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Proteins involved in *Campylobacter jejuni* 81–176 recovery after high-pressure treatment

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Campylobacteriosis has been recognized as the major bacterial food-borne infection worldwide. *Campylobacter*, especially *C. jejuni,* contaminate mainly poultry meat. Although more sensitive than other food-borne pathogens to many stresses, *C. jejuni* can survive food processing and go on to reach its final reservoir (the human gut). Genomic analyses of this organism indicate a lack of genes described in other gram-negative bacteria to overcome stresses. The high-pressure recovery response of *C. jejuni* 81–176 was analyzed from two-dimensional electrophoretic profiles of the cytoplasmic proteome. The main cellular mechanisms controlling the down- and upregulated proteins are discussed.

Keywords: *Campylobacter jejuni*; high pressure; recovery; protein synthesis; metabolic pathway

Introduction

Campylobacter jejuni is the most frequent human food-borne pathogen worldwide. This microorganism is responsible for campylobacteriosis in humans: a disease ranging from a self-limiting gastroenteritis to a more serious systemic infection. In addition, a small proportion of patients, including children, can develop serious complications like neuroparalytic diseases known as Guillain–Barré or Miller–Fisher syndromes. This zoonotic pathogen is transmitted by food of animal origin, especially poultry.[1] In Europe, the prevalence of campylobacterioses is around 63/100,000, as compared to 52/100,000 for salmonelloses. [2]

However *C. jejuni* appears to be a more fragile organism than other bacterial pathogens (particularly to heat, oxidation, and acidity).[3] Moreover, this microorganism is unable to grow in the presence of air or at a temperature lower than 30° C. *C. jejuni* cannot multiply in food where it endures all environmental stresses, but the great campylobacteriosis incidence indicates its great survival rate.[4] On the other hand, genomic analyses of *C. jejuni* have shown a lack of general well-described bacterial resistance gene products, such as RpoS, for stationary phase and the general stress responses, SoxRS and OxyR for the oxidative stress response, RpoH for the heat shock response, and CspA for the cold shock response.[5] The absence of DNA repair genes (*ada*, *phr*, *vsr*, *mutH*, *mutL,* and *sbcB*) and SOS response genes (*lexA*, *umuC,* and *umuD*) has also been reported.[6] This indicates that *C. jejuni* must have unknown resistance mechanisms to respond to environmental stresses.

The effect of high pressure (HP) on *C. jejuni* has not received much study. This pathogen was shown to be rather sensitive to HP as compared to other food-borne pathogenic bacteria: the bactericidal effect was obtained at 400 MPa.[7,8] Its sensitivity depends on strain, medium (food or broth), and growth phase.[9,10] The inactivation of the *C. jejuni* strain EB1410/02 has been modeled as a function of pressure, temperature, and time in poultry meat slurries with a first-order kinetic model.[11] Another study comparing *C. jejuni* strains NCTC 11168 and 81–176 demonstrated that these two strains, which had similar resistances to HP at 20° C, had

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different sensitivities to the same pressure treatment performed at 37° C. The sensitivity of strain 81–176 was not changed with an increase of temperature from 20° C to 37° C when HP treatment was performed in a pH 7.0 buffer.[12] However, the physiological mechanisms involved in *C. jejuni* HP response are still unknown.

The purpose of this study was to better understand how *C. jejuni* recovers after a sublethal HP treatment by comparing the cytoplasmic proteome of pressurized and unpressurized cells.

Material and methods

Strain and growth conditions

C. jejuni 81–176 strain, a particularly virulent strain whose genome has been sequenced, was selected.[13] It was stored at −80° C in Brain and Heart Infusion (BHI) (Oxoid, Basingstoke Hampshire, UK) supplemented with 15% glycerol (Nutri-Service, Villefranche-de-Rouergue, France). Prior to each experiment, cells were subcultured onto Karmali agar (Oxoid) for 48 h, then one grown colony was suspended in 50 mL of BHI and incubated for 24 h. Cultures were obtained by inoculating 500 mL of BHI with 5 mL of the last subculture and incubated for 16 h under 110 rpm shaking. All subcultures and cultures were grown at 42° C in microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N_2).

Pressurization and recovering conditions

Stationary phase-cultured cells were harvested by centrifugation at 7000 × *g* for 20 min at 20° C and suspended in 500 mL of phosphate buffer at pH 7.0 (0.2 mol/L^{-1} Na_2HPO_4 [Merck, Darmstadt, Germany] and 0.2 mol/L^{-1} NaH_2PO_4 [Merck]) in sterile polyethylene bags (AES, Combourg, France) which were sealed under sterile conditions.

Pressurization was carried out in a thermostated vessel of a 3.5 l reactor unit (ACB Pressure Systems, Nantes, France). In a previous study, it was demonstrated that *C. jejuni* strain 81–176 started to be inactivated after 200 MPa for 10 min HP treatment at either 20° C and 37° C.[13] Consequently, a pressure treatment of 220 MPa for 10 min at 37° C was applied to the cells: the cell reduction after treatment was less than 0.5 Log (CFU/mL) as calculated from plate counts. The control culture was subjected to the same conditions without HP treatment, that is, 10 min at 37° C under atmospheric pressure. After HP treatment, cells were harvested and suspended in preheated BHI during 1 h, at 42° C under microaerophilic conditions and 110 rpm shaking.

Protein extraction

Cells were retrieved by centrifugation at 7000 *g* for 20 min and washed consecutively with 200 mM glycine solution (Sigma-Aldrich, Saint-Quentin Fallavier, France) and 100 mM Tris-HCl pH 7.0 solution (Sigma-Aldrich). Pellets were resuspended in 10 mL of a 10 mM Tris-HCl pH 7.0 solution and cells were disrupted by series of 6 × 30 sec sonication with 6-min intervals on ice (Vibracell 72434, Bioblock Scientific, Illkirch, France). To eliminate cell debris, samples were centrifugated twice at 10,000 *g* for 20 min at 4° C. Then, cytoplasmic proteins were separated from membrane fractions by ultracentrifugation at 188,000 × *g* for 1 h at 4° C. Then, the cytoplasmic protein fraction in the supernatant was treated with protease inhibitor cocktail tablets COMPLETE (Roche Diagnostics, Mannheim, Germany) and nuclease solution with final concentrations of 0.06 mg/mL RNAse and 0.12 mg/mL DNAse (Sigma-Aldrich). Protein samples were dialyzed using cellulose membrane tubing with a cut-off at 12,000 Da (Sigma-Aldrich) against MilliQ water at 4° C (800 mL water for 5 mL sample) in shaking conditions, during 3 days by refreshing the dialyze bath each day. Purified protein samples were then aliquotted and stored at −80° C. Total protein concentration was determined using the Micro BCA Protein Assay Kit (Perbio Science, Brebieres, France).

Two-dimensional gel electrophoresis

A quantity of 100 μg of protein was concentrated using Concentrator 5301 (Eppendorf, Le Pecq, France), at room temperature, to reduce the solution volume to 15 μL ± 5 μL. Then, each sample was diluted with 275 μL of a 6 mol/L^{-1} urea, 2 mol/L^{-1} thiourea, 4% CHAPS, 0.4% DTT, a few grains of Bromophenol blue (BB) (Sigma-Aldrich) and 2% Biolyte 3/10 (Bio-Rad, Marnes la Coquette, France) solution. Proteins in the rehydratation solution were absorbed overnight by a 17 cm pH 4–7 IPG strip (Bio-Rad). Then, the Iso-ElectroFocalization (IEF) was performed using the Bio-Rad IEF program as

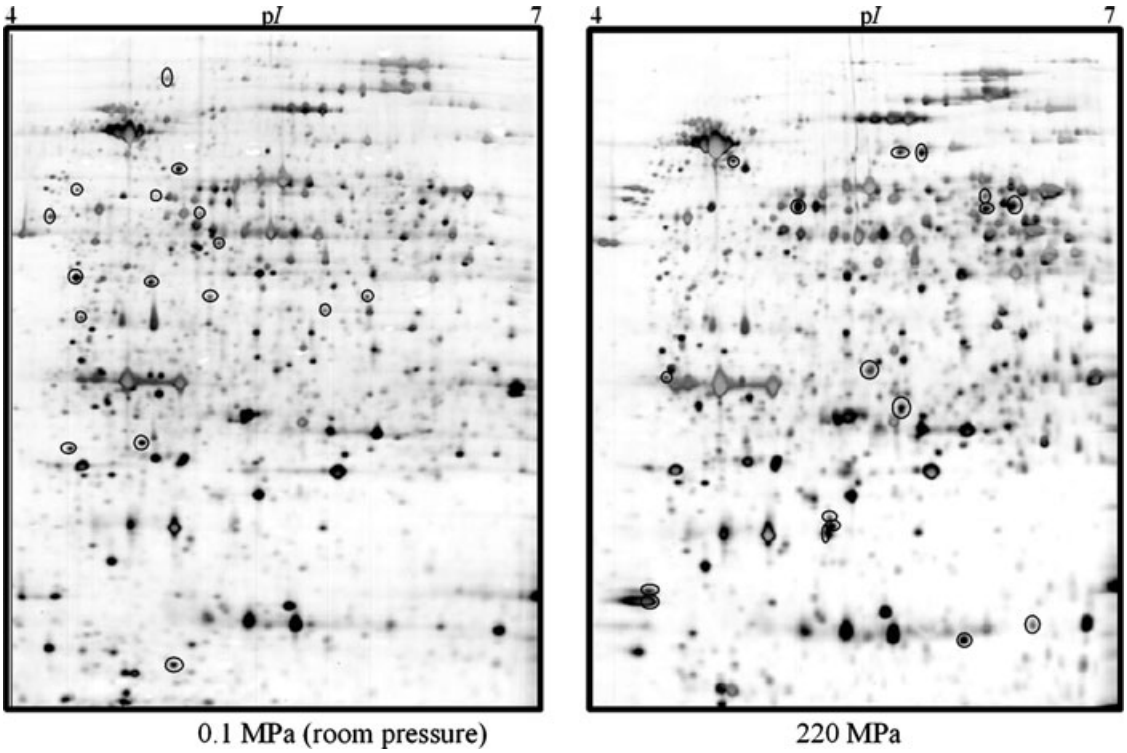

Figure 1. Two-dimensional electrophoresis gels of *C. jejuni* 81–176 obtained after 1h recovering from HP treatment with 16 repressed proteins (0.1 MPa gel) and 17 overexpressed proteins (220 MPa gel).

follows: from 50 to 250 V for 3 h, from 250 to 6000 V for 3 h, and at 6000 V until reaching 54,000 Vh. Finally, each strip was soaked for 20 min in equilibration buffer (1.5 mL of a 6 mol/L^{-1} urea, 2% SDS, 0.05 mol/L^{-1} Tris-HCl (pH 8.8), 30% glycerol, and a few grains of BB solution) supplemented with 2% DTT and subsequently for 20 min in equilibration buffer with 4% iodoacetamide (Bio-Rad). The second dimension was performed in 12% acrylamide gels (20 cm × 20 cm × 0.1 cm) covered with 1% low-melting point agarose (Biorad, Hercules, CA, USA) and run at 40 mA/gel at 14° C using a Protean II xi cell (Biorad) until the migration reached the base of the gels. Proteins in gels were finally silver stained and scanned with a GS-800 densitometer (Bio-Rad) operated with the QuantityOne software (Bio-Rad) at the resolution of 42.3 μm.

Image and statistical analysis

The image analysis was performed using the Progenesis Samespots software (NonLinear Dynamics, Newcastle upon Tyne, UK). For the statistical analysis of the results, two independent experiments were performed, with three technical replicates for each of them.

Differences between the two conditions from the independent experiments and replicates were validated by principal component analysis (PCA) and differences among matched spot intensities were statistically validated by performing an ANOVA (at a 5% significance level).

When spots of interest were located, two-dimensional electrophoresis gels were performed again using 500 μg of proteins and were stained with BioSafe colloidal Coomassie blue (Bio-Rad). Visible spots of interest were picked up and the corresponding proteins were analyzed after trypsin digestion by LC-MS/MS (LTQ-Orbitrap Discovery) at the PAPPSO platform of the INRA Center in Jouy-en-Josas (France). Resulting peptides were analyzed with Bioworks 3.3.1 SP1 software (Thermo Fisher Scientic, Cergy-Pontoise, France) searched against the draft of the genome of *C. jejuni* sequenced strains.

Table 1. Metabolic pathways influenced by HP in *C. jejuni* 81–176 after 1 h of recovery

Metabolic pathways	
Repressed after HP recovery	Overexpressed after HP recovery
Energy metabolism	Carbohydrate synthesis and energy metabolism
Nucleotide metabolism	Fatty acids synthesis
Translation	Translation
Amino acid metabolism	Amino acid metabolism
Metabolisms of cofactors and vitamins	Signal transduction
Motility and chemotaxis	Stress responses

The functional categories for proteins classification were those used by the Kyoto Encyclopedia of Genes and Genomes.[14]

Results and discussion

The recovery response of *C. jejuni* after HP treatment was analyzed by comparing electrophoretic profiles of the cytoplasmic proteome of *C. jejuni* 81–176 with or without 220 MPa pressurization. Replicability and reproducibility of the gels were insured by an available dynamic range over 85% and an available intensity levels over 96% as calculated by Samespots software (NonLinear Dynamics). The differences between electrophoretic gels obtained from cells with or without HP treatment were validated by the analyses of a PCA plot that groups proteomic profiles with HP treatment on one side and proteomic profiles without treatment on the other side (data not shown). Among the spots significantly different between the two conditions at a 5% significance level, spots were selected based on a spot intensity variation of at least twofold between the two conditions and variations repeated on at least five of six gels for the same condition. Finally, 33 of these spots were detected on Coomassie blue gels and identified by mass spectrometry. Sixteen proteins were found to be repressed and 17 proteins were overexpressed after HP treatment (Fig. 1). Table 1 represents the metabolic pathways in which the proteins were regulated after HP treatment in *C. jejuni* 81–176.

Repressed proteins were involved in nucleotide metabolism, translation, amino acid metabolism, energy metabolism, metabolisms of cofactors and vitamins, motility, and chemotaxis. Oxygen binding was lowered, which could mean that cells limited oxidative stress caused by oxygen. Since processes, such as motility and chemotaxis, are not critical for survival, their repression could indicate the redirection of energy to recovery processes. The overexpressed proteins during HP recovery were essentially involved in carbohydrate synthesis and energy metabolism, fatty acids synthesis, amino acid metabolism, translation, signal transduction (a *Campylobacter*-specific two-component regulator), and stress responses. The intensified metabolic pathway indicated that cells needed energy to repair HP injuries; the lipidic portion of cell membranes might be damaged, and the biotin carboxylase overexpression could induce repairs or composition changes in cell-membrane fatty acids; amino acid anabolisms and tRNA synthesis could indicate *de novo* protein syntheses, while amino acid catabolism (in particular asparate lysis) would allow energy production by the neoglucogenesis pathway. Indeed, *C. jejuni* does not have a functional glycolytic pathway due to the absence of orthologs of glucokinase and 6-phosphofructokinase.[7] HP stress proteins belonged to general stress and oxidative stress responses.

Compared to *L. monocytogenes*, *E. faecalis*, *E. faecium,* and *L. sakei*, *C. jejuni* had a different proteomic profile during HP recovery.[15] However, some parts of the responses were similar: the evolution of the energy metabolism and carbohydrate transport, the protein synthesis pathway, and the induction of general stress proteins. Among the differences, the induction of cold shock proteins by three of the four Gram-positive bacteria differed from the stress proteins produced by *C. jejuni*, and only *L. monocytogenes* like *C. jejuni* expressed oxidative

stress proteins. A similar comparison was made to the proteomic study of *L. sanfranciscensis*, whose proteomic profile after HP treatment contained significant overlaps with profiles of cold- and NaCl-stressed cells.[16] Studies of the response of *E. coli* to HP showed that there are links between its pressure response and heat shock. Of 55 pressure-induced proteins, 11 were heat shock proteins compared to 4 cold shock proteins and the production of heat shock proteins enhanced *E. coli* resistance to HP.[17,18] The resistance of *E. coli* was largely but not completely related to RpoS activity[19] and a part of its response to HP was an SOS response, which stabilizes and repairs DNA.[20] No orthologs to *rpoS* or to genes associated with the SOS response, such as *lexA*, *umuC*, and *umuD*, were identified in the *C. jejuni* genome.[6] Other unknown mechanisms could provide HP resistance in *C. jejuni*. However, most of the overexpressed proteins detected after HP treatment are not known to play a role in stress resistance. Interestingly, overexpression of oxidative stress proteins involved in the HP recovering response was observed. This is in accordance with the observations of Aertsen *et al*. on *E. coli* after HP treatment.[21] The authors demonstrated that HP treatment induces endogenous intracellular oxidative stress in cells. As a microaerophilic micro-organism, *C. jejuni* is particularly sensitive to oxidative stress. This would explain why oxidative stress proteins are overexpressed after HP recovering response and also why *C. jejuni* is more sensitive to HP than other Gram-negative bacteria, as mentioned in previous studies.[7,8]

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Conflicts of interest

The authors declare no conflicts of interest.

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