Differential gene expression of *Listeria monocytogenes* during high hydrostatic pressure processing

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High hydrostatic pressure processing (HPP) is currently being used as a treatment for certain foods to control the presence of food-borne pathogens, such as *Listeria monocytogenes*. Genomic microarray analysis was performed to determine the effects of HPP on *L. monocytogenes* in order to understand how it responds to mechanical stress injury. Reverse transcriptase PCR analysis of *tufA* and *rpoC* indicated that the reduction of mRNA expression in HPP-treated cells was dependent on intensity and time of the treatment. Treatments of 400 and 600 MPa for 5 min on cells in the exponential growth phase, though leading to partial or complete cellular inactivation, still resulted in measurable relative differential gene expression. Gene set enrichment analysis indicated that HPP induced increased expression of genes associated with DNA repair mechanisms, transcription and translation protein complexes, the septal ring, the general protein translocase system, flagella assemblage and chemotaxis, and lipid and peptidoglycan biosynthetic pathways. On the other hand, HPP appears to suppress a wide range of energy production and conversion, carbohydrate metabolism and virulence-associated genes accompanied by strong suppression of the SigB and PrfA regulons. HPP also affected genes controlled by the pleiotrophic regulator CodY. HPP-induced cellular damage appears to lead to increased expression of genes linked to sections of the cell previously shown in bacteria to be damaged or altered during HPP exposure and suppression of gene expression associated with cellular growth processes and virulence.

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Listeria monocytogenes is a Gram-positive, non-sporulating, rod-shaped, facultative anaerobe that is a member of the phylum *Firmicutes* and causes the disease listeriosis. Listeriosis is an uncommon but very serious condition that has a high mortality rate amongst susceptible individuals and is acquired orally through the consumption of food (Khelef *et al.*, 2006). Due to its environmentally robust and persistent nature, the control and elimination of *L. monocytogenes* currently remains a challenge and a priority for the food industry (Bell & Kyriakides, 2005), especially considering the increasing consumer demand for convenience foods that often have varied and minimal conventional post-lethality processing treatments. This concern has led to various technology-driven innovations in food processing, including the application of thermal and non-thermal high hydrostatic pressure processing (HPP) to inactivate and kill pathogenic

bacteria present in food or in raw ingredients (Toepfl *et al.*, 2006).

High pressure damages cellular membranes, resulting in leakage of cell contents; oligomeric proteins and protein complexes also undergo dissociation (Gross & Jaenicke, 1994). In combination these cellular injuries lead to cell inactivation and death. Protein and nucleic acid complexes in the cell with critical functions, e.g. ribosomes and the translation apparatus (Niven *et al.*, 1999) and septal rings (Kawarai *et al.*, 2004) are particularly vulnerable to HPP-induced dissociation. High pressure has been shown to condense nucleoids and aggregate proteins in *Escherichia coli* and *L. monocytogenes* (Mackey *et al.*, 1995; Mañas & Mackey, 2004). At extreme pressures, denaturation of monomeric proteins may occur (Gross & Jaenicke, 1994). Since pressurization tends to cause proteins to become more compact, enzyme activity can be diminished or enhanced, dependent on the intrinsic flexibility of the protein and whether the flexibility is important for the enzymic mechanism.

The tolerance to HPP in *L. monocytogenes* is considerably increased in stationary-growth-phase cells (Mackey *et al.*,

Abbreviations: HPP, high hydrostatic pressure processing; PTS, phosphotransferase system.

The GEO database accession number for the microarray data reported in this paper is GSE9179. The microarray data are also available with the online version of this paper.

1995) and is also cross-protected against high pressure in the presence of NaCl, metal cations and various organic compounds (Hauben *et al.*, 1998; Smiddy *et al.*, 2005; Morales *et al.*, 2006; Koseki & Yamamoto, 2006). It has been generally observed that increasing the osmotic pressure of the medium counteracts the effect of hydrostatic pressure. On a mechanistic level this may be due to hydrostatic and osmotic pressure and/or chemical stabilization having different and often opposing influences on proteins and thus tend to cancel the negative effects of each other out (Martin *et al.*, 2002). It is also possible that pre-exposure to stress 'hardens' the cell against HPP, for example the protective benefit can be due to alterations in the composition and thickness of the cell envelope that increases cell-wall stability (Mañas & Mackey, 2004). The survival of *L. monocytogenes* when exposed to HPP may be influenced by the pre-induction of stress response regulators such as SigB, which can activate several protective genes under stressful conditions. Protection against HPP can, on the other hand, be conferred by mutations in regulators that are also involved in repressing specific sets of stress-response genes (Karatzas *et al.*, 2005).

Though HPP treatments can fully and quite rapidly inactivate *L. monocytogenes*, depending on the pressure applied, the effect of HPP may to an extent be sublethal. Data obtained using microscopy and flow cytometry (Ananta *et al.*, 2004; Ritz *et al.*, 2001, 2006) suggests that at pressure levels of approximately 400 MPa cell integrity is maintained, that only very limited cell destruction occurs and metabolic activity is largely retained for various Gram-positive bacteria, including *L. monocytogenes*. It was concluded that although cell viability was lost, HPP-treated cells were sublethally injured and were potentially capable of recovery back to culturability (Ritz *et al.*, 2006). Physiological studies have also demonstrated that increasing HPP pressure levels results in metabolic indicators (for example the activity of the LmrP membrane transporter) exhibiting an accelerated decline (Kilimann *et al.*, 2005). Over the last several years a number of studies have shown *L. monocytogenes* and other bacteria such as *E. coli* seem to be capable of recovering culturability following HPP exposure (Bozoglu *et al.*, 2004; Bull *et al.*, 2005; Jantzen *et al.*, 2006; Ritz *et al.*, 2006). Though these findings have not been followed up by more mechanistic investigations, they do suggest that HPP exposures cause injuries in cells that may or may not lead to permanent inactivation (death) and subsequently cells may have the capacity to repair and recommence growth assuming the correct conditions are available. This has consequences for the application of HPP in providing safe food with lengthy shelf lives that is otherwise minimally processed.

Various pressure-induced proteins have been found to occur with synthesis maintained or increased relative to other control proteins synthesized at atmospheric pressure (Welch *et al.*, 1993). This suggests that *E. coli* and other

bacteria are able to mount a response to high pressure, though the response is non-specific, reflecting the fact that most bacteria have no specific adaptations to high levels of hydrostatic pressure. Another finding by Welch and colleagues suggests that, although incapable of growth during and after high pressure exposure due to cell injury, a proportion of the cell population appears to be able to maintain cellular activity of some kind, the degree being dependent on the intensity of the pressure exposure. At 70–80 MPa, *E. coli* is no longer able to significantly synthesize RNA (Welch *et al.*, 1993; Yayanos & Pollard, 1969), thus indicating the physiological tolerance limits are well below pressure levels applied during food HPP, which typically range from 300 to 1000 MPa. The exact nature of the specific features and mechanisms of the cellular processes induced during the initial phase and the aftermath of HPP exposure remain currently undefined. It is hypothesized that many of these processes are involved in cell maintenance and repair (Bozoglu *et al.*, 2004) as well as general adaptive responses providing greater cell stability (Mañas & Mackey, 2004).

Various proteomic (Welch *et al.*, 1993; Drews *et al.*, 2002; Aertsen *et al.*, 2004b; Hörmann *et al.*, 2006) and transcriptomic-based studies (Iwahashi *et al.*, 2003; Ishii *et al.*, 2005; Malone *et al.*, 2006) have been performed to date to determine the genetic responses associated with high hydrostatic pressure. As different micro-organisms and different pressure conditions (applied at either non- or partially inactivating levels) have been used, results are difficult to compare. Comparisons of sublethal pressure levels with different growth-suppressive conditions (high NaCl concentrations, high and low temperatures, low pH, stationary growth phase) on *Lactobacillus sanfranciscensis*, did not reveal large differences in protein patterns (Hörmann *et al.*, 2006). In *E. coli*, various genes could be identified that supported barotolerance (Malone *et al.*, 2006), including those encoding thioredoxins, stress-protective DNA/iron-binding protein DPS, cell-division-associated lipoprotein NlpI, nucleoid-associated histone-like proteins (H-NS, StpA), trehalose-6-phosphate synthase (OtsA) and RNA polymerase sigma factors (RpoE, RpoS). In some studies, HPP exposures of *E. coli* and *Saccharomyces cerevisiae* induced heat-shock proteins (Welch *et al.*, 1993; Iwahashi *et al.*, 2003). Heat cross-protects against HPP exposure (Aertsen *et al.*, 2004b), suggesting accumulated cell changes or damage may lead to generalized responses common to other stress-related damage such as heat.

In this study we explored the genetic responses that a *L. monocytogenes* serotype 1/2a isolate induces during HPP exposure levels that result in at least partial cell inactivation, mimicking HPP exposure pressures and exposure times typically used for food processing. The main goal was to determine what type of genetic responses *L. monocytogenes* makes to short-term intense mechanical stress and determine how this relates to what is known about high pressure effects on microbial cells.

METHODS

Strains. Strains used in this study included strain S2542, a comparatively barotolerant strain isolated from salami, and EGD-e (ATCC BAA-679, serotype 1/2a) isolated from rabbit tissue. Both strains were routinely cultured in tryptone soy-yeast extract (TSYE) broth (Oxoid) and on TSYE agar (1.5%, w/v, agar) at 25 °C. The serotype of S2542 was determined using a commercial *Listeria*-specific antigen test kit (Denka-Seiken).

Barotolerance experiment. Bottles containing TSYE broth (1000 ml) were inoculated with strain S2542 to a transmittance of approximately 95%. The strain was cultivated at 15 °C for 48 h (mid-exponential growth phase) or 72 h (stationary growth phase) using an orbital shaker (Ratek Platform Mixer OM6). The incubation temperature was chosen as it mimics typical industrial HPP processing temperature conditions for food products and it overcomes adiabatic heating effects. Cell suspensions were transferred to sterile bags, vacuum-packaged and then heat-sealed. Biological duplicates were tested for every treatment. The samples were pressure-treated at 15 °C using a custom built research-scale HPP unit. For assessment of barotolerance, strain 2542 was subjected to up to 600 MPa for 2–10 min. Pressure increases were approximately 1000 MPa min⁻¹ and the pressure-release time was approximately 5 s. Cells that were exposed to HPP were either in the mid-exponential growth phase or in the stationary growth phase. Adiabatic heating was estimated to raise the temperature of the sample to 25–30 °C during the HPP exposure. Following HPP treatment, strain suspensions were immediately serially diluted in sterile TSYE broth and spread onto TSYE agar using an autoplate (model 4000; Spiral Biotech). Plates were incubated at 25 °C and assessed after 24 h.

DNA microarray-related HPP treatments. For DNA microarray experiments, S2542 cells were harvested during the middle of the exponential growth phase in TSYE broth at 25 °C and subjected to 400 and 600 MPa for 5 min and for 15 min as described above. Adiabatic heating increased the temperature of the samples to 23–27 °C during the HPP exposures. Following HPP, treated samples were immediately frozen in liquid nitrogen. Control samples were held at 15 °C at atmospheric pressure for 5 or 15 min before being frozen. All treatments were performed in triplicate.

DNA extraction and hybridization. Genomic DNA was extracted from strains S2542 and EGD-e using the Marmur extraction protocol, sheared to a mean size of 1.0 kb using sonication, filtered (0.22 µm pore size Millipore disposable filters) and dialysed overnight (2 × SSC buffer: 0.3 M NaCl, 0.03 M trisodium citrate, pH 7.0) at 4 °C. The genomic DNA–DNA hybridization level between the strains was then determined using spectrophotometric renaturation rate kinetics (Huß *et al.*, 1983).

ERIC and BOX PCR. Genomic DNA was extracted as mentioned above. The amplification of enterobacterial repetitive intergenic consensus (ERIC) sequences (primers ERIC1 – 5′-ATGTAAGCT-CCTGGGGATTAC-3′ and ERIC2 – 5′-AAGTAAGTGACTGGGGT-GAGCG-3′) and BOX elements (primer BOX A1R – 5′-CTACGG-CAAGGCGACGCTGACG-3′) by PCR was performed using the method described by Wieser & Busse (2000).

RNA extraction. Cells were defrosted and underwent a 2–3 h (25 °C) enzymic treatment in TE buffer (10 mM Tris/HCl, 1 mM disodium EDTA, pH 7.5) containing 20 mg lysozyme ml⁻¹ and 10 mg proteinase K ml⁻¹. Cells were fully lysed by bead beating using 0.1 mm zirconium-silica sand in RNeasy MIDI RNA extraction kit (Qiagen) lysis buffer containing 0.1% β-mercaptoethanol. RNA was then extracted and purified using the RNeasy kit and subsequently stored at –80 °C. RNA quality and quantity was

assessed with the Agilent Technologies bioanalyser 2100 and RNA LabChip.

Microarray analysis. Strain S2542 RNA samples were hybridized to a custom *L. monocytogenes* strain EGD-e microarray. For each of the treatments, three biological replicate RNA samples were used in the analysis. The array included 2857 × 70 bp oligonucleotides (Operon Technologies), representing all predicted protein-coding genes and pseudogenes of the complete published genome of *L. monocytogenes* EGD-e (GenBank accession no. AL591824; Glaser *et al.*, 2001). Oligonucleotides were arrayed onto glass slides using quill pens at the Australian Genomic Research Facility (Walter & Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia), each spot having a diameter of 12 µm. RNA was converted to cDNA and post-labelled with Cy5 (red) and Cy3 (green) fluorescent dyes using the SuperScript Indirect cDNA Labelling System (Invitrogen). Slides were pre-hybridized in a hybridization solution [25%, v/v, formamide, 5 × SSC buffer (0.75 M NaCl, 0.075 M trisodium citrate, pH 7.0), 0.1% SDS] containing 10 mg BSA ml⁻¹ for 45 min at 42 °C. Slides were then rinsed twice in distilled water and air-dried. Hybridization was performed in a humid hybridization chamber at 42 °C for 16–20 h using a hybridization solution containing 0.42 µg human Cot1 DNA µl⁻¹, 0.62 µg polyA µl⁻¹ and 0.83 µg salmon sperm DNA µl⁻¹. Slides were washed at room temperature in 1 × SSC buffer containing 0.2% SDS for 5 min and again in 0.1 × SSC buffer containing 0.2% SDS for 5 min. The slides were then further washed twice in 0.1 × SSC buffer at room temperature for 2 min. Slides were then dried and subsequently scanned using a GenePix 4000A scanner (Axon Instruments). Downstream processing used the GenePix-Pro software package to generate gpx files from TIFF array images. Normalization of raw data and subsequent statistical testing was performed with the WebArray Online platform (Xia *et al.*, 2005). Within-array normalization used the global LOESS procedure. Between each array, quantile normalization was used to ensure intensities had the same empirical distribution across arrays and across channels. The significance of differential expression was analysed using linear modal statistical analysis (Smyth, 2004). Spacings LOESS histogram analysis (using WebArray) and the program EDGE (v. 1.1.28) (Storey *et al.*, 2005) were used to estimate the conditional false discovery rate (FDR), the expected proportion of false positives conditioned on having *k* ‘significant’ findings (Pounds & Cheng, 2004). A significant alteration in expression was defined as a twofold or greater change. Based on the data, this represented FDR-adjusted significance values of *P* < 0.01–0.02. A total of 25 genes (lmo0037, lmo0241, lmo0266, lmo0795, lmo1012, lmo1083, lmo2105, lmo2226, lmo2236, lmo2581, lmo2667, lmo2691, lmo2692, lmo2702, lmo2713, lmo2714, lmo2752, lmo2757, lmo2761, lmo2769, lmo2773, lmo2778, lmo2792, lmo2811, lmo2837) were identified as consistently giving hybridization levels only equivalent to the background level and thus were not included in any subsequent analyses. Processed microarray data files for both HPP treatments are available as supplementary data files 1 and 2 (available with the online version of this paper) and have been deposited in the GEO database (www.ncbi.nlm.nih.gov/geo/) under accession number GSE9179.

Gene-set enrichment analysis. Gene designations, predicted functions and functional categorization of coded proteins from the *L. monocytogenes* EGD-e genome was based on information obtained from the Kyoto Encyclopedia of Genes and Genomes (www.genome.ad.jp/kegg/), ListiList (<http://genolist.pasteur.fr/ListiList/>) and DAVID (<http://david.abcc.ncifcrf.gov>) bioinformatic databases. Major functional categories (equivalent to Clustered Orthologous Gene classes) and subcategories were defined as listed in Table 2. The gene-by-gene functional designations are shown in the supplementary data files. A *t*-test-based procedure, as described by Boorsma *et al.* (2005), was utilized to score the changes in expression of predefined sets of genes. This score, designated a *T*-value, was only determined

for sets that contained at least 8 genes. The significance of the *T*-value score was established by using the associated two-tailed *P*-value (TDIST function in Microsoft Excel). The determined *P*-value was corrected by Bonferonni adjustment. This involved multiplication of the *P*-value by the number of major gene categories ($n=17$) or by the number of subcategories that occur within a larger category ($n=2-14$). In addition to functional categories, the *T*-value scores for gene sets (regulons) under direct or indirect control of transcriptional regulators SigB (Kazmierczak *et al.*, 2003), PrfA (Milohanic *et al.*, 2003), CodY (Bennett *et al.*, 2007) and RpoN (Arous *et al.*, 2004) were also determined.

Reverse transcriptase-PCR (RT-PCR). To determine gene expression in HPP-treated cells, RT-PCR was utilized. This was done for two reasons: (1) to determine mean mRNA levels for two housekeeping genes in cells subjected to HPP, and (2) to confirm the results of the microarray experiments. Test samples and controls used for both of these experiments were grown and treated as described above. RNA was obtained using the Qiagen RNeasy Micro extraction kit. HPP-treated samples were then compared with controls grown at atmospheric pressure to determine the relative level of mRNA expression. Two housekeeping genes, *tufA* and *rpoC*, were tested with 16S rRNA serving as the calibration standard. Analysis of rRNA levels using the Agilent Bioanalyser 2100/RNA LabChip indicated cellular 16S rRNA was not significantly changed in quantity compared to controls following HPP treatments. For the RT-PCR experiments used to confirm the microarray experiments, mRNA was obtained from cells exposed to 400 MPa for 5 min with 16S rRNA serving as the calibration standard. Oligonucleotide

primers for selected genes were defined using the Primer3 v. 1.0b software (Rozen & Skaletsky, 2000) and are listed in Table 1. RNA was extracted with the mini RNeasy extraction kit (Qiagen) and subsequently treated with RNase-free DNase according to the manufacturer's protocol. PCR samples were set up using a Quantitect multiplex RT-PCR kit (Qiagen) and a pipettor robot (Corbett Research). RNA concentration was estimated using an ND-1000 UV-VIS spectrophotometer (Nanodrop Technologies). RT-PCR was performed using the Rotorgene RG3000A system (Corbett Research). The real-time cycling conditions were as follows: 95 °C for 3 min for the initial activation step, 40 cycles each of denaturing at 95 °C for 15 s, and annealing–extension at 60 °C for 15 s. To confirm that a single PCR product was amplified, melting curve analysis was performed with the following conditions: 95 °C for 1 min, 55 °C for 1 min and 55.0–95.0 °C with a heating rate of 0.5 °C per 10 s. PCR amplicons were electrophoresed on agarose gels to confirm the predicted sizes. Relative gene expression was calculated using the comparative threshold cycle (C_T) method (Livak & Schmittgen, 2001) with PCR efficiency and C_T values automatically determined using software supplied with the Rotorgene RG3000A system.

RESULTS AND DISCUSSION

Strain comparisons

Salami isolate S2542 was found to belong to serotype 1/2a following serological analysis. Hybridization of

Table 1. RT-PCR oligonucleotide primers used in this study

The T_m values of all primers are approximately 60 °C, assuming 50 mM KCl in the reaction volume.

Gene	Gene no.	Forward primer (5'>3')	Reverse primer (5'>3')	DNA product size (bp)
<i>ssb</i>	lmo0045	TTCCTTGAACCGCGTAATTC	TGTCGATTGGCTTACCATCA	208
<i>rpoC</i>	lmo0259	CCGTATGCAAGGGGTAGAAA	TAGCTTCACGGTTGGCTTCT	155
<i>rpoE</i>	lmo2560	GGGAGCGTCTTGTTC AATTT	CCAAGCTCTTCCACGATTTC	235
<i>cspL</i>	lmo1364	CGCAGAAAAAGGATTTGGTT	TGAACGTTAGCTGCTTGAGG	162
	lmo2460	TCTTCCGGAATGACCGTTAC	GGACCCATGGAGTATCATCG	169
<i>ccpA</i>	lmo1599	GGAGCCGTTGATATGAAAA	ATTTCAATTCGCGATTGACC	154
	lmo2072	GATGTGGATCCAGCGAAAGT	TGGTGCACACGAAGTTGTT	227
	lmo2673	GCCAAAGCTTACGGAGAAAGA	AATAACGTCGCAAGGTGCAT	243
<i>fri</i>	lmo0943	TTACTAGCAATCGGCGGAAG	CATTGTCGCCTTCTTTGTCA	190
	lmo0048	GAAAGACCGCTGGAAAGATG	CGTGCAATTCAATGTTTTGG	211
<i>hup</i>	lmo1934	GCAGCGAAAGCAGTAGAAGC	AAGCGCTTTACCAGGTTTGA	195
<i>tufA</i>	lmo2653	TGGCGATGACATTCCTGTAA	CTGGCATCATGAATGGTTTG	152
<i>fus</i>	lmo2654	GTGAAACCCATGAAGGTGCT	TGCATCTAGAACCGCAACAG	203
<i>flaA</i>	lmo0690	CGTGAACAATCAATCCATCG	ACATTGCGGTGTTTGTTT	152
<i>ftsI</i>	lmo2039	AACAACCGTTTTTCGGACAAG	GTACCATGTTCCGCCGCTAAT	237
<i>ftsE</i>	lmo2506	AGGCTAAAGAGCCCACACAA	TCTTCGACGGGAGAAAATTG	222
<i>ftsZ</i>	lmo2032	CAATGAAAGAAGCGGTGGAT	ATTCCCATAAGGGCAGAACC	226
	lmo2674	GCGGCATTTTAGTTTGTGGT	ATTTGTCTACGCGATTTCG	241
<i>pgm</i>	lmo2205	TTGGCATGATGTGGACTTGT	TCAAGCGCCAAGATTATATGA	191
<i>pdhD</i>	lmo1055	AACAGGATCTCGTCCAATCG	CTGGACCACCCTCAAGGATA	191
<i>gadB</i>	lmo2363	AATACCTTGCCCATGCAGTC	AGTGGATATGCGGGAACCTTG	172
<i>iap</i>	lmo0582	GCAGAAGTGAAAACGGAAGC	TGTAGATGGTGCAGGAGCTG	162
<i>inlA</i>	lmo0433	ACCTACGGCACCAACAAAAG	CCATCAACATCAAACGTTGC	165
<i>inlH</i>	lmo0263	ATTTTTCGACAGGGAAAATG	CGGAAGCATGGTATTTTCGT	191
16S rRNA*	–	CCTACGGGAGGCAGCAG	GTATTACGCGGCTGCTG	196

*341–357F and 519–536F primer positions are derived from Lane (1991).

Table 2. Responses of genes to HPP, organized into gene functional categories or regulons

Gene categories and subcategories (no. of genes)	400 MPa, 5 min		600 MPa, 5 min	
	<i>T</i> -value*	<i>P</i> -value†	<i>T</i> -value*	<i>P</i> -value†
PrfA (positive) (64)	−13.52	3.7E-20	−11.19	1.6E-16
SigB (positive) (54)	−10.40	1.6E-13	−8.38	1.9E-10
CodY (negative) (101)	−3.94	0.0004	−3.96	0.0008
CodY (positive) (84)	2.35		1.49	
RpoN (negative) (61)	−1.76		−1.87	
RpoN (positive) (21)	1.32		0.44	
DNA replication/repair/recombination (94)	3.57	0.0096	3.47	0.0135
DNA polymerase (10)	3.43	0.0268	2.56	
DNA replication complex (20)	1.41		0.72	
DNA repair (70)	2.95	0.0130	3.56	0.0021
Nucleotide metabolism (83)	2.81		3.31	0.0237
Nucleotide biosynthesis (23)	−0.03		−0.06	
Nucleotide interconversions (60)	3.45	0.0021	3.99	0.0004
Translation/ribosome (148)	7.11	8.2E-10	8.49	3.6E-13
Ribosomal proteins (58)	6.11	4.0E-07	7.01	1.3E-08
Translation-associated (54)	4.05	0.0007	4.14	0.0005
Translation factors (12)	1.98		3.03	0.0504
tRNA-aminoacyl synthesis (24)	0.08		1.05	
Cell-wall biogenesis (32)	4.01	0.0064	3.55	0.0222
Peptidoglycan biosynthesis (16)	3.41	0.0084	3.50	0.0071
Teichoic acid biosynthesis (16)	2.23	0.0672	1.49	
Flagellar assemblage/motility (27)	4.91	0.0008	2.06	
Signal transduction mechanisms (71)	2.58		2.03	
Chemotaxis (12)	3.67	0.0130	2.30	
Two-component systems (39)	3.38	0.0051	3.10	0.0109
Intracellular trafficking (44)	2.93	0.0920	1.51	
Protein export (19)	2.22		1.93	
Type II secretion (competence) (14)	−0.56		−0.66	
Type III secretion (flagella) (11)	3.52	0.0196	1.20	
Cell division/chromosome partitioning (46)	4.61	0.0006	4.10	0.003
Septal-ring proteins (9)	3.91	0.0006	5.15	0.0013
Lipid biosynthesis (41)	4.77	0.0004	4.10	0.0035
Steroid/terpenoid biosynthesis (16)	2.69	0.0526	1.33	
Fatty acid biosynthesis (16)	3.19	0.0197	3.46	0.0115
Isoprenoid quinone biosynthesis (9)	2.24		2.28	
Energy production and conversion (87)	−5.24	5.7E-07	−4.32	4.1E-05
Oxidative phosphorylation (27)	−3.93	0.0024	−2.88	0.0319
Pyruvate/propanoate metabolism (34)	−2.89	0.0273	−2.86	0.0299
Butanoate metabolism (19)	−3.04	0.0293	−2.67	0.0642
Other/putative energy metabolism-related genes (16)	−1.10		−0.54	
Virulence-related (17)	−4.86	0.0002	−4.45	0.0004
Carbohydrate transport/metabolism (306)	−4.82	3.9E-05	−4.97	1.9E-05
Aminosugars metabolism (15)	0.59		1.77	
Fructose/mannose metabolism (29)	0.90		1.27	
PTSs (89)	−2.97	0.0349	−2.83	0.0515
Glycolysis/gluconeogenesis (36)	−3.36	0.0174	−3.86	0.0043
Nucleotide sugars metabolism (10)	−1.56		−0.89	
Pentose phosphate pathway (34)	−0.60		−0.11	
Starch/sucrose metabolism (29)	−0.78		−0.92	
Non-PTS transporters (33)	−0.56		−1.39	
Other carbohydrate metabolism-related proteins (50)	−2.29		−2.45	
Other catabolism/biosynthesis (61)	−1.96		−3.38	0.0203
Pdu/Eut operons (36)	1.54		0.28	
Inositol/inositol phosphate metabolism (9)	−2.01		−2.94	0.0873
Glycerolipid metabolism (15)	−4.43	0.0027	−2.96	0.0446

Table 2. cont.

Gene categories and subcategories (no. of genes)	400 MPa, 5 min		600 MPa, 5 min	
	<i>T</i> -value*	<i>P</i> -value†	<i>T</i> -value*	<i>P</i> -value†
Glycerophospholipid metabolism (12)	−0.55		−1.37	
Post-translational modification/chaperones (48)	−1.79		−1.35	
Heat-shock proteins (18)	−2.39	0.0876	−2.34	0.0972
Protein folding/processing (18)	−0.50		−0.35	
Folding catalysts (12)	−0.07		0.55	
Transcription (243)	0.16		−0.43	
RNA polymerase (10)	−0.41		0.30	
Transcription-associated (9)	3.83	0.0706	4.50	0.0306
Transcription regulators (Crp/Fnr family) (15)	−3.01		−2.75	
Transcription regulators (BglG family) (14)	−1.34		−1.54	
Transcription regulators (GntR family) (17)	0.90		0.98	
Transcription regulators (LacI family) (12)	0.92		0.42	
Transcription regulators (LysR family) (9)	−0.45		−0.82	
Transcription regulators (MarR family) (13)	−0.06		−0.51	
Transcription regulators (TetR/AcrR family) (11)	0.10		0.48	
Transcription regulators (other families) (66)	1.03		0.55	
Transcription regulators (other/putative) (72)	0.22		−0.42	
Amino acid transport/metabolism (244)	−1.71		−0.11	
Amino acid transport (23)	0.85		1.40	
Compatible solute transport (10)	−2.99		−1.93	
Peptide transport (11)	0.30		1.29	
Alanine/aspartate metabolism (28)	−2.90		−2.27	
Arginine/proline metabolism (22)	−0.75		−0.10	
Aromatic amino acids metabolism (27)	0.99		1.55	
Branched-chain amino acids metabolism (25)	−0.44		−0.54	
Cysteine metabolism (13)	−0.65		−0.70	
Methionine metabolism (13)	−0.16		−0.41	
Glycine/serine/threonine metabolism (26)	0.57		1.60	
Histidine metabolism (14)	−1.38		−1.99	
Lysine biosynthesis (15)	0.83		1.42	
Glutamate metabolism (28)	−1.73		−0.90	
Other/putative amino-acid-related metabolism (22)	−1.17		−1.23	
Coenzyme metabolism (115)	1.50		2.34	
Folate biosynthesis (16)	−0.25		−0.01	
One-carbon pool by folate (11)	0.16		1.09	
Porphyrin/cobalamin biosynthesis (34)	2.08		1.89	
Pantothenate/CoA biosynthesis (15)	2.60		2.18	
Nicotinate/nicotinamide metabolism (13)	−0.42		0.80	
Thiamine metabolism (9)	−0.88		−1.08	
Inorganic ion transport/metabolism (103)	1.38		1.42	
Inorganic ion transport (86)	1.55		1.49	
Inorganic ion metabolism (17)	−0.11		0.11	
Other/putative signal transduction genes (25)	−1.06		−1.12	

**T*-value scores were determined from the expression data (supplementary data files 1 and 2) using the approach of Boorsma *et al.* (2005).

†Significance values are Bonferroni-corrected; *P*-values not shown are >0.1.

genomic DNA of strain S2542 was performed with strain EGD-e to determine genetic similarity. DNA–DNA hybridization values averaged $92 \pm 7\%$ ($n=5$). Profiles for ERIC and BOX element PCR fingerprint patterns were also near identical for these strains (data not shown), confirming that both strains were genotypically very similar.

Barotolerance of *L. monocytogenes* strain S2542

Though no significant inactivation followed 2 min exposures at 450 MPa, an inactivation of 3.1 log units was determined after 5 min exposure (Fig. 1). Stationary-phase cells were comparatively more barotolerant (Fig. 1), exhibiting only a 1.0–1.1 log unit reduction after 5 min.

Fig. 1. Viability of exponential- (□) and stationary-growth-phase (■) *L. monocytogenes* strain S2542 cultures following different HPP treatments.

Complete inactivation and a 5.2 log unit reduction was achieved following 10 min exposure at 750 MPa for cells in exponential and stationary growth phases, respectively. Pressure treatments of 600 MPa resulted in rapid inactivation of both exponential- and stationary-growth-phase cell populations (Fig. 1).

Relative gene expression of *L. monocytogenes* strain S2542 exposed to HPP

To assess the direct effect of HPP on gene expression, the relative levels of mRNA of *tufA* and *rpoC* were assessed by RT-PCR. At HPP exposures of 400 MPa, mRNA levels of *tufA* and *rpoC* increased slightly, but were lower than levels observed at atmospheric pressure (26–35 % reduction) after a 15 min exposure (Fig. 2). A much lower mRNA level was observed when HPP exposure intensity was increased to 600 MPa for 5 min, including a steep decline that occurred with an increased length of HPP exposure (Fig. 2). Extended exposure times combined with an increased pressure level thus appear to result in reduced *tufA* and *rpoC* mRNA levels, indicative of RNA synthesis inhibition due to the effect of HPP (Yayanos & Pollard, 1969); however, the reduction either does not occur or is relatively small within a 5 min timeframe.

To obtain further evidence of the relative differential gene expression responses observed with different procedures, 23 genes were selected that manifested a range of expression levels under the 400 MPa/5 min HPP treatment. It was found that relative changes in gene expression obtained from microarray hybridization and RT-PCR experiments correlated in a linear relationship between the tested genes (Pearson's product moment correlation, 0.968) (Fig. 3).

The 600 MPa/5 min HPP treatment yielded microarray hybridization data with an overall 24 % lower dynamic range compared to the 400 MPa/5 min experiment. However, between the two separate treatments the microarray results correlated when the most differentially expressed genes (>2.0- or -2.0-fold change; $r^2=0.95$) and the total number of genes ($r^2=0.80$) were compared between the treatments. The level of dye label incorporation was found to be poor to negligible for RNA extracted from cells exposed to 400 and 600 MPa for 15 min.

Overall, relative differential gene expression data for HPP treatments of short (5 min) duration were reproducible, suggesting that changes to gene expression were occurring during this time; however, exposures of 15 min led to microarray data of either poor quality or hybridizations that were unsuccessful, possibly due to severe reduction of the total mRNA pools. These changes in mRNA pools are consistent with hydrostatic pressure experiments that showed that RNA synthesis in *E. coli* no longer occurs above about 70–80 MPa (Yayanos & Pollard, 1969). In the Yayanos & Pollard (1969) experiments, RNA synthesis was

Fig. 2. Levels of *tufA* and *rpoC* mRNA in HPP-treated cells relative to atmospheric pressure controls. RNA was obtained from *L. monocytogenes* grown in BHI broth to the mid-exponential growth phase at 25 °C and then subjected to HPP treatment (□, 400 MPa; ■, 600 MPa) for 5 min (bars on left of each panel) or 15 min (bars on right of each panel). Control samples were not subjected to HPP treatment. Values obtained were normalized using 16S rRNA as a calibration standard.

Fig. 3. Comparison between differential responses in gene expression in *L. monocytogenes* 2542 measured by quantitative PCR and microarray hybridization. RNA was obtained from cells grown in BHI broth to the mid-exponential growth phase at 25 °C and then subjected to 400 MPa for 5 min. Control samples were not subjected to HPP treatment.

measured indirectly by following ^{14}C -uracil incorporation over several hours with initial data collected after 50–120 min. In the experiments performed here the time window required to observe declines was much briefer, most probably due to the higher pressure levels applied. Essentially, in our experiments, relative differential gene expression was clearly observed during the short but intense 5 min HPP exposure, since mRNA pools were still maintained at levels comparable to atmospheric controls as determined by RT-PCR analysis of two highly expressed housekeeping genes (Fig. 2).

Differential gene expression induced by HPP on the basis of gene functional classes and regulons

Changes in relative gene expression induced by HPP treatments are detailed in supplementary data files 1 and 2. HPP induced significant gene expression alterations in several gene functional categories and subcategories, as established using the *T*-value scoring procedure of Boorsma *et al.* (2005). The *T*-value scoring results are summarized in Table 2. Significant declines in the expression of the SigB and PrfA regulons were also observed after HPP exposure (*T*-value scores < -8.4 ; Table 2). Genes directly (or indirectly) repressed by pleiotrophic transcriptional regulator CodY were found to be significantly repressed following HPP treatment (*T*-value scores -3.9 to -4.0 ; Table 2). CodY-activated genes showed a weak opposite response. No significant alterations were observed for the RpoN regulon following HPP treatments. This may be due to RpoN only directly controlling a small number of genes (Arous *et al.*, 2004).

Effect of HPP on information-processing and storage genes

HPP-treated cells exhibited increased expression of several genes involved with DNA polymerase and DNA repair as indicated by significant positive *T*-value scores (Table 2). It

has been shown that hydrostatic pressure, depending on the level applied, can impede the DNA replication process, apparently due to dissociative effects on the DNA replication apparatus (Bartlett, 2002) and disassembly of the replication fork (Aertsen *et al.*, 2004a). Thus a compensatory increase in gene expression for DNA polymerase components may be a partial response to HPP disruption of the DNA replication process. This response could also be simply due to high hydrostatic pressure tending to render DNA in a more compact configuration (Barciszewski *et al.*, 1999) or damaging the helix structure, resulting in a reduction in the efficiency of forming DNA polymerase–DNA complexes suitable for the initiation of active replication.

Under HPP there was increased expression of numerous DNA repair genes (see supplementary data files 1 and 2), including homologues of *holB*, *dnaA*, *recDFNU*, *dinG*, *ruvA*, *mutS*, *ssb*, *sbcC*, a DNA ligase (lmo1758) and various DNA exo/excinucleases (Table 2). The upregulation of these genes suggests that HPP directly damages DNA in *L. monocytogenes*. HPP has been shown to create double-stranded breaks in the DNA helix and induce a genuine SOS response in *E. coli* (Aertsen *et al.* 2004a, b; Aertsen & Michiels, 2005).

It was also observed that HPP induced increased expression of *hup* (lmo1934) and *flaR* (lmo1412) (1.7- to 4.0-fold increases), both encoding histone-like proteins that may affect DNA topology. *E. coli* mutants lacking histone-like protein-coding genes *hns* or *stpA* have been shown to exhibit pronounced pressure sensitivity (Ishii *et al.*, 2005; Malone *et al.*, 2006). In *E. coli*, protein HU, which is very similar to the protein encoded by lmo1934, has been shown to alter the conformation of DNA, functioning as a transcriptional repressor/modulator and as an architectural factor affecting transcription at the post-transcriptional level and influencing higher-order nucleoprotein complex assembly, including chromosome compaction. It has been hypothesized that HU has antagonistic activity against

H-NS in *E. coli*, and thus could counteract chromosome compaction (Dame & Goosen, 2002). This functionality may be significant in the context of HPP as the cell nucleoid has been shown to be subjected to considerable condensation by HPP in both *E. coli* and *L. monocytogenes* (Mañas & Mackey, 2004).

HPP appears to lead to upregulation of genes involved in nucleotide metabolism (*T*-value scores 3.45 and 3.99; Table 2), including those encoding proteins involved in nucleotide interconversions (e.g. *drm*, *pdp*, *pnp*, *guaAB*) and salvage reactions (e.g. *upp*, *udk*, *smbA*, lmo1939, lmo1463) (Neidhardt *et al.*, 1996). Since nucleic acid biosynthesis was apparently not affected by HPP (*T*-value scores ~0; Table 2), this may suggest an increased reliance in HPP-damaged cells to recycle nucleotides for RNA synthesis and DNA repair.

HPP-induced changes to transcription-associated genes, including cold-shock proteins

The expression of the RNA polymerase α subunit (lmo2606) and Delta Factor (lmo2560) genes was found to be increased (2.2- to 3.2-fold), possibly suggesting compensation for HPP-induced inhibition of RNA synthesis. Delta Factor occurs only amongst Gram-positive bacteria, and based on *in vitro* studies in *Bacillus subtilis*, seems to have a global effect on gene expression (Gao & Aronson, 2004; Lopez de Saro *et al.*, 1999; Seepersaud *et al.*, 2006), and may play a role in maintaining the efficiency of transcription through improving specificity and by RNA polymerase recycling (Juang & Helmann, 1995; Lopez de Saro *et al.*, 1999). However, the *in vivo* role of Delta Factor is still very poorly understood in *L. monocytogenes*. A number of transcription-associated genes were also observed to show significant upregulation (*T*-value scores 3.83 and 4.50; Table 2), including those encoding proteins directly involved in transcription termination/antitermination activities (e.g. *nusA*, *nusG* and *rho*; increases of 2.0- to 4.3-fold).

Two cold-shock protein genes, *cspB* (lmo2016) and *cspL* (lmo1364), exhibited very strong expression increases (8.6- to 35.3-fold). These genes encode CspA-like RNA-binding proteins that potentially act as RNA chaperones, preventing secondary structure formation during RNA transcription that, if uncorrected, could potentially stall transcription (Jiang *et al.*, 1997). The responses of these particular genes may correspond to the general observation that high hydrostatic pressure stalls transcription elongation complexes (Erijman & Clegg 1998). Cold-shock proteins have been observed previously to be strongly upregulated by HPP treatments and low temperature in *L. monocytogenes* (Wemekamp-Kamphuis *et al.*, 2004). 50S ribosomal protein genes may also contribute as RNA chaperones (Semrad *et al.*, 2004), in particular L13 (lmo2597) and L19 (lmo1787), both of which were significantly upregulated under HPP (increases of 4.7- to 29.1-fold).

HPP effects on heat-shock proteins

Induction of heat-shock protein-coding genes was in general not observed (*T*-value scores -2.39 and -2.34; Table 2), indicating that adiabatic heating did not induce a heat-shock-like response during HPP treatments. Varying between the HPP treatments, certain heat-shock proteins either showed repression (*groEL*, *htrA*, *dnaK*, *clpC* and *clpE*; decreases of -2.2- to -16.5-fold) or only slight increases (*clpQ*, *clpP* and *clpX*; up to a 2.0-fold increase). *L. monocytogenes* Scott A clones possessing mutations in the stress response regulator *ctsR* developed broad stress tolerance, including piezotolerance (Karatzas *et al.*, 2005). Interestingly, mutants of *E. coli* overexpressing heat-shock genes *dnaK*, *lon* and *clpPX* have also been shown to possess enhanced tolerance to HPP treatments. Mild heat also cross-protects the corresponding wild-type strains against HPP (Aertsen *et al.*, 2004b). Thus it is possible that *L. monocytogenes ctsR* mutants may overexpress certain heat-shock proteins that could protect against damage induced by HPP that are otherwise not substantially increased in expression or are even repressed by HPP exposure as observed here.

Effects of HPP on translation-apparatus-related genes

A large number of genes directly involved with translation and ribosomes exhibited increased expression (*T*-value scores 7.11 and 8.49; Table 2). HPP has a well known dissociative affect on ribosomes (Kaletunç *et al.*, 2004). In the experiments performed here, HPP induced increased expression of ribosomal protein genes (*T*-value scores 6.11 and 7.01; Table 2) and translation-associated genes (*T*-value scores 4.05 and 4.14; Table 2), including genes encoding proteins playing roles in tRNA and rRNA functionality, assembly and/or stabilization. Little is known about how specifically HPP affects ribosomes and the resultant responses that are induced, including repair mechanisms or other forms of compensation; however, the very long list of ribosome and translation-associated genes responding to HPP (46–58 genes upregulated >twofold, and 3–7 genes downregulated >twofold) suggests that the response is quite profound.

HPP stimulates the expression of genes associated with flagella, chemotaxis and protein secretion

HPP exposure led to increased expression of several genes encoding proteins associated with protein secretion and trafficking, including *secEG*, *yidC*, *yajC*, *ftsY*, *ffh*, signal peptidase I homologues lmo1269–1271 (increases up to 4.3-fold) and flagella export apparatus genes (*T*-value score 3.52 for the 400 MPa 5 min treatment; Table 2). The other predicted protein secretory pathways in *L. monocytogenes* either had unaltered expression (twin-arginine translocation, holins) or were not significantly expressed (fimbriae

protein export, WXG100 secretion system) in either HPP-exposed or control cells (Table 2). The Sec apparatus is the main protein secretion system in *L. monocytogenes* and is predicted to translocate 508 proteins to the cell membrane, cell wall or extracellularly (reviewed by Desvaux & Hébraud, 2006). The upregulation of Sec apparatus genes may suggest that increased translocation of proteins for the purposes of cell maintenance and repair occurs in HPP-treated cells.

Flagella export apparatus gene upregulation corresponded with upregulation of several other flagella structural component genes (*T*-value scores 4.91 and 2.06; Table 2) and chemotaxis genes (*T*-value scores 3.67 and 2.30; Table 2). The recently identified flagella synthesis anti-repressor gene *gmaR* (lmo0688) (Shen *et al.*, 2006) was upregulated 2.0- to 3.6-fold.

It was also observed that *agrD* (encoding a putative quorum sensory peptide precursor) and *agrB* (putatively involved in AgrD maturation and export) of the accessory gene regulator cluster (lmo0048–0051) had strongly increased expression (up to 8.3-fold). These genes are homologous to the *agr* operon of *Staphylococcus aureus* (Autret *et al.*, 2003), and have been shown to be important for virulence (Autret *et al.*, 2003) and more recently to be linked to sessile growth of *L. monocytogenes* (Rieu *et al.*, 2007). The *agr* signalling system is known to react in a complex manner to environmental cues and probably also acts to regulate biofilm formation in *S. aureus* (Yarwood *et al.*, 2004). A diverse range of proteins are known to be induced when *L. monocytogenes* exists in a surface-associated state (Trémoulet *et al.*, 2002; Helloin *et al.*, 2003); the significance of increased expression of flagella, chemotaxis gene expression and *agr* cluster genes with regard to HPP is difficult to interpret at this time, but hypothetically may represent a general response to mechanical stress damage, leading to maintenance of a planktonic state rather than a sessile state.

HPP effect on genes associated with the septal ring, the cell wall and membranes

Overall, cell-division-associated genes were upregulated by HPP (*T*-value scores 4.61 and 4.10; Table 2). Of the known septal-ring-associated proteins in *E. coli* (Weiss, 2004), expression of homologues to most of these found in *L. monocytogenes* S2542 was observed to increase following HPP treatment. These included *ftsL* (lmo2033), *ftsA* (lmo2034), *ftsI/pbpB* (lmo2039), *ftsQ* (lmo2040), *ftsE* (lmo2506) and *ftsX* (lmo2507). Interestingly, amongst the predicted septal-ring protein genes in *L. monocytogenes*, *ftsZ* (lmo2032), encoding a tubulin-like GTPase that polymerizes to form the basis of the septal ring, did not show differential expression under HPP (confirmed by RT-PCR, Fig. 3). The expression of several other gene homologues encoding proteins possibly associated with mediation of septum placement (*ezrA*, *divIVA*, *minC*) and cell shape (*mreB*, *mreD*, *mpl*) also showed increased

expression (increases up to 11.9-fold; see supplementary data files 1 and 2).

Upregulation of the biosynthesis or modification of cell-wall components (*T*-value scores 4.01 and 3.55; Table 2) was suggested by increased expression of genes associated with biosynthesis of cell-wall polymers, in particular peptidoglycan (e.g. *gcaD*, *glmS*, *murACEG*, *mraY*; increases of 2.0- to 4.4-fold). A gene homologous to *uppS* (lmo1315), encoding a cell-wall component carrier lipid protein required for the assembly of peptidoglycan, teichoic acid and other cell-wall components in bacteria (Apfel *et al.*, 1999), was also upregulated considerably (3.0- to 4.7-fold). This is also suggested by an increase in the expression of several genes associated with synthesis of isoprenyl units (see supplementary data files 1 and 2 for genes categorized under steroid and terpenoid biosynthesis and isoprenoid biosynthesis). Genes of the Opp oligopeptide transporter system (lmo2194–2196) were upregulated under HPP; however, it is unclear what function this transporter system has in *L. monocytogenes*, although it is associated with the cold-stress response and intracellular survival (Tasara & Stephan, 2006). In *Salmonella* Typhimurium, homologous genes have been shown to have a possible role in recycling cell-wall peptides (Hiles *et al.*, 1987).

Several genes associated with fatty acid biosynthesis were found to increase in expression (*T*-value scores 4.77 and 4.10; Table 2). A *fabR*-like transcriptional regulator (lmo1810), involved with global regulation of fatty acid biosynthesis in *B. subtilis* (Schujman *et al.*, 2003), had an up to 7.0-fold increased expression.

At the levels used in this study, HPP has been observed to cause mechanical cell-wall and membrane damage in *E. coli*, leading to loss of viability, especially in actively dividing cells (Alpas *et al.*, 1999). The damage to cell membranes by HPP could be a main cause of cell inactivation or death in Gram-negative bacteria, but whether this is the case in Gram-positive bacteria is uncertain. Stabilization of cell membranes and walls in the stationary growth phase does provide a protective benefit against HPP (Mañas & Mackey 2004); thus it is probably a major factor for the survival of HPP-induced damage. Beyond direct damage to the cell envelope, HPP also appears to interfere with the formation of nascent septal rings as well as other associated cell-wall formation and chromosome segregation processes. HPP has been shown to directly interfere with FtsZ polymerization and septal-ring formation, leading to a filamentous cell morphotype in *E. coli* (Kawarai *et al.*, 2004). As the septal ring is integral to cell division, damage to this system would effectively lead to loss of cell culturability, but re-establishment of the septal ring may allow for cell recovery. The tubulin-like GTPase FtsZ, which forms the backbone of the septal-ring structure, showed significant but unchanged expression in this study, suggesting that the proteins complexing with polymerized FtsZ are perhaps being dissociated by HPP

and, as Kawarai *et al.* (2004) suggested, leading to prevention of complete or new ring structures. Several other genes associated with the regulation of septal-ring position, formation and cell shape, as well as the synthesis or reassembly of cell-wall constituents, in particular peptidoglycan and fatty acids, were observed to have increased expression in this study. Thus cell-wall and membrane damage due to HPP exposure may lead to *L. monocytogenes* compensating by increasing cell division and cell-envelope-associated gene expression, presumably leading to eventual replacement of damaged components.

HPP suppresses genes associated with catabolism and virulence

HPP appeared to suppress many catabolic genes, especially those associated with oxidative phosphorylation (*T*-value scores -3.93 and -2.88 ; Table 2), intermediary metabolism (pyruvate and butanoate metabolic pathways; *T*-value scores -2.67 to -3.04), carbohydrate-associated phosphotransferase system (PTS) transporters (*T*-value scores -2.97 and -2.83), glycolysis (*T*-value scores -3.36 and -3.86) and glycerolipid metabolism (*T*-value scores -4.43 and -2.96).

Reduction in the expression of cytochrome *bo* terminal oxidase (*cyoABCD*), various F_1F_0 -ATPase genes (lmo0088–0092, lmo2528–2530), and NADH oxidase/dehydrogenase (lmo2389, lmo2471) could suggest that HPP causes a reduction in oxidative phosphorylation energy conversion. Amongst intermediary metabolism pathways, several genes were suppressed, including *pdhABCD*, *pflAC*, acetyl-CoA synthase (lmo2720), *ldh*, *alsDS* and pyruvate oxidase (lmo0722). In addition, glycolysis (lmo2455–2457), glycerol metabolism (lmo348, lmo1293, lmo1538, lmo1539, lmo2695, lmo2696) and putative inositol/inositol phosphate metabolism genes (lmo0383–0388) were also repressed. Potentially connected with the downregulation of catabolism genes, it was observed that certain putative repressors of catabolism in *L. monocytogenes* were upregulated. These included a twofold increase in expression of catabolite control protein gene *ccpA* (lmo1599) (Behari & Youngman 1998) and a 3.6- to 5.2-fold increase in the expression of lmo2460, a gene very similar to the *B. subtilis* glycolysis *gap* operon repressor gene *cggR* (Doan & Aymerich 2003). HPP exposure overall repressed PTS transporter gene expression. However, some exceptions were observed – the mannose-specific PTS transporter *mpt* operon (lmo0096–0099) demonstrated a large increase in expression (up to a 15.7-fold increase) and was the most highly expressed amongst the 90 PTS transporter genes present in *L. monocytogenes* (Table 2). PTS-related genes for glucose (lmo1017) and trehalose uptake (lmo1255), as well as the gene encoding a trehalose-6-phosphate hydrolase (lmo1254), which converts trehalose to glucose, were also upregulated (Table 2). On the other hand, many other PTS transporters were downregulated as suggested by their low *T*-value scores. It appears that, when growing in BHI

broth, *L. monocytogenes* actively expresses many of its PTS transporters and other carbohydrate-transporting genes; however, after HPP exposure, active expression of these genes appears to be reduced and this has a knock-on effect on genes involved in energy production/conversion and intermediary metabolism. By comparison, genes encoding proteins involved in amino acid transport and metabolism were less affected by HPP (Table 2).

Repression of several energy production/conversion, carbohydrate and other carbon compound catabolic genes, as well as several PTS transporters, may represent a diminishment of catabolism in cells imposed by HPP treatments. Suppression of F_1F_0 -ATPase by HPP has been observed in *Lactobacillus plantarum* (Wouters *et al.*, 1998) and the genes downregulated in this study encode subunits (γ , ϵ and c chains) that perform the integral proton translocation step that has been observed to be inhibited in *Enterococcus hirae* under mild HPP treatments (Marquis & Bender, 1987). No previous specific molecular-based study has shown that HPP diminishes catabolic gene expression; however, physiological studies have demonstrated that metabolic activity clearly declines (Abe *et al.*, 1999; Ulmer *et al.*, 2000; Kilimann *et al.*, 2005) and cell maintenance energy requirements increase (Bothun *et al.*, 2004). Cell maintenance energy essentially includes any energy consumption that is required to maintain an energized cell membrane, repair cellular components, transport substances in and out of the cell and support cell functions such as motility.

HPP suppresses the SigB and PrfA regulons

The SigB and PrfA regulons include 20 genes that are controlled by both regulators; thus, the simultaneous strong downregulation of these regulons (*sigB* and *prfA* decreases of -2.1 - to -4.4 -fold; Table 2) is not surprising in itself; however, it was interesting to observe that HPP-induced repression also extended to genes that are independently controlled by each regulator. SigB has been shown to be required for effective osmotic- and acid-stress survival (Kazmierczak *et al.*, 2003) and, together with PrfA, helps regulate virulence. PrfA itself is the main regulator of virulence in *L. monocytogenes* (Milohanic *et al.*, 2003). SigB and PrfA (as well as CodY, see below) are also involved in controlling other specific transport and metabolic genes, including many that have unknown functions, and phage-derived genes. As would be expected with the repression of SigB and PrfA regulons, virulence-related functional genes demonstrated significantly reduced expression after HPP exposure (*T*-value scores -4.86 and -4.45 ; Table 2) affecting *hly*, *actA*, *iap* and *bsh*, and genes encoding internalins A, B, G and H (decreases of up to -53 -fold) most strongly.

Perhaps relevant to changes in SigB and PrfA regulon expression, it was also observed that HPP influences the expression of genes within the CodY regulon (Bennett *et al.*, 2007), but these responses are more muted, possibly due to

not all genes being under the direct control of CodY. As observed mainly in *B. subtilis*, CodY helps regulate the transition from rapid exponential growth to the stationary growth phase (Sonenshein, 2005). In *L. monocytogenes*, CodY controls genes associated with amino acid biosynthesis, carbohydrate metabolism and transport, nitrogen uptake, motility and chemotaxis (Bennett *et al.*, 2007). It has been shown that *codY* is derepressed by the stringent response activator *relA* and has a major role in monitoring the energetic capacity and nutritional state of the cell (Sonenshein, 2005).

HPP also differentially affected a large number of transcriptional regulators that still do not have defined roles in *L. monocytogenes* but potentially may be relevant to the regulation of metabolism. HPP strongly repressed all three *uspA* homologues (lmo0515, lmo1580, lmo2673; decrease up to –56-fold) as well as most predicted *crp/fnr* family regulators (lmo0085, lmo0112, lmo0156, lmo0274, lmo0548, lmo0740 and lmo2744; overall *T*-value scores –3.01 and –2.75; Table 2). The Crp/Fnr superfamily of regulators are mainly involved in the regulation of catabolite-sensitive genes in *E. coli* (Zheng *et al.*, 2004), but little is known about their specific roles in *L. monocytogenes* (Bayles & Uhlich, 2006). The repression of the *uspA*-like genes is particularly interesting as they have been linked to a wide range of regulatory roles in *E. coli* that appear to be geared towards adjusting the metabolism of the cell to defend against stress (Nachin *et al.*, 2005).

Conclusions

As shown previously in the literature, HPP inactivation of cellular growth is mainly due to mechanical damage to cell walls and membranes, septal rings, the DNA helix and the ribosome translational apparatus, and also to interference with RNA synthesis. In this study it was shown that in *L. monocytogenes* the expression of genes associated with these HPP damage targets were upregulated (Table 2), indicating the induction of a generalized repair and maintenance response. Sustaining HPP, depending on the pressure level, overcomes the ability of the cells to self-repair, leading to eventual complete inactivation of the cell population (Fig. 1). It was shown that at 600 MPa this is a particularly rapid process and is accompanied by a reduction in mRNA pools of major housekeeping genes, including *tufA* and *rpoC* (Fig. 2). HPP-induced increases in motility- and chemotaxis-related gene expression (Table 2) may be linked to repair processes as well as to changes in the energy capacity of the cell. One hypothesis is that increased flagellar assembly gene expression and chemotaxis is needed for better acquisition of specific nutrients required for biosynthetic pathways. This leads to the interesting observation that HPP extensively represses energy and carbon-compound-catabolic gene expression. Given that CodY gene expression was also affected, this could indicate that HPP forces a switch from active growth dominated by the catabolism of carbohydrates, as is the case in *L.*

monocytogenes in the mid-exponential growth phase, to a state where cell repair is more paramount. The connection between catabolic gene repression and virulence caused by HPP thus seems logical in this scenario since the virulence functionality of *L. monocytogenes* (and other functions controlled by the SigB and PrfA regulons) essentially represent adaptations for maintaining successful and continued cell division under different environmental pressures.

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