mixing the samples were further subjected to spore coat isolation & protein extraction as described previously (Abhyankar et al., 2013, 2011). The isolated coat material was freeze-dried overnight and immediately used for mass spectrometric analysis.

2.5. Sample preparation for MS analysis

The freeze dried samples were reduced with 10 mM dithiothreitol in 100 mM NH$_4$HCO$_3$ (1 h at 55 °C) followed by a reaction with 55 mM iodoacetamide in 100 mM NH$_4$HCO$_3$ for 45 min at room temperature in the dark. The samples were directly digested for 18 h at 37 °C with trypsin (Trypsin gold Promega, Madison, WI) using a 1:60 (w/w) protease: protein ratio. The tryptic digests were desalted using Omix µC18 pipette tips (80 µg capacity, Varian, Palo Alto, CA) according to the manufacturer's instructions. The copy number of proteins does not change after the on-set of maturation of released spores at *t* is 0 h. Differences in protein ^{14}N/^{15}N isotopic ratio in course of maturation time are the result of changes of accessibility of spore protein lysine and arginine residues to the active site of the protease trypsin. The ^{14}N/^{15}N isotopic ratio is a measure of the spore protein digestion efficiency.

2.6. LC-FT-ICR MS/MS analysis

LC-MS/MS data were acquired with a Bruker ApexUltra Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 7 T magnet and a nano-electrospray Apollo II DualSource™ coupled to an Ultimate 3000 (Dionex, Sunnyvale, CA, USA) HPLC system. Samples containing up to 60 ng of the tryptic peptide mixtures were injected as a 10 µl 0.1% TFA, 3% ACN aqueous solution and loaded onto a PepMap100C18 (5-µm particle size, 100-Å pore size, 300-µm inner diameter × 5 mm length) precolumn. Following injection, the peptides were eluted via an Acclaim PepMap 100 C18 (3-µm particle size, 100-Å pore size, 75-µm inner diameter × 250 mm length) analytical column (Thermo Scientific, Etten-Leur, The Netherlands) to the nano-electrospray source. Gradient profiles of up to 120 min were used from 0.1% formic acid/3% CH$_3$CN/97% H$_2$O to 0.1% formic acid/50% CH$_3$CN/50% H$_2$O at a flow rate of 300 nL/min. Data dependent Q-selected peptide ions were fragmented in the hexapole collision cell at an Argon pressure of 6 × 10^{-6} mbar (measured at the ion gauge) and the fragment ions were detected in the ICR cell at a resolution of up to 60,000. In the MS/MS duty cycle, 3 different precursor peptide ions were selected from each survey

Table 1
^{14}N/^{15}N isotopic ratios of identified spore coat and crust proteins from *B. subtilis* PY79.

	Protein	Description	Mass (Da)	^{14}N/^{15}N ratios[a]			
				Day 2	Day 4	Day 6	Day 8
	CotZ	**Spore coat protein Z**	**17,093**	**1.42**	**0.86**	**0.79**	**0.69**
Crust	**CotY**	**Spore coat protein Y**	**18,728**	**1.35**	**0.85**	**0.83**	**0.65**
	CgeA	Protein CgeA	14,149	1.19	1.07	1.28	1.12
	CotG	**Spore coat protein G**	**24,399**	**1.98**	**0.75**	**0.64**	**0.34**
	YurS	**Uncharacterized protein**	**10,483**	**1.92**	**1.05**	**0.86**	**0.51**
	CotI	Spore coat protein I	41,447	1.53	1.10	1.06	0.98
	CotU	**Uncharacterized protein**	**11,612**	**1.46**	**0.96**	**0.67**	**0.31**
	CotW	Spore coat protein W	12,486	1.44	1.13	1.02	0.89
	CotA	Spore coat protein A	58,690	1.30	1.07	1.00	0.90
	CotSA	Spore coat protein SA	43,056	1.30	0.99	0.98	0.86
	CwlJ	Cell wall hydrolase	16,680	1.26	1.01	1.13	1.08
Outer	**CotB**	**Spore coat protein B**	**42,946**	**1.23**	**0.81**	**0.82**	**0.56**
Coat	CotS	Spore coat protein S	41,286	1.22	0.99	0.94	0.74
	CotE	**Spore coat protein E**	**21,078**	**1.20**	**0.83**	**0.78**	**0.67**
	CatX	Catalase X	62,383	1.15	1.26	0.79	1.05
	CotR	Putative sporulation hydrolase	35,335	1.15	0.99	1.03	0.90
	CotX	Spore coat protein X	18,989	1.15	0.93	0.92	0.89
	SleB	Spore cortex-lytic enzyme	34,569	1.08	1.02	1.00	1.11
	CotC	**Spore coat protein C**	**8868**	**0.99**	**0.81**	**0.68**	**0.37**
	CotQ	Uncharacterized FAD-linked oxidoreductase	50,167	0.91	0.81	0.78	0.59
	Gpr	Germination protease	40,315	1.45	1.00	1.14	0.92
	SafA	SpoIVD-associated factor A	43,429	1.37	1.21	1.09	1.11
	YxeE	Uncharacterized protein	14,705	1.34	1.37	1.08	1.08
	Tpx	Probable thiol peroxidase	18,318	1.32	1.12	1.14	1.05
	YtfJ	Uncharacterized spore protein	16,336	1.20	0.99	0.99	1.04
	LipC	Spore germination lipase	24,038	1.19	1.08	0.97	0.85
	Tgl	Protein-glutamine gamma-glutamyl transferase	28,392	1.15	0.99	0.96	0.97
	DacF	D-alanyl-D-alanine carboxypeptidase	43,327	1.03	1.05	0.99	1.00
	SodM	Superoxide dismutase [Mn]	22,476	1.03	1.01	0.99	0.99
Inner	OxdD	Oxalate decarboxylase	44,113	1.01	1.01	1.07	0.92
Coat	YaaH	Spore germination protein	48,607	1.00	1.00	1.00	1.00
	CotF	Spore coat protein F	18,714	0.97	0.98	1.00	0.94
	CotJB	Protein CotJB	10,214	0.97	0.99	1.17	0.97
	CotJC	Protein CotJC	21,993	0.95	1.05	1.07	1.01
	SodF	Probable superoxide dismutase [Fe]	33,513	0.92	0.93	1.03	1.01
	YdhD	Putative sporulation-specific glycylase	47,411	0.92	0.92	0.87	0.95
	CotH	Inner spore coat protein H	42,843	0.90	0.94	0.99	0.69
	CotJA	Protein CotJA	9790	0.89	1.06	1.03	0.98
	GerQ	Spore coat protein	20,263	0.84	0.99	1.06	1.02
	SpoIVA	Stage IV sporulation protein A	55,197	0.83	1.09	1.08	1.06
Other	YqfX	Uncharacterized protein	13,893	1.29	1.16	1.05	1.07
Coat	YabP	Spore protein	11,396	1.23	1.19	1.23	1.13
Proteins	YpeB	Sporulation protein	51,210	1.22	1.12	1.13	1.09
	YhcM	Uncharacterized protein	17,020	1.19	1.18	0.92	1.01
	YkuD	Putative L,D-transpeptidase	17,910	1.16	1.02	1.28	1.23
	YrkC	Uncharacterized protein	21,299	1.14	1.02	1.38	0.86
	YisY	AB hydrolase superfamily protein	30,540	1.09	1.04	1.08	1.00
Other	YhcQ	Spore coat protein F-like protein	25,246	1.08	1.07	0.98	0.96
Coat	YckD	Uncharacterized protein	12,782	1.05	1.01	1.08	1.15
Proteins	YodI	Uncharacterized protein YodI	9245	1.02	0.88	0.82	0.78
	YhbB	Uncharacterized protein	36,095	0.94	1.03	1.05	1.10
	YhxC	Uncharacterized oxidoreductase	31,307	0.90	0.99	0.85	0.79
	YjqC	Uncharacterized protein	31,403	0.87	0.87	0.80	0.79
	YdcC	Sporulation protein	38,170	0.76	1.01	1.01	1.02
	YrbF	UPF0092 membrane protein	9897	1.63	1.21	1.13	1.20
	YyxA	Uncharacterized serine protease	42,762	1.44	1.20	1.55	2.35
	OppA	Oligopeptide-binding protein	61,543	1.36	1.38	1.89	1.42
	YkzQ	Uncharacterized protein	8889	1.35	1.19	1.77	0.81
	YaaQ	Uncharacterized protein	11,959	1.31	1.36	1.42	1.28
	YmfF	Probable inactive metalloprotease	48,794	1.28	0.88	0.81	0.86
	YkuS	UPF0180 protein	8716	1.27	1.19	1.04	0.89
	YuzA	Uncharacterized membrane protein	8518	1.25	1.05	1.01	1.00
	YkuU	Thioredoxin-like protein	20,787	1.19	1.06	1.12	1.18
	YrzQ	Uncharacterized protein	5039	1.19	1.06	1.06	1.06
	YkuJ	Uncharacterized protein	9296	1.17	0.97	0.91	1.58
	YhcN	Lipoprotein	21,061	1.16	1.12	0.98	0.98
Putative	YfkD	Uncharacterized protein	29,929	1.16	0.95	1.13	1.07
Spore	YfkO	Putative NAD(P)H nitroreductase	25,669	1.16	1.03	1.08	0.92
Coat	YhfW	Putative Rieske 2Fe−2S iron−sulfur protein	58,264	1.15	1.05	1.16	0.97
Proteins	YsdC	Putative amino peptidase	39,249	1.13	1.00	1.01	0.98
	YmfH	Uncharacterized zinc protease	49,089	1.09	1.06	1.08	1.04
	YwfI	UPF0447 protein	29,886	1.08	1.04	1.36	1.23
	YqfA	UPF0365 protein	35,676	1.07	0.95	0.89	0.93

Table 1 (*continued*)

	Protein	Description	Mass (Da)	^{14}N/^{15}N ratios[a]			
				Day 2	Day 4	Day 6	Day 8
	YmxG	Uncharacterized zinc protease	46,137	1.05	1.11	1.13	0.78
	YqgO	Uncharacterized protein	6907	1.05	0.94	0.98	0.90
	YjdH	Uncharacterized protein	15,249	1.04	0.93	1.28	1.00
	YurZ	Uncharacterized protein	13,898	1.00	1.15	0.79	1.00
	AtcL	Calcium-transporting ATPase	98,677	0.99	1.05	1.05	0.97
	YgaK	Uncharacterized FAD-linked oxidoreductase	51,661	0.98	0.97	1.03	0.72
	YqiG	Probable NADH-dependent flavin oxidoreductase	40,894	0.97	1.13	1.60	1.01
	DacB	D-alanyl-D-alanine carboxypeptidase	43,561	0.93	1.07	1.07	1.21
	YhcB	Uncharacterized protein	19,400	0.93	1.02	1.53	1.11
	YxeD	Uncharacterized protein	13,793	0.81	0.80	0.77	0.88
	Fer	Ferredoxin	9092	0.58	0.51	0.62	0.33
Core[b]	SspA	Small, acid-soluble spore protein A	7066	1.21	1.20	0.98	1.15
Proteins	SspB	Small, acid-soluble spore protein B	6975	0.78	0.75	0.61	0.71

[a] Normalized ratios of all the identified coat proteins in one biological replicate are tabulated. The coat and crust proteins speculated to take part in spore maturation are highlighted in bold. The results for replicate 2 and 3 can be found in the Supplementary data.

[b] Core proteins SspA and SspB are not involved in maturation and serve as additional internal control.

MS. The MS/MS duty cycle time for 1 survey MS and 3 MS/MS acquisitions was about 2 s. Instrument mass calibration was better than 1 ppm over a m/z range of 250–1500. Raw FT-MS/MS data were processed with the MASCOT DISTILLER program, version 2.4.3.1 (64 bits), MDRO 2.4.3.0 (MATRIX science, London, UK), including the Search toolbox and the Quantification toolbox. Peak-picking for both MS and MS/MS spectra were optimized for the mass resolution of up to 60,000. Peaks were fitted to a simulated isotope distribution with a correlation threshold of 0.7, with minimum signal to noise of 2. The processed data, from the three independent biological replicates, were searched with the MASCOT server program 2.3.02 (MATRIX science, London, UK) against a complete *B. subtilis* 168 ORF translation database (Uniprot 2011 update, downloaded from http://www.uniprot.org/uniprot). The parameters for Quantification using ^{15}N-Metabolic labeling were used. Trypsin was used as enzyme and 2 missed cleavages were allowed. Carbamidomethylation of cysteine was used as a fixed modification and oxidation of methionine as a variable modification. The peptide mass tolerance was set to 30 ppm and the peptide fragment mass tolerance was set to 0.03 Da.

Using the quantification toolbox, the isotopic ratios for all identified proteins were determined as an average of the isotopic ratios of the corresponding light over heavy tryptic peptides. Selected critical settings were: require bold red: on, significance threshold: 0.05: Protocol type: precursor; Correction: Element ^{15}N; Value 99.4; Report ratio L/H; Integration method: Simpson's integration method; Integration source: survey; Allow elution time shift: on; Elution time delta: 20 s; Std. Err. Threshold: 0.15, Correlation Threshold (Isotopic distribution fit): 0.98; XIC threshold: 0.1; All charge states: on; Max XIC width: 200 s; Threshold type: at least homology; Peptide threshold value: 0.05; unique pepseq: on.

2.7. Data normalization

In order to correct for the possible errors in the 1:1 mixing of the ^{14}N cultures with the ^{15}N reference cultures the protein isotopic ratios were normalized for the data set of each time point by setting the ratio for a normalization protein to 1. The protein for normalization was chosen to be YaaH. This protein is localized in the inner spore coat and it is one of the hydrolases important during germination. The expression levels of YaaH, during sporulation, have been found to be constant in a previous study (Nicolas et al., 2012). Since, germination requires complete hydrolysis of cortex peptidoglycan (Setlow, 2003) the likelihood of YaaH to be involved in inter-protein cross-linking is therefore minimal. Also the relative

peptide ratios for the protein were found to be stable over the duration of 8 days in our work. The identified proteins and their respective isotopic ratios over the period of eight days are mentioned in Table 1.

2.8. Slide preparation for time-lapse microscopy and data analysis

A special microscope slide with a closed air containing chamber developed by Pandey and co-workers was used for phase contrast image acquisition (Pandey et al., 2013). The slides were prepared as described by the authors. 1 μl of spore solution was loaded on a thin agarose pad made by using two siliconized (24 × 32 mm) cover slips. The agarose-medium pad was placed in an upright position on the Gene Frame® and pressure was applied for complete sealing. This chamber was used for time-lapse microscopy. Time-lapse series were made, using a temperature-controlled boxed incubation system for live imaging set at 37 °C and observing the specimens with a 100X/1.3 plane Apochromatic objective (Axiovert-200 Zeiss, Jena, Germany). Phase-contrast time-lapse series were recorded at a sample frequency of 1 frame per min for 5 h for control and 10 h for heat-treated (85 °C for 10 min spores). In each field of view, on average 8 spores were identified and followed in time. Maximally 9 areas (fields of view) were recorded in parallel. This resulted in the analysis of approximately 70 spores from the start of each imaging experiment. One biological replicate for control and stress condition was performed. Spore germination times (speed with which phase bright spores turn phase-dark) were analyzed using a semi-automatic image analysis macro called Spore Tracker (Pandey et al., 2013), a plugin for ObjectJ (http://simon.bio.uva.nl/objectj), which runs under ImageJ (http://rsb.info.nih.gov/ij). The germination time (speed) was marked by Spore Tracker (Pandey et al., 2013). Frequency distribution plots of the germination times of individual spores from day 2, day 4, day 6 and day 8 were generated. Differences in the variance between different sample day and treatment were tested with one-way ANOVA test and student's *T*-test was performed to test differences in the averages.

3. Results

3.1. Protein digestion efficiency during spore maturation reflects the extent of protein cross-linking

Protein digestion efficiency during spore maturation was monitored for 89 proteins over a period of eight days, relative to metabolically ^{15}N labeled coat proteins of reference mature spores.

The numbers of the identified proteins were plotted as a function of their $^{14}N/^{15}N$ isotopic ratios for each time point (Fig. 1). After release of spores by lysis of the mother cell protein copies do not change anymore. The protein $^{14}N/^{15}N$ isotopic ratios are a measure for the protein digestion efficiency in course of maturation time. All the protein ratios in our datasets appeared to be normally distributed (Fig. 1). The distribution for the young (two-day old) spores showed a distinct group of proteins with isotopic ratios near to as well as > 1.0. The smaller groups (highlighted green and blue, Fig. 1(A)) were centered more towards the ratio values of ~1.5 and 2.0 whereas the larger group (highlighted red, Fig. 1(A)) was more centered around ratios of 1.0–1.2. The farthest cluster (cluster highlighted green, Fig. 1(A)) comprised of outer coat proteins CotG and YurS as well as outer coat and crust proteins such as CotB, CotC, CotU, CotY, and CotZ (cluster highlighted blue, Fig. 1(A)). The inner coat proteins such as CotF, CotJA, CotJC, CwlJ, SleB, YdhD, Tgl were identified in the larger cluster (clusters highlighted red and in gray band, Fig. 1(A)). As the maturation period increased, the distributions for relatively matured (four-day, six-day) and matured (eight-day) spores demonstrated a considerable shift towards the ratios of 1.0 and the two clusters (highlighted blue and red, Fig. 1(A)) dissolved to some extent giving a unimodal distribution for 4-day mature spores. For six-, eight- day matured spores, the above mentioned clusters reappeared however they were now centered on ratios lower than 1.0 or <0.3 (Fig. 1(A)). This maturation behavior has been reproduced for all the three replicates. For instance, the digestion efficiency (the $^{14}N/^{15}N$ isotopic ratios) for CotG decrease in course of time in all three replicates but the actual $^{14}N/^{15}N$ ratios are different. Thus it appears from the results shown in Supplementary Tables S1–S3 that the maturation timings

relative to the analytical reference (eight-day ^{15}N spores) may vary from one replicate to the other. The cytosolic proteins were also identified but they were not calculated for their $^{14}N/^{15}N$ ratios (Supplementary Table S4).

3.2. Young spores show enhanced protein digestion efficiency from inner to outer coat to crust proteins

The digestion efficiency appeared likely to depend on the cross-linking of the spore outer coat and crust proteins. Proteins CotG, YurS, CotU, CotI, CotZ, CotY, CotB and CotC were identified to become more protease resistant over time thereby playing a role in spore maturation. Proteins CotG, CotC, CotU have been identified to be cross-linked in the spore coat (Henriques et al., 1998; Isticato et al., 2004, 2008). Also CotX, CotY and CotZ are said to be a part of the insoluble cluster of spore coat proteins (Zhang et al., 1993). In our analyses it appeared that the outer coat proteins such as CotG, CotU and crust proteins such as CotY and CotZ are more efficiently digested in young (2-day) spores. Proteins CotG and CotU appeared to be more difficult to digest in 8-day mature spores whereas the inner coat and spore morphogenetic proteins SpoIVA and SafA appeared to be digested with similar efficiencies in query (^{14}N) and reference (^{15}N) spores over the period of eight days as the $^{14}N/^{15}N$ ratios stabilized around 1.0 in the mature spores (Fig. 2). Protein CotC, a CotU (YnzH) homologue, also seemed to be involved in the maturation behavior albeit to a lower extent as compared to CotU. The spore maturation protein CgeA, which is localized in the crust layer (Imamura et al., 2011), was identified and did not show any appreciable change in its digestion efficiency (Table 1). No other member of the CgeABCDE family was identified. The crust proteins
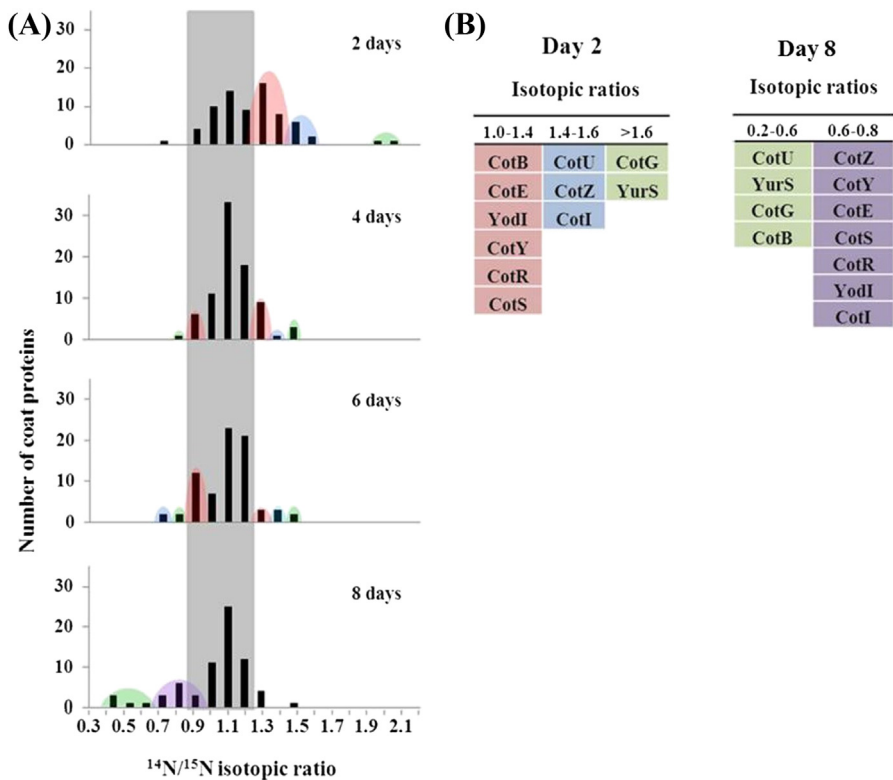

Fig. 1. Protein digestion efficiency as a function of $^{14}N:^{15}N$ ratios during spore maturation. (A) Three groups (discussed in Section 3.1) of identified proteins are indicated by red, blue and green highlighted regions. The $^{14}N/^{15}N$ isotopic ratios are a measure for protein digestion efficiencies. The proteins that showed uniform digestion efficiencies over an eight day period are shaded in gray bar. (B) The proteins, from the three groups (red, blue, green), observed for change in their digestion efficiencies when compared to day 2 and day 8 spores are shown. The color in each column represents the group of the protein in Fig. 1(A). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
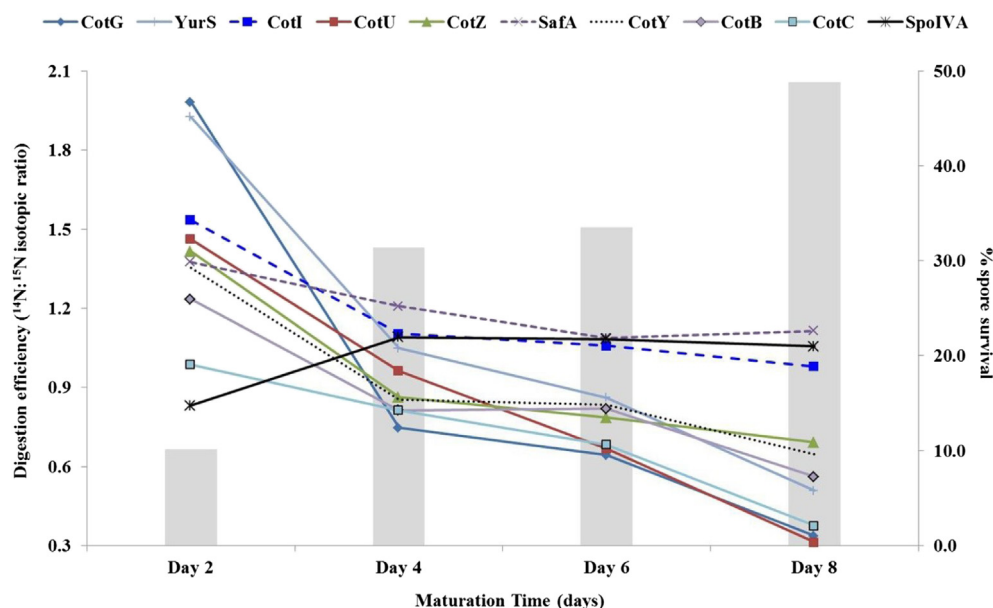
Fig. 2. Isotope ratios, averaged over the identified peptides, from the crust, outer coat and inner coat proteins against the % survival of thermally stressed young to old spores. The primary Y-axis shows the change in the digestion extent of proteins as a function of $^{14}N/^{15}N$ ratios (line plots) while the secondary Y-axis shows the increase in the thermal resistance of spores as estimated by % survival rate (gray bars) of thermally stressed spores on tryptic soy agar plate after incubation for 24 h at 37 °C. Compared to younger spores reduced digestion extent for crust and outer coat proteins and higher thermal resistance ($P < 0.05$) is seen in more mature spores. Proteins CotG, YurS, CotI, CotU, CotB, CotC are localized in the outer coat while SafA, SpoIVA are inner coat proteins. CotY and CotZ are located in the crust.

CotY and CotZ seemed to be affected by spore maturation in their digestion patterns. CotX however did not show an appreciable change in its digestion efficiency over the period of eight days. Manual inspection of identified peptides from individual proteins showed that the outer coat and the crust proteins carried a large variation in the digestion efficiencies of the peptides. As seen in Fig. 3, the coat protein YaaH and inner coat protein CotJC showed uniform digestion efficiencies for all the peptides used for quantification. Contradictorily for most of the peptides identified from the outer coat and crust proteins (represented in Fig. 3) the digestion efficiencies for each peptide varied to a great extent. In most cases, the peptides that showed the highest digestion efficiencies in the young spores were seen to be least efficiently digested in the older spores.

3.3. Spore maturation is coupled to protein cross-linking and spore thermal resistance

From the assessment of the viable counts of thermally stressed spores at 85 °C for 10 min it appeared that 8-day or mature spores had acquired more heat resistance when compared to 2-day or younger spores confirming the previous notion (Sanchez-Salas et al., 2011) (Fig. 3). In fact, the decrease in the protein digestion efficiency synchronized well with the increase in the thermal stress resistance of spores ($P < 0.05$).

3.4. Spore maturation and heterogeneity in spore germination times

Analysis by Spore Tracker live imaging tool showed that there was a slight, but significant ($P < 0.05$) delay in the germination times of 8-day mature spores when compared to 2-day old spores (Fig. 4). In the un-stressed condition, the young spore population appeared to be a mixed population where in most spores germinated fast while some germinated slower. On the contrary, the 8-day mature spore population was more homogeneous in their on

average delayed germination times. The thermally stressed young as well as mature spores showed a delay in germination times. For young spores the shift in germination times was larger but this shift was small for the un-stressed and stressed mature spores (Fig. 4, Supplementary Figures F1–F4).

4. Discussion

A recent study concluded that spore maturation, after the spore's release from the mother cell, is an important factor in acquiring wet-heat resistance in spores (Sanchez-Salas et al., 2011). On the same lines, our research has allowed us to identify a group of spore outer coat and crust layer proteins affected, most likely due to the extent of their cross-linking, during spore maturation. In our research, the ^{15}N-labeled 8-day mature spores were used as the reference to which the spores prepared in ^{14}N-labeled medium were compared. The query spores were harvested at different time points and mixed with the reference spores in 1:1 ratio based on the OD_{600} of samples. Since no new proteins are synthesized per se once the spore is released from the mother cell, the relative composition of spore coat protein in spores is constant. However, we show that the digestion efficiency id est $^{14}N/^{15}N$ ratios of proteins and peptides thereof display a characteristic distribution when 2-day, 4-day, 6-day and 8-day query (^{14}N) spores are compared to the 8-day mature reference (^{15}N) spores. Also, the proteins from 8-day query spores should exhibit the same digestion efficiencies ($^{14}N/^{15}N$ is 1) as in the 8-day reference spores. However, the lack of this similarity in ratios suggests that maturation in the query and reference spores may have progressed to different extents. Nevertheless, a detailed analysis of the peptides identified from the proteins facilitates the use of $^{14}N/^{15}N$ ratios as a measure of the extent to which these proteins are digested.

Our research clarifies that the inner spore coat proteins are not affected during spore maturation. As seen in Table 1, the $^{14}N/^{15}N$ ratios for most of the inner coat proteins, averaged over all the identified peptides used for quantification are close to 1.0. Manual

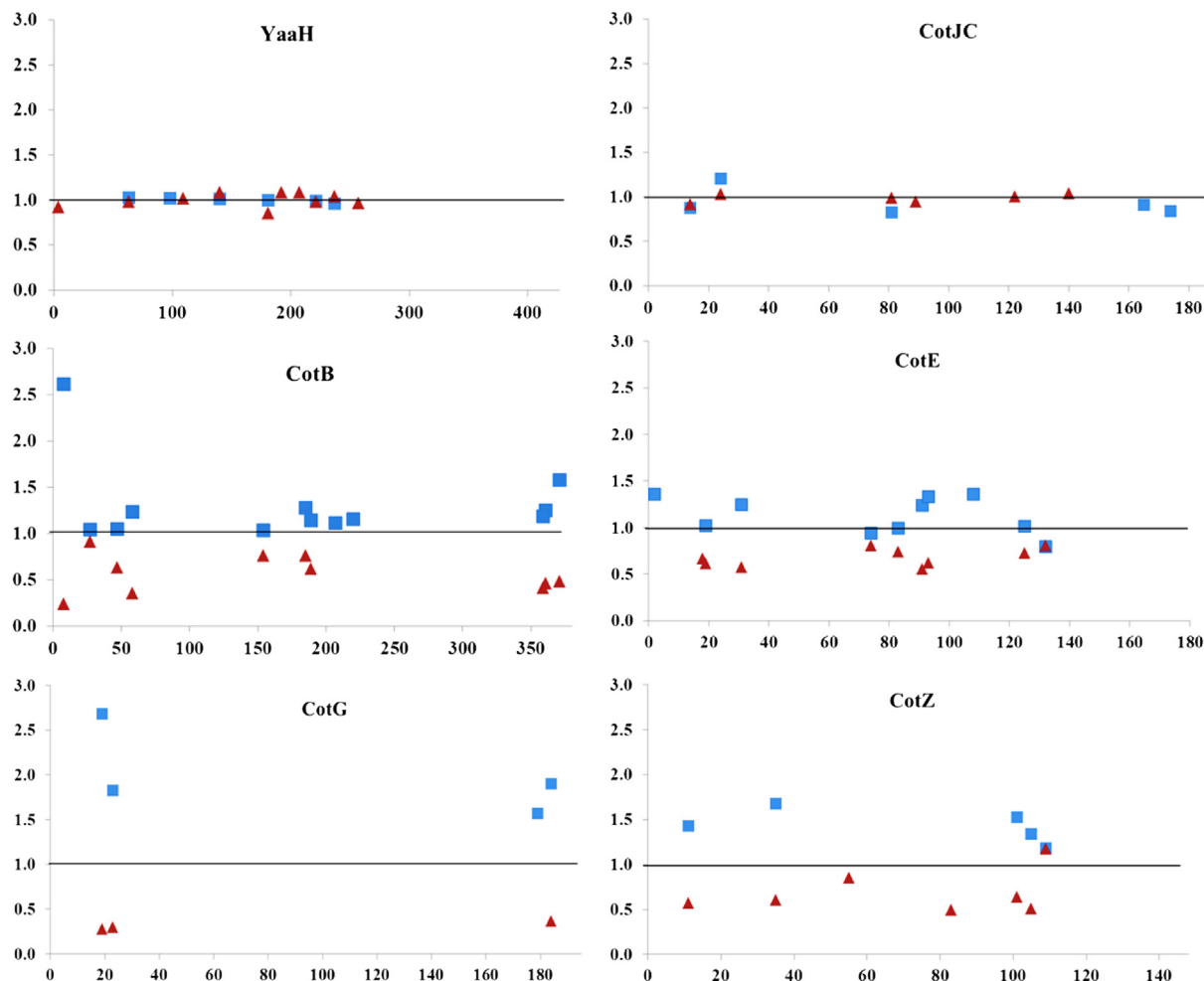**Fig. 3.** Digestion efficiency of the peptides from the spore coat proteins. The *x*-axis shows the position of the identified peptide within the protein and the *y*-axis shows the digestion efficiency of the peptides over time compared to the ^{15}N-labeled reference spores. Symbol (■) represents peptides identified from 2-day young spores while (▲) represents the same identified peptides from 8-day mature spores. Proteins YaaH and CotJC represent the inner coat proteins, CotB, CotE, CotG represent the outer coat and CotZ represents the crust proteins.
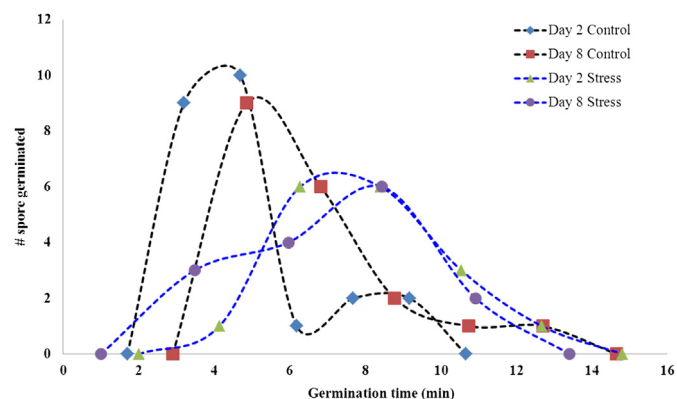

Fig. 4. Heterogeneity in the germination times of young and mature spores. A frequency distribution of number of spores germinating at different germination times is plotted. A slight but significant delay in the germination times (speed) of young and mature un-stressed spores can be seen (black lines). No significant difference in the germination times of thermally-stressed spores is seen (blue lines; see Section 4). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

inspection of the peptides identified from the inner coat proteins, exemplified by CotJC in Fig. 3, clearly shows that the inner coat proteins are digested to the same extent in both the query and the reference spores. The inner coat morphogenetic proteins SpoIVA and SafA, as shown in Fig. 2, are indeed involved in the morphogenesis of the inner coat and are affected in their digestion efficiencies in young spores. However, as spores mature these proteins show digestion efficiencies that coincide in the ^{14}N and ^{15}N spores. Based on these results it is possible that the identified putative spore coat proteins, such as YsdC, YqgO, YxeD, with ^{14}N/^{15}N ratios near to the window in which the inner coat proteins fall, may also be localized in the inner coat.

Compared to the inner coat, the outer coat and crust proteins are critical for spore maturation. Most of the outer coat proteins show decreasing ^{14}N/^{15}N ratios, averaged over the identified peptides, when compared between 2- and 8-day old spores (Table 1). Proteins CotG, YurS, CotU, CotI, CotZ, CotY, CotB and CotC are the most affected proteins with regards to their protease resistance over time. Incidentally these proteins are rich in tyrosine, glutamine, lysine and/or cysteine. This fact coincides with the predicted dityrosine, ε-(γ)-glutamyl-lysine and disulfide linkages in the spore surface layer proteins (Henriques and Moran, 2007). The strongest maturation effect is seen in case of protein CotG, an outer coat protein which carries 9 repeats of 13 amino acid residues -H/Y-KKS-Y-R/

C-S/T-H/Y-KKSRS- at the central core of the protein (Sacco et al., 1995). These repeats are rich in lysine and tyrosine. In our previous study (Abhyankar et al., 2011) as well as in the current study we could not identify any peptides from this region. This may be due to two reasons — abundance of lysine giving rise to small peptides that escape the mass spectrometric detection and/or the tyrosine residues that could be cross-linked via an oxidation dependent mechanism mediated by SodA also present in the spore coat (Henriques et al., 1998). Nevertheless, we identify the peptides from the C- & N-termini of CotG which show a significant decrease in their digestion efficiencies from two- to eight-day spores (Fig. 3). YurS is an uncharacterized protein that appears to be involved in spore coat maturation in this study. This protein is a product of gene *yurS* which is reported to be co-transcribed with another coat protein gene *sspG* (Bagyan et al., 1998). SspG was a putative coat protein identified from our previous study. We did not identify it in the current study suggesting a strain-dependent variation in coat protein composition. Protein CotU (YnzH) also appears to participate in spore maturation. Though we identified only the C-terminal peptides the digestion efficiencies of these peptides are significantly lower in eight-day spores when compared to two-day spores. Similarly, the spore morphogenetic protein CotE, responsible for building of the outer coat layer, became more protease resistant in eight-day old spores. CotC, CotE, CotG, CotU are all known to be involved in inter-protein cross-links previously (Henriques et al., 1998; Isticato et al., 2004, 2008; Kim et al., 2006). Thus the decrease in the digestion efficiencies of these proteins over the time is indicative of spore maturation coupled to the progress in protein—protein cross-linking. Protein CotB, which is dependent on CotG for its assembly, is rich in serine residues at its C-terminal. Serine residues are target for glycosylation. Glycosylation is a post-translational modification and could also be involved in spore maturation. We did not identify any peptide from this region. This is possible since this region is also rich in lysines giving rise to small peptides undetectable for the mass spectrometer. It is also likely that CotB carries glycosyl moieties in this region and due to lack of suitable modification information we do not identify the peptides. Yet, CotB also showed the same behavior with less digestion efficiency in eight-day spores suggesting its involvement in spore maturation. Outer coat protein CotA is an abundant protein that encodes for a copper-dependent laccase (Hullo et al., 2001). Laccases can also mediate the protein—protein cross-linking via an oxidation based reaction (Elegir et al., 2007; Steffensen et al., 2008). Although no such evidence for CotA has been obtained, our results show that spore maturation also has a minor effect on the digestion efficiency of CotA. Its role in enhancing structural integrity of spores needs to be studied in more detail.

The difference in the extent of inter-protein cross-linking amongst different spores from a single population could also be a contributing factor to the inherent heterogeneity in spore germination. Therefore we analyzed young (two-day) and mature (eight-day) spores for their germination behavior. In the current context, young spores may show heterogeneity in the germination times as optimum maturation has not yet been achieved. In contrast, eight-day spores appeared to have a more homogenous spore germination behavior. Also, the germination times measured in our analysis differed significantly (*P*-value <0.05) when young (2-day) and 4-day, 6-day and 8-day mature spores were compared (Supplementary Figs. F1–F4). Generally the more mature the spores became the slightly longer it took on average for germination to complete. This may have a relation with the inferred increased coat protein cross-linking. Thermal treatment led to an even further increase in the germination time. This suggests that the limiting factor for germination to proceed is different in thermally damaged spores compared to non heat stressed spores. Whether this hypothesis is true needs to be confirmed by further experiments in which different spore layers/ compartments are probed for their functionality before and after a thermal challenge. Finally, transcriptional analyses of outgrowing young and mature spores with or without a pre-exposure to heat stress could help reveal the differences in the two spore populations.

5. Conclusion

The spore outer coat and the crust layers are favored targets for spore maturation with proteins CotG, YurS, CotU, CotI, CotZ, CotY, CotB and CotC being critical for the process and likely subjected to cross-linking. The inner coat proteins are not involved in spore maturation except in the young spores where SpoIVA and SafA play a pivotal role in stabilizing this layer before the spore is released out of the mother cell. The level of enhancement of digestion of individual spore proteins in young spores may be an indication of their location in the spore layers.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2014.03.007.

References

Abhyankar, W., Hossain, A.H., Djajasaputra, A., Permpoonpattana, P., Ter Beek, A., Dekker, H.L., Cutting, S.M., Brul, S., de Koning, L.J., de Koster, C.G., 2013. In Pursuit of protein targets: proteomic characterization of bacterial spore outer layers. J. Proteome Res. 12, 4507–4521.

Abhyankar, W., Ter Beek, A., Dekker, H., Kort, R., Brul, S., de Koster, C.G., 2011. Gel-free proteomic identification of the *Bacillus subtilis* insoluble spore coat protein fraction. Proteomics 11, 4541–4550.

Bagyan, I., Setlow, B., Setlow, P., 1998. New small, acid-soluble proteins unique to spores of *Bacillus subtilis*: identification of the coding genes and regulation and function of two of these genes. J. Bacteriol. 180, 6704–6712.

Elegir, G., Bussini, D., Antonsson, S., Lindstrom, M.E., Zoia, L., 2007. Laccase-initiated cross-linking of lignocellulose fibres using a ultra-filtered lignin isolated from kraft black liquor. Appl. Microbiol. Biotechnol. 77, 809–817.

Henriques, A.O., Melsen, L.R., Moran, C.P., 1998. Involvement of superoxide dismutase in spore coat assembly in *Bacillus subtilis*. J. Bacteriol. 180, 2285–2291.

Henriques, A.O., Moran Jr., C.P., 2007. Structure, assembly, and function of the spore surface layers. Annu. Rev. Microbiol. 61, 555–588.

Hullo, M.F., Moszer, I., Danchin, A., Martin-Verstraete, I., 2001. CotA of *Bacillus subtilis* is a copper-dependent laccase. J. Bacteriol. 183, 5426–5430.

Imamura, D., Kuwana, R., Takamatsu, H., Watabe, K., 2011. Proteins involved in formation of the outermost layer of *Bacillus subtilis* spores. J. Bacteriol. 193, 4075–4080.

Isticato, R., Esposito, G., Zilhão, R., Nolasco, S., Cangiano, G., De Felice, M., Henriques, A.O., Ricca, E., 2004. Assembly of multiple CotC forms into the *Bacillus subtilis* spore coat. J. Bacteriol. 186, 1129–1135.

Isticato, R., Pelosi, A., Zilhão, R., Baccigalupi, L., Henriques, A.O., De Felice, M., Ricca, E., 2008. CotC-CotU heterodimerization during assembly of the *Bacillus subtilis* spore coat. J. Bacteriol. 190, 1267–1275.

Kim, H., Hahn, M., Grabowski, P., McPherson, D.C., Otte, M.M., Wang, R., Ferguson, C.C., Eichenberger, P., Driks, A., 2006. The *Bacillus subtilis* spore coat protein interaction network. Mol. Microbiol. 59, 487–502.

Kort, R., O'Brien, A.C., van Stokkum, I.H.M., Oomes, S.J.C.M., Crielaard, W., Hellingwerf, K.J., Brul, S., 2005. Assessment of heat resistance of bacterial spores from food product isolates by fluorescence monitoring of dipicolinic acid release. Appl. Environ. Microbiol. 71, 3556–3564.

Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., Setlow, P., 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. 64, 548–572.

Nicolas, P., Mader, U., Dervyn, E., Rochat, T., Leduc, A., Pigeonneau, N., Bidnenko, E., Marchadier, E., Hoebeke, M., Aymerich, S., Becher, D., Bisicchia, P., Botella, E., Delumeau, O., Doherty, G., Denham, E.L., Fogg, M.J., Fromion, V., Goelzer, A., Hansen, A., Hartig, E., Harwood, C.R., Homuth, G., Jarmer, H., Jules, M., Klipp, E., Le Chat, L., Lecointe, F., Lewis, P., Liebermeister, W., March, A., Mars, R.A.,

Nannapaneni, P., Noone, D., Pohl, S., Rinn, B., Rugheimer, F., Sappa, P.K., Samson, F., Schaffer, M., Schwikowski, B., Steil, L., Stulke, J., Wiegert, T., Devine, K.M., Wilkinson, A.J., van Dijl, J.M., Hecker, M., Volker, U., Bessieres, P., Noirot, P., 2012. Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. Science 335, 1103–1106.

Pandey, R., Ter Beek, A., Vischer, N.O.E., Smelt, J.P.P.M., Brul, S., Manders, E.M.M., 2013. Live cell imaging of germination and outgrowth of individual *Bacillus subtilis* spores; the effect of heat stress quantitatively analyzed with Spore-Tracker. PLoS ONE 8, e58972.

Sacco, M., Ricca, E., Losick, R., Cutting, S., 1995. An additional GerE-controlled gene encoding an abundant spore coat protein from *Bacillus subtilis*. J. Bacteriol. 177, 372–377.

Sanchez-Salas, J.L., Setlow, B., Zhang, P., Li, Y.Q., Setlow, P., 2011. Maturation of released spores is necessary for acquisition of full spore heat resistance during *Bacillus subtilis* sporulation. Appl. Environ. Microbiol. 77, 6746–6754.

Setlow, P., 2003. Spore germination. Curr. Opin. Microbiol. 6, 550–556.

Setlow, P., 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J. Appl. Microbiol. 101, 514–525.

Setlow, P., 2007. I will survive: DNA protection in bacterial spores. Trends Microbiol. 15, 172–180.

Steffensen, C.L., Andersen, M.L., Degn, P.E., Nielsen, J.H., 2008. Cross-linking proteins by laccase-catalyzed oxidation: importance relative to other modifications. J. Agric. Food Chem. 56, 12002–12010.

Xu, H., He, X., Gou, J., Lee, H.Y., Ahn, J., 2009. Kinetic evaluation of physiological heterogeneity in bacterial spores during thermal inactivation. J. Gen. Appl. Microbiol. 55, 295–299.

Zhang, J., Fitz-James, P.C., Aronson, A.I., 1993. Cloning and characterization of a cluster of genes encoding polypeptides present in the insoluble fraction of the spore coat of *Bacillus subtilis*. J. Bacteriol. 175, 3757–3766.