Effects of high-pressure carbon dioxide on proteins and DNA in *Escherichia coli*

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Protein changes in *Escherichia coli*, when subjected to high-pressure carbon dioxide (HPCD) at 10 MPa and 37°C for 5–75 min, were assessed using the Bradford method, 2D electrophoresis (2-DE) and liquid chromatography-electrospray ionization-MS-MS (LC-ESI-MS-MS). The changes in DNA in *E. coli* under the same conditions were also investigated by using flow cytometry with propidium iodide and acridine orange, agarose gel electrophoresis (AGE) and the comet assay. The results showed that HPCD induced leakage loss of the proteins and DNA of *E. coli* as a function of treatment time. With regard to the protein changes, 182 proteins in the 2-DE profile were not found in the HPCD-treated *E. coli*. Among 20 selected protein spots exhibiting significant changes in intensity, 18 protein spots were identified as 15 known proteins and two as hypothetical proteins. These proteins were involved in cell composition, energy metabolism pathways, nucleic acid metabolism, global stress regulation and general metabolism. The DNA denaturation of *E. coli* induced by HPCD was demonstrated in this study for the first time to our knowledge, and the denaturation was enhanced by increasing treatment time. However, HPCD did not cause DNA degradation, as suggested by both AGE analysis and the comet assay.

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INTRODUCTION

High-pressure carbon dioxide (HPCD) has been proposed as an alternative non-thermal pasteurization technique for foods. So far, over 100 papers using HPCD as a pasteurization method have been published. Most research focuses primarily on the bactericidal effects and inactivation kinetics of HPCD and, to a lesser extent, the mechanisms of microbial inactivation. Some reviews have summarized the possible pasteurization mechanisms of HPCD, such as: (1) a decrease in extracellular and intracellular pH; (2) damage to the cell structure, including loss of cell membrane integrity and removal of vital constituents from cells or cell membranes; (3) the direct inhibition effect of molecular CO₂ and HCO₃[−] on cell metabolism, including key enzyme inactivation; and (4)

disorder of intracellular electrolyte balance (Damar & Balaban, 2006; Garcia-Gonzalez *et al.*, 2007).

Since the growth and multiplication of micro-organisms occurs within a narrow range of physico-chemical conditions, environmental changes, such as heat, cold, pressure, acids, alkalis, salts and ions can affect viability, protein stability and protein expression in microbial cells. The effects of HPCD pasteurization on micro-organisms have been ascribed to the interaction of anaerobic environment, acidification, high pressure and mild heat, and it is a novel and effective stress against micro-organisms in foods, medicines and other materials. The protein changes in micro-organisms subjected to HPCD have not been extensively investigated. Using SDS-PAGE and 2D electrophoresis (2-DE), White *et al.* (2006) demonstrated no appreciable degradation of *Salmonella typhimurium* proteins following HPCD at 35°C and 9.52 MPa for 1 h, but did not identify any protein spots in 2-DE gels. Kim *et al.* (2009) showed that 2-DE images of untreated and HPCD-treated *S. typhimurium* at 40°C and 10.0 MPa for 30 min differed significantly, and 33 spots were found to exhibit

Abbreviations: AGE, agarose gel electrophoresis; AO, acridine orange; 2-DE, two-dimensional electrophoresis; DFI, DNA fragmentation index; FCM, flow cytometry; HPCD, high-pressure carbon dioxide; LC-ESI-MS-MS, liquid chromatography-electrospray ionization-MS-MS; PI, propidium iodide.

large changes in intensity following HPCD treatment. Eleven downregulated protein spots were identified by using MALDI-TOF MS, which were found to be enzymes involved in cell metabolism (Kim *et al.*, 2009). These controversial observations suggest that the effects of HPCD on the proteins of micro-organisms need more investigations. Many proteins involved in cell composition, energy metabolism, nucleic acid metabolism and regulators responding to a variety of stresses in micro-organisms exhibit different responses under different types of stress. For example, Kirkpatrick *et al.* (2001) claimed that treatment of *Escherichia coli* cells with acetic acid increased the expression levels of 37 proteins, such as autoinducer synthesis protein LuxS, and also repressed 17 proteins, including phosphotransferase. To our knowledge, no study has carried out a proteomic analysis of *E. coli* proteins subjected to HPCD stress. Therefore, as 2-DE coupled with MS is a powerful technique for analysing complex protein mixtures (Han & Lee, 2006), a proteomic 2-DE method coupled with liquid chromatography-electrospray ionization MS-MS (LC-ESI-MS-MS) was applied to elucidate the mechanism of microbial response to HPCD in this study.

The effects of HPCD on DNA in micro-organisms have not been studied. DNA damage is a general phenomenon that spontaneously alters coding properties or the normal functions of DNA during replication or transcription (Nickoloff & Hoekstra, 1998). DNA damage includes both denaturation and degradation of DNA. DNA denaturation results from the rupture of the hydrogen bond of the double helix (Bacolla & Wells, 2004), while DNA degradation results from the rupture of the phosphodiester bond in the primary structure (Murray & Geftter, 1974). DNA damage can be introduced by both endogenous cellular processes, including oxidation, alkylation and hydrolysis, and exogenous agents such as heavy metals, UV light, ionizing radiation and other bulky chemical adducts (Castellani *et al.*, 1988; Nickoloff & Hoekstra, 1998). DNA degradation occurred as micro-organisms were exposed to high hydrostatic pressure (HHP) of approximately 100–600 MPa (Xu, 2007). We presumed that HPCD would induce DNA damage in micro-organisms. Firstly, high pressure in HPCD was applied in a way similar to HHP, though treatment conditions were more moderate. Secondly, HPCD caused a pH decrease in the suspending medium and the interior of microbial cells due to carbonic acid formation on dissolution of HPDC, as shown in previous studies (Spilimbergo *et al.*, 2005, 2010). The pH decrease, both externally and internally of microbial cells, would induce DNA denaturation as DNA is sensitive to extreme acidic environments. Moreover, Garcia-Gonzalez *et al.* (2010), in their analysis of membrane damage of *E. coli*, *Listeria monocytogenes* and *Saccharomyces cerevisiae* cells subjected to HPCD, speculate that HPCD-induced damage to nucleic acids during cell inactivation is due to the lower level of uptake of propidium iodide (PI).

This work aimed to assay the changes in the proteins and DNA of *E. coli* responding to HPCD stress, and to analyse the effects of HPCD on the proteins and DNA of *E. coli*

after HPCD treatment. This study provides a new insight into the underlying molecular inactivation mechanism of micro-organisms subjected to HPCD.

METHODS

Bacterial strain and culture conditions. *E. coli* strain CGMCC1.90 was obtained from the China General Microbiological Culture Collection Center (CGMCC, Beijing, China), and was maintained on slants of nutrient agar (NA; Beijing Aoboxing Biological Technology). A subculture was prepared by inoculating 300 ml nutrient broth (NB; Beijing Aoboxing Biological Technology), which was incubated at 37 °C for 12 h to obtain cells in the early stationary growth phase. Cells were harvested, washed twice with physiological saline (PS; pH 6.8), and then resuspended in PS.

HPCD processing. Cell suspensions (15 ml) were transferred to single sterile plastic tubes and stored at 2 °C before HPCD treatment. Samples were treated in an 850 ml pressure vessel with a batch HPCD system (Liao *et al.*, 2007). The experiments were performed at 37 °C and 10 MPa for 5–75 min. A control experiment performed under atmospheric pressure without CO₂ was included. Before each sample was treated, the pressure vessel was sanitized for 20 min with 50 p.p.m. chlorine dioxide (Beijing Techgreen), and rinsed three times with sterile water. As the pressure vessel was being heated to the required temperature (37 °C), the loosely capped plastic tubes containing the cell suspensions were placed in the pressure vessel. Once the pressure vessel was sealed, the CO₂ inlet valve was opened and, using a plunger pump, was pressurized to the required pressure level (10 MPa). Following the HPCD treatments, the vessel was depressurized and treated samples were removed immediately. Approximately 45 ml of untreated or treated *E. coli* samples were stored at –80 °C before subsequent protein extraction, agarose gel electrophoresis (AGE) and comet assay; 5 ml of each sample was stored at 2 °C for flow cytometry (FCM) analysis immediately. All HPCD processing was done at least in triplicate.

Extraction and quantification of proteins. Cell suspensions were thawed and centrifuged at 4 °C and 14 000 g for 10 min, the supernatant was collected for quantification of proteins in suspension using the Bradford assay. The cell pellets were resuspended in a 10-fold volume of lysis buffer solution containing 40 mM Tris/HCl, 7 M urea, 2 M thiourea, 2 M CHAPS, 20 mM DTT and 1 mM EDTA, and 10 µl protease inhibitor cocktail. The mixtures were sonicated for 3 s following 10 s of immersion in an ice-water bath until the suspensions were limpid, and then 50 µl nuclease stock solution was added to each sample suspension. The suspensions were centrifuged at 4 °C and 14 000 g for 10 min. The supernatant fractions were extracted from the homogenized samples and used for 2-DE detection, with protein loading being normalized based on quantitative analysis using the Bradford assay.

Analysis of leakage of nucleic acids using A₂₆₀. The culture broths of the untreated and HPCD-treated samples were centrifuged at 4 °C and 10 000 g for 10 min, and the A₂₆₀ of the supernatant was measured with a Cary 50 spectrophotometer (Varian) and presented as a mean value of multiple measurements.

2-DE analysis. The first-dimension separation was carried out by using 18 cm pI 3–10 ready strips (Bio-Rad). Aliquots (200 µg) of protein from each sample were mixed with 800 µl buffer (8 M urea, 2 M CHAPS, 0.15% Bromophenol blue, 1 M DTT, 20 mM IPG buffer). Solutions were loaded onto pH gradient (IPG) strips and allowed to rehydrate for 2 h, followed by active rehydration at 30 V for 10 h. The voltage was linearly increased from 500 to 8000 V over

4 h; and the focusing was considered complete after 81.8 kV. The strips were then equilibrated for 15 min in SDS-DTT equilibration solution [pH 8.8, 50 mM Tris/HCl, 6 M urea, 34.5 % (v/v) glycerol, 3.7 % (w/v) SDS, 20 mM DTT] and then in SDS/iodoacetamide equilibration solution [pH 8.8, 50 mM Tris/HCl, 6 M urea, 34.5 % (v/v) glycerol, 3.7 % (w/v) SDS, 100 mM iodoacetamide]. For the second dimension, 13 % acrylamide gels were used. The strips were embedded by using 1 % (w/v) low-melt agarose on top of the acrylamide gel. The gels were run at 40 mA for 40 min, followed by a run at 60 mA for 5 h using a PROTEAN II xi Cell (Bio-Rad). The gels were stained by using the EMBL silver-staining method as follows: gels were first fixed for 20 min (50 % methanol, 5 % acetic acid), washed twice with water, sensitized for 1 min [0.02 % (w/v) sodium thiosulfate], washed with water, stained at 4 °C for 20 min [0.1 % (w/v) silver nitrate] then washed twice with water, coloured in a mixture of 0.04 % (v/v) formol and 2 % (w/v) anhydrous sodium carbonate until the protein spots appeared distinct, and finally, the reaction was terminated by immersing the gels in 5 % (v/v) acetic acid solution for 30 min. Images were digitized with an Image Scanner (Amersham Pharmacia Biotech) and the data processing was carried out using Image Master 2-D Platinum software (Amersham Pharmacia Biotech).

LC-ESI-MS-MS analysis. To evaluate the effects of HPCD treatments on proteins of *E. coli*, 27 spots exhibiting significant changes of greater than 50 % of their initial intensity were selected and identified. The selected protein spots were excised from the gels, enzymically digested and subsequently washed with acetonitrile to remove the SDS, salt and stain. Washed spots were freeze-dried to remove the solvent, rehydrated with trypsin (0.01 µg µl⁻¹), and then incubated in 25 mM ammonium hydrogen carbonate for 12 h at 37 °C. The tryptic peptides were extracted from the gels by using acetonitrile. Samples were then concentrated and desalted on a Symmetry C18 precolumn and further peptide separation was achieved on a BEH C18 column from the nano ACQUITY UPLC system (Waters Micromass). The column outlet was directly coupled to and analysed by Synapt high definition mass spectrometry (Waters Micromass). For protein identification, peptide data from the spots were analysed automatically by database matching against the NCBI protein database (NCBI), using the MASCOT database search engine (Matrix Science; www.matrixscience.com).

Analysis of DNA content by using FCM with PI. A 1 ml sample of the untreated or HPCD-treated cell suspension was centrifuged at 4 °C and 10 000 g for 10 min, as the supernatant was removed and then the pellet was resuspended in 3 % (v/v) glutaraldehyde solution in 0.1 M PBS (pH 7.2) and fixed for 30 min. After fixation, cells in suspension were pelleted, the glutaraldehyde was removed and 70 % cold ethanol was added slowly. The cells were placed at 4 °C for 4 h, then the cell suspension was centrifuged at 4 °C and 10 000 g for 10 min, the pellet was washed twice with 0.2 M Tris/HCl (pH 7.0) and resuspended in 1.0 ml 0.2 M Tris/HCl (pH 7.0). A 50 µl volume of RNase (1 mg ml⁻¹) was added to the suspension and incubated at 37 °C for 1 h to remove RNA, then the cells in suspension were pelleted and washed with 0.2 M Tris/HCl (pH 7.0) once and resuspended in 1.0 ml 0.2 M Tris/HCl (pH 7.0). Finally, 10 µl 0.1 mg PI ml⁻¹ (Sigma Aldrich) was added and incubated for 15 min at room temperature. Samples were placed in 12 × 75 mm plastic tubes and analysed with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems), the red fluorescence of PI was collected in the FL3 channel with a 670 nm long-pass filter. Data were collected using CellQuest software (Becton Dickinson).

Analysis of DNA denaturation by using FCM with acridine orange (AO). The preprocessing, including fixation with glutaraldehyde solution, penetration with cold ethanol solution and removal of RNA, was the same as above. After that, 10 µl 0.1 mg AO ml⁻¹ (Sigma Aldrich) was added and incubated for 15 min at room

temperature, the pellets were then washed twice with 0.2 M Tris/HCl (pH 7.0) and resuspended in 1.0 ml 0.2 M Tris/HCl (pH 7.0) before detection by FCM. AO is a type of cationic dye and can penetrate the cell membrane. The denaturation sites of DNA are characterized by photosensitivity, AO intercalated with double-stranded DNA emits green fluorescence and AO associated with single-stranded DNA emits red fluorescence when excited with blue light at 488 nm (Evenson *et al.*, 2002). DNA damage was expressed as the DNA fragmentation index (DFI), which is the ratio of red to total amount of red plus green fluorescing cells in an individual sample.

Analysis of DNA degradation by using AGE analysis and comet assay

AGE analysis. Before the AGE analysis, DNA of untreated and HPCD-treated *E. coli* cells was extracted and purified from each 5.0 ml suspension with a bacterial gDNA kit (BioMIGA) according to its protocol, and the final extraction volume was 100 µl for each sample. For the electrophoresis analysis, 5 µl purified DNA extract was solubilized in a 5 µl 6 × loading buffer (30 % glycerol, 0.25 % cyanol xylene, 0.25 % bromophenol blue) and DNA fragments were separated by migration on a 1.0 % agarose gel dissolved in TBE buffer (89 mM Tris/borate, 2 mM EDTA, pH 8.3) in the presence of 0.5 µg ethidium bromide ml⁻¹. Migration was performed over 40 min at 70 V using a DYY-6C electrophoresis system (Beijing Luyi Instrument Factory). The DNA fragments were visualized under UV using Image Master VDS (Pharmacia Biotech).

Comet assay. Comet assay was carried out according to Tice *et al.* (2000) with some modifications, which were developed as a new approaching for analysing DNA damage for use in biological research by Ostling & Johanson (1984). Untreated and HPCD-treated cell suspensions were adjusted to 10⁷–10⁸ c.f.u. ml⁻¹ with a final volume of 1 ml. One slide was precoated with 50 µl 0.7 % normal-melting-point agarose as the first layer, 20 µl prepared cell suspension was mixed with 80 µl 1 % low-melting-point agarose dissolved in PBS at 37 °C, which acted as the second layer. The first two layers were coated with 50 µl 0.7 % normal-melting-point gel, which acted as the comet gel. This was immediately covered with a 22 mm square slide. After solidifying at 4 °C for 5 min, the gel was lysed in lysozyme (10 µg ml⁻¹) at 37 °C for 20 min and incubated in a mixed solution overnight at 4 °C in the dark. The solution used was comprised of 2.5 M NaCl, 10 mM Tris/HCl, 100 mM EDTA, 10 % dimethylsulfoxide and 1 % Triton X-100, and was adjusted to pH 10.0. The gel was washed with distilled water for 10 min and digested in 0.1 mg proteinase K ml⁻¹ for 30 min. The gel on the slide was transferred to a jar containing alkaline electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH >13) at 4 °C for 30 min. After DNA unwinding, all slides were transferred to an electrophoresis tank containing the same alkaline buffer and subjected to voltage of 25 mV for 15 min at room temperature with an 0888 comet electrophoresis system (Research Bio-Lab). After electrophoresis, all the slides were neutralized with neutralization buffer (0.4 M Tris/HCl, pH 7.5), rinsed in distilled water and dehydrated with cold ethanol for 10 min. Finally, DNA was stained by adding 50 µg gelred ml⁻¹ (60 µl per slide) for 20 min, and the images were visualized under a fluorescence microscope (Nikon UFX-II). For each slide, a minimum of 50 cells were scored. Two slides were taken for each sample. All experiments were done in triplicate.

RESULTS AND DISCUSSION

HPCD-induced loss of proteins in *E. coli* cells

The protein content in *E. coli* cells and the suspension was quantified by using the Bradford assay. As shown in Fig. 1,

Fig. 1. Effect of HPCD at 10 MPa and 37 °C on the protein content of *E. coli* cells (grey) and the supernatants (white). U, Untreated. Error bars, SD.

the protein content in the untreated *E. coli* cells and the supernatant was 45.88 and 7.43 $\mu\text{g ml}^{-1}$, respectively. Compared with the untreated *E. coli* cells, the protein content decreased in the HPCD-treated *E. coli* cells and increased in the supernatant with increasing treatment time, confirming that HPCD induced protein loss from *E. coli* cells. Moreover, the HPCD-treated *E. coli* cells showed that increasing the treatment time decreases total proteins. In previous investigations, HPCD caused damage to the cytoplasmic membrane (Ballestra *et al.*, 1996; White *et al.*, 2006; Kim *et al.*, 2009; Spilimbergo *et al.*, 2009). Therefore, the protein loss in the HPCD-treated *E. coli* cells in this

study was mainly ascribed to leakage of proteins into the media due to damage to the cytoplasmic membrane.

2-DE analysis of proteins from the HPCD-treated *E. coli* cells

Based on the loss of proteins in the HPCD-treated *E. coli* cells, the proteins of the untreated and HPCD-treated *E. coli* cells were further analysed at pI 3.0–10.0 and molecular mass of approximately 14.4–97 kDa using 2-DE. As shown in Fig. 2(a), a total of 1344 protein spots appeared in the 2-DE gel images of the untreated *E. coli* cells, and most proteins fell into the pI 4.0–8.0 and molecular mass 10–60 kDa range (Fig. 3). Fig. 2(b) shows the 2-DE protein profiles of the HPCD-treated *E. coli* cells. Compared with the untreated cells, the HPCD-treated *E. coli* cells showed 1162 protein spots in the 2-DE profiles, which exhibited a significant reduction in the number of proteins. The reduction in number of proteins in the HPCD-treated *E. coli* cells was ascribed to two main factors. One was the leakage loss of intracellular proteins in the HPCD-treated *E. coli* cells, as previously discussed. In this study, the ratio of extracellular proteins to total proteins in *E. coli* cells increased by almost 13% after HPCD treatment as detected with the Bradford assay, reflecting the reduction of protein spots in the 2-DE gel. The second was that the survival ratio of *E. coli* cells was reduced to 72.82% after HPCD treatment, and the dead cells could not synthesize the proteins, which also caused a reduction in the number of proteins. Moreover, the matched ratio of the proteins in HPCD-treated *E. coli* cells was 72.1% when compared with the untreated *E. coli* cells as a reference in the 2-DE gels; there were 28.5% and 14.2% upregulated and down-regulated protein spots in these gels, respectively. Similarly,

Fig. 2. 2-DE of whole proteins from the untreated (a) or HPCD-treated (b; 37 °C and 10 MPa for 15 min) *E. coli* cells. The red circles and numbers indicate the identified protein spots.

Fig. 3. Molecular mass and pI distribution of protein spots in 2-DE images of the untreated (black bars) and HPCD-treated (hatched bars) *E. coli* cells.

Kim *et al.* (2009) revealed a visual difference in the protein profile of *S. typhimurium* in a suspended medium subjected to HPCD at 40°C and 10 MPa for 30 min. The significant HPCD-induced loss of these protein spots could negatively affect the survival of *E. coli* cells in this study. However, White *et al.* (2006) concluded that there was no effect on the protein profile of *S. typhimurium* in response to HPCD pasteurization at 35 °C and 9.66 MPa for 1 h.

Identification of selected differentially expressed protein spots by LC-ESI-MS-MS

As shown in Fig. 2, 20 protein spots (marked with red circles) from the 2-DE gels of *E. coli* cells exposed to HPCD showing changes in intensity greater than 50% were selected for identification by LC-ESI-MS-MS analysis. Table 1 shows the identified proteins and the organisms from which they originated, together with the MASCOT database search score, the sequence coverage, and the values for the calculated and expected pI and molecular mass. The number of matched peptides was between 1 and 43, with up to 79% sequence coverage and a MASCOT database search score up to 1666. Thirteen (2, 3, 4, 5, 6, 7, 9, 13, 14, 15, 18, 19, 20) of the 20 spots showed a good correlation between the calculated and expected pI and molecular mass (Table 1). The remaining seven protein spots were identified based on comparison with sequences from different organisms, showing more variable score results and larger differences between the calculated and expected pI/molecular masses. Fifteen proteins were identified, and the other two were identified as hypothetical proteins (spots 12 and 18). The hypothetical proteins were predicted from nucleic acid sequences which have not been shown to exist *in vivo* by experimental protein chemical evidence and have not been functionally characterized (Lubec *et al.*, 2005). Some identified proteins were present in more

than one spot. For example, spots 14, 16 and 17 were identified as glutamate and aspartate transporter subunits. Two factors could explain this situation: one is the proteolytic degradation of proteins during the second dimension of 2-DE, followed by the generation of several subunits; the other is that these proteins could be products of different genes (Jorge *et al.*, 2005) or post-translational protein modifications (Nawrot *et al.*, 2007).

The above-identified 15 proteins were further classified into five general groups according to their functions.

The first group of proteins identified in *E. coli* cells was involved in cell composition, including a lipoprotein mutant (spot 1), OmpW (spot 4) and OmpA (spot 7). The lipoprotein mutant is a form of lipoprotein that lacks a diglyceride residue and contains a free thiol group that forms disulfide bridges between lipoprotein molecules upon oxidation (Suzuki *et al.*, 1976). OmpA and OmpW are the outer-membrane proteins of a large array of Gram-negative bacteria. The major physiological functions of OmpA include maintenance of structural integrity and morphology of the cells and porin activity, as well as a role in conjugation and bacteriophage binding. OmpA-deficient *E. coli* exhibits reduced growth (Jeannin *et al.*, 2002). OmpW is involved in protection of bacteria against various forms of environmental stress and in the transport of small hydrophobic molecules across the bacterial outer membrane (Hong *et al.*, 2006). Lipoprotein mutant and OmpA of *E. coli* were downregulated, while OmpW was upregulated following HPCD treatment. The outer membrane is an important barrier for Gram-negative bacteria. However, HPCD induced significant damage to the outer membrane of *E. coli* cells, which was observed in our earlier study using double SYTO 9 and PI stains (Liao *et al.*, 2010). We

Table 1. Proteins that were differentially expressed from untreated and HPCD-treated *E. coli* cells identified by LC-ESI-MS-MS

Spot*	Identified protein†	NCBI Inr accession no.	Molecular mass (kDa)‡	pI§	Matched peptides	Sequence coverage (%)¶	Score#	Up/down-regulation
1	Lipoprotein mutant (<i>E. coli</i>)	gil223967	15.0/8.3	4.7/8.7	2	33	149	Down
2	Autonomous glycyl radical cofactor GrcA (<i>E. coli</i> O157:H7 EDL933)	gil15803106	15.4/14.3	4.9/5.1	13	74	539	Down
3	Inorganic pyrophosphatase (<i>E. coli</i> CFT073)	gil26251130	23.4/19.7	4.9/5.0	12	54	329	Down
4	Chain A, outer-membrane protein W (<i>E. coli</i>)	gil88192831	21.2/21.7	5.8/6.2	10	49	430	Up
5	50S Ribosomal protein L10 (<i>E. coli</i> O157:H7 EDL933)	gil15804575	17.2/17.7	9.3/9.0	16	58	554	Down
6	Chain A, the crystal structure of Dps (<i>E. coli</i>)	gil3660175	27.8/18.7	6.6/5.7	16	79	541	Down
7	Outer-membrane protein A (<i>E. coli</i> O157:H7 EDL933)	gil15800816	46.5/37.2	6.2/6.0	30	56	945	Down
8	Putative glutathione peroxidase (<i>E. coli</i> CFT073)	gil26247961	24.4/20.4	4.7/4.8	11	60	227	Up
9	Histidine ABC transporter, periplasmic histidine-binding protein (<i>E. coli</i> 101-1)	gil194436225	31.3/28.4	5.3/5.3	8	35	189	Up
10	Chain I, β -galactosidase (<i>E. coli</i>)	gil1311063	15.4/116.3	7.5/5.3	2	3	117	Up
11	Chain I, β -galactosidase (<i>E. coli</i>)	gil1311063	15.2/116.3	8.2/5.3	1	2	85	Up
12	Hypothetical protein Z4452 (<i>E. coli</i> O157:H7)	gil15803639	20.7/11.0	9.0/9.1	9	37	300	Down
13	Glucosamine-6-phosphate deaminase (<i>E. coli</i> O157:H7 EDL933)	gil15800380	31.5/29.7	7.6/6.8	9	45	411	Up
14	Glutamate and aspartate transporter subunit (<i>E. coli</i> O157:H7 EDL933)	gil15800369	32.6/33.3	8.3/8.6	43	71	1666	Up
15	Glutathione ABC transporter, periplasmic glutathione-binding protein GsiB (<i>E. coli</i> B7A)	gil191166211	53.6/56.4	8.1/8.2	15	48	504	Up
16	Glutamate and aspartate transporter subunit (<i>E. coli</i> O157:H7 EDL933)	gil15800369	14.2/33.3	5.5/8.6	5	16	332	Up
17	Glutamate and aspartate transporter subunit (<i>E. coli</i> O157:H7 EDL933)	gil15800369	12.8/33.3	6.2/8.6	3	14	332	Up
18	Hypothetical protein Z4380 (<i>E. coli</i> O157:H7 EDL933)	gil15803570	13.5/11.5	6.6/5.8	7	32	275	Up
19	Thioredoxin-dependent thiol peroxidase (<i>E. coli</i> O157:H7 EDL933)	gil15803003	17.6/17.6	5.2/5.0	18	64	599	Up
20	Starvation-inducible DNA-binding protein (<i>E. coli</i>)	gil384221	19.2/18.7	6.6/6.2	3	25	143	Up

*Assigned spot numbers are indicated in Fig. 2.

†Identified homologous proteins and organisms from which they were obtained.

‡Calculated and expected molecular masses, respectively, of identified proteins are given. Calculated values were calculated by using standard molecular mass markers. Expected values were retrieved from the protein database.

§Calculated and expected pIs, respectively, of identified proteins. Calculated values were calculated by using standard molecular mass markers. Expected values were retrieved from the protein database.

||Number of matched peptides by using the Mascot search data (www.matrixscience.com).

¶Amino acid sequence coverage for the identified proteins.

#Mascot search probability based Mowse score. Ion score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Individual ion scores >34 indicate identity or extensive homology ($P < 0.05$). Protein scores are derived from ion scores as a non-probabilistic basis for ranking protein hits.

concluded that the changes in lipoprotein mutant, OmpA and OmpW cause the damage to the outer membrane.

The second group of proteins identified was involved with regulators responding to varied stresses, including autonomous glycyl radical cofactor (spot 2), a putative glutathione peroxidase (spot 8) and thioredoxin-dependent thiol peroxidase (spot 19). Autonomous glycyl radical cofactor is correlated with cell adaptation as a radical domain of pyruvate and formic acid lyase, making the cells of the micro-organism able to adapt to atypical situations more easily (Mangalappalli-Illathu *et al.*, 2008). Putative glutathione peroxidase and thioredoxin-dependent thiol peroxidase are involved in oxidation regulation. Glutathione peroxidase catalyses the reduction of hydrogen peroxide, organic hydroperoxides and lipid peroxides by reduced glutathione, and helps to protect the cells against oxidative damage (Flohé & Günzler, 1984). Thioredoxin-dependent thiol peroxidase belongs to the peroxiredoxin family of anti-oxidant enzymes. This enzyme is very important in organisms, as it catalyses the reduction of H₂O₂ or alkyl hydroperoxides and produces water or corresponding alcohols, protecting the organism from oxidation. Autonomous glycyl radical cofactor was downregulated, while both putative glutathione peroxidase and thioredoxin-dependent thiol peroxidase were upregulated following HPCD treatment.

The third group of proteins identified was involved in energy metabolism, including histidine ATP-binding cassette (ABC) transporter (spot 9), β -galactosidase (spots 10 and 11), glucosamine-6-phosphate deaminase (spot 13), and glutathione ABC transporter (spot 15). Histidine ABC transporter and glutathione ABC transporter belong to the ABC transporter family; its function is to transfer substances that are foreign to the organism from the inside to the outside of the cell, including heavy metals, anions, sugars, amino acids, phospholipids and proteins (Bozdech *et al.*, 1996). β -Galactosidase has two catalytic activities: one is to hydrolyse the disaccharide lactose to galactose plus glucose and the other is to convert lactose to allolactose (which is the natural inducer for the *lacZ* operon) (Jacobson *et al.*, 1994; Matthews, 2005). Glucosamine-6-phosphate deaminase is involved in the pentose phosphate pathway and the glycolytic pathway. In the present study, all four proteins were upregulated following HPCD treatment. The results indicated that HPCD-treated *E. coli* cells exhibit stress adaptation in the form of accelerated energy metabolism.

The fourth group of proteins identified was involved in general metabolism, including 50S ribosomal protein L10 (spot 5) and glutamate and aspartate transporter subunits (spots 14, 16 and 17). 50S Ribosomal protein L10 cooperates with L11, L1, L12 and the β and β' subunits of RNA polymerase, forming the *rplKAJE-rpoBC* gene cluster (Downing *et al.*, 1990). After the transcription of the polycistronic mRNA from this cluster, RNase III excises the mRNA of ribosomal proteins and the polymerase subunits, which are then translated independently

(Downing & Dennis, 1987); the process is regulated by the cluster. Glutamate and aspartate transporter subunits are responsible for the transcription of L-glutamate, L-aspartate and D-aspartate during protein synthesis (Slotboom *et al.*, 1999). 50S Ribosomal protein L10 was downregulated following HPCD treatments, which might have synchronized the synthesis of rRNA and indirectly controlled protein synthesis. Glutamate and aspartate transporter subunits were upregulated following HPCD treatments.

The fifth group of proteins identified in *E. coli* cells was involved in nucleic acid metabolism, including inorganic pyrophosphatase (spot 3), the crystal structure of DNA protection during starvation (Dps, spot 6) and starvation-inducible DNA-binding protein (spot 20). Inorganic pyrophosphatase catalyses the reversible transfer of the phosphoryl group from a polyphosphate to water and the reaction provides a thermodynamic pull for such vital biosynthetic processes as the syntheses of proteins and nucleic acids (Kornberg, 1962). Inorganic pyrophosphatase was downregulated following HPCD treatments, resulting in the accumulation of pyrophosphate followed by inhibition of nucleic acid synthesis. Dps protects DNA by its ability to chelate ferrous iron and its direct association with DNA (Almirón *et al.*, 1992; Zhao *et al.*, 2002), which was downregulated following HPCD treatments. Starvation-inducible DNA-binding protein is essential for DNA replication, recombination and repair (Whittier & Chase, 1983) and is a helix destabilizing protein (DNA unwinding protein) (Bobst *et al.*, 1985). Following HPCD treatments, the *E. coli* DNA-binding protein was upregulated, which could induce the denaturation of DNA. The differential expression of the above-mentioned three proteins was very complicated, which would inhibit nucleic acid synthesis and induce DNA damage, but it was difficult to foretell the change in DNA and the extent of DNA damage of *E. coli* cells subjected to HPCD. So, we adopted some novel methods to measure this and we discuss the effect of HPCD on DNA structure in the following section.

Effects of HPCD on DNA in *E. coli* cells

HPCD-induced DNA loss in *E. coli* cells. Similar to the leakage loss of the proteins as observed above, the nucleic acids in the HPCD-treated *E. coli* cells also leaked into the media as suggested by a significant increase in A₂₆₀ with increasing treatment time from 5 min to 75 min ($P < 0.05$) (data not shown). For a more precise analysis of DNA loss, FCM combined with PI was used. The untreated and HPCD-treated *E. coli* cells were preprocessed, including fixation, permeation and elimination of RNA, then stained with PI. The fluorescence intensity of PI was proportional to the DNA content of *E. coli* cells. As shown in Fig. 4, there was no significant difference among the DNA histograms corresponding to the untreated *E. coli* cells, *E. coli* cells treated at 37 °C for 15 min, and HPCD-treated *E. coli* cells at 10 MPa and 37 °C for 5 or 15 min. All the cells were

Fig. 4. FCM analysis combined with PI staining of the DNA content of *E. coli* cells that were untreated or HPCD-treated at 10 MPa and 37 °C for between 5 and 75 min. M1, PI-negative; M2, PI-positive.

distributed at high fluorescence channels and exhibited a unimodal distribution indicating a relatively higher DNA content. However, the distribution profile of DNA content of the HPCD-treated *E. coli* cells was visibly changed when the treatment time was ≥ 30 min. A noticeable bimodal distribution of DNA content emerged and the subpopulation with relatively lower fluorescence corresponding to cells with lower DNA content was seen in the *E. coli* cells treated with HPCD for 45 min. The two peaks of the bimodal distribution were almost equal when the *E. coli* cells were treated with HPCD for 75 min. The results indicated that the

DNA content of *E. coli* cells decreased with increasing treatment time. Some researchers also claimed that the amount of chromosomal DNA in *E. coli* K-12 cells subjected to HPCD (4 MPa, 35 °C, 1 min) is greatly reduced (Watanabe *et al.*, 2007), which agrees with our results.

DNA denaturation analysis with FCM combined with AO.

The DNA integrity of an organism is fundamental to its survival (Steinert, 1999). The secondary structure of DNA is the double helix, and the rupture of the hydrogen bond of the double helix would lead to denaturation of DNA

Fig. 5. FCM analysis combined with AO staining of *E. coli* cells that were untreated or HPCD-treated at 37 °C and 10 MPa for 5–75 min. The R1 region shows no or slight fluorescence, corresponding to cell fragments; the R2 region shows only red fluorescence, corresponding to cells with totally denatured single-stranded DNA; the R3 region shows both green and red fluorescence, corresponding to cells with partially denatured DNA; the R4 region shows only green fluorescence, corresponding to cells with intact DNA.

(Bacolla & Wells, 2004). In this study, AO was used to stain untreated and HPCD-treated *E. coli* cells, followed by FCM to determine HPCD-induced DNA denaturation in *E. coli* cells. FCM dot plots of green fluorescence (FL1) against red fluorescence (FL3) of *E. coli* cells are shown in Fig. 5. The plots are divided into four regions corresponding to four different subpopulations: the R1 region shows no or slight fluorescence, corresponding to cell fragments; the R2 region shows only red fluorescence, corresponding to cells with totally denatured single-stranded DNA; the R3

region shows both green and red fluorescence, corresponding to cells with partially denatured DNA, which was in an intermediate state of double-stranded and single-stranded; the R4 region shows only green fluorescence, corresponding to cells with intact DNA. *E. coli* cells with intact DNA in the untreated sample made up 65.8 %, while only 0.03 % of the subpopulation was characterized by red fluorescence. For *E. coli* cells that were exposed to 37 °C and atmospheric pressure for 15 min, the subpopulation emitting only red fluorescence (R1) did not change, while

Fig. 6. AGE profiles of DNA in *E. coli* cells that were untreated or HPCD-treated at 10 MPa and 37 °C for between 5 and 75 min. Lanes: 1, untreated; 2, 5 min; 3, 15 min; 4, 30 min; 5, 45 min; 6, 60 min; 7, 75 min; 8, 0.1 MPa, 37 °C and 15 min.

the subpopulation with intact DNA decreased to 37.9% and that with partially denatured DNA increased by 26.3%. Compared with the untreated *E. coli* cells and *E. coli* cells exposed to 37 °C and atmospheric pressure for 15 min, the subpopulation in the HPCD-treated *E. coli* cells that emitted only red fluorescence also showed no change. However, the subpopulation with intact DNA decreased and that with partially denatured DNA increased. These results indicated that HPCD induced the partial unwinding of double-stranded DNA into single-stranded DNA and that denaturation of DNA in the HPCD-treated *E. coli* cells had occurred. The DNA damage was expressed as DFI, which is the ratio of red to total amount of red plus green fluorescing cells in an individual sample (Evenson *et al.*, 2002). The DFI in this study was 0.19 in the untreated *E. coli* cells and increased to 0.25–0.42 in HPCD-treated *E. coli* cells, suggesting that HPCD induced DNA damage. The denaturation of DNA in the HPCD-treated *E. coli* cells was associated with the upregulated starvation-inducible DNA-binding protein. Watanabe *et al.* (2007) found that when subjected to HPCD treatment, cytoplasmic pH is reduced to around pH 5.2, and nuclear DNA becomes entangled in coagulated cytoplasmic proteins, and then the efficiency of DNA recovery from the acidified extracts was low. The reduction of cytoplasmic pH was also directly or indirectly proven in

some other research (Spilimbergo *et al.*, 2005; Watanabe *et al.*, 2005), which also possibly involved inducing the denaturation of DNA.

DNA degradation analysis with AGE and comet assay.

The primary structure of DNA is its base sequence; rupture of the phosphodiester bond in its primary structure would lead to degradation of DNA (Murray & Geftter, 1974). AGE analysis was used to verify whether HPCD induced DNA degradation or not. As shown in Fig. 6, there was only one band present in all the AGE profiles of the untreated and HPCD-treated *E. coli* cells and the migration of each band was similar, indicating that HPCD did not induce DNA degradation of *E. coli* cells. However, the band in *E. coli* cells exposed to HPCD for 30–75 min was not as clear as that in the untreated *E. coli* cells and *E. coli* cells exposed to HPCD for 5–15 min, indicating that HPCD for 30–75 min resulted in a noticeable loss of DNA in *E. coli* cells, which supported the FCM and A_{260} results described above. To support the AGE analysis, further the comet assay was applied in this study. The comet assay is a relatively fast, simple and sensitive technique for the analysis of DNA damage in all cell types, which is based on quantification of the denatured DNA fragments migrating out of the cell nucleus during electrophoresis under alkaline conditions (Liao *et al.*, 2009) and the DNA tailing combined with the tail length represents the extent of DNA damage (Olive *et al.*, 1990). As shown in Fig. 7, the comet assay also showed that there was no significant difference among the HPCD-treated *E. coli* cells and the untreated *E. coli* cells. *E. coli* is a prokaryote, and therefore lacks a nuclear membrane and nucleolus but exhibits a nucleus area. All the photomicrographs displayed clear nucleus areas without any DNA tailing. Similar to the AGE analysis, the comet assay indicated that HPCD did not induce DNA degradation and the DNA primary structure remained intact. Watanabe *et al.* (2007) also claimed that DNA degradation was not clearly observed in *E. coli* K-12 cells subjected to HPCD at 4 MPa and 35 °C for 1 min. The degradation of DNA involves the rupture of the phosphodiester bond, which might be much easier for HPCD treatment as moderate parameters were used; for example, the temperature was 37 °C. Furthermore, some

Fig. 7. Comet assay profiles of DNA in *E. coli* cells that were untreated (1) or HPCD-treated at 10 MPa and 37 °C for 5–75 min (2, 5 min; 3, 15 min; 4, 30 min; 5, 45 min; 6, 60 min; 7, 75 min). 8, Treatment was 0.1 MPa, 37 °C for 15 min.

upregulated proteins can protect against the degradation of DNA during HPCD treatment, which may be investigated further.

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