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ORIGINAL PAPER

Gene expression profiling of a pressure-tolerant *Listeria monocytogenes* Scott A *ctsR* deletion mutant

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Abstract *Listeria monocytogenes* is a food-borne pathogen of significant threat to public health. High hydrostatic pressure (HHP) treatment can be used to control *Listeria monocytogenes* in food. The CtsR (class three stress gene repressor) protein negatively regulates the expression of class III heat shock genes. A spontaneous pressure-tolerant *ctsR* mutant 2-1 that was able to survive under HHP treatment has been identified previously. So far, there is only limited information about the mechanisms of survival and adaptation of this mutant to high pressure. Microarray technology was used to monitor the gene expression profiles of the *ctsR* mutant 2-1 under HHP treatment. Compared to pressure-treated *L. monocytogenes* Scott A wild type, 17 genes were up-regulated (>2-fold increase) in the *ctsR* mutant 2-1, whereas 58 genes were down-regulated (<−2-fold decrease). The entire *clpC* operon was up-regulated in the *ctsR* mutant 2-1, indicating that the mutant CtsR protein was not a functional repressor. The increased levels of expression of stress-related genes in *ctsR* mutant 2-1 may contribute to its survival under high pressure. The reduced expression levels of the genes related to virulence, flagella synthesis, and cell division in the *ctsR* mutant 2-1 correlate with its characteristics (elongated cells, reduced virulence, and absence of flagella). The gene expression changes determined by microarray assays were confirmed by real-time reverse transcriptase PCR analyses. This study enhances our understanding of how *Listeria monocytogenes* survives under HHP and may contribute to the design of effective and economically feasible HHP treatment in food processing.

Keywords *Listeria monocytogenes* Scott A · Microarray and real-time PCR · High hydrostatic pressure (HHP) treatment · *ctsR* mutant

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Introduction

Listeria monocytogenes, a Gram-positive bacterium, is of major concern to the food industry. This bacterium is pathogenic to both humans and animals, particularly susceptible individuals such as pregnant women, newborns, people over 65 years old, and immunocompromised patients. *L. monocytogenes* is widely distributed in the environment, including soil and food. Well-documented outbreaks of listeriosis have been associated with the

consumption of contaminated food products including ready-to-eat (RTE) meats [9]. Because *L. monocytogenes* can survive in foods under very harsh conditions, including high acidity and low temperature, it is very difficult to eliminate this pathogen from foods and/or food processing plants.

High pressure processing has been utilized commercially for processing of RTE meats to control *L. monocytogenes* and extend product shelf life. The advantage of this technology is to inactivate microorganisms without significant deterioration of food quality. High pressure resulted in changes in viability, morphology, and physiology in bacteria such as *Escherichia coli* and *L. monocytogenes* [23, 29, 32]. However, the molecular survival mechanisms of *L. monocytogenes* under pressure remain unknown. Microarrays have been used to study differential gene expression of *L. monocytogenes* and *E. coli* during high pressure processing and some important genes have been identified [4, 12, 22].

The *ctsR* gene encodes a transcriptional regulator that represses the class III heat shock genes. CtsR protein is encoded by the first gene of the *clpC* operon that includes *ctsR*, *mcsA*, *mcsB*, and *clpC* [18]. Under non-stressed conditions, the CtsR protein acting as a dimer negatively regulates *clpC*, *clpP*, and *clpE* genes by binding specifically to the regulatory regions of these genes [26, 27]. Under stressed conditions, CtsR protein is inactivated by McsB phosphorylation [10, 17, 19] and degraded by the ClpCP protease and ClpE [25], resulting in the elevation of these Clp proteases [20]. The elevated Clp proteases specifically degrade misfolded proteins generated by stress [19]. The CtsR protein, which is highly conserved among Gram-positive bacteria [6], has three functional domains: a dimerization domain, a helix-turn-helix DNA binding domain, and a putative heat-sensing domain. Mutational analysis of this gene indicated that the *N*-terminus of the CtsR protein is highly conserved and is important for dimerization and heat sensing [7]. Using microarray analysis, Hu et al. identified genes that were regulated by the CtsR protein in the *ctsR* deletion strain [11]. Furthermore, a *ctsR* deletion strain in *L. monocytogenes* did not affect its virulence, whereas overexpression of CtsR decreased virulence [27].

CtsR has been shown to be related to high pressure because several pressure-tolerant mutants contained mutations in this gene [13–16, 33]. The *L. monocytogenes* Scott A *ctsR* mutant AK01 containing a glycine deletion was immotile and showed more resistance to heat, acid, and H_2O_2 than the wild type. The *L. monocytogenes* Scott A *ctsR* mutant 2-1 exhibited a 100-fold higher level of viability than the wild type when exposed to 450 MPa. The *ctsR* mutant 2-1 had a deletion in the *ctsR* gene that resulted in production of truncated CtsR of 20 amino acids compared to a CtsR of 152 amino acids in the wild type [13]. The *N*-terminal truncation of CtsR in mutant 2-1 lacked the DNA binding domain. The mutant 2-1 was less virulent, immotile, heat and acid resistant, and sensitive to nisin [13]. However, the mechanism of its survival under pressure is unclear. In this study, microarray was used to study the gene expression of *ctsR* mutant 2-1 under pressure. The genes that were differentially regulated in *ctsR* mutant 2-1 were verified with real-time PCR assays. This study highlights the importance of CtsR in pressure tolerance and will contribute to the appropriate design of effective and feasible high hydrostatic pressure (HHP) treatments.

Materials and methods

Bacterial strains and HHP treatments

The *ctsR* mutant 2-1 of *L. monocytogenes* Scott A [19] and wild-type *L. monocytogenes* strain Scott A were streaked onto a Brain Heart Infusion (BHI) (Fluka BioChemika, catalog# 53286, Sigma–Aldrich St. Louis, MO) agar plate from a glycerol stock culture (stored at −80°C) followed by incubation at 30°C overnight. A single colony was picked from the plate, inoculated into 20 ml of BHI broth, and grown at 30°C with agitation at 170 rpm. A 7.5-ml aliquot of this overnight culture was used to inoculate 142.5 ml of BHI broth. After growth at 30°C for 3 h [optical density at 600 nm (OD_{600}) 0.7 to 0.8], 150 ml of the suspension was centrifuged at 5,000 rpm for 8 min at room temperature. The pellets resuspended in 25 ml BHI were transferred to sterile nylon-polyethylene bags (Prime Source Packaging Ltd., Houston, TX) and vacuum-sealed to 950 mbar using a vacuum-packaging unit (Ultravac-500, Bunzl/Koch). The sealed bags were then transferred to a second nylon-polyethylene bag and vacuum-sealed again to 950 mbar. The samples were treated (450 MPa, 3 min) using high pressure equipment at the Eastern Regional Research Center (2-L capacity; Model 2L; Avure Technologies, Kent, WA). Biological duplicates were tested for wild type and *ctsR* mutant 2-1. After pressure treatment, 2 ml of the suspension was used for plate counting; the rest of the suspension was centrifuged at 4,500 rpm for 5 min at room temperature. The pellets were resuspended in 1.5 ml RNAlater prior to RNA isolation.

Scanning electron microscopy

A single colony was picked from the plate and inoculated into 20 ml of BHI broth and grown at 30°C with agitation at 170 rpm. A 7.5-ml aliquot of this overnight culture was used to inoculate 142.5 ml of BHI broth. After growth at

30°C for 3 h (OD_{600} 0.7 to 0.8), 50-µl aliquots of bacterial suspensions were deposited on glass coverslips and, after 60 s, the coverslips were immersed into a multiwell plate with 2-ml volume of a fixative solution containing 2.5% glutaraldehyde/0.1 M imidazole buffer solution (pH 7.2), then covered and sealed in a polyethylene bag for at least 2 h before further processing. Subsequently, the fixative solution was exchanged with imidazole buffer, and the samples were dehydrated by exchange with 2-ml volumes of graded ethanol solutions (50%, 80%, and absolute); two changes at each concentration. Finally the coverslips were critical point dried from liquid CO_2 in a DCP-1 Critical Point Dryer (Denton Vacuum, Inc., Cherry Hill, NJ). The coverslips were mounted on Al specimen stubs with carbon adhesive tabs, and edges were painted with colloidal silver adhesive (Electron Microscopy Sciences, Hatfield, PA), then sputter coated with a thin layer of gold using a Scancoat Six Sputter Coater (BOC Edwards, Wilmington, MA). Digital images of topographical features of the bacteria samples were collected using a Quanta 200 FEG environmental scanning electron microscope (FEI Co., Inc., Hillsboro, OR) operated in the high vacuum/secondary electron imaging mode at an accelerating voltage of 10 kV and instrumental magnification ×25,000.

RNA isolation, microarray chip design, hybridization, and data analysis

Total RNA was isolated using the Ambion RiboPure™-Bacteria Kit (Ambion, Austin, Texas; catalog# 1925) according to the manufacturer's instructions with the following modification: RNA samples were incubated for 2.5 h at 37°C for DNase I treatment. The concentration and purity of RNA were evaluated using an Agilent 2100 bioanalyzer (Agilent Technologies, Wilmington, DE) and absorbance readings at 260 nm and 280 nm using the Nanodrop®ND100 UV–Vis spectrophotometer (Nonodrop Technologies, Wilminton, DE).

A whole genome microarray was constructed to include 35-mer oligonucleotides representing the 2,847 open reading frames (ORFs) identified based on the annotated genome for *L. monocytogenes* strain F2365 (accession# AE017262; 24). For each ORF, two unique probes were selected to be specific as judged by pairwise BLASTN [2]. The probes were designed to have similar annealing stability, i.e., a T_m of 72°C, as judged by a nearest neighbor thermodynamic model [1]. Probes that had significant secondary structure ($T_m >$ 45°C), significant repeat structure, and/or guanine-cytosine content (GC%) outside of the range 35–65% were rejected. Each probe was custom synthesized in duplicate by Combimatrix (Combimatrix, Mukilteo, WA).

To save on the cost of microarray chips, the balanced block design [8] with dual-labeled microarrays was used in this study. Dye swap experiments were performed to eliminate the dye bias caused by Alexa 555 and Alexa 647. Two biological (two independent RNA sources) and two technical (same RNA samples divided into two aliquots) replicates were included to ensure accurate measurements. Ten micrograms of total RNA was reverse transcribed into cDNA and labeled with Alexa Fluor dyes (either Alexa Fluor 555 or Alexa Fluor 647) using Superscript Reverse Transcriptase III (Invitrogen Inc., Carlsbad, CA). The fluorescence incorporation in the cDNA was measured using a Nanodrop Spectrophotometer. Equal amounts (50–100 pmol) of Alexa Fluor 555 and 647 labeled probes were mixed and used for microarray hybridization. All samples were hybridized twice with one experiment (chip 1) using Alexa Fluor 555 to label the cDNA from wild type and Alexa Fluor 647 to label cDNA from *ctsR* mutant 2-1; in the reciprocal experiment (chip2), Alexa Fluor 647 was used to label the cDNA from wild type and Alexa Fluor 555 to label the cDNA from *ctsR* mutant 2-1. The expression ratio of a particular gene was calculated as follows: [chip1 (Alexa Fluor 555/647) + chip2 (Alexa Fluor 647/555)]/2. Each experiment was performed in duplicate. Microarray hybridization and washing were performed according to the CustomArray 12 K microarray protocols provided by Combimatrix. The microarray slide was scanned at 5-µm resolution by the ScanArray ExpressHT microarray scanner (Packard Bioscience, Biochip Technologies, Billerica, MA). The intensity of the signal was quantified by Microarray Imager software provided by Combimatrix.

Microarray data were analyzed using the software package BRB-ArrayTools (version 3.4), developed by the Biometric Research Branch of the US National Cancer Institute (http://linus.nci.nih.gov/BRB-ArrayTools.html) according to the instructions provided in the software package. The lowest 5% of the signals were used as background. The median of the entire array was used for data normalization. A minimum threshold of a 2-fold change in gene expression and $P < 0.01$ were used as the cutoff values.

cDNA synthesis, primer design, and real-time PCR analysis

Synthesis of cDNA was carried out using Invitrogen's SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA; catalog# 18080-400) following manufacturer's instructions. Reactions were prepared for each RNA sample using 1 µg of DNase I treated RNA, including reactions with and without reverse transcriptase (negative control). cDNA synthesis was performed on Applied Biosystems GeneAmp®PCR System 9600. Primers were designed using Primer3 (v.0.4.0) software and selected

based on the gene sequences of *L. monocytogenes* F2365 strain (GenBank accession# AE017262). Primer sequences were checked using the National Center for Biotechnology Information (NCBI) BLASTN program against the non-redundant (nr) database which indicated that the primer sequences showed homology to the *L. monocytogenes* F2365 strain (GenBank accession# AE017262). Primers were ordered from IDT (www.idtdna.com) and are listed in Tables 1 and 2. The housekeeping gene (*spoG*) was used as the internal control gene for real-time PCR analysis (primer sequences 5′TGACGGTGAATTCCGTGATA3′; 5′TCA GCAGAAACGGATTCAGA3′) because this gene had the least variation among other housekeeping genes. PCR was performed in a 96-well plate on an Applied Biosystems 7500 Real-Time (ABI, Carlsbad, CA) PCR System in a 50-µl total volume and contained 25 µl Power SYBR Green PCR Master Mix (ABI), 1.25 µl of each primer at 10 µM, 0.5 µl of cDNA and nuclease-free water (Ambion). Thermal cycling parameters were 50°C for 2 min for 1 cycle, an initial denaturation at 95°C for 10 min for 1 cycle, followed by 35 cycles of 95°C for 15 s and 55°C for 1 min. Fluorescence data were collected at the 55°C annealing step. The final step was a dissociation curve of 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s. Results were visualized using the 7500 System SDS Software provided with the thermocylcer. To determine relative gene expression, the value of the internal control gene was subtracted from the pressure treated samples. The ΔCt, ΔΔCt, and the 2^{-fx} values were calculated as previously described [21].

Microarray data accession number

The microarray data were deposited into the Gene Expression Omnibus (GEO) database under accession number GSE22819 (www.ncbi.nlm.nih.gov/geo).

Results

Scanning electron microscopy of the *ctsR* mutant 2-1

To examine the morphology of *ctsR* mutant 2-1, wild-type and *ctsR* mutant 2-1 cells grown to exponential phase (OD_{600} 0.7) were subject to scanning electron microscopy (SEM) analysis. Compared to the wild-type *L. monocytogenes*, the mutant 2-1 cells lacked flagella and became 5–10-fold longer (Fig. 1).

Up-regulated genes in *L. monocytogenes ctsR* mutant 2-1 strain under pressure treatment

A total of 17 genes were expressed at higher levels in the *ctsR* mutant 2-1 than in the wild type under pressure treatment (Table 3). These genes grouped into the following categories: genes encoding for transport and binding, signal transduction, cellular processes, transcriptional regulator, energy metabolism, protein fate, and hypothetical proteins of unknown functions.

In *L. monocytogenes*, the *clpC* operon contains four genes: *ctsR*, *mscA*, *mscB*, and *clpC* (*LMOf2365_lmo0241* to

Table 1 Oligonucleotides used for real-time PCR to evaluate up-regulated genes

Gene	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)
LMOf2365_lmo0057	TTGATGATGTTTGTGCTTGC	CTTCACCGATTAAAGGCAAA	145
LMOf2365_lmo0058	ATCCATGAAAGTTGCGGATT	ATTTTCGTTTTTCTCTTGCATT	100
LMOf2365_lmo0667	CGCTCATTATTTTGCCTGTT	CGACAAGCTCTTCTGTTTCA	114
LMOf2365_lmo0241	AAGTAAACGTGGTGGTGGTG	GCCACCATTAATCTTGCTTC	189
LMOf2365_lmo0242	GATTTGGTTAAAGCGATGGA	AGCTGGCAATTCTCACAAAC	110
LMOf2365_lmo0243	TGAATATGGTGCGGTTCTTT	CTGCCTCTAACGCATCAAAT	122
LMOf2365_lmo0244	TCCAACGTGTGATTGAAGTG	CAATTTGTTGCGCTAGTCCT	111
LMOf2365_lmo0442	ATTGAGTGGGAATCACTGGA	AGTTTCGTTGCAATTTCTGC	116
LMOf2365_lmo0443	GTAGAAACACAAGGCGCAAC	TTCCAGCACGACTTTACCAT	135
LMOf2365_lmo0444	GCGCCAATTAGTGGTTTTTA	TAGCTTGAACCAGCCATTTC	149
LMOf2365_lmo0445	TGAAAACGGAGAAATCAAGC	AACATCCAGCAGTTCGCTAC	149
LMOf2365_lmo1018	TCACCGTATTTTCGTCCAGT	CAGAAGCACTAACAGCAGCA	141
LMOf2365_lmo1272	GTTTTCGTGCCGTCTTCTAA	CAGCGTTTACTTGCTGGTTT	128
LMOf2365_lmo2097	TCATTCTGCCGTTTTTCTTC	GGTCCAGCTTGGTATCTCAC	102
LMOf2365_lmo2147	TGGAATTATGGGACCATCTG	TTGTCCGGAAATCACAATCT	100
LMOf2365_lmo2148	TTTGCACTTCTCGGACTTTC	TTGCCGAAAACGATATTTGT	122
LMOf2365_lmo2620	CTTATACGGCGTTCCTTCAA	CGGGGATTTTGAAACCTACT	109

Table 2 Oligonucleotides used for real-time PCR to evaluate down-regulated genes

Gene	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)
LMOf2365_lmo0113	ATTCACGTCTCTTGCATGGT	TTACGAAGGTCGTCTTTTGC	106
LMOf2365_lmo0114	GGTAGTTGCCGTTGGTTATG	AGAGCACCAATTGCGATAAG	126
LMOf2365_lmo0115	CGGCTACAAAGCTGGTTCTA	ATTTACCCATCTCTGCACCA	124
LMOf2365_lmo0143	CCTTAATGAAGACGCGGATA	CCATTTGGTAGCGTTCATTC	113
LMOf2365_lmo0376	GCAGCACTAGAAGCAAAAGC	GAGGCGTTCATACCAGAAGA	104
LMOf2365_lmo0566	TACCAAATGGTGGTGCTTTT	TTCCACCATCGCAATAATTT	122
LMOf2365_lmo0710	ACTGGCATAAGCGAAATCAG	AAATGCCAAGAATTGGAACA	111
LMOf2365_lmo0729	GATGTCCTCGAAGTGGAAAA	TAACGATTCCGAAGTTCTCG	112
LMOf2365_lmo0730	CAAGAGGCTTACAACGCAGT	TTTTCGTGCGCTAATTTTTC	119
LMOf2365_lmo0731	AGCACCACTCGAACTCAAAG	TGTGGGTTCTTTCGGTGTAT	133
LMOf2365_lmo0739	AAATTTCGCAGTCCAAACAG	TCAGTCAGGGAAGGAGTTTG	121
LMOf2365_lmo0740	GCGGAAAATGCCATTATAGA	AAAAGCCAATTGCATTTCTG	111
LMOf2365_lmo0742	TTGCAAGAAACAAACAGCAA	TGGATTTACGTTCGGAAAAG	136
LMOf2365_lmo0744	CGGGCAAGAACTTTACGATA	ATCACTCCCTCGTAGCCTTC	147
LMOf2365_lmo0745	AAAGCACCGAACATTTACCA	GCAGCATTGCGACTTTTATT	145
LMOf2365_lmo0746	ATCTTCAAACAGCCAACCAA	GAGCCAAGTGATTCCTCAAA	100
LMOf2365_lmo0747	ATCCGAAATCACACCATTTG	ACCTGCTTCATTTGCGTTAG	130
LMOf2365_lmo0749	CAACAACAAGTCACCGATCA	ATCTTGGCCTGTGTATGGAA	144
LMOf2365_lmo0750	CCGCGCTAGATATTTTACGA	TCGATGCTTCTTCTGGTTTC	120
LMOf2365_lmo0751	CCAGTTGCTTTCGACAAACT	AATCGAGTCGCATATCGAAG	133
LMOf2365_lmo0753	GTTGATGTACGGCTCGAATC	CCATATTTGCGAACATTTCC	150
LMOf2365_lmo0754	CAAGCCAGCTTCAAAACCTA	TTGCATGTGGGTATCATTTG	134
LMOf2365_lmo0777	TGTTCGTGCAAAAATAAGCA	CTTTTTGCATTCTGCCAAGT	146
LMOf2365_lmo0808	CGCTGTTAAAGGGCTTGATA	GCATTTTGAACAGCTTTCGT	150
LMOf2365_lmo0847	TCCAAATAGCGAAGAAATGG	TACTTACCCGCGAAGCATAC	107
LMOf2365_lmo1090	TTGTAGTTCCAGACGCAACA	TCCATTGAATCGGTCACTTT	109
LMOf2365_lmo1093	TGAATTCCGCAAATACCCTA	GTTGCGTCGACAACTTTCTT	111
LMOf2365_lmo1191	CTGGAAGCTGAAATTCTGGA	CTGATTCGATTTGCTCGTTT	136
LMOf2365_lmo1193	TCGCTGAATTAGCTCGAATC	CGTTTATAAGCATGCGCTTT	130
LMOf2365_lmo1201	TCCTAATTGCGCTTATCCTG	ACGGGTTTATTTCCACTTCC	123
LMOf2365_lmo1248	AAACCGTCATTGCTGATGAT	TCCGGTTCAACACCTTCTAA	148
LMOf2365_lmo1311	TAAGCGCCGAGTGATTAGAG	AACGCTCTGTCCAAATCAAG	132
LMOf2365_lmo1322	AGCCGGAACATTAACTGCTA	CTTTATGCGGCGTTACATCT	146
LMOf2365_lmo1365	CTTTGGATTTGTGGAGGATG	TGCCTCAAAAACTTTTCCTG	127
LMOf2365_lmo1377	TGCGGTTGTTATTGATGTTG	CGCAAGTAGCATTGTGATTG	148
LMOf2365_lmo1379	CGAAAATGGTAGTGCCTCAC	CCATTCGTTTTTCTGCTGAT	107
LMOf2365_lmo1442	CTTTCACTGTGGCTGGTTCT	TTGAGCGGAGAAGTTTTCAC	100
LMOf2365_lmo1622	CGGCTTTGCTTTGTTTAGAA	GGTGTAGCCAAAGGAACAAA	115
LMOf2365_lmo1623	TCGCTTCGTTTTGCTCTACT	GTGGATCCAGTTTTTGATGC	115
LMOf2365_lmo1664	TATCCACTTGTTCCGCTGTT	GCCATTGTTATTCCACAAGC	145
LMOf2365_lmo1665	AGTTGATAATCGCGCAACTC	AGCAGGAGCACAAGATGAAC	132
LMOf2365_lmo1690	TGTTCGTGTATTTCCCTGGT	TTAGTGGAAGCTACCGATGC	132
LMOf2365_lmo1781	CATGCCATCTTGTTCATCAG	CATGCAATCGACGTATCAAA	102
LMOf2365_lmo1812	TTCCGGCAAGAGATTTAATG	TAGCGAATGCAGTGAAACAA	123
LMOf2365_lmo1848	CCAAGTGGTGAGGCATAATC	GAACGGACGTAGTCGCTAAA	127
LMOf2365_lmo1875	TTGTCTGGATCCGCTTTTAC	AGGCAAGACGTCTGAAACAG	105
LMOf2365_lmo1937	ATGACCGTATCGTGTGCTTT	CGAAAAACCGAAGAAAACAA	129
LMOf2365_lmo2045	CGCGATTTTACGAGCTTTAG	CATTAATCGTGGCACAAACA	137

Table 2 continued

Gene	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)
LMOf2365_lmo2071	GATCAGCAGGGTTGAAAGAA	GATGACAACTGTGGATGCAA	116
LMOf2365_lmo2072	TGTAAATTTGCGCTTGTACG	CATAGAGAAGCGGAACAACC	142
LMOf2365_lmo2229	TTTCAAACCAGCTTTCCAAG	CCTGCAAACAACCTTGTACC	132
LMOf2365_lmo2290	GGCCAATGAGATGAATGAAA	TTCATGGGGATAAGACCCTA	129
LMOf2365_lmo2300	CCTGCTTAACCCAGAGACAA	CGAACGCTTAGAAGTTCGAG	139
LMOf2365_lmo2479	CCAGTTGGTCGCTCCTACTA	CTTGAAATGGGGACGATATG	148
LMOf2365_lmo2495	TTTTACGCCCCAGTTGTTAG	GTTGAAGGATACGGACAAGC	111
LMOf2365_lmo2534	TTGAAATGCGACTTCACTCA	CGGAGAAGTACTGCGTCTGT	120
LMOf2365_lmo2670	GCTTACAACGGCCAATCTTA	TTTCGAATGACTTGTGCGTA	137

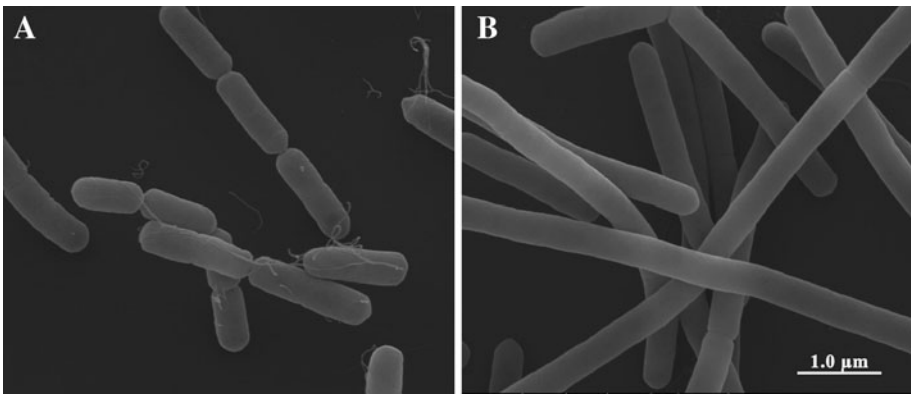

Fig. 1 Morphological characteristics of exponentially grown cells of *L. monocytogeneces* Scott A wild type (**a**) and *ctsR* mutant 2-1 with scanning electron microscopy (SEM). *Bars* 1 µm

LMOf2365_lmo0244). *LMOf2365_lmo0241* encodes for the CtsR protein that negatively regulates the clpC operon by binding to their promoter regions [27]. *LMOf2365_lmo0242* encodes for a UVR domain protein that shows homology with McsA (modulator of ctsR repressor), whereas *LMOf2365_lmo0243* encodes for a ATP:guanido phosphotransferase family protein that is homologous to MscB. *LMOf2365_lmo0244* encodes for a ClpATPase [28]. In this study, we have shown that the entire *clpC* operon containing mutant CtsR protein is induced under high pressure.

In *ctsR* mutant 2-1, *LMOf2365_lmo0241* (encodes ctsR) is up-regulated 6.3-fold and 10.2-fold by microarray and real-time PCR assays, respectively. *LMOf2365_lmo0243* and *LMOf2365_lmo0244* (ClpC ATPase) are also up-regulated (Table 3). *LMOf2365_lmo0242* is also up-regulated (10.1-fold by microarray assay, 5.2-fold by real-time PCR assay). The observed derepression of the *clpC* operon may indicate that the *ctsR* mutant 2-1 strain is mutationally compromised in the production of functional CtsR. In addition to the *clpC* operon, another heat shock gene that was highly induced under pressure was *LMOf2365_lmo1018* (39.5-fold in microarray assay and 68.6 in real-time PCR assay) (Table 3). FM2365_lmo1018 encodes for a ATP-binding subunit ClpE, which is also negatively regulated by CtsR [5] and is responsible for CtsR degradation under heat stress in *B. subtilis* [14].

Other very highly induced genes are genes encoding putative accessory gene regulator proteins B and D (*LMOf2365_lmo0057*, *0058*; 15.3–28.7-fold by microarray assay and 68.6–45.3-fold by real-time PCR assay) (Table 3). In *Staphylococcus*, these *agr* genes are in the same operon and AgrB is responsible for cleaving AgrD to generate the autoinducing peptide [30]. Although up-regulation of the *agr* operon is related to biofilm formation [31], the ability of biofilm formation between the wild type and *ctsR* mutant 2-1 revealed no difference (data not shown).

PTS system fructose-specific phosphotransferase operon (*LMOf2365_lmo0442–0445*) was up-regulated under pressure (Table 3). ABC transporter system (*LMOf2365_lmo2147, 2148*) was up-regulated moderately (Table 3). The function of these genes under high pressure is unknown.

Down-regulated genes in *ctsR* mutant 2-1 under pressure treatment

A total of 58 genes appear to be repressed in the *ctsR* mutant 2-1 under high pressure treatment (Table 4). These down-regulated genes encode proteins involved in cellular processes,

Table 3 Genes up-regulated in *L. monocytogenes* strain Scott A *ctsR* mutant 2-1 under pressure treatment (450 MPa, 3 min) as identified by microarray and real-time PCR analysis

Category/gene	Function[a]	Fold change[b] Microarray[c]	RT-PCR[d]
Genes encoding proteins involved in transport and binding, and signal transduction			
LMOf2365_lmo1272	PTS system; trehalose-specific; IIBC component	2.6	2.3
LMOf2365_lmo2147	ABC transporter; ATP-binding protein	2.2	1.3
LMOf2365_lmo2148	ABC transporter; permease protein	2.2	1.9
LMOf2365_lmo0442	PTS system; fructose-specific; IIA component	3.0	5.7
LMOf2365_lmo0443	PTS system; fructose-specific; IIB component	3.3	9.2
LMOf2365_lmo0444	PTS system; fructose-specific; IIC component	3.1	8.0
Genes encoding proteins involved in cellular processes			
LMOf2365_lmo0058	Putative accessory gene regulator protein D	28.7	45.3
LMOf2365_lmo0244	ClpC ATPase	6.1	5.5
LMOf2365_lmo0057	Putative accessory gene regulator protein B	15.3	68.6
Genes encoding proteins of regulatory functions			
LMOf2365_lmo0241	Transcriptional regulator CtsR	6.3	10.2
Genes encoding hypothetical or unknown function proteins			
LMOf2365_lmo0243	ATP:guanido phosphotransferase family protein	5.2	10.2
LMOf2365_lmo2620	Phosphotriesterase family protein	2.2	2.6
LMOf2365_lmo0667	Conserved hypothetical protein	4.3	4.3
LMOf2365_lmo2097	Conserved hypothetical protein	2.1	2.4
LMOf2365_lmo0242	UVR domain protein	10.1	5.2
Genes encoding proteins involved in energy metabolism			
LMOf2365_lmo0445	Glycosyl hydrolase; family 38	3.7	10.2
Genes encoding proteins involved in protein fate			
LMOf2365_lmo1018	ATP-dependent Clp protease; ATP-binding subunit ClpE	39.5	68.6

Only the genes that met the stringent criteria for being up-regulated in the *cts*R mutant of *L. monocytogenes* Scott A (i.e., fold change > 2-fold; $P < 0.01$) are listed here

PTS phosphoenolpyruvate-dependent sugar phosphotransferase, *ABC* ATP-binding cassette, *Clp* caseinolytic protease

[a] Gene functions are based on annotations provided by TIGR (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi)

[b] Fold change indicates the transcript ratios between the *cts*R mutant and wild type as determined by microarray and real-time PCR

[c] Numbers are average values from four independent experiments

[d] Numbers are average values from three independent experiments

transport and binding, cell envelope, regulatory functions, energy metabolism, biosynthesis of cofactors, protein synthesis, DNA and amino acid biosynthesis. Additional down-regulated genes in the *ctsR* mutant 2-1 encode hypothetical proteins and proteins with unknown functions (Table 4).

Expression of genes related to flagella synthesis (*LMOf2365_lmo0729*, *0742*, *0744*, *0746*, *0747*, *0749*, and *0750*) was reduced significantly in the *ctsR* mutant 2-1 under pressure (Table 4); this correlates with the absence of flagella (Fig. 1) and immotile characteristics in *ctsR* mutant 2-1 [13]. Consistent with our findings, flagella mRNA and protein were also reduced in the AK01 mutant [15]. In addition, genes encoding for cell division proteins (*LMOf2365_lmo2045*, *2072*, *2479*) were down-regulated in the *ctsR* mutant 2-1 under pressure. The reduced transcript levels of these genes correlate with the elongated cells in *ctsR* mutant 2-1 (Fig. 1). Most importantly, the gene encoding for a internalin family protein (*LMOf2365_lmo1812*) was down-regulated moderately (2.5-fold by microarray assay, 3.3-fold by real-time PCR assay), indicating that the *ctsR* mutant 2-1 may have reduced virulence. This notion is further supported by the fact that the *ctsR* mutant 2-1 displayed reduced virulence compared to the wild type [13].

The PTS system mannose-specific operon (*LMOf2365_lmo0113*, *0114*, *0115*) was down-regulated (2–5-fold by microarray assay, 133–303-fold by real-time PCR assay) in *ctsR* mutant 2-1 under pressure. Why this operon is down-regulated remains unknown.

Discussion

The pressure-tolerant *ctsR* mutant 2-1 was heat and acid resistant, nisin sensitive, and non-motile with low

Table 4 Genes down-regulated in *L. monocytogenes* strain ScottA *cts*R mutant 2-1 under pressure treatment (450 MPa, 3 min) as identified by microarray and real-time PCR analysis

Category/gene	Function[a]	Fold change[b]	
		Microarray[c]	RT-PCR[d]
Genes encoding hypothetical proteins			
LMOf2365_lmo0143	Hypothetical proteins: conserved	−2.5	−10.0
LMOf2365_lmo0710	Hypothetical proteins: conserved	−2.0	−5.0
LMOf2365_lmo0730	Hypothetical proteins: conserved	−2.5	−5.0
LMOf2365_lmo0731	Hypothetical proteins: conserved	−3.3	−5.0
LMOf2365_lmo0739	Hypothetical proteins: conserved	−3.3	−5.0
LMOf2365_lmo0740	Hypothetical proteins: conserved	−3.3	−5.0
LMOf2365_lmo0745	Hypothetical proteins: conserved	−5.0	−10.0
LMOf2365_lmo0751	Hypothetical proteins: conserved	−3.3	−5.0
LMOf2365_lmo0754	Hypothetical proteins: conserved	−3.3	−10.0
LMOf2365_lmo0808	Hypothetical proteins: conserved	−2.0	−3.3
LMOf2365_lmo1193	Hypothetical proteins: conserved	−2.0	−3.3
LMOf2365_lmo1322	Hypothetical proteins: conserved	−2.0	−2.5
LMOf2365_lmo1442	Hypothetical proteins: conserved	−2.0	−3.3
LMOf2365_lmo1622	Hypothetical proteins: conserved	−2.5	−5.0
LMOf2365_lmo1623	Hypothetical proteins: conserved	−2.5	−5.0
LMOf2365_lmo1664	Hypothetical proteins: conserved	−2.0	−3.3
LMOf2365_lmo1690	Hypothetical proteins: conserved	−2.5	−5.0
LMOf2365_lmo1937	Hypothetical proteins: conserved	−2.0	−2.5
LMOf2365_lmo2290	Hypothetical proteins: conserved	−2.5	−5.0
LMOf2365_lmo2534	Hypothetical proteins: conserved	−2.0	−3.3
LMOf2365_lmo1191	Hypothetical proteins: conserved	−2.0	−2.5
Genes encoding proteins involved in cellular processes			
LMOf2365_lmo0742	Putative flagellar hook-associated protein FlgL	−3.0	−10.0
LMOf2365_lmo0744	Putative flagellar protein FliS	−5.0	−10.0
LMOf2365_lmo0746	Flagellar basal-body rod protein FlgB	−10.0	−10.0
LMOf2365_lmo0747	Flagellar basal-body rod protein FlgC	−5.0	−5.0
LMOf2365_lmo0749	Flagellar M-ring protein FliF	−3.3	−5.0
LMOf2365_lmo0750	Flagellar motor switch protein FliG	−2.5	−5.0
LMOf2365_lmo1812	Internalin family protein	−2.5	−3.3
LMOf2365_lmo2045	Cell division protein DivIVA	−2.5	−3.3
LMOf2365_lmo2072	Cell division protein; FtsL family	−2.0	−3.3
LMOf2365_lmo2423	Carboxylesterase	−2.0	−2.5
LMOf2365_lmo2479	Cell division ABC transporter; permease protein FtsX	−2.0	−3.3
Genes encoding proteins involved in transport and binding			
LMOf2365_lmo0113	PTS system; mannose-specific; IIAB component	−5.0	−303
LMOf2365_lmo0114	PTS system; mannose/fructose/sorbose family; IIC component	−2.0	−133
LMOf2365_lmo1090	ABC transporter; substrate-binding protein	−3.3	−10.0
LMOf2365_lmo1875	ABC transporter; manganese-binding protein	−2.0	−3.3
LMOf2365_lmo2229	Oligopeptide ABC transporter; oligopeptide-binding protein	−2.0	−3.3
LMOf2365_lmo0115	System; mannose/fructose/sorbose family; IID component	−5.0	−133
Genes encoding unknown functions proteins			
LMOf2365_lmo0729	Flagellar motor switch domain protein	−2.5	−3.3
LMOf2365_lmo0847	Putative phosphatase	−2.0	−3.3
LMOf2365_lmo1248	HAM1 family protein	−2.0	−2.5
LMOf2365_lmo1665	Helicase; Snf2 family	−2.0	−2.5
LMOf2365_lmo2495	LysM domain protein	−2.0	−3.3

Table 4 continued

Category/gene	Function[a]	Fold change[b]	
		Microarray[c]	RT-PCR[d]
Genes encoding proteins involved in cell envelope			
LMOf2365_lmo0753	Transglycosylase; SLT family	−3.3	−5.0
LMOf2365_lmo1093	*N*-Acetylmuramoyl-L-alanine amidase; family 4	−2.5	−5.0
LMOf2365_lmo2071	Penicillin-binding protein	−2.0	−3.3
LMOf2365_lmo2670	*N*-Acetylmuramoyl-L-alanine amidase; family 4	−2.5	−5.0
Genes encoding proteins involved in regulatory functions			
LMOf2365_lmo0777	Transcriptional regulator; Crp family	−2.0	−2.0
LMOf2365_lmo1848	Putative protein kinase	−2.0	−3.3
Genes encoding proteins involved in energy metabolism			
LMOf2365_lmo0376	Fumarate reductase; flavoprotein subunit	−3.3	−3.3
LMOf2365_lmo1365	Glycine cleavage system T protein	−2.5	−10.0
Genes encoding proteins involved in biosynthesis of cofactors			
LMOf2365_lmo1201	Cobalamin biosynthesis protein CobD methylenetetrahydrofolate	−2.0	−5.0
LMOf2365_lmo1377	Dehydrogenase/methenyltetrahydrofolate cyclohydrolase	−2.5	−2.5
Genes encoding proteins involved in protein synthesis			
LMOf2365_lmo1311	tRNA delta(2)-isopentenylpyrophosphatetransferase	−2.5	−2.5
LMOf2365_lmo1781	Glutamyl-tRNA(Gln) amidotransferase; C subunit	−2.0	−2.5
Genes encoding proteins involved in DNA metabolism			
LMOf2365_lmo1379	Exodeoxyribonuclease VII; small subunit	−2.5	−5.0
LMOf2365_lmo2300	ATP-dependent nuclease; subunit A	−3.3	−5.0
Genes encoding proteins involved in amino acid biosynthesis			
LOMf2365_lmo0566	Putative *N*-carbamoyl-L-amino acid amidohydrolase	−2.5	−5.0

Only the genes that met the stringent criteria for being down-regulated in the *cts*R mutant of *L. monocytogenes* Scott A. (i.e., fold change <−2-fold; $P < 0.01$) are listed here

[a] Gene functions are based on annotations provided by TIGR (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi)

[b] Fold change indicates the transcript ratios between the *cts*R mutant of *L. monocytogenes* Scott A and wild type as determined by microarray and real-time PCR, negative values indicate transcript levels that are lower in *cts*R mutant compared to the wild type. (e.g., −2.0-fold lower transcript level in *cts*R mutant as compared to the wild type)

[c] Numbers are average values from four independent experiments

[d] Numbers are average values from three independent experiments

invasiveness of human cells compared with the wild type [13]. To identify genes that are differentially expressed in *ctsR* mutant 2-1 under HPP treatment, RNA isolated from *L. monocytogenes* Scott A *ctsR* mutant strain 2-1 was labeled and subjected to microarray experiments. The total RNA isolated from *L. monocytogenes* Scott A wild type strain held under same conditions was used as a control. A minimum threshold of a 2-fold change in gene expression with a *P* value of less than 0.01 was used as the cutoff value. All of the genes identified by microarray analysis that were differentially expressed in *ctsR* mutant 2-1 were confirmed by quantitative reverse transcriptase real-time PCR (qRT-PCR). Only genes that were up- and down-regulated in both microarray and qRT-PCR assays are presented here. Compared to the wild type, 17 up-regulated and 58 down-regulated genes were identified in the *ctsR* mutant 2-1. Identification of these *ctsR*-related genes reveals some molecular mechanisms responsible for the observed phenotypes in *ctsR* mutant 2-1.

Our SEM results showed that the *ctsR* mutant 2-1 has elongated cells and absence of flagella. These observations are similar to those obtained with AK01, a previously identified pressure-resistant *ctsR* mutant [14]. Although both AK01 and *ctsR* mutant 2-1 had mutations in *ctsR* gene, AK01 has a single codon deletion (Glycine) [15] and the *ctsR* mutant 2-1 had a truncated protein with only 20 amino acids [13]. Different mutations in the *ctsR* gene can result in different phenotypes. For example, deletion of the entire *ctsR* gene in *L. monocytogenes* only resulted in partial reduced swarming ability compared to the wild type [11], indicating that the flagella were not lost in the *ctsR* deletion mutant. In another study, a *ctsR* mutant with a

55-bp deletion showed flagella and normal cell size. This mutant also displayed reduced colony sizes on BHI plate and was classified as small-colony variants (SCVs) [33].

Because the mutant CtsR protein only contained the dimerization domain and no DNA binding domain, it lost the ability to bind to DNA target sequences and to repress the CtsR-regulated genes. Clp proteases of *B. subtilus* have been shown to be directly involved in degradation of misfolded proteins [19]. The increased expression level of *clpC* and *clpE* may contribute to stress tolerance (to heat, acid, and high pressure) in the *ctsR* mutant 2-1 probably by preventing the accumulation of misfolded and damaged proteins, which might have toxic effects on the cell. The increased expression of *clp* genes in the *ctsR* mutant 2-1 may also assist its survival under high pressure. In addition, the ClpE protein has also been shown to be indirectly involved in cell division and virulence in *L. monocytogenes* [26].

Under non-stressed condition, the *ctsR* mutant 2-1 *ctsR* gene was also up-regulated at the transcriptional level (3-fold in microarray, data not shown). The transcript levels of *LMOf2365_lmo0242–0244* were also increased (6–8-fold in microarray, data not shown), indicating that the repressor function of CtsR was at least partially lost. Consistent with our results, the expression of the *clpC* operon was also increased in *L. monocytogenes ctsR* deletion mutant under normal conditions [11, 27]. Our results are also in agreement with a recent study showing that the *clpC* transcripts were increased in three different *ctsR* mutants under non-stress conditions [33]. An *L. lactis ctsR* mutant lacking the putative DNA binding domain also showed increased expression (3–8-fold) of *clp* genes [34].

Consistent with the observation of elongated cells and absence of flagella in *ctsR* mutant 2-1, expression of genes related to cell division and flagella synthesis was reduced (Table 4). Transcripts of genes encoding proteins involved in cell envelope were also reduced moderately in *ctsR* mutant 2-1 under pressure, indicating that the mutant CtsR protein may interact indirectly with the membrane proteins.

The *LMOf2365_lmo1812* encoding for a internalin family protein was reduced moderately in the *ctsR* mutant 2-1. This correlates with the reduced invasiveness of human cileocecal adenocarcinoma cells in *ctsR* mutant 2-1 [13]. Consistent with our study, AK1 showed significantly attenuated virulence compared with the wild-type strain [15]. In another study, Hu et al. [11] showed that even though the *ctsR* deletion did not exhibit reduced invasion efficiency, the Δ*ctsR* Δ*sigB* strain showed significantly lower invasion efficiency than either the parent strain or the Δ*sigB* strain, indicating that interactions between the *ctsR* and σ^B contribute to invasiveness [11].

Our results showed that *agrD* and *agrB* of the *agr* operon had strongly increased expression (Table 3). These genes are homologous to the *agr* operon of *Staphylococcus aureus* and have been shown to be important for virulence [3] and biofilm formation in *L. monocytogenes* [31]. It is possible that the mutant CtsR protein interacts with ArgB and D proteins either directly or indirectly. Alternatively, the *argD* and *argB* genes were also shown to be induced under high pressure treatment in wild-type *L. monocytogenes* [4], suggesting that this may represent a general response to mechanical stress damage.

The transport of carbohydrates in bacteria is accomplished by the PTS and ATP-binding cassette (ABC) transporters. High pressure treatment in *ctsR* mutant 2-1 repressed and activated different PTS and ABC transporters. For example, PTS-related genes for fructose (*LMOf2365_lmo0442–0445*) and trehalose (*LMOf2365_lmo1272*) were highly expressed. On the other hand, mannose-specific PTS system (*LMOf2365_lmo0113–0115*) was repressed. The relationship between the mutant CtsR protein and the altered expression levels of the PTS and ABC transporters remains unclear.

A problem observed during high pressure treatment is that a small portion of a bacterial population can be relatively resistant after a certain pressure is applied. This phenomenon is called the tailing effect [24], and it is a big challenge for the food industry to prevent this effect. The existence of pressure-tolerant mutants could be an explanation for the tailing phenomenon [14]. A majority of the pressure-resistant mutants contained mutations in the *ctsR* gene [16, 33], indicating the involvement of this gene in high pressure treatment. Understanding how bacteria survive under high pressure may help food processors develop effective preservation strategies to better manage pathogens in food. For example, the *ctsR* mutant 2-1 also displays sensitivity to nisin, suggesting that combination of nisin and HPP treatments may inhibit growth of *L. monocytogenes*. Our study can be extended to evaluate the gene expression profiling of different bacterial pathogens in different high pressure treatments. This study not only provides new insights into the survival and growth of *L. monocytogenes* under high pressure, but also helps identify target genes for future functional genomics experiments. Most importantly, this study enhances our understanding of how *L. monocytogenes* survives under HHP and may contribute to the design of safe, accurate, and economically feasible HHP treatment in food processing.

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