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Proteomic Analysis by Two-Dimensional Differential Gel Electrophoresis (2D DIGE) of a High-Pressure Effect in *Bacillus cereus*

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High hydrostatic pressure (HHP) is a new method used to reduce or eliminate microorganisms that are present in food. Proteins are known to be the most important target of high pressure in living organisms. The main goal of this investigation was focused on the changes that occur on the proteins of *Bacillus cereus* under HHP stress conditions. The two-dimensional differential gel electrophoresis (2D DIGE) technique allows for a simultaneous resolution of thousands of proteins based on fluorescent prelabeling of the samples with spectrally resolvable fluorescent CyDyes. The results of proteomics profiling show an average of 1300 spots being detected. The analysis revealed 75 spot proteins whose abundance is modified after the application of high pressure, of which 66 were decreased after the HHP treatment. Among them, flagellin was the protein that changed the most. The differential expression of some proteins after HHP treatment at 700 MPa may suggest a reduction of virulence and protective response against oxidative stress in flagellated *Bacillus*.

KEYWORDS: *Bacillus cereus*; DIGE; high hydrostatic pressure; mass spectrometry

INTRODUCTION

Currently, there is a trend in the food industry toward the use of other methods of food preservation that are different to the conventional ones, such as heating; the purpose of these new preservation methods is presenting foods with the sensorial properties that are closer to the raw product to consumers. At the same time, it remains of crucial importance that the final product is pathogen-free food. The use of high hydrostatic pressure (HHP) technology as a new method for food preservation has been under research in the last 2 decades. The Japanese pioneered the preservation of foods (yogurt, jam, etc.) under HHP. Today, pressurized products (dry and cooked ham, oyster juice, etc.) are developed in many countries. HHP inhibits the growth of microorganisms by inactivating key enzymes and modifying both the microbial cell walls and membrane. A HHP above 300 MPa is generally effective in reducing the capability of growing in most vegetative bacteria, yeast, and molds. However, Gram-positive bacteria, such as *Bacillus cereus*, are quite resistant to this treatment and are capable of resisting pressures above 1000 MPa at 25 °C (*1*). Pressure on its own is not effective in killing *B. cereus*, and thus, the combination of pressure and heat is required. At present, some available data indicate that endospores of *Bacillus* and *Clostridium* species are inactivated by pressure treatments ranging from 600 to 700 MPa at temperatures between 60 and 80 °C, after 16 min of pressure holding time (*2*, *3*). However, to preserve the sensorial properties in some pressurized foods, such as fruit and vegetables, it is necessary to keep the temperature at around 30 °C or lower.

The primary aim of treating food with HHP in most cases is to reduce or eliminate the relevant food-borne microorganisms that may be present. Proteins are known to be the most important target of high pressure in living organisms (*4*). In general, the lethal effect of HHP includes a number of different processes particularly related to the damage of key enzymes that are involved in DNA replication and transcription (*5*).

The main goal of this research is therefore focused on the changes that occur in the proteins of *B. cereus* under stressful conditions. Two-dimensional gel electrophoresis (2D GE) is a powerful technology for protein abundance studies. It allows simultaneous resolution of thousands of proteins. The principle of 2D GE is based on the separation of proteins according to their charge in the first dimension by isoelectric focusing (IEF) and then to their size in the second dimension by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Despite being a well-established technique for protein analysis, traditional 2D GE is time-consuming and labor-intensive. Many gels have to be run, analyzed, and compared. In addition, the lack of reproducibility between gels leads to significant system variability, making it difficult to distinguish between system variation and induced biological change.

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The Unlu et al. (*6*) research group were the first to describe a method, the two-dimensional differential gel electrophoresis (2D DIGE), based on fluorescent prelabeling of the samples with spectrally resolvable fluorescent CyDyes (Cy2, Cy3, and Cy5; GE Healthcare). The use of fluorescent dyes not only increases the sensitivity and the dynamic range of the detection but also allows loading up to three different samples in the same 2D GE. The use of the pool internal standard sample allows for a reduction of intergel variations and diminishes false positives, thereby improving the accuracy of the image analysis comparison.

We have used 2D DIGE technology to study the effect of HHP on the proteomics profiling of *B. cereus*. We have quantified differences and identified some of the proteins that are differentially expressed. The profiling analysis revealed protein patterns that modified their abundance after the treatment and were grouped into different clusters.

MATERIALS AND METHODS

Sample Preparation. The *B. cereus* (CECF 148) strain was obtained from the Spanish type culture collection. Four cultures of 200 mL were grown in brain heart infusion (BHI) at 30 °C of a stock from an isolated colony. The samples were centrifuged for 10 min at 5000*g*. The supernatant was discarded, and the pellet was suspended in peptone. Aliquots of 2 mL were prepared in vials for the HHP treatment.

An experimental machine (Unipress Equipment Division, Warsaw, Poland) was used to pressurize the samples at 700 MPa for 30 min, and the high pressure vessel used was thermo-regulated at 20 °C. Both pressure and temperature were monitored by a pressure gauge (type PR811, Asco Instruments, Chateaufort, France) and a Laboratory note book program (Laboratory Technologies Corp., Wilmington, MA) interfaced to a personal computer. Parts **A** and **B** of **Figure 1** depict the conditions of the treatment (temperature/time and pressure/time) of two replicates. The pressure was reached in 73 s; the holding time was 30 min; and the release to the atmospheric pressure was achieved in 140 s.

After the treatment, the samples remained at 4 log units/mL and were centrifuged at 10000*g* for 10 min. The proteins were extracted from the *Bacillus* pellet with extraction buffer [30 mM Tris at pH 8.5, 7 M urea, 2 M, thiourea, 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), proteases inhibitor (Roche)] and precipitated with 10% TCA and acetone (at −20 °C), after which they were suspended in the extraction buffer (without protease inhibitors).

Protein Labeling. Four biological replicates were used from the control and treated samples, thus generating eight individual samples. The samples were distributed across four DIGE gels, with the internal standard pooled sample also present, according to the experimental design unique to this technique (**Figure 2A**).

Proteins in each sample were fluorescently labeled following the protocol of the manufacturer. Briefly explained, 400 pmol of Cy dye in 1 μL of anhydrous *N*,*N*-dimethylformamide (DMF, Sigma) was used per 50 μg of protein. After 30 min of incubation on ice in the dark, the reaction was quenched with 10 mM lysine for 10 min.

Two-Dimensional Gel Electrophoresis and Image Acquisition. Labeled samples were combined according to the experimental design, and an equal volume of 2× hydratation buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (w/v) dithiothreitol (DTT), and 4% pharmalytes at pH 3−11) was added for the cup loading process.

The 2DE was performed using GE Healthcare reagents and equipment. For the first dimension, 24 cm IPG strips in the pH range of 4−7 were used. They were previously hydrated overnight with 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 100 mM DeStreak, and 2% pharmalytes at pH 4−7. IEF was performed at 20 °C using the following program: 120 V for 1 h, 500 V for 1 h, 500−1000 V for 2 h, 1000−4500 V for 6 h, and 4500 V for 12 h. Subsequently, strips were equilibrated for 12 min in reducing solution [6 M urea, 50 mM Tris-HCl at pH 6.8, 30% (v/v) glycerol, 2% (w/v) SDS, and 2% (w/v) DTT] and then for 5 min in alkylating solution [6 M urea, 50 mM Tris-HCl at pH 6.8, 30% (v/v) glycerol, 2% (w/v) SDS, and 2.5% (w/v) iodoacetamide]. The second-dimension SDS−PAGE was run on homogeneous 10% T and 2.6% C polyacrylamide gels casted in low-fluorescent glass plates. Electrophoresis was carried out at 20 °C, 2 W/gel for 18 h, using an Ettan-Dalt six unit.

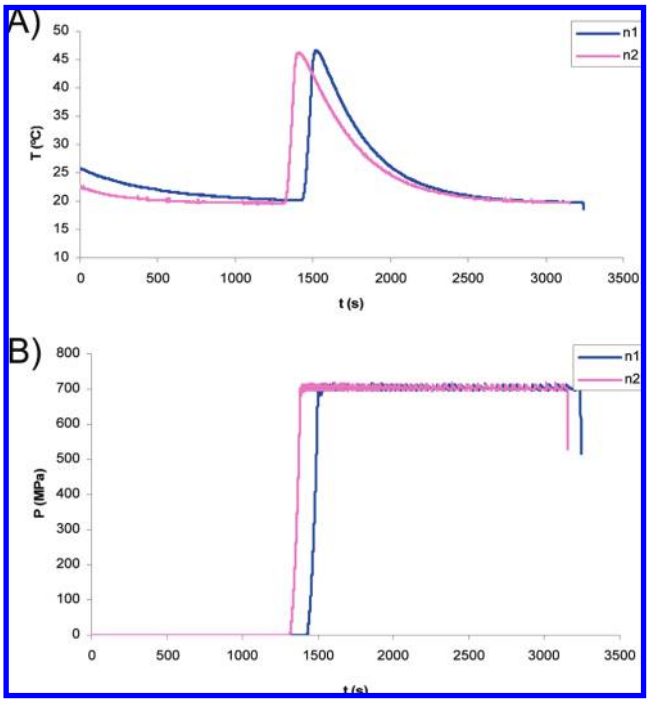

Figure 1. (A) Temperature and (B) pressure conditions of the high-pressure treatment of *B. cereus*.

Proteins were visualized using a Typhoon 9400 scanner (GE Healthcare) with CyDye filters. For the Cy3, Cy5, and Cy2 image acquisition, 532/580, 633/670, and 488/520 nm excitation/emission wavelengths were used, respectively, and acquired with a 100 μm pixel size.

DIGE Data Analysis. Image analysis was carried out with DeCyder differential analysis software version 6.5 (GE Healthcare) (*7*). The differential in-gel analysis (DIA) module was used to assign spot boundaries and to calculate parameters such as normalized spot volumes. The intergel variability was corrected by matching and normalizing it with the internal standard spot maps in the biological variation analysis (BVA) module. A control versus treated comparison was carried out. The average ratio and unpaired Student's *t* test were also calculated. To reduce the false positives in the *p*-value calculation, the false discovery rate (FDR) was applied (*8*). Those protein spots with 1.5-fold as a threshold in the average ratio and with *p* values under 0.05 were considered to be differentially expressed with statistical significance in the comparison. An unsupervised multivariate analysis was performed using the extended data analysis (EDA) module. Proteins of interest were used as a subset for this analysis. A principal component analysis (PCA) was performed following the nonlinear interactive partial least-squares method. The hierarchical clustering analysis (HCA) used Euclidean and average linkage for distance calculation. The number of K-means clustering was established as 4, when taking into account the HCA results. The Euclidean distance was selected as the similarity measure.

Protein Identification by Mass Spectrometry. The total protein profile was detected by staining the DIGE gels with

A)

	Cy5	Cy3	Cy2
Gel 1	Control 1	Treated 4	Internal Standard
Gel 2	Control 2	Treated 1	Internal Standard
Gel 3	Treated 2	Control 3	Internal Standard
Gel 4	Treated 3	Control 4	Internal Standard

B)

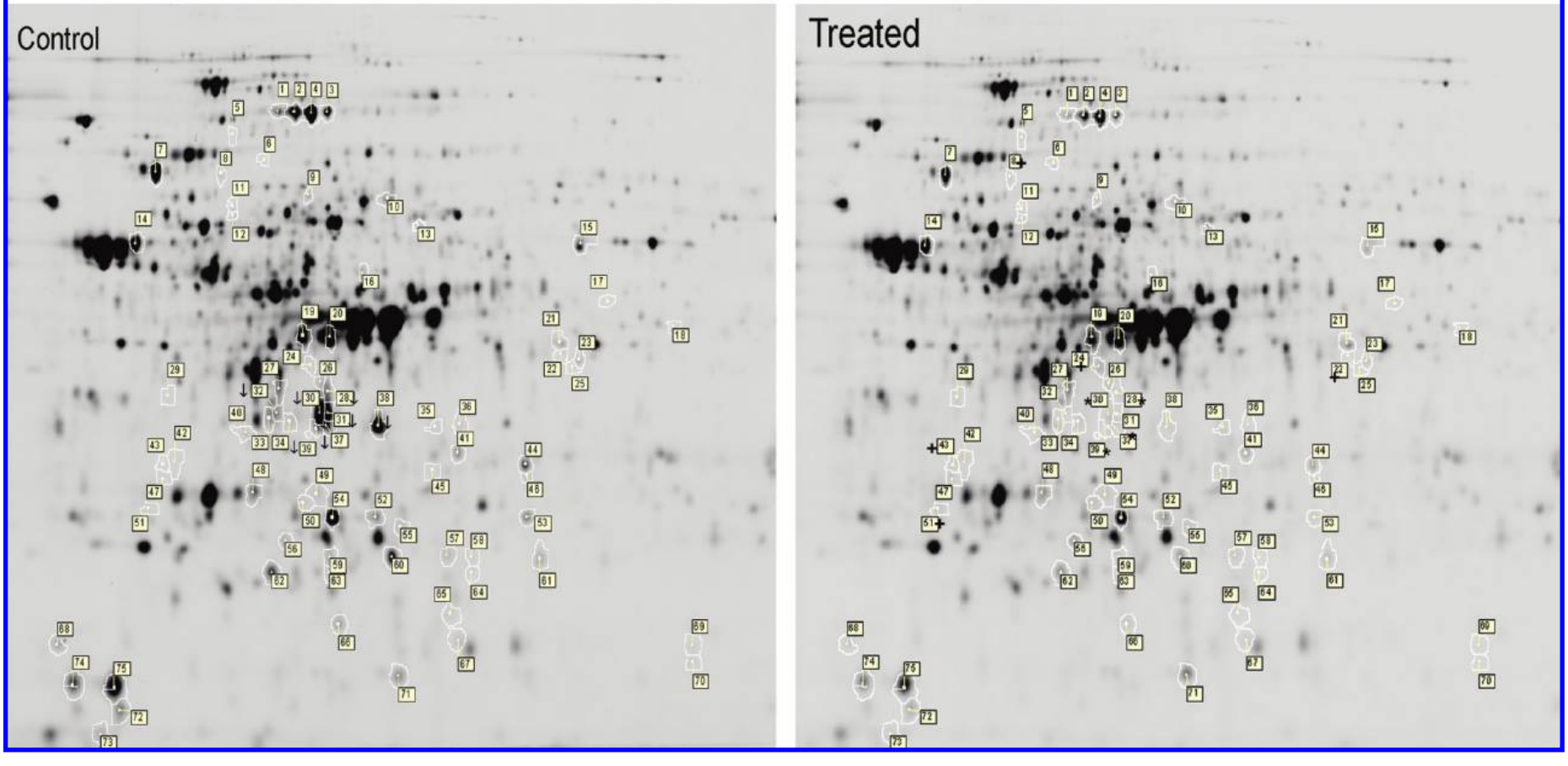

Figure 2. Experimental design and 2D DIGE maps of the control and HHP-treated samples of *B. cereus*. (**A**) Experimental design indicating the CyDye labeling in each of the four replicates for each condition (indicated as "1−4") and the samples that are mixed in each of the gels. (**B**) Proteins differentially expressed are shown in the gels by the spot master number. Special protein spots have been highlighted (∗, protein spots that disappear after HHP treatment; +, protein spots that appear after HHP treatment; ↓, 10-fold or greater decrease).

Colloidal Coomassie Blue (CCB). The changes observed by 2D DIGE analysis were aligned with CCB profiling, and spots considered to be of interest were excised and in-gel-digested (*9*). Briefly, they were then washed with water and dehydrated with 75% acetonitrile (ACN). They were reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide. Finally, samples were digested with 12.5 ng μL^{-1} sequencing-grade trypsin (Roche Molecular Biochemicals) in 25 mM ammonium bicarbonate (pH 8.5) overnight at 37 °C. After digestion, the supernatants were collected and 1 μL was spotted onto a matrix-assisted laser desorption ionization (MALDI) target plate and allowed to air-dry at room temperature. Then, 0.5 μL of a 3 mg mL^{-1} of α-cyano-4-hydroxycinnamic acid matrix (Sigma) in 50% ACN and 0.1% trifluoroacetic acid (TFA) was added to the dried peptide-digested spots and allowed to air-dry again.

Mass spectrometry analyses were performed in a MALDI−TOF/TOF spectrometer 4700 proteomics analyzer (PerSeptives Biosystems, Framingham, MA). The instrument was operated in reflector mode, with an accelerating voltage of 20 000 V. All mass spectra were internally calibrated using autodigested trypsin peptides. The mass fingerprint spectra with a signal-to-noise ratio greater than 20 were collated and represented as a list of monoisotopic molecular weights. Some proteins were subjected to MS/MS sequencing analyses to be more confident of the identification. The suitable precursors for MS/MS sequencing analyses were selected, and fragmentation was carried out using the acquisition method in the 1 kV ion reflector mode, collision induced dissociation (CID) on, and precursor mass window ±10 Da.

For protein identification, the nonredundant National Center for Biotechnology Information (NCBI) database (Feb 27, 2008; 6 189 142 sequences; 2 116 873 858 residues) was searched using MASCOT 2.1 (www.matrixscience.com) through the Global Protein Server version 3.5 (Applied Biosystems). The Mascot search parameters were (1) species, all; (2) allowed number of missed cleavages, 1; (3) fixed modification, carbamidomethyl cystein; (4) variable modifications, methionine oxidation; (5) peptide tolerance, ±50−100 ppm; (6) MS/MS tolerance, ±0.3 Da; and (7) peptide charge, +1. In all identified proteins, the probability score was greater than the one fixed by Mascot as being significant, that is, a p value under 0.05. The parameters for the combined search (peptide mass fingerprint and MS/MS spectra) were the same as described above.

RESULTS AND DISCUSSION

Identification of High-Pressure Protein Expression Changes in *B. cereus*. In the field of food preservation, the most commonly encountered stresses in food are high or low temperature, high salt, and low pH. The stress response to each of these factors has been characterized in some detail for important food-borne pathogens, such as *Salmonella*, *Escherichia coli*, and *Lysteria monocytogenes*. However, a number of new techniques for food preservation that cause other types of stress are emerging. In this study, we focus on the treatment by high hydrostatic pressure and its impact on the protein abundance in *B. cereus* cells.

The *B. cereus* bacteria was chosen because of a previous work (data not shown), which described the growth capacity of *Bacillus* after 700 MPa as remaining at about 20%. According to Préstamo et al. (*1*), *B. cereus* can resist pressures of up to 1000 MPa.

For the differential expression analysis, we used DIGE technology to compare the control and HHP-treated bacteria in four biological replicates of *B. cereus*. The 2D DIGE technique enables the use of an experimental design that would not be possible using the traditional 2D GE. A key aspect of this experimental design is the internal standard sample. It was formed by mixing aliquots of every individual sample, which were all included in equal amounts in the experiment. The internal standard sample was then labeled with Cy2 dye and run on each gel. After this, there is a reciprocal labeling of the samples. Half of the samples of each condition were labeled with Cy3 dye, and the other half were labeled with Cy5 dye, to avoid any possible bias derived from the labeling efficiency. To conclude, two samples from different replicates (Cy3 and Cy5) and an aliquot of the internal standard pool (Cy2) were separated by 2DE in each one of the four gels. A scheme with the experimental design and the distribution of the samples in the 2D DIGE is shown in **Figure 2A**.

The proteomic profiles show an average of 1600 spots detected in the DIA module of DeCyder software. A total of 1300 spots were matched in the BVA module. This study reveals a group of proteins that undergoes modifications after pressurization. We detected 76 protein spots that showed differential ratios with a 1.5-fold or greater change in abundance and consistent differences between the two conditions (Student's t test, $p < 0.05$) (see **Figure 2B**). A total of 57 protein spots decreased in abundance in the treated samples. They represent $^2/_3$ of the total protein spots that changed their abundance after HHP treatment. Four of these spots disappeared completely (labeled with ∗ in **Figure 2B**), and seven of them showed a 10-fold or greater decrease in abundance after high-pressure application (labeled with ↓ in **Figure 2B**). A total of 19 protein spots increased in abundance in the treated samples; a total of 5 of them appeared after pressure treatment (labeled with + in **Figure 2B**).

Among these 76 protein spots, the 19 most intense protein spots were excised from 2D GE gels staining with CCB. The excised spots were subjected to tryptic digestion and peptides, and the results were analyzed by mass spectrometry (MALDI−TOF/TOF). **Table 1** summarizes those identified proteins with the average ratio and the statistical data from each comparison as well as the data from Mascot identifications. These proteins were classified according to the biological functions as described in the protein knowledge base (UniProtKB) (www.uniprot.org).

It is well-known that pressure exerts different effects on organisms. Some of the key pressure-sensitive processes that have been described in *E. coli* include motility, substrate transport, cell division, growth, DNA replication, translation, transcription, and viability (*10*). We have observed that the treatment by HHP has different effects on proteins involved in different biological processes. Our data show that phosphofructokinase and piruvate kinase, two enzymes that play an important role in glycolysis, change their abundance.

Phosphofructokinase is an important control point in the glycolytic pathway, because it is one of the irreversible steps and has key allosteric effectors, AMP and fructose 1,6-biphosphate. Phosphofructokinase converts fructose 6-phosphate into fructose 1,6-biphosphate. Piruvate kinase catalyzes the last step of glycolysis: it converts phosphoenolpyruvate into pyruvate and ATP. Decreased pyruvate kinase activity and increased phosphofructokinase activity result in an accumulation of phosphoenolpyruvate (*11*). In *B. cereus*, phosphoenolpyruvate can be converted to oxaloacetate and it can enter the tricarboxilic acid cycle via phosphoenolpyruvate carboxylase activity (*12*). Therefore, a much higher phosphoenolpyruvate concentration is thought to favor its diminution via tricarboxilic acid cycle for further processing. We suggest alternative routes for glucose use

Table 1. Summary of the Identified Proteins, Classified According to the Biological Function, with the Average Ratio and Statistical Data from the Comparison, the Data from Mascot Identifications, and the Number of the Cluster Where Each Spot Has Been Grouped

master number	protein ID	name	average ratio[a]	*t* test[a]	score[b]	number of peptides[c]	percent coverage[d]	cluster[e]
		Carbohydrate Catabolic Process						
2	gil30022674	pyruvate kinase	−2.29	0.015	252	65/27	62	1
3	gil30022674	pyruvate kinase	−2.16	0.014	187	65/24	47	1
4	gil30022674	pyruvate kinase	−2.26	0.011	281	65/30	63	1
21	gil47530139	phosphofructokinase	1.71	0.0096	78	23/4	17	3
48	gil168134547	deoxyribose−phosphate aldolase	−1.85	0.015	99	22/7	51	1
		Amino Acid Biosynthetic Process						
20	gil30018617	carbamate kinase	−2.14	0.016	219	65/19	80	1
		Nucleotide Metabolic Process						
14	gil30022172	pyrimidine−nucleoside phosphorylase	−1.66	0.022	171	65/18	59	1
71	gil30019663	nucleoside diphosphate kinase	1.89	0.0096	137	22/6	33	3
56	gil30260255	hypoxanthine−guanine phosphoribosyltransferase	1.63	0.028	131	37/5[f]	26	3
		Cellular Protein Metabolic Process						
19	gil30021914	elongation factor Ts	−1.9	0.032	219	65/24	67	1
		Carbohydrate Transport						
60	gil30023351	PTS system; glucose-specific IIA component	−4.53	0.0037	109	19/7	46	1
		Ciliary or Flagellar Motility						
30	gil30019803	flagellin	−30.24	0.0013	145	35/18	50	4
38	gil30019805	flagellin	−41.25	0.0013	154	48/17	47	4
32	gil30019803	flagellin	−18.21	0.011	186	37/5[g]	25	4
15	gil30019797	flagellar hook protein FlgE	−3.16	0.0075	89	31/16	54	1
		Protein Refolding						
7	gil30018515	chaperonin GroEL	−2.36	0.028	327	65/30	62	1
		Unknown						
41	gil30022069	tetrahydrodipicolinate *N*-acetyltransferase	−1.59	0.022	166	37/5[h]	30	1
54	gil34395846	superoxide dismutase [Mn] 1	−1.64	0.016	347	82/8	57	1
44	gil47567981	phage shock protein A, putative	−2.99	0.016	87	22/7	51	1
62	gil29899113	hypothetical protein BCp0002	−2.48	0.012	226	47/6[i]	40	1

[a] Average volume ratio treated/control and *t* test *p* value, quantified by DeCyder BVA module. [b] Score: Mascot MS protein score or MS/MS, obtained from MALDI−TOF/TOF spectra. In all of the cases, a probability score of <0.05 was obtained. [c] Number of peptide mass values searched/matched. [d] Amino acid sequence coverage for the identified proteins or secuence identified by MS/MS. [e] Number of the cluster in the EDA module. [f] Combined identification with MS/MS sequence and score [IVTLLDKPTGR(28) and ILKDLDTSVEGR(58)]. [g] Combined identification with MS/MS sequence and score [TGDAALGSISNILLR(64) and SINSAADDAAGLAIATR(92)]. [h] Combined identification with MS/MS sequence and score [SGVLFGEWSEIK(29), HIVDYVVENDRR(21), and GAVVAAGAVVTEDVPPYTVVAGTPAR(76)]. [i] Combined identification with MS/MS sequence and score [EVFFSSIQATDR(58), EVFFSSIQATDRK(61), and MYNAINSSENALADHR(58)].

after the HPP treatment, coupling glycolysis to the tricarboxilic acid cycle.

Among the identified proteins, flagellin is the one that changes the most. According to Ghelardi et al. (*13*), flagellin is a protein that forms the filament in bacterial flagellum and is present in large amounts in nearly all flagellated bacteria. The flagellum is composed of three parts, basal body, a hook, and a filament (*14*). The basal body anchors the flagellum to the bacterial cell wall and cell membrane. The hook is the flexible coupling structure between the basal body and the filament. The filament is the rod that provides motility. It is made of multiple copies of a flagellin, which is also responsible for eliciting the immunological reaction (*15*). Bacterial swarming is an organized form of multicellular translocation across solid surfaces. Bacteria that are capable of swarming produce highly organized communities initially consisting of vegetative cells. It has been suggested that swarming uses a strategy developed by flagellated microorganisms to ensure their rapid expansion and colonization in their natural environment. *B. cereus* presents swarming motility, which may be coupled with virulence in *B. cereus* (*16*) and is also related to the early stages of infection in different strains of *B. cereus* (*17*, *18*). Ramarao and Lereclus (*19*) describe a relationship between the lack of flagella and the lower production of secreted factors, where FlhA appears to be an essential virulence factor.

Flagellin has been associated with the increase of a protective effect against oxidant stress, by increasing Hsp25 in *Salmonella* (*20*). Taking into account all of these data, the reduction in the abundance of flagellin, and even the disappearance of some isoforms of this protein, our study may suggest that the effect of HHP in the viability of *B. cereus* causes a diminution in virulence and its ability to respond to this stress.

Another one of the identified proteins in *B. cereus* was superoxide dismutase (Sod). Sods have been considered to be key enzymes against oxidative stress. Four classes of superoxide dismutases have been defined according to their metal cofactor, which can be either iron, manganese, cooper/zinc,

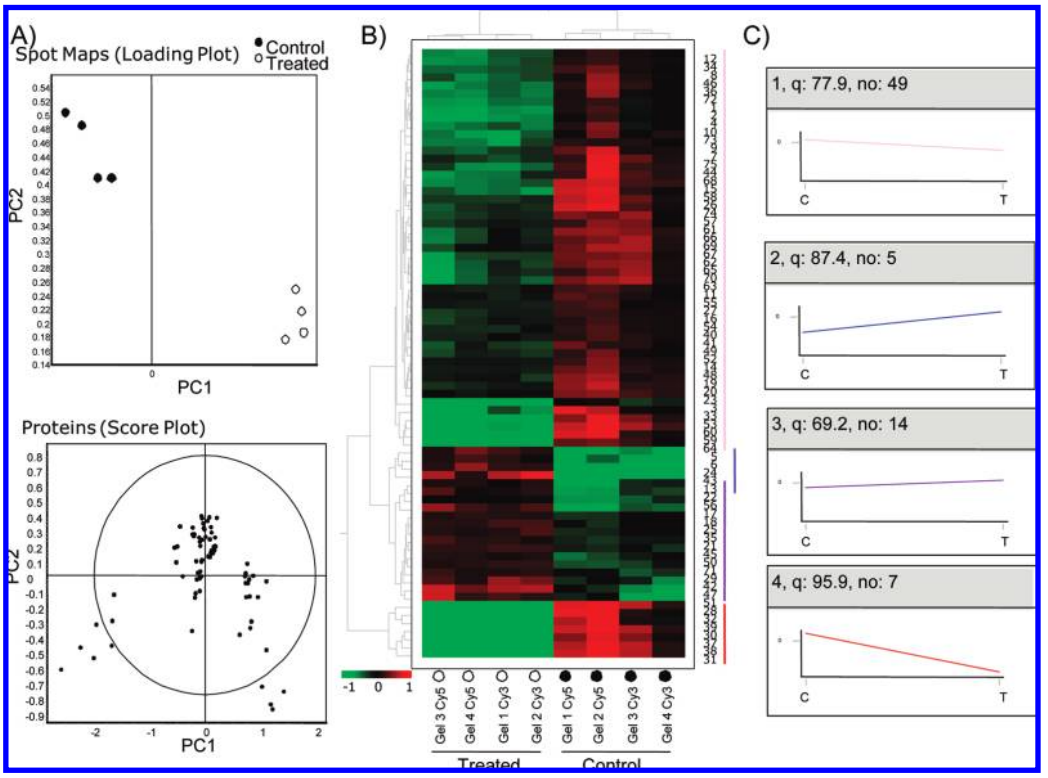

Figure 3. Unsupervised multivariate analysis of data from the 2D DIGE experiment. (A) PCA loading and score plots show the clustering of the eight individual Cy3- and Cy5-labeled DIGE spot maps and the subset of proteins whose ratios varied 1.5-fold or more and in which $p < 0.05$ respectively, in the two principle components. (B) Hierarchical clustering settings are Pearson distance measurements and average linkage. The dendrogram of eight individual spot maps clustering is shown at the bottom, and that of individual proteins is shown on the left, with relative expression values being displayed in a heat map. (C) K-means validated score was 0.314. For each cluster quality measure (q), the number of spot proteins in the cluster is displayed.

or nickel (*21*). Among the others Sods, the MnSods play a major role in protection against oxidative stress in *B. cereus*. Two distinct MnSods have the antioxidant function in *B. cereus*, but MnSod2 encoded by *sodA2* plays a more important role in antioxidant stress than MnSod1 as *sodA1*, which is expressed at a lower level. The MnSod found in this investigation matches with the *sodA1* more than *sodA2* (*22*). Our data show a diminution of MnSod that could be justified mainly by a metabolism reduction in the active population (*23*) and by cellular death (80%), processes that are known to occur in HHP treatment (*24*). It seems that HHP treatment could reduce the protective effect against oxidative stress. More studies, including the identification of new proteins related to HHP stress, are needed to elucidate the relationship between the endogenous oxidative stress and the cellular high-pressure resistance.

In general, we could say that the effect of HHP may specifically damage different processes that are responsible for the protective effect that the cell develops for a stress situation.

Clustering Analysis Based on 2D DIGE Patterns. To obtain an in-depth study of the data obtained in the differential expression analysis from the 2D DIGE experiment, a pattern analysis was carried out to find protein and spot maps with similar abundance profiles using multivariate methods.

The principal component analysis (PCA) reduces the dimensionality of the multidimensional analysis and displays the two principal components that distinguish the two largest sources of variation within the data set. In **Figure 3A**, the data sets from the four replicates were grouped together with the first component (PC1) in the loading plot. In the score plot, nine protein spots were detected out of the ellipse with a 95% significance level. These outliers correspond to the most strongly differentiated spots in abundance: 31 (−68.22), 38 (−41.25), 30 (−30.24), 37 (−28.2), and 39 (−18.41) in the left-down quadrant and 43 (7.14), 24 (5.65), 5 (4.64), and 6 (3.74) in the right-down quadrant of the score plot (**Figure 3A**).

Hierarchical clustering analysis (HCA) is based on similarities of abundance patterns in spot maps and protein spots. When HCA was applied to the abundance profile for each analysis, the control and treated replicates clustered together. The values displayed in an expression matrix (heat map) are represented in a standardized log abundance scale ranging from −1 (green) to +1 (red) (**Figure 3B**).

Another statistical tool, K-means clustering, shows the abundance relationship with the treatment reinforcing the HCA view (**Figure 3C**). A K-mean cluster analysis was used to place the proteins with differing abundance into four clusters. In clusters 1 and 4, protein spots in which abundance decreased in HHP-treated samples were grouped together, with the decrease being higher in cluster 4 than in cluster 1. Clusters 2 and 3 contain the protein spots, in which abundance increased after HHP treatment, with the increment being higher in cluster 3 than in cluster 2. **Table 1** shows the clusters into which identified proteins are grouped.

To summarize, the 2D DIGE approach shows a higher confidence differential protein profile than that obtained by the classical 2D GE strategy. At the conclusion of the present study, 1300 spots have been matched in the 2D DIGE gels and 75 differentially expressed proteins resulted when HHP was applied, of which 66% were decreased after pressurization. Flagellin was the protein that changed the most (by decreasing), which could be related to the loss of virulence in

B. cereus. In general, it could be said that the HHP treatment at 700 MPa produces an important diminution of several of the proteins identified, with this effect being quite remarkable in flagellin.

Some genome level analyses of gene expression at high pressure have already begun. However, proteins are the primary targets in the study of pressure behavior in organisms. The DIGE methodology is a promising approach to attempt to gain a better understanding of the behavior of proteins under HPP.

Extensive studies of all of these differentially expressed proteins are needed to understand the effect of HHP on the microorganisms present in food. The goal of these studies will be to identify new targets to improve food preservation.

ABBREVIATIONS USED

HHP, high hydrostatic pressure; Cy, cyanine dyes; DIA, differential in-gel analysis; BVA, biological variance analysis; EDA, extended data analysis. The DIGE and MALDI-TOF/TOF analysis were carried out in the Proteomic Facility UCM-PCM, a member of ProteoRed network.

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