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Proteomic Analysis of Cross Protection Provided between Cold and Osmotic Stress in *Listeria monocytogenes*

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Abstract

Listeria monocytogenes is a Gram-positive, foodborne pathogen responsible for approximately 28% of all food-related deaths each year in the United States. *L. monocytogenes* infections are linked to the consumption of minimally processed ready-to-eat (RTE) products such as cheese, deli meats, and cold-smoked finfish products. *L. monocytogenes* is resistant to stresses commonly encountered in the food-processing environment, including low pH, high salinity, oxygen content, and various temperatures. The purpose of this study was to determine if cells habituated at low temperatures would result in cross-protective effects against osmotic stress. We found that cells exposed to refrigerated temperatures prior to a mild salt stress treatment had increased survival in NaCl concentrations of 3%. Additionally, the longer the cells were pre-exposed to cold temperatures, the greater the increase in survival in 3% NaCl. A proteomics analysis was performed in triplicate in order to elucidate mechanisms involved in cold-stress induced cross protection against osmotic stress. Proteins involved in maintenance of the cell wall and cellular processes, such as penicillin binding proteins and osmolyte transporters, and processes involving amino acid metabolism, such as osmolyte synthesis, transport, and lipid biosynthesis, had the greatest increase in expression when cells were exposed to cold temperatures prior to salt. By gaining a better understanding of how this pathogen adapts physiologically to various environmental conditions, improvements can be made in detection and mitigation strategies.

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ASSOCIATED CONTENT

S Supporting Information

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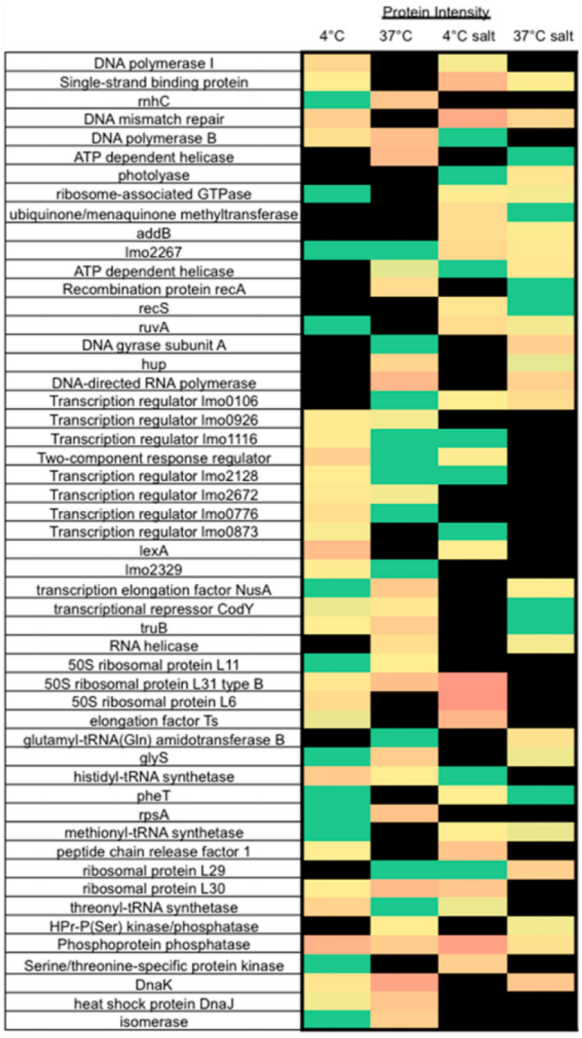

Keywords

Listeria monocytogenes; cold stress; salt stress; cross protection; proteomics

INTRODUCTION

Listeria monocytogenes is a deadly foodborne pathogen of vital importance to public health and the food industry. Infections with *L. monocytogenes* are more common among the elderly, pregnant women, infants, or immunocompromised individuals and are primarily associated with ingestion of contaminated food products.[1] Much care is taken to ensure the safety of ready-to-eat products and proper preservation of these products, including the application of salts and low temperature to improve shelf life.[2] However, the psychrophilic and halophilic nature of this pathogen enables it to persist and grow at refrigerated temperatures and moderate salt concentrations.[1]

It has previously been shown through transcriptome analysis that acclimation of *L. monocytogenes* to low temperatures induces cross protection to high salt concentrations.[3,4] The ability of *L. monocytogenes* to adapt to osmotic stress has been proposed to be lineage-specific, with lineages I and III exhibiting survival advantages at 37 °C over lineage II strains under osmotic stress.[5] The adaptation of *L. monocytogenes* to cold or osmotic stress revealed an overlap in adaptive mechanisms used by the pathogen, including increased expression genes and proteins related to the transport of compatible solutes (*betL*, *opuC*, and *gbu* operons[6–8]), alterations in cell membrane fluidity,[9] and sequestration and storage of iron by ferritin.[10,11] The expression of cold shock proteins (Csps) has also been found to be essential for the growth of *L. monocytogenes* at both low temperatures and high salt concentrations,[4,12] suggesting cold stress induces cross protection against high osmotic conditions. Additionally, osmotic resistance is enhanced in cold temperatures, with resistance being potentially mediated by enhanced expression of peptidoglycan synthesis genes, general stress response genes, and cation transporter genes.[3]

However, the increase in survival due to cross-protective mechanisms may differ between lineages. A previous study identified that variations exist in the survival of *L. monocytogenes* and *L. innocua* following sequential exposure to stressors that were not necessarily related to genetic lineages.[13] In this previous study, the genetic lineage II strain EGDe actually had increased survival following sequential exposure to several stressors, including cold temperatures and 6% NaCl. Therefore, the purpose of this current study was to expand upon these previous findings by determining the mechanisms that enhance the adaptation of *L. monocytogenes* to a minimal osmotic stress (3% NaCl) following exposure to low temperatures utilizing a proteomics-based approach. These mechanisms were found to involve variations in the expression of several proteins associated with cell wall architecture and synthesis. Gaining a better understanding of the mechanisms involved in adaptation to stresses typically encountered in the food-processing environment could potentially aid in the development of mitigation strategies to reduce or eliminate this pathogen in ready-to-eat products.

MATERIALS AND METHODS

Bacterial Growth Conditions and Survival Analysis

The *L. monocytogenes* strain EGDe (serovar 1/2a) was routinely cultured in brain heart infusion (BHI) medium at 37 °C. Fresh cultures were incubated overnight at 37 °C in BHI broth, after which cultures were diluted 1:100 (~1×10^6 CFU/mL) in 10 mL of BHI (pH 7.4) and incubated at either 4 or 37 °C. A 1 mL aliquot of cells was removed each hour for up to 6 h, pelleted at 10,000 × *g* for 2 min, and then resuspended in BHI supplemented with 3% NaCl. Cultures were then incubated at 37 °C for 4 h. Aliquots were removed for viable plate analysis hourly following resuspension in media supplemented with 3% NaCl. At least three independent replicates were performed.

Purification of Proteins

Cultures of *L. monocytogenes* EGDe were incubated for 4 h at either 4 or 37 °C in BHI, after which cells were pelleted by centrifugation at 10,000 × *g* for 10 min and resuspended in an

equal volume of BHI supplemented with 3% NaCl and incubated at 37 °C for another 4 h. Methods previously described by our group were used to isolate proteins after growth at 4 and 37 °C for 4 h and the subsequent exposure to 3% NaCl for 4 h.[14,15,16] Briefly, aliquots collected were pelleted by centrifugation at 10,000 × *g* for 10 min, and resulting pellets were resuspended in 4 mL of a lysis solution [2% Triton X-100, 2.6 mg/mL sodium azide, 0.1 M Tris pH 8.0, and 8 mM phenylmethanesulfonyl fluoride (PMSF)]. Cells were incubated at 37 °C for 20 min with the addition of 20 mg/mL of lysozyme and sonicated (Fisher Scientific Model 100 Sonic Dismembrator, setting 3) for four 30 s pulses on ice, with 1 min cooling between pulses. Following the addition of 85 μg/mL DNase I and 20 μg/mL RNase A, samples were incubated at 37 °C for 30 min and centrifuged at 6,200 × *g* for 10 min at 10 °C to pellet the cell debris. An equal volume of 50% trichloroacetic acid (TCA) was added to the supernatant of each sample, and proteins were precipitated at −20 °C overnight. The precipitated proteins were pelleted by centrifugation at 6,200 × *g* for 10 min at 10 °C, washed with ice-cold acetone (Chromosolv for HPLC, Sigma Aldrich), and dried at room temperature.

The proteins were subsequently resuspended in 0.5 mL of solubilization solution (7 M urea, 20 mM Tris-Cl, pH 8.0, 5 mM EDTA, 5 mM $MgCl_2$, 4% CHAPS, 1 mM PMSF) and quantitated using the 2-D Quant Kit (GE Healthcare Life Sciences). An aliquot of 0.1 mg of proteins was precipitated once more with 50% TCA at −20 °C, followed by centrifugation and a wash with ice-cold acetone. Samples were resuspended in 0.1 mL of 100 mM ammonium bicarbonate and 5% acetonitrile, then treated with 5 mM dithiothreitol for 10 min at 65 °C, 10 mM iodoacetamide for 30 min at 30 °C, and finally digested with 2 μg of sequencing-grade trypsin at 37 °C for 16 h. Peptides were desalted using a peptide macrotrap (Michrom Bioresources, Inc.), dried at room temperature, and stored at −80 °C until further processing. Proteins were isolated from three independent experiments.

Protein Analysis

Desalted peptides were resuspended in 250 μL of 5 mM monosodium phosphate in 25% acetonitrile adjusted to a pH of 3 using formic acid and processed using a strong cation exchange (SCX) macrotrap (Michrom Bioresources, Inc.) according to the manufacturer's instructions. Cleaned samples were dried and resuspended in 40 μL of 2% acetonitrile and 0.1% formic acid; ~50 μg of each sample was transferred to low retention HPLC vials for analysis using mass spectrometry.

Peptide mass spectrometry was accomplished using an EASY-nLC (Thermo Scientific) high performance liquid chromatography machine (HPLC) coupled with an LTQ Velos (Thermo Scientific) linear ion trap mass spectrometer. The Easy-nLC was configured for reverse phase chromatography using a Hypersil Gold KAPPA C18 column (Thermo Scientific) with a flow rate of 333 nL/min. Peptides were separated for mass spectrometry analysis using an acetonitrile gradient starting at 2% ACN, 0.1% FA and reaching 50% ACN, 0.1% FA in 120 min, followed by a 15 min wash of 95% ACN, 0.1% FA. Column equilibration was handled automatically using the EASY-nLC. The eluate from the HPLC was fed directly to the LTQ Velos for nanospray ionization followed by MS/MS analysis of detected peptides. The LTQ Velos was configured to perform 1 ms scan followed by 20 MS/MS scans of the 20 most

intense peaks repeatedly over the 135 min duration of each HPLC run. Dynamic exclusion was enabled with a duration of 5 min, repeat count of 1, and a list length of 500. The collected spectra were subsequently analyzed using the X!tandem search algorithm.[17]

Raw spectral data from the LTQ Velos were converted to mzML format using the msConvert tool form the ProteoWizard software project because X!tandem cannot read the Thermo raw format directly.[18] The FASTA database used for peptide spectrum matching (target database) was the *Listeria monocytogenes* strain EGD-e RefSeq protein database from the National Center for Biotechnology Information (accession no. 61583). X!tandem was configured to use tryptic cleavage sites with up to two missed cleavages. Precursor and fragment mass tolerance were set to 1000 and 500 ppm, respectively. Four amino acid modifications were included in the database search: single and double oxidation of methionine and both carboxymethylation and carboxamidomethylation of cysteine. A decoy search was also performed using a randomized version of the target database with the same search parameters as above. The search results were filtered using previously described methods.[19,20] A decoy score distribution was created and each match from the target database was evaluated as a possible outlier and assigned a probability of being correct. Peptides from the target database were accepted if the probability of being correct was 95% or higher. A list of proteins and identified peptides was generated for each replicate of a given treatment.

Protein Comparison

Protein differential expression between treatments was evaluated on the basis of peptide spectral intensity. The raw spectral data were converted to the MS1 tab delimited format using the MakeMS2 tool available from the MacCoss lab at the University of Washington.[21] The intensities for each peptide elution peak were identified from the associated MS1 file using Perl script and summed. For each identified protein, the peptide intensities were combined and organized by experimental replicate. Differential expression was evaluated using Monte Carlo resampling techniques to compare the replicate intensities between treatments. Each comparison used 1 million iterations and was assigned a *p*-value based on the number of times each test favored one treatment over another ($p < 0.05$ indicated significance). Functional classifications were assigned based on ListiList categories (http://genolist.pasteur.fr/ListiList/).

RESULTS

Prolonged Exposure to Low Temperatures Increases Resistance to 3% NaCl

To determine if exposure to cold temperatures could enhance osmotic protection, we analyzed the viability of EGDe after exposure at 4 °C and a subsequent salt exposure. EGDe exposed to cold temperatures had increased resistance to osmotic stress in comparison to cells incubated at 37 °C (Figure 1). Exposure to cold temperatures resulted in a significant increase in the fold change ($p < 0.0001$) of EGDe's growth in 3% NaCl within the 6 h time period tested (Figure 1).

Cold and Osmotic Stress Protein Expression Profile of EGDe

In order to investigate how exposure to cold temperatures can induce cross protection against salt stress, the protein expression of EGDe incubated at either 4 or 37 °C for 4 h prior to exposure to 3% NaCl for 4 h was analyzed by LTQ mass spectrometry in triplicate. ListiList categories were used to classify each of the differentially expressed proteins into functional groups.[15,16] A total of 299 significantly differentially expressed proteins were identified.

Differentially Expressed Proteins Associated with Cell Envelope and Cellular Processes (Category 1)

Cell wall associated proteins and proteins involved in membrane bioenergetics that increased in expression following exposure to cold temperatures prior to salt included the invasion associated protein (gi: 16802625) and the H+ transporting ATP synthase chain subunits a and c (gi: 16412019 and 16412022). The cell division protein FtsA (gi: 16411503) also increased in expression, while the cell division proteins FtsZ and FtsW (gi: 16411502 and 16804724) decreased in expression in cold-stressed cells exposed to osmotic stress (Table 1).

The acquisition of nutrients, as well as osmoprotectants, from the environment requires proteins involved in binding and transporting substrates. Such proteins that increased in expression when cold-stressed cells were exposed to salt were those similar to the pheromone ABC transporter binding protein OppA (gi: 16411666), phosphotransferase system (PTS) fructose-specific enzyme IIABC component (gi: 16411823), a putative fructose-like permease EIIC subunit 2 (gi: 16802674), PTS mannose-specific enzyme IIAB (gi: 18140806), cellobiose phosphotransferase enzyme IIB (gi: 16804720), and an ABC transporter/ATP-binding protein and permease (gi: 16803545 and 16803102) (Table 1).

Cells exposed to cold stress prior to osmotic stress had an increase in expression of proteins involved in secretion, such as foldase protein PrsA 2 (gi: 16804258). Cell surface proteins, such as ActA (gi: 16409569), InlC (gi: 16411240), a hypothetical cell wall associated protein (gi: 16409952), a peptidoglycan binding protein (gi: 16802207), and a protein similar to internalin (gi: 16803329) also increased in expression.

Differentially Expressed Proteins Associated with Metabolic Pathways (Category 2)

Adaptation to osmotic stress requires a shift in expression of proteins involved in specific metabolic pathways and metabolism of amino acids and nucleic acids. Cells previously exposed to low temperatures prior to osmotic stress increased in expression of proteins involved in fermentation, such as L-lactate dehydrogenase (gi: 16409575), bifunctional acetaldehyde-CoA/alcohol dehydrogenase (gi:16803674), and phosphotransacetylase (gi: 16804142) (Table 2). Numerous glycolytic enzymes also increased in expression, such as enolase (gi: 16411943), glyceraldehyde 3-phosphate dehydrogenase (gi: 16411947), glucose-6-phosphate isomerase (gi: 16804405), phosphoglyceromutase (gi: 16804494), phosphoglycerate kinase (gi: 16804496), and pyruvate formate-lyase (gi: 227478797). The glycolytic enzyme pyruvate carboxylase (gi: 16803112) had reduced expression (Table 2) following subsequent exposure to NaCl.

Metabolism of alternative carbohydrates is advantageous during stress conditions. Proteins involved in utilization of different sugars were increased in cold-adapted salt-stressed cells and included α-mannosidase (gi: 16411468), phosphofructokinase (gi: 16411565), fructose-1-phosphate kinase (gi: 16804374), sorbitol dehydrogenase (gi: 16804701), glucose kinase (β-glucoside kinase) (gi: 16804801), and 6-phospho-β-glucosidase (gi: 16802579).

Numerous proteins important in the metabolism of nucleic acids increased in expression as well, including a protein similar to phosphopentomutase Drm (gi: 16411407), dihydroorotase PyrC (gi: 16803877), anaerobic ribonucleoside triphosphate reductase (gi: 16802325), and adenylate kinase (gi: 16804649). Cold-adapted salt-stressed cells also had an increased expression of branched-chain α-keto acid dehydrogenase E3 subunit (gi: 16410787), a protein involved in the metabolism of lipids.

Differentially Expressed Proteins Associated with Information Pathways (Category 3) or Other Functions (Category 4)

In order to adapt to stressful environments, bacteria must be able to efficiently repair damaged DNA and synthesize mRNA and proteins. Proteins involved in mismatch repair, homologous recombination, and the SOS response increased in expression in cold-stressed cells when exposed to osmotic shock. These included single-stranded binding protein (gi: 16802093), DNA mismatch repair MutS (gi: 16410832), Holliday junction DNA helicase RuvA (gi: 16410962), and ATP-dependent deoxyribonuclease (gi: 16804306) (Table 3). Proteins involved in base excision repair and nucleotide excision repair decreased in expression in cold-adapted salt-stressed cells: DNA polymerase I (gi: 16410994) and putative DNA polymerase β similar to *B. subtilis* YshC (gi: 16803271). Proteins involved in transcriptional regulation, such as the SOS response regulator LexA (gi: 16410718) and the transcriptional regulator LacI family (16804167), had a reduced expression in cold-adapted salt-stressed cells (Table 3).

There was an increased expression of proteins involved in translation and ribosome assembly in cold-adapted salt-stressed cells, such as the elongation factor Ts (gi: 16803697), peptide chain release factor 1 (gi: 16804581), 50S ribosomal protein L30 (gi: 16412102), 50S ribosomal protein L31 type B (gi: 16804586), and 50S ribosomal protein L6 (gi: 16804655). In cold-adapted salt-stressed cells, the methionyl-tRNA-synthetase (gi: 16802223) and phenylalanyl-tRNA synthetase β subunit (gi: 16803647) increased in expression, while the histidyl-tRNA synthetase (gi: 16410949) and threonyl-tRNA synthetase (gi: 16410988) decreased in expression. Salt-stressed cells that received no cold pretreatment had an increased expression of aspartyl/glutamyl-tRNA synthetase subunit B (gi: 16411208) and a reduced expression of glycyl-tRNA synthetase β chain (gi: 16803498).

There was an increase in the expression of the general stress response protein Ctc (similar to *Bacillus subtilis*) (gi: 16409576) and tRNA modification GTPase MnmE (gi: 16412311) in cold-exposed salt-stressed cells. In cold-adapted salt-stressed cells phage related proteins, such as gp20 bacteriophage A118 (gi: 16411753) and antigen A (gi: 16802166), increased in expression, while proteins similar to a bacteriophage protein (gi: 16411783) and a bacteriophage minor tail protein (16802169) had reduced expression.

DISCUSSION

Previous studies have examined the mechanisms *L. monocytogenes* use to adapt to growth at low temperatures or when exposed to osmotic stress.[6–8] It has recently been proposed that exposure to cold stress may induce cross protection against subsequent exposure to salt stress,[4] yet the mechanisms that allow for this protection have not been fully elucidated. *L. monocytogenes* is routinely subjected to multiple stresses within the food-processing environment either concomitantly or sequentially; it is important to discern the mechanisms this organism uses to withstand these conditions in order to eliminate its presence. Therefore, the purpose of this study was to examine the relationship between mechanisms involved in survival in cold temperatures in providing cross protection against osmotic stress. A concentration of 3% NaCl was selected as this is typically the minimum concentration that is used as brine in the food industry, particularly in the smoked finfish industry. A low concentration of NaCl was therefore used in this study to characterize the initial response to this condition while also minimizing cell death.

Though our time course was limited to 6 h of pretreatment to cold temperatures, it did appear as though the longer cells were exposed to cold temperatures, the higher the degree of cross protection against osmotic stress, therefore indicating that exposure to low temperatures may induce cross protection against osmotic stress in *L. monocytogenes.* To examine the physiological response, the variation in the expression of the proteome was examined for cells preconditioned at either 4 or 37 °C for 4 h prior to exposure to the minimal stress of 3% NaCl for an additional 4 h; this time point was chosen because this was when cells that had received a cold-stress pretreatment began to exhibit an increase in viability following osmotic stress. Adaptation to stressful conditions requires energy in the form of ATP, and the increase in the expression of the H^+-transporting ATP synthase chain proteins indicates that the oxidative phosphorylation pathway is active. Increased expression of enzymes involved in oxidative phosphorylation is a common component of the stress-adaptation response of *L. monocytogenes*. The need for increased uptake of carbohydrates and production of ATP are required to fuel the high-energy demands of the cell for repair of damaged DNA, proteins, and lipids.

Another mechanism used by bacteria to adapt to growth at low temperatures and salt stress is the accumulation of osmolytes from the surrounding environment. Transporters, such as ABC transporter OppA, are involved in the uptake of oligopeptides as a means of accumulating proline, isoleucine, and valine to serve as cryoprotectants or osmolytes.[22,23] The fact that OppA is required for growth at low temperatures and its potential role in the uptake of osmolytes strongly suggests that it is involved in cross protection of cold-stressed *L. monocytogenes* against osmotic stress.

The increased expression of PTS proteins involved in uptake of fructose, mannose, and cellobiose in cold-exposed salt-stressed cells suggests their importance in energy production or as osmoprotectants.[8,24] This is interesting considering recent evidence that suggests that salt-stressed *L. monocytogenes* cells have reduced cell growth as a result of decreased expression of PTS genes. The decreased expression of PTS enzyme II cytoplasmic components associated with the uptake of glucose, fructose, mannose, and cellobiose were

found to be dependent on the concentration of NaCl.[25] This is congruent with the reduced expression of PTS enzyme II components responsible for the uptake of mannose and fructose in cells exposed to salt stress without a cold pretreatment. Exposure of *L. monocytogenes* to low temperatures has previously been shown to induce the expression of PTS-associated proteins, indicating their need for production of complex macromolecules and energy.[7,8]

There was an increase in the expression of proteins secreted by the Sec system (invasion associated protein (Iap) or autolysin, OppA, and enolase).[26] Iap has an important role in the degradation of cell wall components and aids in the invasion of nonphagocytic cells. Mutants exhibiting a rough phenotype have reduced expression of Iap but are capable of directional gliding motility toward uncolonized areas of agar media.[27] This phenotype could provide *L. monocytogenes* a competitive advantage in the environment, particularly those associated with food-processing, allowing for increased acquisition of nutrients and colonization of numerous niches in processing plants.

There was an increase in expression of proteins involved in pyruvate metabolism, which implies that the culture was exposed to oxygen-limited conditions and may in part be due to the cultures being incubated statically. Under hypoxic conditions, glucose and other sugars are processed by fermentation and the pentose phosphate pathways. Two enzymes important in the non-oxidative branch of the pentose phosphate pathway were expressed in the cold-adapted salt-stressed cells. The increased expression of 6-phosphogluconate dehydrogenase indicates 6-phosphogluconate is converted into ribulose-5-phosphate, CO_2, and NADPH. Given that BHI is a complex medium, carbohydrates other than glucose are available to be transported into the cell and metabolized. Turnover of glycoproteins such as mannose glycopeptides and 2-*O*-α-mannosyl-glycerate (α-MG) by α-mannosidase allows release of α-D-mannose residues that can be transported into the cell by PTS mannose-specific enzyme IIABC components and PTS fructose-specific enzyme IIABC, respectively, and converted into D-mannose-6-phosphate and shunted into glycolysis. It has previously been shown α-MG serves as an important compatible solute in response to osmotic stress in algae, cyanobacteria, aerobic heterotrophic bacteria, thermohalophilic bacteria, and hyperthermophilic archaea.[28–30] Most mesophilic bacteria utilize neutrally charged compatible solutes, while thermophilic eubacteria and archaea utilize negatively charged compatible solutes. It would be interesting to determine if *L. monocytogenes* is capable of utilizing α-MG as a carbon source or as a compatible solute under temperature or osmotic stress conditions.

An important metabolite utilized by *L. monocytogenes* cold-stressed cells is fructose.[31] Cold-exposed salt-stressed cells had an increased expression of PTS fructose-specific IIABC, fructose-like permease EIIC subunit, and fructose-1-phosphate kinase responsible for the uptake of D-fructose along with its shunting into central carbon metabolism. Metabolism of sugar alcohols such as D-sorbitol by sorbitol dehydrogenase results in the production of D-fructose (shunted into glycolysis as previously mentioned) and NADH. Sorbitol could possibly serve as an osmoprotectant in *Listeria* given its roles as such in various species of archaea, bacteria, plants, and fungi.[32,33] Previous examination of the metabolic profile of cold-adapted *L. monocytogenes* cells indicate a decrease in expression

of sorbitol and sorbitol-6-phosphate in comparison to cells grown at 37 °C.[31] Conversion of sorbitol into fructose by sorbitol dehydrogenase could explain the reduced amount of sorbitol and higher level of fructose present in cold-stressed cells. Cellobiose PTS enzyme IIB component and β-glucoside kinase are involved in the transport of the disaccharide cellobiose into the cell, phosphorylated, and directed toward central carbon metabolism.

The increase in expression of proteins involved in the synthesis of precursors for nucleic acids and the overexpression of double-strand break repair proteins supports the conclusion that exposure of cells to salt stress induces some type of DNA damage.[34] One possible mechanism for repair of double-strand breaks is through the tethering of DNA fragments by DNA polymerase X (*B. subtilis* YshC) as a means of preserving chromosomal integrity. This mechanism of salt -induced DNA damage has been shown to occur in eukaryotic cells.[35,36] The increased expression of the RecBCD homologue in *B. subtilis* AddAB, RuvA, SSB, and RecA suggest homologous recombination is involved in repair of double stranded DNA damage.[37] Increased expression of SufB supports the need for iron clusters because the nuclease domain of AddAB contains an iron-sulfur cluster that is required for proper binding and processing of broken DNA.[38]

Adaptation to stressful conditions requires proper synthesis of mRNA and proteins, increased stability of proteins, proper folding, and subsequent secretion of response proteins. Transcription elongation factor GreA is required for the resumption of elongation following transcriptional arrest.[22] Translation elongation factors EF-TU and -G have been shown to possess chaperone-like functions by aiding in proper protein folding and interacting with improperly folded proteins.[39,40] General stress protein Ctc in *L. monocytogenes* may interact with ribosomes as a sensor for osmotic stress.[41] It has also been shown to be required for growth in media in the absence of any osmoprotectants.[6,42]

Numerous physiological mechanisms are involved in cold-stress-induced cross protection against osmotic stress in *L. monocytogenes*. Expression of proteins involved in uptake of a variety of organic molecules aids in shunting available nutrients toward central carbon metabolism and the accumulation or synthesis of compatible solutes. Synthesis of fatty acids from amino acid precursors is needed to adjust membrane fluidity and stabilize the integrity of the cell. DNA repair mechanisms are also required to repair damaged DNA. A better understanding of the physiological responses of this pathogen to adaptation to various strategies in place in the food-processing environment will hopefully aid in implementing feasible listericidal strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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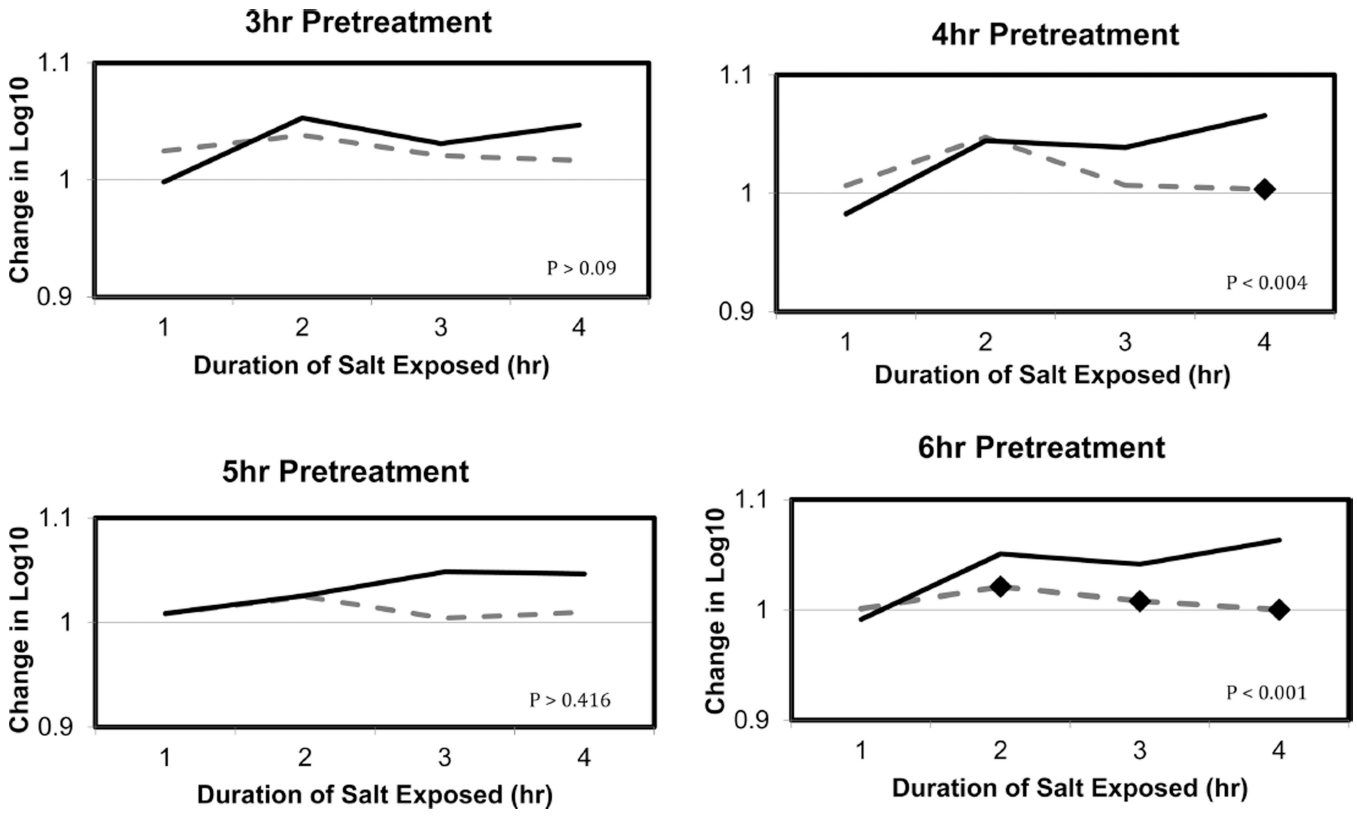

Figure 1.

Tolerance to salt increased for EGDe exposed to 4 °C. Fold change in $\log_{10}$ CFU/mL for EGDe grown for 3, 4, 5, or 6 h at either 37 °C (dashed lines) or 4 °C (solid lines) prior to a 4 h treatment in 3% NaCl.

Table 1

Cell Envelope Associated Proteins (ListiList Category 1) with Differential Expression between Treatments[a]

			intensity				*p*-value			
gi no.	**protein**	**ListiList**	**4 °C**	**37°C**	**4 °C salt**	**37 °C salt**	**4 to 4 °C salt**	**4 to 37 °C**	**4 °C salt to 37 °C salt**	**37 to 37 °C salt**
16802625	invasion associated protein	1.1	0.0000	6.8099	6.8360	7.1841	0.0206	-	-	-
16409456	similar to MptC	1.2	6.7586	6.7747	5.2658	4.5423	NS	NS	-	0.0079
16410404	PTS phosphocarrier protein Hpr	1.2	6.6204	7.0356	8.2533	8.2297	0.0327	-	-	NS
16410405	PTS enzyme I	1.2	6.2438	6.0000	6.8722	7.6487	NS	-	0.0412	0.0176
16410642	similar to *B. subtilis* YdgH	1.2	6.9560	7.1084	7.0663	0.0000	NS	NS	0.0203	0.0019
16411053	similar to multidrug-efflux transporter	1.2	7.0510	6.3653	7.2640	6.5801	NS	NS	0.0014	NS
16411174	similar to PTS lichenan-specific enzyme IIB	1.2	0.0000	7.0617	0.0000	5.7445	-	0.0325	-	0.0399
16411454	similar to PTS mannose-specific enzyme IIC	1.2	6.7404	5.9139	6.5757	0.0000	NS	-	0.0122	-
16411514	similar to transporter binding protein	1.2	7.1926	6.7399	7.5163	5.8352	NS	NS	0.0233	-
16411654	similar to FurA	1.2	4.7994	0.0000	0.0000	6.3280	-	-	0.0350	0.0350
16411666	similar to OppA	1.2	6.6197	7.6957	7.8619	7.8669	0.0474	NS	-	NS
16411823	similar to PTS fructose-specific IIABC	1.2	6.4731	7.6951	7.9466	8.0758	0.0450	0.0064	-	NS
16802674	putative fructose-like permease EIIC subunit 2	1.2	0.0000	0.0000	7.2327	0.0000	0.0108	-	0.0108	-
16802675	putative fructose-like phosphotransferase EIIB	1.2	0.0000	6.4162	0.0000	0.0000	-	0.0231	-	0.0230
16802687	similar to amino acid transporter	1.2	5.2100	0.0000	0.0000	6.3805	NS	-	0.0112	0.0111
16803102	similar to permease	1.2	6.6757	6.5914	6.5034	0.0000	NS	NS	<0.0001	0.0098
16803545	similar to ABC transporter ATP-binding protein	1.2	6.0356	5.9003	7.0098	0.0000	0.0169	-	0.0076	-
16804537	similar to phosphate ABC transporter binding protein	1.2	7.4625	5.8699	6.1640	0.0000	NS	-	0.0185	-
16804720	similar to cellobiose phosphotransferase enzyme IIB component	1.2	0.0000	5.9132	7.3123	5.6812	0.0204	-	0.0228	-
18140806	similar to PTS system mannose-specific IIAB	1.2	0.0000	7.6286	7.7607	7.9634	<0.0001	NS	-	NS
16412018	similar to H+-transporting AtpG	1.4	0.0000	6.7863	0.0000	0.0000	-	0.0294	-	0.0294
16412019	similar to H+-transporting AtpA	1.4	6.6325	7.5308	8.3146	8.1094	0.0266	0.0450	-	NS
16412022	similar to H+-transporting AtpE	1.4	0.0000	0.0000	6.6495	5.9374	0.0134	-	0.0461	-
16802062	AA3-600 quinol oxidase subunit I QoxB	1.4	6.6654	6.5351	7.1047	0.0000	NS	-	0.0246	-
16804258	similar to PrsA2	1.6	5.6330	4.6566	7.8584	7.3901	0.0089	-	-	NS
16411502	highly similar to FtsZ	1.7	7.0514	6.8614	6.2807	5.9949	0.0327	NS	-	-

			intensity				*p*-value			
gi no.	**protein**	**ListiList**	**4 °C**	**37°C**	**4 °C salt**	**37 °C salt**	**4 to 4 °C salt**	**4 to 37 °C**	**4 °C salt to 37 °C salt**	**37 to 37 °C salt**
16411503	highly similar to FtsA	1.7	7.4381	6.9844	6.7043	0.0000	NS	NS	0.0246	-
16804724	similar to FtsW	1.7	7.2647	0.0000	0.0000	5.7918	0.0278	0.0280	-	-
16409569	ActA	1.8	5.5168	0.0000	7.1229	7.7576	0.0164	-	-	NS
16409952	hypothetical cell wall associated protein	1.8	6.4446	0.0000	7.8056	5.2354	0.0098	-	0.0074	-
16802207	peptidoglycan binding protein	1.8	0.0000	5.5687	7.0563	0.0000	0.0140	-	0.0141	-
16803329	internalin protein, peptidoglycan bound	1.8	6.4058	6.1610	6.2332	0.0000	NS	-	0.0191	-
16411240	Internalin C	1.9	0.0000	0.0000	6.7818	6.2085	0.0448	-	-	-

[a]Expression levels with no significant difference between a given treatment are indicated by NS. 4 °C = cells exposed to 4 °C for 4 h; 37 °C = cells exposed to 37°C for 4 h; 4 °C salt = cells exposed to 4 °C for 4 h followed by exposure to salt; 37 °C salt = cells exposed to 37°C followed by exposure to salt.

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Table 2

Intermediary Metabolism Proteins (ListiList Category 2) with differENtial Expression between Treatments[a]

			intensity				*p*-value			
gi no.	**protein**	**ListiList**	**4 °C**	**37°C**	**4 °C salt**	**37 °C salt**	**4 to 4 °C salt**	**4 to 37 °C**	**4 °C salt to 37 °C salt**	**37 to 37 ° C salt**
16409575	similar to L-lactate dehydrogenase	2.1.1	6.9612	7.3634	7.9398	7.9802	0.0033	NS	-	NS
16409725	dihydroxyacetone kinase	2.1.1	0.0000	6.6876	7.8031	0.0000	-	0.0020	-	0.0020
16410835	pyruvate formate-lyase	2.1.1	0.0000	6.8677	7.7523	7.2528	0.0498	0.0099	-	NS
16411468	α-mannosidase	2.1.1	5.7918	6.4030	6.6137	0.0000	0.0374	-	0.0150	-
16411565	similar to 1-phosphofructokinase	2.1.1	0.0000	6.1179	6.7230	0.0000	0.0111	-	0.0111	-
16412162	similar to ribose 5-phosphate epimerase	2.1.1	6.7400	6.1520	5.1173	6.4804	0.0086	NS	-	NS
16802066	β-glucosidase	2.1.1	7.8661	0.0000	7.0558	0.0000	NS	NS	0.0158	-
16802579	6-phospho-β-glucosidase	2.1.1	0.0000	0.0000	7.0197	7.1229	0.0030	-	-	-
16803211	NADPH-dependent butanol dehydrogenase	2.1.1	6.8034	5.9597	6.0998	0.0000	0.0486	0.0345	-	-
16803215	similar to ethanolamine ammonia-lyase	2.1.1	6.7624	5.7603	5.5218	0.0000	0.0258	0.0326	-	-
16803219	acetaldehyde dehydrogenase/alcohol dehydrogenase	2.1.1	6.9123	6.0708	7.6642	0.0000	0.0027	-	0.0002	-
16803416	6-phosphogluconate dehydrogenase	2.1.1	6.7825	6.6058	8.2542	7.6424	0.0092	-	0.0476	NS
16803610	pyruvate kinase	2.1.1	6.9556	8.1493	8.6512	8.3909	0.0099	NS	-	NS
16803674	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	2.1.1	6.3356	7.6512	8.0228	7.7441	0.0088	NS	-	NS
16803907	pyruvate phosphate dikinase	2.1.1	6.2712	6.5881	7.3780	7.0393	-	-	-	0.0363
16804142	phosphotransacetylase	2.1.1	6.1527	7.4961	7.8103	7.0716	0.0007	<0.0001	0.0048	0.0095
16804374	fructose-1-phosphate kinase	2.1.1	5.9542	6.8914	7.0088	3.9846	0.0445	NS	0.0300	NS
16804701	sorbitol dehydrogenase	2.1.1	5.9253	5.8023	7.0092	5.1989	0.0167	NS	0.0105	-
16804801	similar to xylose operon regulatory protein and to glucose kinase	2.1.1	0.0000	5.5560	6.8148	5.4736	0.0432			
16804818	similar to β-glucosidase	2.1.1	7.3414	6.1402	7.0027	6.9850	-	0.0009	-	0.0267
227478797	pyruvate formate-lyase	2.1.1	4.4422	6.5867	7.6970	7.5296	0.0177	-	-	NS
16409932	phosphoglycerate mutase	2.1.2	6.7814	6.8841	6.5850	0.0000	NS	NS	0.0238	0.0171
16411943	enolase	2.1.2	0.0000	7.8602	7.4834	8.1384	0.0217	NS	-	NS
16411947	glyceraldehyde 3-phosphate dehydrogenase	2.1.2	7.2901	8.0892	8.5794	8.3793	0.0070	NS	-	NS
16803112	pyruvate carboxylase	2.1.2	7.6587	7.3676	0.0000	6.8210	0.0167	NS	-	NS
16804405	glucose-6-phosphate isomerase	2.1.2	0.0000	7.1315	7.9954	7.4979	0.0415	NS	-	NS

			intensity				*p*-value			
gi no.	**protein**	**ListiList**	**4 °C**	**37°C**	**4 °C salt**	**37 °C salt**	**4 to 4 °C salt**	**4 to 37 °C**	**4 °C salt to 37 °C salt**	**37 to 37 ° C salt**
16804494	phosphoglyceromutase	2.1.2	7.4374	7.9937	8.4495	7.5346	0.0005	NS	0.0007	NS
16804496	phosphoglycerate kinase	2.1.2	4.8207	7.7023	7.6283	7.8756	0.0146	-	-	NS
16410414	similar to N-acyl-L-amino acid amidohydrolases	2.2	5.0345	0.0000	5.7760	6.8229	0.0352	-	-	-
16411443	similar to 3-isopropylmalate dehydratase	2.2	7.7082	0.0000	5.5451	0.0000	0.0092	0.0089	-	-
16411545	probable *O*-sialoglycoprotein endopeptidase Gcp	2.2	7.5966	5.1507	6.6183	6.4445	NS	NS	-	<0.0001
16411560	similar to argininosuccinate synthase	2.2	0.0000	6.5992	7.5140	6.4491	0.0149	<0.0001	0.0249	NS
16411902	similar to aminotransferase; Fe-S cluster assembly protein	2.2	6.9243	7.5226	7.6740	6.8930	0.0495	NS	0.0469	NS
16412034	threonine synthase	2.2	6.9294	7.4119	8.3031	8.4006	0.0187	NS	-	NS
16412249	similar to glutamine amidotransferase	2.2	0.0000	0.0000	7.2733	0.0000	0.0166	-	0.0167	-
16412325	phosphoserine aminotransferase	2.2	6.2299	0.0000	7.4518	7.3295	0.0076	-	-	-
16802491	glutamate decarboxylase	2.2	5.0580	6.7632	5.0575	6.4716	-	-	0.0166	-
16803257	*endo*-1,4-β-glucanase and to aminopeptidase	2.2	6.5306	7.0107	0.0000	6.7576	-	NS	0.0362	NS
16803394	aminopeptidase P	2.2	0.0000	0.0000	7.4421	6.9641	0.0093	-	-	NS
16803660	dipeptidase PepV	2.2	6.3184	7.9841	8.1506	7.9454	0.0091	0.0231	-	NS
16803704	*S*-adenosylmethionine synthetase	2.2	0.0000	6.7040	7.4543	6.0209	0.0373	-	0.0433	NS
16804022	dihydroxy-acid dehydratase	2.2	6.7569	5.9028	0.0000	6.5999	0.0091	0.0249	0.0251	NS
16804026	2-isopropylmalate synthase	2.2	6.4251	0.0000	0.0000	7.2174	0.0196	0.0197	-	NS
16411289	highly similar to carbamoyl-phosphate synthetase (catalytic subunit)	2.3	5.8757	0.0000	7.0540	0.0000	NS	-	0.0397	-
16411407	phosphopentomutase Drm	2.3	5.1172	7.4228	7.7811	6.8321	0.0099	NS	0.0206	NS
16802325	anaerobic ribonucleoside triphosphate reductase	2.3	6.2082	6.6751	6.8456	6.6630	0.0156	-	-	NS
16803877	dihydroorotase PyrC	2.3	5.5521	5.7992	6.8805	6.2331	0.0313	-	-	-
16804649	adenylate kinases	2.3	6.5162	7.3866	7.7739	7.6280	0.0282	NS	-	NS
16410787	similar to branched-chain α-keto acid dehydrogenase E3 subunit	2.4	0.0000	6.6942	7.1144	6.8158	0.0216	0.0228	-	NS

[a] Expression levels with no significant difference between a given treatment are indicated by NS.

Table 3

Information Pathway Proteins (ListiList Category 3) and Proteins with Other Functions (ListiList Category 4) with Differential Expression between Treatments[a]

			intensity				*p*-value			
gi no.	**protein**	**ListiList**	**4 °C**	**37°C**	**4 °C salt**	**37 °C salt**	**4 to 4 °C salt**	**4 to 37 °C**	**4 °C Salt to 37 °C salt**	**37 to 37 °C salt**
16410994	DNA polymerase I	3.1	6.8723	6.5453	5.6347	6.5548	0.0118	NS	-	NS
16802093	similar to single-strand binding protein	3.1	6.0229	0.0000	7.5454	5.9570	0.0430	-	0.0422	-
16409964	DNA photolyase	3.2	0.0000	6.6394	0.0000	6.5368	-	-	0.0180	NS
16410832	DNA mismatch repair MutS	3.2	7.0763	7.0809	7.8285	6.8644	0.0287	NS	0.0192	NS
16803271	DNA polymerase β, similar *B. subtilis* YshC	3.2	6.6778	7.3774	0.0000	0.0000	0.0057	0.0411	-	-
16410827	RecA	3.3	6.1232	6.7226	7.5282	0.0000	-	NS	-	0.0380
16410962	RuvA	3.3	0.0000	0.0000	6.7651	5.5560	0.0354	-	0.0453	-
16411395	RecS	3.3	6.2345	6.5578	6.5471	0.0000	NS	-	0.0456	-
16411738	ATP-dependent deoxyribonuclease B	3.3	6.3311	6.4461	6.7314	5.8742	NS	-	0.0192	-
16804306	ATP-dependent deoxyribonuclease A	3.3	0.0000	0.0000	6.8177	6.4622	0.0291	-	-	<0.0001
16804794	RecQ	3.3	6.4065	4.9159	0.0000	6.4819	-	-	0.0015	0.0019
16410718	LexA	3.5.2	7.4147	7.2348	6.2958	6.8476	<0.0001	NS	-	NS
16804167	similar to transcription regulator, LacI family	3.5.2	6.4056	0.0000	0.0000	0.0000	0.0072	0.0072	-	-
16803697	elongation factor Ts	3.5.3	5.1279	7.7511	7.5649	7.9317	0.0088	-	-	NS
16412102	50S ribosomal protein L30 RpmD	3.7.1	6.2986	7.4704	7.2308	7.6548	0.0251	0.0001	-	NS
16412112	50S ribosomal protein L29 RpmC	3.7.1	0.0000	0.0000	0.0000	6.9926	-	-	0.0319	0.0319
16804586	50S ribosomal protein L31 type B RpmE2	3.7.1	6.5147	7.3646	8.1881	7.7370	0.0029	0.0221	-	NS
16804655	50S ribosomal protein L6 RplF	3.7.1	6.7922	7.7788	8.2158	7.9844	0.0219	NS	-	NS
16803498	glycyl-tRNA synthetase β chain	3.7.2	0.0000	7.0592	5.8591	5.2545	-	-	-	-
16410949	histidyl-tRNA synthetase	3.7.2	7.1119	6.1159	0.0000	0.0000	0.0233	0.0389	-	-
16410988	threonyl-tRNA synthetase	3.7.2	6.9629	0.0000	5.2213	6.1896	0.0374	0.0349	-	-
16411208	aspartyl/glutamyl-tRNA aminotransferase subunit B	3.7.2	6.6991	0.0000	6.2265	6.6142	NS	-	NS	0.0070
16802223	methionyl-tRNA synthetase	3.7.2	0.0000	5.4281	6.1184	5.0938	0.0039	-	0.0089	-
16803647	phenylalanyl-tRNA synthetase β subunit	3.7.2	0.0000	5.5149	6.2381	0.0000	<0.0001	-	<0.0001	-
16804581	peptide chain release factor 1 Prf1	3.7.5	6.1543	7.6104	7.2635	6.6608	0.0153	NS	NS	NS

			intensity				*p*-value			
gi no.	**protein**	**ListiList**	**4 °C**	**37°C**	**4 °C salt**	**37 °C salt**	**4 to 4 °C salt**	**4 to 37 °C**	**4 °C Salt to 37 °C salt**	**37 to 37 °C salt**
16409576	similar to *B. subtilis* general stress protein Ctc	4.1	0.0000	7.3261	6.8294	7.7079	0.03612	0.0109	NS	NS
16411753	protein gp20 bacteriophage A118	4.3	7.3442	0.0000	6.3662	4.9735	NS	-	0.0398	-
16411783	similar to bacteriophage protein	4.3	6.7270	0.0000	0.0000	6.6206	0.0276	0.0276	NS	-
16802169	similar to bacteriophage minor tail proteins	4.3	6.7460	0.0000	0.0000	6.0269	0.0015	0.0016	NS	-
16412311	tRNA modification GTPase MnmE	4.5	6.4817	0.0000	7.4959	5.5128	0.0088	-	0.0039	-
16802166	antigen A LmaA	4.5	4.7242	0.0000	6.5650	7.2069	0.0080	-	NS	NS

[a]Expression levels with no significant difference between a given treatment are indicated by NS.