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REGULAR ARTICLE

Functional proteomics and correlated signaling pathway of the thermophilic bacterium *Bacillus stearothermophilus* TLS33 under cold-shock stress

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The thermophilic bacterium *Bacillus stearothermophilus* TLS33 was examined under cold-shock stress by a proteomic approach to gain a better understanding of the protein synthesis and complex regulatory pathways of bacterial adaptation. After downshift in the temperature from 65°C, the optimal growth temperature for this bacterium, to 37°C and 25°C for 2 h, we used the high-throughput techniques of proteomic analysis combining 2-DE and MS to identify 53 individual proteins including differentially expressed proteins. The bioinformatics database was used to search the biological functions of proteins and correlate these with gene homology and metabolic pathways in cell protection and adaptation. Eight cold-shock-induced proteins were shown to have markedly different protein expression: glucosyltransferase, anti-sigma B (σ^B) factor, Mrp protein homolog, dihydroorthase, hypothetical transcriptional regulator in FeuA-SigW intergenic region, RibT protein, phosphoadenosine phosphosulfate reductase and prespore-specific transcriptional activator RsfA. Interestingly, six of these cold-shock-induced proteins are correlated with the signal transduction pathway of bacterial sporulation. This study aims to provide a better understanding of the functional adaptation of this bacterium to environmental cold-shock stress.

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1 Introduction

Prokaryotic microorganisms can be classified according to their temperature ranges of growth. Thermophiles are heat-loving microorganisms with an optimum growth above 50°C, whereas mesophiles grow between 10°C and 50°C [1]. Psychrophiles and psychrotrophs grow at or near 0°C but are differentiated by their optimal and maximal growth temperatures. Despite their differences, common physiological changes occur within all these microorganisms in response to temperature change [1–3]. Study of the cold-shock response is important for understanding cell growth at, or cell tolerance to, low temperature. A downshift in temperature causes a transient induction of a large number of proteins, termed cold-induced proteins (CIPs), but also repression of many proteins that are synthesized under normal condition [4]. Some of these cold-induced proteins are essential for various levels of cellular physiology including metabolism, transcription, translation and protein folding [5, 6]. The most extensive studies on low-temperature adaptation of prokar-

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yotes have been conducted with *E. coli* [7–12] and *B. subtilis* [13–16]. Adaptation and protein expression in response to cold-shock stress has been investigated for several bacteria, such as *B. caldolyticus* [17], *B. caldotenex* [18], *Helicobacter pylori* [19], *Mycoplasma genitalium* [19], *Thermotoga maritime* [20], and *B. stearothermophilus* [21, 22]. Proteomic analysis combining 2-DE, MS and bioinformatics is a powerful approach for studying the alteration of protein expression in organisms under different environmental conditions. These techniques have also been applied to explore structures or functions of specific proteins from microorganisms in sequenced genomes, such as *E. coli* [23, 24], *B. subtilis* [25, 26], and *Saccharomyces cerevisiae* [27].

In this study, we used a proteomic approach combining 2-DE and MS to analyze the differentially expressed proteins of the thermophilic bacterium *B. stearothermophilus* TLS33 under cold-shock stress. After downshift in the temperature from the optimal growth temperature at 65°C to 37°C and 25°C, 2-DE results showed the differential protein expressions at these three temperatures, and image analysis software was used to compare the protein spots on 2-D images and to detect the spots representing differentially expressed protein. We identified a number of protein spots that were differentially expressed when the bacterium encountered in the cold-shock environment. We then used the bioinformatics database to search for protein and gene correlation, biological functions and the relationship of the proteins in signaling pathway of sporulation. Interestingly, we found that eight cold-shock-induced proteins, which differ from those in *B. subtilis* and *E. coli*, play an important role in cell adaptation under cold-shock stress. Moreover, we suggest a model of sigma (σ) signal transduction network correlated with the observed cold-shock-induced proteins in an attempt to further understand the bacterial adaptation under cold-shock stress.

2 Materials and methods

2.1 Bacterial growth and cold-shock experiment

The thermophilic bacterium *B. stearothermophilus* TLS33 was isolated from soil at hot springs in Chiang Mai, Thailand. The bacteria were cultured in 50 mL media containing 0.1% yeast extract, 0.1% tryptone and 0.1% base mixture pH 7.2 (0.8 g/L Titriplex I, 0.36 g/L $CaSO_4.2H_2O$, 1 g/L $MgCl_2.6H_2O$, 0.2 g/L NaOH and 4 mL 0.01 M iron III citrate) [28]. The cultures were incubated in a water bath at 65°C with shaking at 200 rpm (in triplicate for each experiment). After reaching an OD_{620} of 0.5 (the mid-log phase of this bacterium), cold-shock was initiated by transferring the culture flasks to the water baths at 37°C or 25°C. The cell growth profile under cold-shock stress was examined for up to 8 h. For all analytical procedures, the cells cultured 65°C (control), 37°C and 25°C were harvested by centrifugation at 12 000 × *g* at 4°C for 30 min.

2.2 Sample preparation

Cell pellets were resuspended in TE buffer, containing 20 mM Tris-HCl pH 8.0 and 10 mM EDTA, and disrupted on ice by sonication (five cycles of 45 s). Cell debris was removed by centrifugation at 12 000 × *g* at 4°C for 20 min. The sample solutions were precipitated by addition of 10% TCA and 0.1% DTT and stored overnight at −20°C to precipitate the proteins and to remove the salt and nucleic acid. The suspension was centrifuged at 20 000 × *g* for 30 min. Protein pellets were resuspended in ice-cold acetone containing 0.1% DTT and stored at −20°C for at least 30 min. The protein suspension was centrifuged again at 20 000 × *g* for 30 min and the pellets were resuspended in ice-cold acetone without DTT. The protein suspension was stored at −20°C for 30 min and then centrifuged at 20 000 × *g* for 30 min. The obtained pellets were immediately dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% IPG buffer pH 4–7 and 65 mM DTT) [29]. Each sample was sonicated and centrifuged, and the protein concentration was determined using a PlusOne™ 2-D Quant Kit (Amersham Biosciences, Uppsala, Sweden) with bovine albumin (BSA) as a standard.

2.3 2-DE analysis

Each sample was applied onto IPG strips (18 cm, pH 4–7 L; Amersham Biosciences) with a final concentration of 100 μg protein in 350 μL. IPGphor IEF (Amersham Biosciences) was performed under following condition: IPG strips were rehydrated passively and/or actively for 12 h at 30 V followed by ramping to 250 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 3500 V for 3 h, and focusing at 8000 V for up to 7.5 h. After IEF, the IPG strips were equilibrated in equilibration buffer I (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 2% DTT and a trace of bromophenol blue) for 15 min, and then subsequently alkylated in equilibration buffer II (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 2.5% w/v iodoacetamide and a trace of bromophenol blue) for 15 min. Each equilibrated IPG strip was placed on top of the linear gradient of 10–20% polyacrylamide gel (185 × 200 × 1.5 mm) and covered with 0.5% agarose. The second-dimensional separation was performed using the Protean xi Multi-Cells (Bio-Rad) and carried out at 45 mA *per* gel at 15°C until the bromophenol blue dye front reached the bottom of the gel. At the end of each run, the 2-D gels were fixed in 10% methanol/7% acetic acid for 30 min and subsequently stained using the SYPRO Ruby method [30]. The stained gels were scanned using a Typhoon 9200 image scanner (Amersham Biosciences).

2.4 Comparative image analysis

The statistical data (spot detection, spot editing, pattern matching, up- and down-regulations) were acquired and analyzed using the ImageMaster 2D elite software package (Amersham Biosciences) with a high image quality TIF for-

mat (600 dpi). Statistical analyses were performed in triplicate of three gels from each experimental growth condition to determine spots showing reproducible changes. For gel-to-gel comparison, the 2-D image of cell at 65°C was set as the reference gel image. Before matching the images, background was subtracted (using the lowest-on-boundary method) and normalization was performed to correct for the differences in protein spot intensity. The reference gel image was matched to another gel image, and the matching was manually edited to ensure correct spot matches and to ensure more consistent determination of spot volume. Matched spot data (spot number and volume) was exported to an Excel table (Microsoft) for calculation of spot volume and construction of graphs to compare protein expression. The quantification of each spot was expressed as percent volume, where %V = spot volume/volumes of all spots resolved in the gels.

2.5 Protein digestion

Protein spots were manually excised from the polyacrylamide gels and transferred to 500-μL siliconized Eppendorf tubes. The gel pieces were washed twice with 200 μL 50% ACN/25 mM ammonium bicarbonate buffer pH 8.0 for 15 min each. The gel pieces were then washed once with 200 μL 100% ACN and dried using a SpeedVac concentrator. Dried gel pieces were swollen in 10 μL 25 mM ammonium bicarbonate containing 0.1 μg trypsin (sequencing grade; Promega, Madison, WI, USA). Gel pieces were then crushed with a siliconized blue stick and incubated at 37°C for at least 16 h. Peptides were subsequently extracted twice with 50 μL 50% ACN/5% TFA, and the extracted solutions were combined and dried using SpeedVac concentrator. The peptides or pellets were then resuspended in 20 μL 0.1% TFA and the suspended solutions were purified using ZipTip C18 (Millipore, Bedford, MA, USA). Briefly, 10 μL sample was drawn up and down in the ZipTip ten times, and the ZipTip was washed with 10 μL 0.1% formic acid by drawing up and expelling the washing solution three times. The peptides were eluted with 5 μL 75% ACN/0.1% formic acid.

2.6 MALDI-MS analysis

The samples were mixed in a ratio of 1:1 with matrix solution (5 mg/mL CHCA in 50% ACN, 0.1% TFA and 2% ammonium citrate) and spotted onto the 96-well plate of a PerSeptive Biosystems Voyager DE-RP MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA). Adrenocorticotropic hormone (ACTH), 1 pmol/μL, was used as an external calibration. MS analysis was performed using an acceleration voltage of 20 kV [31]. PMF spectra obtained from each digested protein were searched against protein PMF databases *via* the programs Protein Prospector MS-FIT (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) and MASCOT (http://www.matrixscience.com). Search parameters allowed for oxidation of methionine, carbamidomethylation of cysteine, one missed trypsin cleavage, and 50 ppm mass accuracy. Peptides in the mass range of 1000–3500 Da were selectively searched in the database. The remaining autodigestion trypsin and keratin peaks were removed from the mass list before database searching. Protein identification was repeated at least once more using spots from different gels.

2.7 Bioinformatics for functional properties

The SubtiList (http://genolist.pasteur.fr/SubtiList) supplementing with EMBL/GenBank/DDBJ databanks, COGs (http://www.ncbi.nlm.nih.gov/COG), Swiss-Prot (http://us.expasy.org/sprot), TrEMBL (http://www.expasy.ch/sprot) and Micado (http://www-mig.jouy.inra.fr/bdsi/Micado) were searched for sequence homology and biological functions corresponding to identified proteins. In addition, the DIP database (http://dip.doe-mbi.ucla.edu) was used for determining protein-protein interactions.

3 Results and discussion

3.1 Growth profile of *B. stearothermophilus* TLS33 under cold-shock

To investigate transient metabolic adaptation for bacterial survival and its physiological activity, the proteome of thermophile *B. stearothermophilus* TLS33 under cold-shock stress was studied immediately after transferring the bacterial cultures from their optimal growth temperature at 65°C to 37°C or 25°C, which represent the temperature of normal bacterial growth temperature and room temperature, respectively. The growth profile of this bacterium is shown in Fig. 1. We chose the mid-log phase of the bacterial growth at 24 h (OD_{620} ~0.5, T=0 h) for cold-shock experiment because at this time

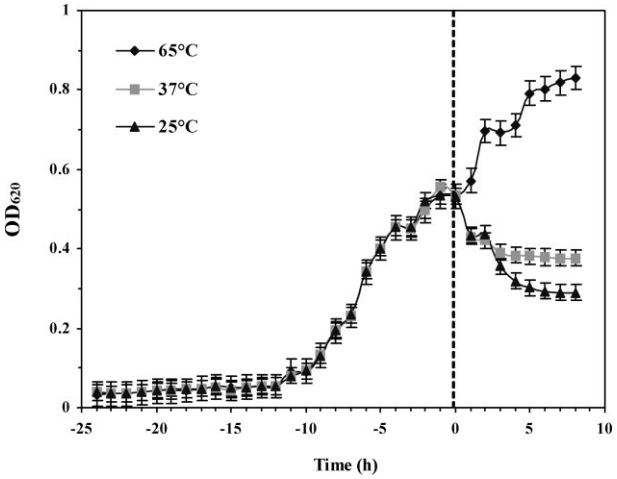

Figure 1. Growth profile of *B. stearothermophilus* TLS33 under cold-shock stress at 37°C and 25°C cultured for 32 h. Cold-shock stress was induced after the 24 h of culture (T = 0 h, starting time of cold-shock stress) for 8 h (T = 8 h).

bacterial cells begin to synthesize differential proteins and other components, such as primary and secondary metabolites, leading to more resistance to the non-optimal environmental conditions. Within the first 2 h after cold-shock at 37°C or 25°C, the bacterial growth rate decreased, and reached a constant rate by 4 h. In contrast, bacterial growth at 65°C increased over the 8-h period (T=+8 h). Thus, 2 h after cold shock was considered the appropriate time point for proteomic analysis of this bacterium response to cold-shock stress in this study. We investigated a large number of proteins for which the synthesis and expression levels were changed after cold-shock, including those involved in bacterial adaptation to cold-shock stress and sporulation [28, 32–35].

3.2 2-DE analysis

The cell extracts obtained after a temperature downshift from 65°C to 37°C or 25°C for 2 h or after incubation at 65°C for 2 h (control) (all performed in triplicate) showed reproducible 2-D gel patterns, analysis of which demonstrated the differential protein synthesis of this bacterium under cold-shock stress (Fig. 2). The protein pattern at 65°C showed a number of proteins within a narrow p*I* range of 4–7 and with molecular mass of more than 25 kDa, while the protein patterns at 37°C and 25°C showed fewer proteins. Few proteins with a p*I* in the range 3–4 or 7–11 were observed in any cell extract from any of the experimental temperatures. Thus, we focused our analysis on the proteins in the narrow p*I* range of 4–7, similar to other reports showing that a number of proteins in *B. subtilis* were restricted to a rather narrow p*I* range of 4–7 and a molecular mass range of 5–100 kDa [35–37]. Moreover, the 2-D gel patterns were shown to have a low resolution of protein separation, leading to a low yield of proteins. This may be caused by the low solubility of intracellular proteins from thermophilic cells, which may be due to (1) the high residue hydrophobicity and more charged amino acids, especially Glu, Arg and Lys, necessary to conserve its protein function in high temperature, and (2) the proteins in the cell extracts were precipitated by TCA, which may have resulted in protein aggregation due to the low pH and difficulty with resolubilizing protein precipitate completely. However, we attempted to resuspend the precipitated proteins in lysis buffer homogeneously prior to protein analysis using 2-DE. Although the protein distributions at the three different temperatures appeared similar on 2-D gel patterns, the exact number of protein spot for each temperature were compared on triplicate 2-D gels and quantitated using ImageMaster 2D elite software. The total number of protein spots on 2-D gels detected after culture at 65°C, and after cold shock at 37°C and 25°C were 191, 116 and 115, respectively. The pair-wise comparisons of protein spots from the different temperature groups: (1) 37°C and 65°C, (2) 25°C and 65°C, and (3) 37°C and 25°C, showed the matched proteins of 66, 57 and 75 spots with 34.55%, 29.84% and 64.65% matching, respectively. A twofold increase or decrease in protein synthesis of the differentially expressed

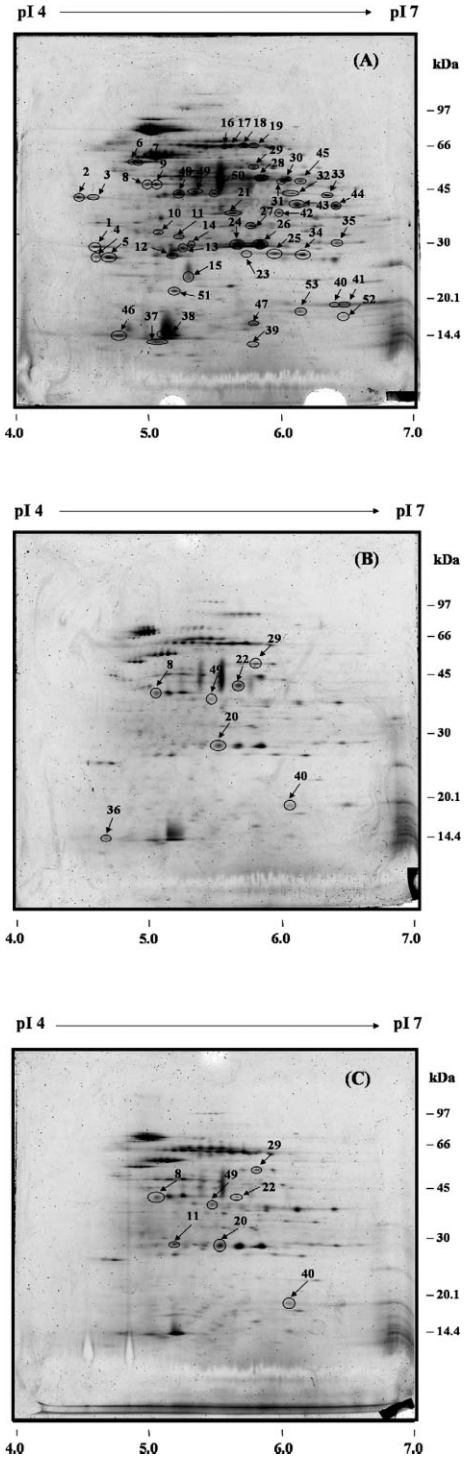

Figure 2. 2-D gel images of cell extracts from *B. stearothermophilus* TLS33 at three different temperatures; (A) 65°C, (B) 37°C, (C) 25°C. The arrows show the spots of proteins, which were identified by MALDI-TOF MS.

protein under cold-shock stress was classified as up-regulation or down-regulation, respectively. The pair-wise comparisons of the proteins in the different temperature groups 1–3,

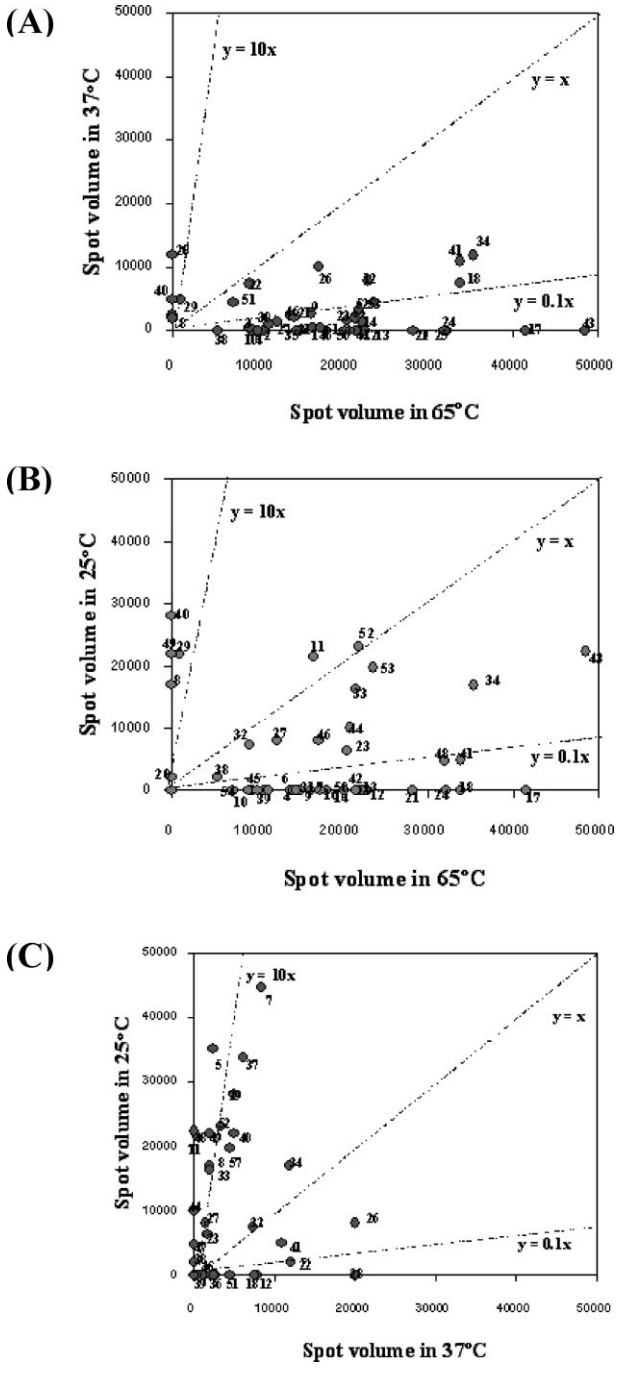

Figure 3. The increase and decrease of protein levels from *B. stearothermophilus* TLS33 after temperature downshift from 65°C to 37°C and to 25°C, demonstrated using ImageMaster 2D Elite software. (A) Comparison between the temperatures at 37°C and 65°C; (B) comparison between the temperatures at 25°C and 65°C; (C) comparison between the temperatures at 25°C and 37°C.

as mentioned above, showed 44, 25 and 10 up-regulated proteins, and 20, 29 and 12 down-regulated proteins, respectively. Furthermore, the differential protein levels were also evaluated using a linear scatter plot, in which y = 10x (up-regulation or increase of protein abundance), y = x (equal) and y = 0.1x (down-regulation or decrease of protein abundance), as shown in Fig. 3A–C. Fifty-three intracellular proteins were detected in the cells of this thermophilic bacterium at these three temperatures and these might represent the housekeeping proteins. Interestingly, only eight major cold-shock-induced proteins were markedly changed by the cold-shock stress.

3.3 Protein identification and differential protein synthesis

Individual protein spots were excised from the 2-D gels, subjected to in-gel digestion and analyzed by MALDI-TOF MS. A total of 53 intracellular proteins were recognized on the basis of the tryptic mass profile comparisons using the SwissProt and NCBI non-redundant database with MS-FIT and MASCOT software (Table 1). In addition, the SubtiList database (http://genolist.pasteur.fr/SubtiList) and EMBL/GenBank/DDBJ databases were used to determine the correlations at the genome level, accession numbers, description and functional categories of the identified proteins (Table 2). Moreover, the intracellular proteins from *B. stearothermophilus* TLS33 were compared with the complete genomes of *B. subtilis* and *B. halodurans* based on the COG (Clusters of Orthologous Groups of protein) database using the COGNITOR program (http://www.ncbi.nlm.nih.gov/COG), which represents a phylogenetic classification in functional annotation (Table 3). The results of the database search showed that most of intracellular proteins from *B. stearothermophilus* TLS33 had biological functions related to cellular process and metabolism, indicating the adaptation or maintenance of this bacterium under cold-shock stress. We found that eight cold-shock-induced proteins had markedly changed at the different temperatures: glucosyltransferase, anti-sigma B (σ^B) factor (RsbT), Mrp protein homolog, dihydroorthase, hypothetical transcriptional regulator in the FeuA-SigW intergenic region, RibT protein, phosphoadenosine phosphosulfate reductase, and prespore-specific transcriptional activator. Only glucosyltransferase, Mrp protein homolog, dihydroorthase, RibT protein, phosphoadenosine phosphosulfate reductase, and prespore-specific transcriptional activator were observed at 37°C and 25°C. Using Image Master 2D Elite software, we also classified the major cold-shock-induced proteins into three groups, depending on the protein synthesis level at different temperatures (Fig. 4). The first group of the protein synthesis at 37°C and 25°C comprised glucosyltransferase, Mrp protein homolog, dihydroorthase, phosphoadenosine phosphosulfate reductase, and prespore-specific transcriptional activator RsfA. The second group of the protein synthesis at only 37°C comprised RibT protein. The last group of the protein synthesis at 65°C and 25°C comprised anti-σ^B factor (RsbT) and the hypothetical transcriptional regulator in FeuA-SigW intergenic region. In addition, the cold-shock-induced proteins at 37°C were Mrp protein homolog, dihydroorthase and RibT pro-

Table 1. Protein identification of intracellular proteins in *B. stearothermophilus* TLS33, after temperature downshift to 37°C and 25°C, by MS-FIT and MASCOT software, based on Swiss-Prot database (http://www.expasy.ch/sprot)

Spot no.	Protein name	Accession no.	Mol. mass (Da) / p*I*	No. peptide matched	4 sample matched peptides
1	Pts system, fructose-specific IIB component (EIIB-FRU) (Fructose-permease IIB component) (Phosphotransferase enzyme II, B component) (P18) (PTFB)	P26380	35 051 / 4.37	9	MMNIVLAR IDDRFIHGQILTRWIK VHAA-DRIIVVSDDIAQDEMR KTLILSVAPSNVK
2	Stage III sporulation protein AE (S3AE)	P49782	39 191 / 4.55	7	TAASLETDK IGEFWNDIMTEYGGLLPESQK GSLME-FINGDK SFSPQEWLK
3	Inosine-5′-monophosphate dehydrogenase (IMP dehydrogenase) (IMPDH) (IMPD) (Superoxide-inducible protein 12, SOI12) (IMDH)	P21879	39 191 / 4.62	12	DVDLSVELTKTLK GVITNPFFLTPDHQVFDAEHLMGK SGVPIVNNEEDQK LVGIITNRDLRFISDYSMK
4	COMF operon protein 3 (CMF2)	P39147	29 901 / 4.76	9	ALFLLKPDEK VCYSCRSKLK EVWRTRIR SDFSSTFSKVYPDK
5	Unknown protein	–	28 100 / 4.64	–	
6	Flagellar protein (FLIT)	P39740	58 100 / 4.64	14	SMLSHIQNTPESDELLK SIATELQQMKR RVMHTTYLNPYNNITIDGTYYDKR QSIA-TELQQMKRKR
7	DNA topoisomerase I (Omega-protein) (Relaxing enzyme) (Untwisting enzyme, Swivelase) (TOP1)	P39814	58 250 / 4.55	26	TIERYLGKKYK SQMGVDIEQNFEPK NPALPFTTSTLQQEAARK EGTVGLITYMR
8	Probable poly (glycerol-phosphate) alpha-glucosyltransferase (Teichoic acid biosynthesis protein) (TAGE)	P13484	48 700 / 4.44	18	QIPDMDYYFISGGLPSNYGGLTK LFGEECNQNTFFLTFR NNEMSVVYYGDGETIR MYLQEYINDQNQVYLDK
9	Translation initiation factor (IF-2)	P17889	48 100 / 4.64	29	NMDLEVNNHMAMLEEK VVEGE-AGGITQHIGAYQIEENGK ITFLDTPGHAAFTTM-RAR AAEVPIIVAVNK
10	Putative peptidase in gcvT-spoIIIAA intergenic region (YQHT)	P54518	36 067 / 5.10	12	YMTGFTGSAGLAVISGDK SSLPHGVASDK TVAVGQPSDQLK EIYQVVFDAQALGVA-HIKPGMTGK
11	Anti-sigma B factor (RSBT)	P42411	34 803 / 5.14	10	MNDQSCVR IMTEWDIVAAR ELGFGTVDQAR IT-TAISELAR QLGR
12	Extragenic suppressor protein suhB homolog (SUHB)	Q45499	31 533 / 5.20	11	KWIREAGAR ITQSMHESLTIETK SNPNDLVTNIDK NFAISIGIFENGEGK
13	Unknown protein	–	33 103 / 5.30	–	
14	Hypothetical 28.6-kDa protein in recQ-cmk intergenic region precursor (YPBG)	P50733	34 187 / 5.53	9	MYATAKGNHLK THTFPLSKMK LVHFGVPIVFVWGNNDYEVR NAAYLISNGYGTTK
15	Unknown protein	–	24 187 / 5.53	–	
16	GTP-binding protein (LEPA)	P37949	69 284 / 5.49	26	NFSIIAHIDHGK EQLLDSMDLER QEVEDVIGLDA-SEAVLASAK VPAPTGDPEAPLK
17	2,3-Bisphosphoglycerate-independent phosphoglycerate mutase (BPG-independent pgaM) (LEPA)	P39773	69 284 / 5.61	12	NETVGNAVALAK AIQISNTFTNKDFR TYINQLNDQIK SALDVVDDSYANGIYDEFVIPSVITK
18	Unknown protein	–	69 486 / 5.72	–	–
19	Peptide methionine sulfoxide reductase (Protein-methionine-s–oxide reductase) (Peptide met (O) reductase) (MSRA)	P54154	69 284 / 5.81	10	EIATFAGGCFWCMVKPFDEQPGIEK VVSGYTGGH-TENPTYEEVCSETTGHR AEPFYEAEGYHQHFYK NPAHYQRYRTGSGR
20	MRP protein homolog (MRP)	P50863	28 628 / 5.55	11	ALERVGVPYFVSEKPEELK EADA-FILPGVGSFGDAMDNLGYTK GKAVRLKAE-DEKGNKLK EPFLQRPLGELDAVK

Table 1. Continued

Spot no.	Protein name	Accession no.	Mol. mass (Da) / p*I*	No. peptide matched	4 sample matched peptides
21	Hypothetical 58.2-kDa protein in kdgT-xpt intergenic region (YPWA)	P50848	38 614 / 5.59	12	TGAPK ELIDVLYER ELSLYFLQELGYDFDGGR AIFSNEVSVEDLPSLWNQK
22	Dihydroorotase (Dhoase) (PYRC)	P25995	39 144 / 5.66	14	NGWILNENGEK VTGETITAIGK LDATDNETVIDAK GGYTTVAAMPNTRPVPDTK
23	Transcription anti-termination protein nusG (NUSG)	Q06795	31 924 / 5.63	9	VIDGPFANFTGSIEEIDYDK VFVNMFGR ETPVELEFTQIDK ANLEKR
24	Ferrichrome transport ATP-binding protein (FHUC)	P49938	33 229 / 5.67	10	ITTLIGPNGCGK STILKTMSRIMR WALEETGMAEYAERPIEALSGGQR EGTALEVMTPDILK
25	Protein export protein prsA precursor (PRSA)	P24327	30 331 / 5.85	8	EVIAKTDAGDVTK GELYTNMK EQVKYELLTQKAAK EYSTDSSASK
26	Unknown protein	–	32 802 / 6.16	–	
27	Amidotransferase hisH (HIS5)	O34565	35 465 / 5.84	11	GKAVRLKAEDEKGNKLK LSFHNESPLLTK TEQGYAYFVHSYYIDGMEENALLASADYGVR SSTVGMSILTQFTKMAAEQKVK
28	Hypothetical 35.0-kDa protein in rapJ-opuAA intergenic region (YCEB)	O34504	43 485 / 5.75	9	RYWFAEHHSTK GLASTAPEIMIAR IAAQTNTIR QLEALYPNR
29	Hypothetical transcriptional regulator ybbB in feuA-sigW intergenic region (ORF3) (YBBB)	P40408	57 121 / 4.55	18	MQNAVIYQPVQIEYLK ETGQDIFTPCTESELAK LMNVSHIENLAVR LQELAALWNESSQLSQLK
30	Unknown protein	–	58 250 / 6.07	–	
31	Glycine betaine/carnitine/choline transport ATP-binding protein opuCA (OPCA)	O34992	54 178 / 5.85	12	CVIEVQSLIYDLFTASLSDQTDTHSAIEK WTSYYYQHYSTDIPVQLSAYR LQELAALWNESSQLSQLK MQNAVIYQPVQIEYLK
32	Penicillin-binding protein 1A/1B (PBP1) [Includes: penicillin-insensitive transglycosylase (Peptidoglycan Tgase, PBPA)	P39793	49 081 / 6.01	10	AQPGSTIKPILDYGPVIENK QSVDGGSYSEIQNSSAK WLADYDQQTTAAK TGTGQLAQVPGVEVAGK
33	PBSX phage terminase small subunit (XTMA)	P39785	43 484 / 6.14	14	LSLYFDLFPDQFK DSGLVDGTIVTEAK TIGIWK SFNATQSAIK
34	Probable amino-acid ABC transporter ATP-binding protein in bmrU-ansR intergenic region (YQIZ)	P54537	29 604 / 6.05	15	ENIGMVFQHFHLFPHK CLNLLEKPNGGTITIK DTEITKPK EVLQVMK
35	Hypothetical 19.1-kDa protein in sigD-rpsB intergenic region precursor (ORFC) (YLXL)	P40405	32 236 / 6.30	10	MSTLLWLLSFMLHGVLLYAVIILYTR QILEETENTLAAFLLELK ASSASQSDEESQK TEIELFLK
36	RIBT protein (RIBT)	P17622	18 139 / 4.60	12	EDEDIVGAIGVEK DYETDTDR HQGIGK QMMDALK
37	PAL-related lipoprotein precursor (SLP)	P39910	18 200 / 4.77	13	AVFPMLIIVFALSGCTLSTI EGWEDEIETVPTLMVVDQR EDIIKPLQHVLSK DYPEQIDK
38	Hypothetical protein YVYF (YVYF)	2897794	17 648 / 5.15	14	
39	Unknown protein	P21468	12 806 / 5.44	10	DGFYQIVNVQSDAAAVQEFDR FNNVLTSNGAEITGTK LAYEINDFR ISDDIIR
40	Phosphoadenosine phosphosulfate reductase (PAPS reductase, Thioredoxin dependent) (PADOPS reductase) (3′-Phosphoadenylsulfate reductase) (PAPS sulfotransferase) (CYH1)	P94498	22 031 / 5.95	9	MLTYDNWEEPTITFPEDDPYK KPDLTLEEQAEEHGDK DAEIVFLDTGLHFK EALSGHPAWLSGLR
41	Unknown protein	–	20 546 / 6.05	–	
42	Hypothetical 73.2-kDa protein in sodA-comGA intergenic region (YQGS)	P54496	38 549 / 6.14	11	GAVFFTNAGNQYMAAPEILK ALADSNSLTEIENYVTANAK DGSFITDQVVYTDGACYDK NVILVSLESTQSFVINEK

Table 1. Continued

Spot no.	Protein name	Accession no.	Mol. mass (Da) / p*I*	No. peptide matched	4 sample matched peptides
43	Aspartokinase 2 (Aspartokinase II) (Aspartate kinase 2) [contains: aspartokinase II alpha subunit; Aapartokinase II beta subunit] (AK2)	P08495	40 764 / 6.32	13	ITDIDTSVLADQLEK GGSDTTAVALAAALK GHQVVVVVSAMGK GIAFEDQITR
44	Hypothetical 45.3-kDa protein in prkA-cspB intergenic region (ORF4) (YHBH)	P45742	43 986 / 5.25	17	HVGQGDGESQVGDVVAR TWNDITKPESK GE-SGGTICSSVYR ELELPNLQQK
45	Unknown protein	O07636	56 284 / 5.35	9	GNVTYPITIDPSVWIFDDR NAEPNS-SATQCVFVTSSGK NEESGVSHQFS LMNGSFAMR
46	Hypothetical 17.9-kDa protein in nprE-pycA intergenic region (YLAL)	–	18 200 / 4.77	–	
47	Stage III sporulation protein AH	P49785	17 587 / 5.64	9	TVVTETADDDLFTTYR TQGYEDALVNAEGDK EEL-NAIVSSDDATAK MTALSEVEGTGK
48	(D49467) Unnamed protein product	P39807	56 667 / 5.20	9	DQMDHLNHEDALK DTYYAYNTK LQTVCQACIK MGELANCPK
49	Prespore specific transcriptional activator (RSFA)	P39650	58 758 / 5.34	11	QDAWSEENDLLLAETVLR FLQNYEGNHEQSSALK LVLFEEDEHASPSFK TIQEDYETLVK
50	Glucose-6-phosphate isomerase (GPI) (Phosphoglucose isomerase) (PGI) (Phosphohexose isomerase) (PHI) (G6PI)	P80860	58 515 / 5.52	21	TGAGSDFLGWVDLPEHYDK DVMDLLEDVDFSIN-VISK GNPQVIFIGNNISSSYMR ALTFFPTE-HELTYLR
51	Unknown protein	–	20 249 / 5.07	–	
52	Hypothetical 21.0-kDa protein in lysS-mecB intergenic region (YACH)	P37569	17 461 / 5.70	10	ELESLIHQEEFENAAHVR MICQECHERPATFHFTK VNMFALLGKPGFEEK STDSEEEQEVNK
53	Molybdopterin-gianine dinucleotide biosynthesis protein B	O31704	18 445 / 5.87	9	LIELYQFLETDCLLIEGFK MAL-VRPFPIVQVVGFQNSGK EDLEALQAVNIIAIIYR AAGADVTAVEGAGVLQLTAR

tein, while the cold-shock-induced proteins at 25°C were glucosyltransferase, anti-σB factor, and hypothetical transcriptional regulator in FeuA-SigW intergenic region, phosphoadenosine phosphosulfate reductase and prespore specific transcriptional activator. This result suggested that the temperatures of cold-shock experiment highly influenced the levels of the protein synthesis.

3.4 Cold-shock effect on sporulation signaling pathway of *B. stearothermophilus* TLS33

To gain a better understanding of the bacterial adaptation under cold-shock stress, advanced bioinformatics based on database searches was used to thoroughly search the specific cold-shock-induced proteins and the relationship between their functions and signal transduction pathways of bacterial adaptation when the bacterium was subjected cold-shock stress. Only six proteins, TagE, RsbT, MrpA, PyrC, YbbB and RsfA, were shown to have the functions correlated to the signaling pathway of sporulation. Regarding sporulation in *B. subtilis*, it has been reported that these proteins were also correlated with the σF and σG factors, which are involved in the 'Forespore' stage of the sporulation process [40]. We propose that the functions of the cold-shock-induced proteins in the sporulation signaling pathway corelates to five routes. In the first route, RsbT is up-regulated when the bacterium is cold-shocked at only 25°C. Generally, RsbT controls the early sporulation in vegetative cell cycle of *Bacillus* sp. by coordination with the σ^B factor [41–45]. When the bacterium is exposed to stress, RsbT from the upstream module is triggered to inactivate the principal negative regulator of RsbS by phosphorylation, and then to activate RsbU. Subsequently, PP2C phosphatase can dephosphorylate the phosphorylated form of RsbV (RsbV-P) to its dephosphorylated form (RsbV) in the downstream module (Fig. 5A). RsbV forms a complex with RsbW and forces the release of σ^B. Thus, RsbT is linked to the upstream and downstream modules, which activates RsbU and stimulates its enzymatic properties towards its substrate [46–53]. In the second route, TagE has two activities in stressed cells, in which it activates the PAS-RsbP to form a complex with RsbW, and alternately activates the σ^F/σ^G in the forespore via RsbW/σ^B route (Fig. 5B). Only this route is activated when the bacterium is cold-shocked at 37°C or 25°C. We suggest that this route may be dormant at 65°C because the protein has not been observed. It has been reported that TagE is involved in the activation of PhoP∼P

Table 2. Protein and gene identification, description, and functional category of intracellular proteins in *B. stearothermophilus* TLS33, after downshift temperatures at 37°C and 25°C, by SubtiList and EMBL/GenBank/DDBJ databanks and COG databases

Spot no.	Protein ID/no.	Gene name/no.	Description	Functional category
1	PTFB (P26380)	*lev*E (X56098)	PTS fructose-specific enzyme IIB component	Transport/binding proteins and lipoproteins
2	S3AE (P49782)	*spoIII*AE (U35252)	Mutants block sporulation after engulfment	Sporulation
3	IMDH (P21879)	*gua*B (X55669)	Inosine-monophosphate dehydrogenase	Metabolism and transport of nucleotides and nucleic acids (purine biosynthesis)
4	CMF3 (P39147)	*com*FC (Z18629)	Late competence gene	Transformation /competence
5	Unknown	–	–	–
6	FLIT (P39740)	*fli*T (Z31376)	Flagellar protein	Mobility and chemotaxis
7	TOP1 (P39814)	*top*A (L27797)	DNA topoisomerase I	DNA packing and segregation / DNA replication, recombination
8	TAGE (P13484)	*tag*E (X15200)	UDP-glucose: polyglycerol phosphate glucosyltransferase	Cell wall
9	IF2 (P17889)	*inf*B (M34836)	Initiation factor IF-2 (GTPase)	Initiation, translation factors and enzymes involved in translation
10	YQHT (P54518)	*yqh*T (D84432)	Unknown; similar to Xaa-Pro dipeptidase	Protein modification
11	RSBT (P42411)	*rsb*T (L35574)	Positive regulator of sigma-B activity (switch protein/serine-threonine kinase)	Adaptation to atypical conditions
12	SUHB (Q45499)	*suhB* (AF012285)	Archaeal fructose-1,6-biphosphate and related enzyme of inositol monophosphatase family	Carbohydrate transport and metabolism
13	Unknown	–	–	–
14	YPBG (P50733)	*ypb*G (L47648)	Unknown; similar to unknown proteins	From other organisms
15	Unknown	–	–	–
16	LEPA (P37949)	*lep*A (X91655)	GTP-binding protein	Elongation
17	PMGI (P39773)	*pgm* (L29475)	Phosphoglycerate mutase	Carbohydrate transport and metabolism (gluconeo-genesis)
18	Unknown	–	–	–
19	MSRA (P54154)	*msr*A (L77246)	Peptidyl methione sulfoxide reductase	Detoxification, post-translational modification, protein turnover, chaperone
20	MRP (P50863)	*mrp* (X74737)	Multiple resistance and pH homeostasis	Transport/binding proteins
21	YPWA (P50848)	*ypwA* (L47838)	Unknown; similar to carboxypeptidase	Metabolism of amino acids and related molecule
22	PYRC (P25995)	*pyr*C (BG10714)	Dihydroorthase (Pyrimidine biosynthesis)	Metabolism of nucleotides and nucleic acids
23	NUSG (Q06795)	*nus*G (D13303)	Transcription anti-termination factor	Termination
24	FHUC (P49938)	*fhu*C (X93092)	Ferrichrome ABC transporter (ATP-binding protein)	Transport/binding proteins and lipoproteins
25	PRSA (P24327)	*prs*A (X57271)	Protein secretion (post-translocation molecular chaperone), phosphoribosylpyrophosphate synthetase	Protein secretion, nucleotide and amino acid transport and metabolism
26	Unknown	–	–	–

Table 2. Continued

Spot no.	Protein ID/no.	Gene name/no.	Description	Functional category
27	HIS5 (O34565)	*his*H (AF017113)	Amidotransferase	Amino acid transport and metabolism (histidine bio-synthesis)
28	YCEB (O34504)	*yce*B (AB000617)	Unknown; similar to unknown proteins	From other organisms
29	YBBB (P40408)	*ybb*B (L19954)	Unknown; similar to transcriptional regulator (AraC/XylS family)	Regulation
30	Unknown	–	–	–
31	OPCA (O34992)	*opu*CA (AF009352)	Glycine betaine/carnitine/ choline ABC trans-porter (ATP-binding protein)	Transport/binding proteins and lipoproteins
32	PBPA (P39793)	*pon*A (U11883)	Penicillin-binding proteins 1A/1B	Cell wall
33	XTMA (P39793)	*xtm*A (Z70177)	Phage PBSX terminase (small subunit)	Phage-related functions, DNA replication, recombination and repair
34	YQIZ (P54537)	*yqiZ* (D84432)	Unknown; similar to amino acid ABC transporter (ATP-binding protein)	Transport/binding proteins and lipoproteins
35	YLXL (P40405)	*ylx*L (Z99112)	Unknown; similar to unknown proteins	From *B. subtilis*
36	RIBT (P17622)	*rib*T (L09228)	Reductase	Metabolism of coenzymes and prosthetic groups, riboflavin biosynthesis
37	SLP (P39910)	*slp* (M57435)	Small peptidoglycan-associated lipoprotein, starvation-inducible outer membrane lipoprotein	Transport/binding proteins and lipoproteins, cell envelope biogenesis, outer membrane
38	YVYF (P39807)	*yvy*F (L14437)	Unknown; similar to flagellar protein	Mobility and chemotaxis
39	Unknown	–	–	–
40	CYH1 (P94498)	*cys*H (U76751)	Phosphoadenosine phosphosulfate	Amino acid transport and metabolism, coenzyme metabolism (FAD biosynthesis)
41	Unknown	–	–	–
42	YQGS (P54496)	*yqg*S (D84432)	Unknown; similar to putative molybdate binding protein	From other organisms
43	AK2 (P08495)	*lys*C (J03294)	Aspartokinase II (alpha and beta subunits)	Amino acid transport and metabolism (threonine and methionine biosynthesis)
44	YHBH (P45742)	*yhb*H (Z99108)	Ribosome-associated protein Y (PSrp-1)	Translation, ribosomal structure and biogenesis
45	Unknown	–	–	–
46	YLAL (O07636)	*yla*L (Z99111)	–	–
47	AH (P49785)	*spoIII*AH (Z99116)	–	–
48	Unnamed protein (BAA24873)	–	Unknown; similar to flagellar proteins	Mobility and chemotaxis
49	RSFA (P39650)	*rsf*A (X73124)	Probable regulator of transcription of sigma-F-dependent genes, leucine zipper motif	Sporulation
50	G6PI (P80860)	*pgi* (Z93936)	Glucose-6-phosphate isomerase	Carbohydrate transport and metabolism (glycolysis, gluconeogenesis)
51	YQBP (P45932)	*yqb*P (D32216)	Unknown; similar to phage-related protein	Phage-related functions
52	YACH (P37569)	*yac*H (D26185)	Unknown	–
53	MOBB (O31704)	*mob*B (AF012285)	Molybdopterin-guanine dinucleotide biosynthesis	Metabolism of coenzymes and prosthetic groups

Table 3. Comparison of the functional systems of the *Bacillus* sp. in protein-coding genes using the COG and SubtiList databases. The pathway and functional systems are classified from COG database using COGNITOR program

Functional systems	*B. subtilis*	*B. halodurans*	*B. stearothermophilus* TLS33[a)]
Information storage and processing			
1. Translation, ribosomal structure and biogenesis	152	153	3 (*inf*B, *yhb*H, *ybb*B)
2. Transcription	272	269	2 (*nus*G, *rsf*A)
3. DNA replication, recombination and repair	131	227	2 (*top*A, *xtm*A)
Cellular processes			
1. Cell division and chromosome partitioning	31	32	1 (mrp)
2. Posttranslational modification, protein turnover, chaperones	87	84	2 (*msr*A, *yqh*T)
3. Cell envelope biogenesis, outer membrane	161	115	6 (slp, *spo*IIIAE, *pon*A, *tag*E, *yqiZ*, *rsf*A)
4. Cell motility and secretion	90	90	3 (*lep*A, *fli*T, *yvy*F)
5. Inorganic ion transport and metabolism	148	146	2 (*fhu*C, *opu*CA)
6. Signal transduction mechanisms	122	135	–
Metabolism			
1. Energy production and conversion	164	158	–
2. Carbohydrate transport and metabolism	271	262	3 (*suh*B, pgm, pgi)
3. Amino acid transport and metabolism	293	284	6 (*prs*A, *his*H, *cys*H, *lys*C, *lev*E, *ypw*A)
4. Nucleotide transport and metabolism	82	73	2 (*prs*A, *gua*B)
5. Coenzyme metabolism	109	109	5 (*fhu*C, *cys*H, *mob*B, *opu*CA)
6. Lipid metabolism	84	89	–
7. Secondary metabolites biosynthesis, transport and catabolism	128	110	–
Poorly characterized			
1. General function prediction only	332	329	3 (*com*FC, *ybb*B, *rsb*T)
2. Function unknown	226	246	5 (*ypb*G, *yce*B, *yqg*S, *yhb*H, *yla*L)
Not in COGs	1221	1155	–

a) Some identification

under conditions of phosphate starvation since the cold-stress regulon was under the control of the alternative signaling pathway of σ^B [54–56]. In the third route, YbbB activates FeuA to form a complex with RsbW, leading to release the σ^B from RsbW-σ^B complex and subsequently convert to σ^F or σ^G in the forespore (Fig. 5C). In the fourth route, RsfA is also activated when the bacterium is cold-shocked at 37°C or 25°C. RsfA can directly activate σ^F/σ^G in the forespore (Fig. 5D). The activation of σ^F in the forespore could lead to transcription of SpoIIR and SpoIIQ, and disrupt, without preventing, the formation of stress-resistant spores. σ^G could then activate transcription in the engulfed forespore of a large set of genes [40, 57]. In the fifth route, MrpA and PyrC proteins are correlated in the signaling pathway of bacterial sporulation, in which they are down-regulated when the bacterium is cold-shocked at 37°C or 25°C (Fig. 5E). Both of MrpA and PyrC indirectly activate a transcription factor σ^H, leading to σ^F/σ^G production in the forespore stage. The MRP protein homolog is the multi-resistance protein and functions as an Na^+/H^+ antiporter in pH homeostasis, by which it could influence to the post-translational regulation control of σ^H in the early sporulation in cell cycle [58, 59]. On the other hand, PyrC or dihydroorthase could function in the regulation of pyrimidine biosynthesis and the control of gene expression [60]. According to the correlation of the cold-shock-induced proteins in signaling pathway of sporulation,

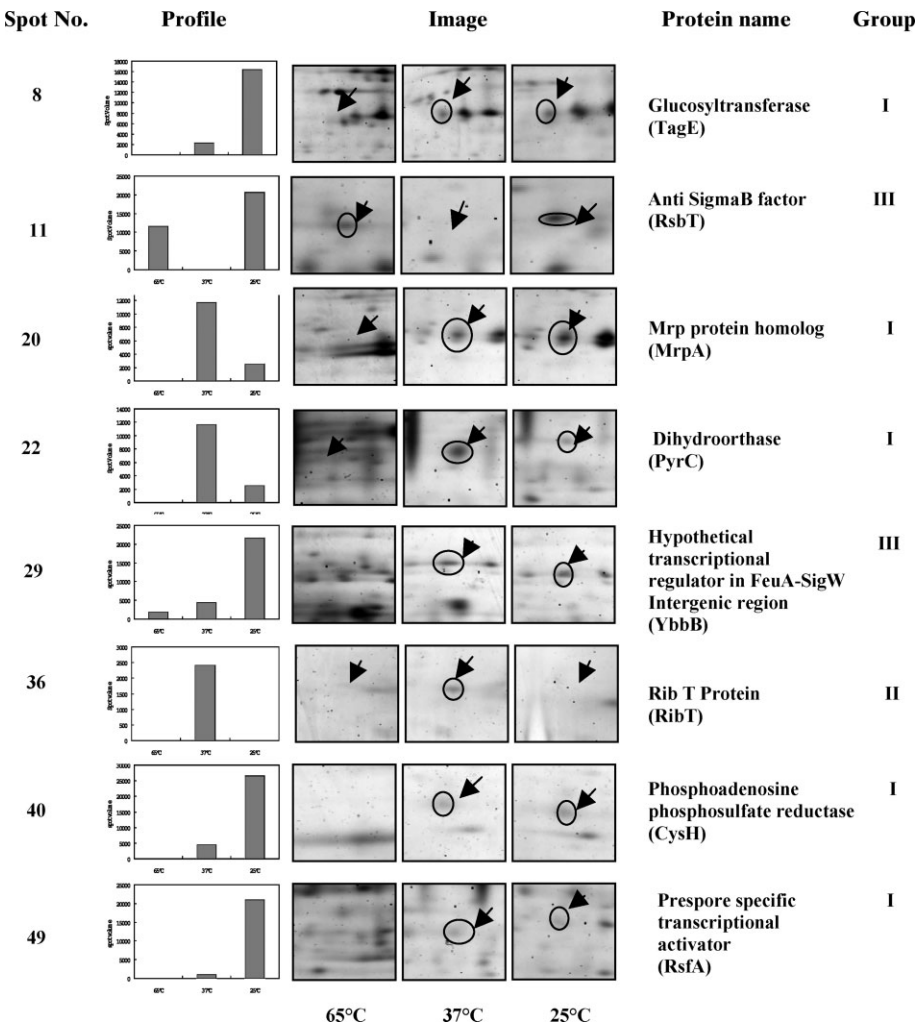

Figure 4. Image analysis of eight major cold-shock-induced proteins of *B. stearothermophilus* TLS33 under cold-shock stress using ImageMaster 2D Elite software. The arrows on 2-D gels show the situation of protein spots. Three groups of cold-shock-induced proteins are classified by ImageMaster 2D Elite software. (I, protein synthesis at 37°C and 25°C; II, protein synthesis at 37°C; III, protein synthesis at 65°C and 25°C).

we presume that up- and down-regulation of these proteins are involved in the σ^F/σ^G production in the forespore (Fig. 6). Thus, the forespore is also considered as an event proceeding bacterial sporulation in the environmental cold-shock stress.

These observations demonstrate the correlation of six major cold-shock-induced proteins when the bacterium encounters cold-shock stress (Fig. 7). TagE, YbbB, RsfA and RsbT, the upstream proteins, are activated in the low temperature at 37°C or 25°C, whereas MrpA and PyrC are inactivated, representing downstream proteins. Surprisingly, RsbT is observed at 65°C and 25°C, but not at 37°C. We suggest that normally, RsbT would be inactive at 65°C, the optimal temperature for this bacterium, and associated with a negative regulator RsbU [49]. To explain the fact that RsbT is not observered by proteomic analysis at 37°C, two hypotheses can be proposed, linking RsbT to upstream and downstream modules of the sporulation signaling pathway. First, the 2-h cold-shock stress at 37°C may initiate the bacterial shock response in which RsbT can form a complex in the upstream module or be subjected to the translational regulation [46]. Second, the temperature at 37°C may not be low enough to induce this cold-shock-induced protein [61]. Although RibT and CysH can be observed at 37°C and 25°C, they are not involved in the signaling pathway of sporulation. RibT protein generally functions as a reductase enzyme in riboflavin or vitamin B_2 biosynthesis and reduction metabolism [62, 63]. However, the function of RibT, which preferentially appeared at only 37°C, has not yet been clarified in *Bacillus* sp. and other bacterial species [64–69]. Likewise, CysH protein, observed at the low temperatures, has also been demonstrated to be involved in the regulation of the sulfur starvation [70]. Thus, RibT and CysH are not part of this signaling pathway. Although other related proteins involved in the TagE, YbbB, RsfA, MrpA and PyrC routes at 65°C can not be identified, this may be due to the limitation of the proteomic analysis in this study. This failure may be caused by post-translational modifications, such as glycosylation or phosphorylation, or by the regulation of gene expression; for example, *tag*E, *ybb*B and *rsf*A genes are turned on at 37°C, while *mrp*A and *pyr*C genes are turned off. If this proposal is valid, it is necessary to determine the unknown genes involved in the upstream module of the

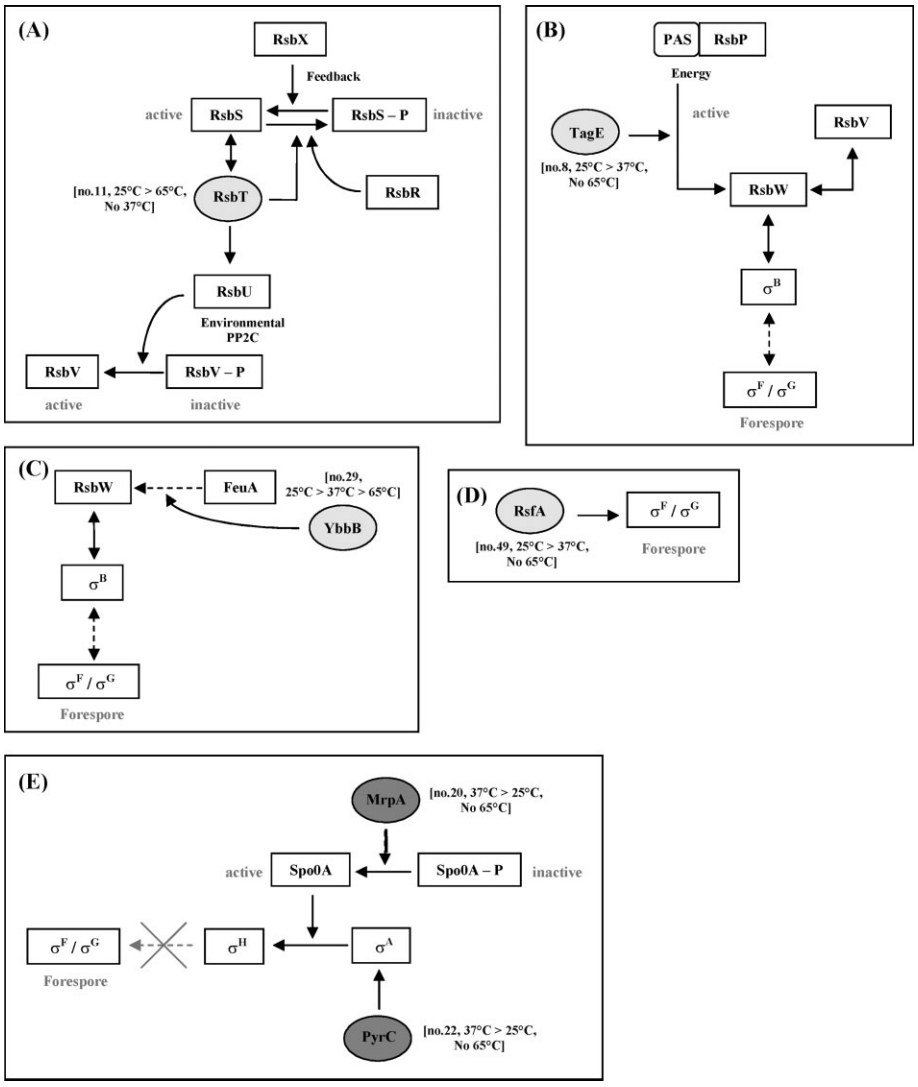

Figure 5. Correlation of the cold-shock-induced proteins in signal transduction pathway of *B. stearothermophilus* TLS33 sporulation under cold-shock stress. (A) RsbT route; (B) TagE route; (C) YbbB route; (D) RsfA route; (E) MrpA and PyrC routes.

signaling pathway. The proteome of *B. stearothermophilus* TLS33 under cold-shock stress is being studied further to obtain a more complete understanding of the biological events.

4 Concluding remarks

In summary, this is the first report of the thermophilic bacterium *B. stearothermophilus* TLS33 proteome, which correlates the signaling pathway of bacterial sporulation, under cold-shock stress. Using a proteomic approach combining 2-DE and MALDI-TOF analysis, individual proteins were identified and shown to have biological functions in the metabolic system of bacterial cell adaptation. Interestingly, six cold-shock-induced proteins were shown to correlate with the sigma B protein, which plays an important role in the signal transduction pathway of sporulation in this bacterium. Thus, this study adds to our understanding of bacterial adaptation under cold-shock stress. However, this thermophile needs to be studied further both with regard to its the biological functions and mechanisms, also at the gene expression level, under different stresses, as well as to the proteins that play an important role in the industrial and pharmaceutical applications.

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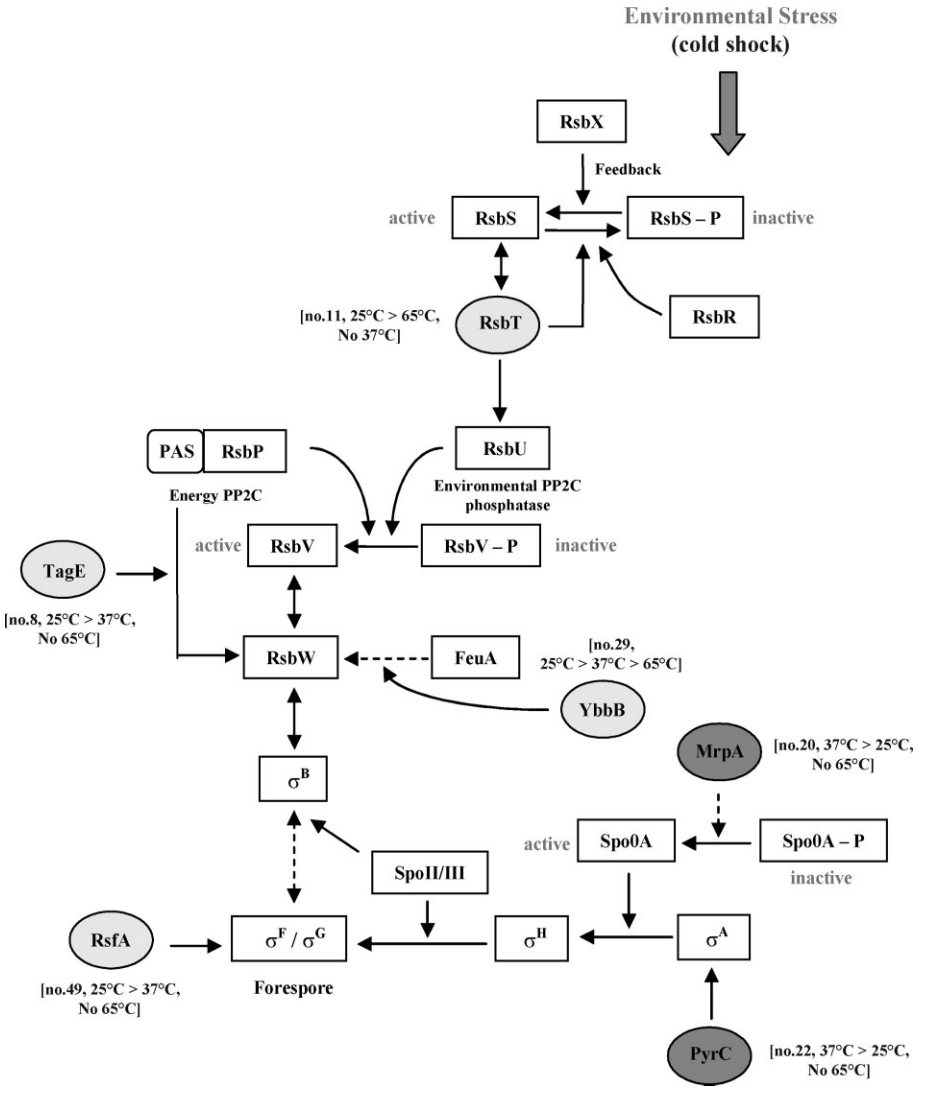

Figure 6. Model summarizing the signal transduction pathway of *B. stearothermophilus* TLS33 sporulation under cold-shock stress.

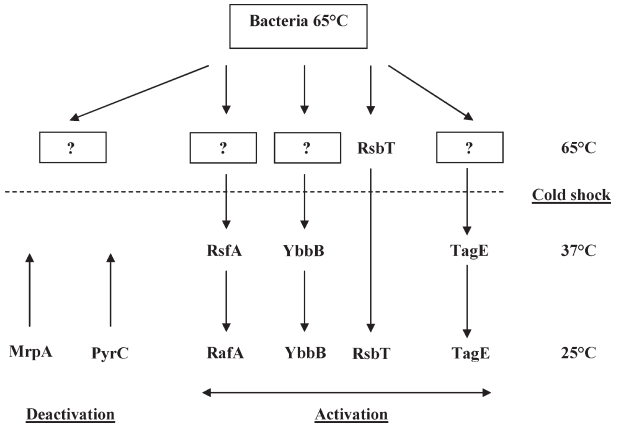

Figure 7. Up- and down-regulations of the six major cold-shock-induced proteins when *B. stearothermophilus* TLS33 encounters cold-shock stress. The direction of arrows represents the change of the protein expression.

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