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Gene expression profiling of a nisin-sensitive *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. Nisin is the only bacteriocin that can be used as a food preservative. Due to its antimicrobial activity, it can be used to control *L. monocytogenes* in food; however, the antimicrobial mechanism of nisin activity against *L. monocytogenes* is not fully understood. The CtsR (class III stress gene repressor) protein negatively regulates the expression of class III heat shock genes. A spontaneous pressure-tolerant *ctsR* deletion mutant that showed increased sensitivity to nisin has been identified. Microarray technology was used to monitor the gene expression profiles of the *ctsR* mutant under treatments with nisin. Compared to the nisin-treated wild type, 113 genes were up-regulated (>2-fold increase) in the *ctsR* deletion mutant whereas four genes were down-regulated (<−2-fold decrease). The up-regulated genes included genes that encode for ribosomal proteins, membrane proteins, cold-shock domain proteins, translation initiation and elongation factors, cell division, an ATP-dependent ClpC protease, a putative accessory gene regulator protein D, transport and binding proteins, a beta-glucoside-specific phosphotransferase system IIABC component, as well as hypothetical proteins. The down-regulated genes consisted of genes that encode for virulence, a transcriptional regulator, a stress protein, and a hypothetical protein. The gene expression changes determined by microarray assays were confirmed by quantitative real-time PCR analyses. Moreover, an in-frame deletion mutant for one of the induced genes (*LMOf2365_1877*) was constructed in the wild-type *L. monocytogenes* F2365 background. Δ*LMOf2365_1877* had increased nisin sensitivity compared to the wild-type strain. This study enhances our understanding of how nisin interacts with the *ctsR* gene product in *L. monocytogenes* and may contribute to the understanding of the antibacterial mechanisms of nisin.

Keywords *Listeria monocytogenes* Scott A · Microarray and quantitative real-time PCR · Nisin

Introduction

Listeria monocytogenes is a Gram-positive bacterium that is capable of growth at low temperatures and is known for its ability to adapt to various environmental conditions, such as acidity and high salt. Because of this, as well as its wide distribution in the environment and food, it is of great concern to the food industry [15]. *L. monocytogenes* can cause sporadic or epidemic cases of food-borne listeriosis, which can lead to high fatality rates in the elderly, pregnant women, newborns, and immunocompromised individuals. Although there has been a decline in the number of outbreaks of listeriosis, outbreaks still occur. General incidence rates are about 2,500 cases per year in the United States, leading to about 30 % of all deaths caused by

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food-borne pathogens [15]. Cantaloupe was implicated in a recent major multistate outbreak of food-borne listeriosis caused by two serotypes (1/2a and 1/2b) of *L. monocytogenes* [5, 6, 13].

In addition, illness due to *L. monocytogenes* can produce a large economic burden. Between 1993 and 1998, the pathogen was responsible for 71 % of all food recalls in the US [10]. It is found in raw and processed foods, milk, dairy products, meats and meat products, fresh produce, fresh juices, seafood, and fish [10, 15, 33]. A zero-tolerance policy is in effect for *L. monocytogenes* in ready-to-eat foods [15]. Due to the survival of *L. monocytogenes* under food-related and stress conditions, it is difficult to eliminate the bacteria from foods and food-processing plants.

One commercially employed method for control of *L. monocytogenes* is through the use of nisin. Nisin is one of the most well-known and studied lantibiotics, a heterogeneous group of anti-bacterial proteins produced by lactic acid bacteria. Nisin is a 34-amino-acid cationic peptide that is ribosomally synthesized [3]. It is well known to be inhibitory to a wide range of Gram-positive bacteria, including *Staphylococcus aureus*, *Streptococcus pneumoniae*, and vancomycin-resistant enterococci, in addition to showing antimicrobial effects against *L. monocytogenes* at different temperatures [1]. Nisin works in two fashions, first by forming pores in the bacterial cell membrane and secondly by inhibiting peptidoglycan synthesis [1, 3, 10]. Nisin has been used for over 50 years in food preservation and is the only natural food-grade antibacterial agent approved for use by the EU [3].

Spontaneous nisin resistance occurs in *L. monocytogenes*, and is strain- and environmental condition-specific. Rates of resistance have been detected at frequencies of 10^{-2} through 10^{-8}. Most nisin resistance is developed upon repeated exposure to increasing concentrations of the lantibiotic, but resistance is usually not stable and is lost once nisin pressure is removed [3, 15]. Many genes have been associated with the resistance, including *telA*, *virR*, *mprF*, *dltA*, *galKMT*, and *ahrC*. Various regulators have also been found to play a role in resistance, including LisRK, LiaRS, VirRs, and σ^B. Cell membrane composition adaptations are thought to be the main explanation for development of resistance, but they cannot fully explain the phenomena [3, 8, 10, 15, 18, 35].

The class III stress gene repressor (*ctsR*) regulates the expression of genes encoding the Clp ATPases and ClpP protease. CtsR is a DNA-binding protein encoded by the first gene of the *clpC* operon and acts as a repressor of class III genes that encode ClpC, ClpP, and ClpE [11, 12, 30]. These Clp ATPases are all required for survival and growth under stress, including high temperature. *ctsR* mutations often show increases in heat resistance and general stress tolerance. Mutant *ctsR* strains in *Lactobacillus plantarum* show changes in the cell envelope that indicate a role for *ctsR* in cell wall integrity [12].

A naturally occurring *ctsR* mutant was obtained in *L. monocytogenes* Scott A, which had an increase in pressure tolerance, exhibiting 100-fold higher levels of viability than the wild type when exposed to 450 MPa [21, 28, 29]. This mutant, *ctsR* 2-1, had a deletion in the *ctsR* gene that resulted in the production of a truncated CtsR composed of only 20 amino acids, as compared to 152 amino acids in the wild type [21]. This mutant was also found to be less virulent, non-motile, heat and acid resistant, and sensitive to nisin. Gene expression profiling for this mutant under pressure treatments indicated changes in gene expression that are consistent with the loss of the repressor function of CtsR. Expression of other genes was also changed, including the moderate reduction of expression in genes that encode proteins involved with the cell envelope, indicating that CtsR may interact with membrane proteins in some fashion [28, 29].

To identify genes that show changes in expression in the *ctsR* mutant 2-1 under nisin treatment, both the *ctsR* mutant and the *L. monocytogenes* Scott A wild type were treated with nisin (20 μg/ml), RNA was isolated and labeled, and microchip arrays of the whole genome of *L. monocytogenes* were performed. These results were verified using quantitative real-time PCR (qRT-PCR). This work was conducted to enhance our understanding of nisin interaction with the *ctsR* gene product in *L. monocytogenes* and to contribute to our understanding of the antibacterial mechanisms of nisin.

Materials and methods

Bacterial strains and nisin treatments

The *ctsR* mutant 2-1 of *L. monocytogenes* Scott A [21] 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, USA) agar plate from a glycerol stock culture (stored at −80 °C) followed by incubation at 37 °C overnight. A single colony was picked from the plate, inoculated into 5 ml of BHI broth, and grown at 37 °C with agitation at 200 rpm. The overnight cultures were diluted 1:10 in BHI and grown at 37 °C until the OD_{600} reached 0.3. Nisin (containing 2.5 % pure nisin, balance sodium chloride, and denatured milk solids, activity of 1×10^6 IU/g, according to the manufacturer) from *Lactococcus lactis* was purchased from Sigma-Aldrich (N5764). Different concentrations of nisin (0, 5, 10, 25, 50, 150, 300 μg/ml) dissolved in 0.02 N acetic acid were added to the log-phase cells and incubated at 37 °C for 24 h with agitation at 200 rpm. Aliquots

(100 µl) were removed from each concentration and viable plate counts were performed by serial dilution and plating onto BHI agar. All experiments were performed in triplicate and from three independent cultures.

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

Listeria monocytogenes cultures treated with nisin (20 µg/ml) for 24 h were centrifuged at 4,500 rpm for 5 min at room temperature. The pellets were resuspended in 0.5 ml RNA later followed by RNA isolation. Total RNA was isolated as described [27]. A whole-genome microarray was used and microarray data were analyzed as described previously [27].

cDNA synthesis, primer design, and qRT-PCR analysis

Synthesis of cDNA and real-time qPCR analysis were carried out as described [28]. Primers designed using Primer3 are listed in supplemental Tables 1 and 2.

Construction of in-frame deletion mutant (ΔLMOf2365_1877) in *L. monocytogenes* F2365

The deletion mutant of *LMOf2365_1877* (Δ*LMOf2365_1877*) was constructed using allelic-exchange mutagenesis as previously described [7]. Briefly, splicing by overlap extension (SOE) PCR (Table 1 lists the primers used) was used to construct a Δ*LMOf2365_1877* allele with an in-frame 708-bp deletion, with the 1877 open reading frame cloned into pKSV7. The pKSV7 containing the deletion fragment was electroporated into *L. monocytogenes* F2365 as described [32], and transformants were serially passaged at 40 °C in BHI with chloramphenicol (10 µg/ml) to select for cells in which the plasmid had integrated into the chromosome by homologous recombination. Colonies obtained during subsequent passages at 30 °C in BHI without chloramphenicol were screened for chloramphenicol sensitivity (indicating a second homologous event with loss of the plasmid). Chloramphenicol-sensitive colonies were then screened by colony PCR to identify isolates with the Δ*LMOf2365_1877* allele. The chromosomal deletion was confirmed by PCR amplification and direct sequencing of the PCR product with primers LMOf2365_1877SOEA and LMOf2365_1877SOED.

Nisin inhibition assay

Listeria monocytogenes F2365 wild-type and Δ*LMOf2365_1877* mutants were used for growth assays. To make a log-phase culture, one *L. monocytogenes* colony was inoculated into 5 ml of BHI and grown at 37 °C with agitation at 200 rpm overnight. A 50-µl aliquot of overnight culture was added into 5 ml of BHI and grown at 37 °C with agitation at 200 rpm for 3 h until an OD_{600} of 0.4 was obtained. Growth assays were performed in a 96-well plate format using log-phase bacteria. The lid of the microtiter plate was pre-treated with 0.05 % Triton X-100 in 20 % ethanol to eliminate liquid condensation. After 15–30 s, the treatment solution was poured off and the cover was leaned against a vertical surface and allowed to air-dry [4]. Nisin, at a concentration of 250 µg/ml in BHI, was used for growth studies, and 0.02 N HCl was used as a negative control. The plate was placed into a Safire II spectrophotometer (Tecan) at 37 °C, with OD readings at λ 600 nm taken every 2 h for 16 h.

Microarray data accession number

The microarray data have been deposited into the Gene Expression Omnibus database under accession number GSE41891 (www.ncbi.nlm.nih.gov/geo).

Results

The *ctsR* mutant 2-1 was more sensitive to nisin treatment when compared to the wild type

A nisin resistance assay was performed to study the nisin sensitivity of the *ctsR* mutant 2-1. As shown in Fig. 1, the *ctsR* mutant 2-1 was more sensitive to nisin treatments compared to the wild type. This result was consistent with that from the previous study [21]. Samples from the 20 µg/ml nisin treatment (sublethal dose) were used for microarray and real-time qPCR assays. To identify genes that show changes in expression in the *ctsR* mutant 2-1 under nisin treatment, both the *ctsR* mutant 2-1 and the Scott A wild type were treated with nisin. The RNA was isolated and labeled, and microchip arrays of the whole genome of *L. monocytogenes* were performed. A minimum threshold of a twofold change in gene expression with a p value of <0.01 was used as the cut-off value. All of the genes identified by microarray analysis that were differentially expressed under nisin treatment were confirmed by qRT-PCR (see Supplemental Tables 1 and 2 for primer sequences). Only genes that were induced or repressed by both microarray and qRT-PCR assays are presented.

Induced genes in *L. monocytogenes ctsR* mutant 2-1 strain under nisin treatment

The 113 genes that were induced under nisin treatment are shown in Table 2. These genes were grouped into the

Table 1 Strains, plasmids, and primers used in this study

E. coli strains		
TOP10	Competent cells	Invitrogen
DH5α	Competent cells	Invitrogen
Plasmids		
pKSV7	Temperature-sensitive integration vector; Cm^r	Gift from S. Kathariou
L. monocytogenes strains		
L. monocytogenes Scott A	Parent strain	Gift from R. D. Joerger
L. monocytogenes Scott A	*ctsR* mutant 2-1	
LMOf2365	Wild-type serotype 4b strain, genome sequenced	Nelson et al. [31]
Δ*LMOf2365_1877*	1877 deletion	This study
Primers		
LMOf2365_1877SOEA	5′GG**GGTACC**ACTCCGCACCGCAAGCAC3′	This study
LMOf2365_1877SOEB	5′GTGGGAAAAGGGTGGTTTAGATGTAATA GATGTTGTTTTTAGAAGG3′	This study
LMOf2365_1877SOEC	5′CATCTAAACCACCCTTTTCCCAC3′	This study
LMOf2365_1877SOED	5′GC**TCTAGA**GAATTAGCTAAAACGCTTG3′	This study

Restriction sites (KpnI and XbaI) are highlighted in *bold*. Regions overlapping complementary to SOEC primer are *underlined*

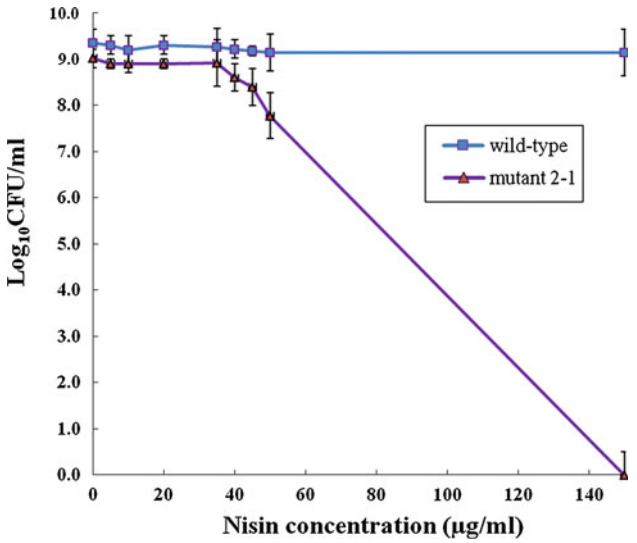

Fig. 1 The survival of wild-type *L. monocytogenes* Scott A and the *ctsR* mutant 2-1 of *L. monocytogenes* Scott A in the presence of nisin. Exponential phase cells of *L. monocytogenes* ($OD_{600} = 0.3$) were treated with different concentrations of nisin (0, 5, 10, 20, 35, 40, 45, 50, 150 μg/ml) for 24 h, and variable cell counts were measured under each concentration. Data presented here are the averages of three independent experiments with standard deviations

following categories: genes encoding for transport and binding, transcriptional regulator, proteins in amino acid biosynthesis and energy metabolism, protein synthesis, toxin production and resistance, cell division, and hypothetical proteins.

Of the transporters whose transcript levels were up-regulated, *LMOf2365_1877* (manganese ABC transporter; ATP-binding protein) showed an over twofold induction in the microarray assay and a >24-fold induction by qRT-PCR assays (Table 2). This gene was also highly induced in *L. monocytogenes* present in milk [27] but inhibited in ready-to-eat meat [2]. Genes related to the cell envelope (*LMOf2365_0056, 2240, 2457, 2610*) were moderately induced. Since nisin targets the cells by forming pores on the membrane and inhibiting peptidoglycan synthesis, the induction of these genes in the *ctsR* mutant 2-1 is not surprising.

Other very highly induced genes encoded putative accessory gene regulator proteins B and D (*LMOf2365_0057, 0058*). These genes were induced 2.5 and 3.1-fold in the microarray assay, respectively, and 8.3 and 4.4-fold in the qRT-PCR assay, respectively. These two genes were also induced in the *ctsR* mutant 2-1 under pressure treatment [28], indicating that they may be involved in general stress response. Another gene that was induced by nisin treatment was *LMOf2365_2147* (2.6-fold in the microarray assay and 5.7-fold in the qRT-PCR assay), which encodes for an ABC transporter. This gene was also induced in the *ctsR* mutant 2-1 under pressure treatment [28].

Repressed genes in *ctsR* mutant 2-1 under nisin treatment

A total of four genes were repressed in the *ctsR* mutant 2-1 under nisin treatment (Table 3). These genes encode for a transcriptional regulator (*LMOf2365_2233*), a universal stress protein (*LMOf2365_1602*), a hypothetical protein (*LMOf2365_2819*), and a virulence factor (*LMOf2365_0213*). *LMOf2365_0213*, encoding for listeriolysin O, is down-regulated (−2.5-fold in the microarray and −10.0-fold in qRT-PCR assays), indicating that the *ctsR* mutant 2-1 may be less virulent under nisin treatment.

Table 2 Genes up-regulated in *L. monocytogenes* strain Scott A *ctsR* mutant 2-1 under nisin treatment as identified by microarray and qRT-PCR analysis

Category/gene	Function[a]	Fold change[b]	
		Microarray[c]	RT-PCR[d]
Purines, pyrimidines, nucleosides, and nucleotides: nucleotide and nucleoside interconversions			
LMOf2365_2584	Adenylate kinase	2.2	6.5
Amino acid biosynthesis: pyruvate family			
LMOf2365_0999	Branched-chain amino acid aminotransferase	3.0	7.7
Amino acid biosynthesis: aspartate family			
LMOf2365_0624	*O*-acetylhomoserine (thiol)-lyase	2.4	1.7
Fatty acid and phospholipid metabolism: biosynthesis			
LMOf2365_1834	Acyl carrier protein	2.3	5.9
Energy metabolism: other			
LMOf2365_1159	Propanediol utilization protein PduA	2.3	11.3
LMOf2365_1160	Propanediol utilization protein PduB	2.1	10.6
LMOf2365_1162	Propanediol utilization: dehydratase, medium subunit	2.0	8.9
LMOf2365_1163	Propanediol utilization: dehydratase, small subunit	2.0	11.3
LMOf2365_1164	Propanediol utilization: diol dehydratase reactivation	2.1	11.3
LMOf2365_1165	PduH protein	2.4	13.5
LMOf2365_1166	Active propanediol utilization protein PduK	2.0	8.3
LMOf2365_1167	Putative propanediol utilization protein PduJ	2.3	17.8
LMOf2365_1170	Propanediol utilization protein PduM	2.6	9.5
LMOf2365_1171	Propanediol utilization: polyhedral bodies	3.3	12.6
LMOf2365_1173	CoA-dependent propionaldehyde dehydrogenase	2.2	10.2
LMOf2365_1174	Propanol dehydrogenase	2.6	16.0
Energy metabolism: fermentation			
LMOf2365_1425	Formate acetyltransferase	2.1	2.6
LMOf2365_1656	Aldehyde-alcohol dehydrogenase	2.6	2.4
LMOf2365_1946	Formate acetyltransferase	2.9	5.9
Energy metabolism: electron transport			
LMOf2365_2184	Putative thioredoxin	2.1	1.9
LMOf2365_2697	Cytochrome d ubiquinol oxidase, subunit II	2.0	2.0
LMOf2365_2698	Cytochrome d ubiquinol oxidase, subunit I	2.0	2.5
Energy metabolism: pyruvate dehydrogenase			
LMOf2365_1075	Dihydrolipoamide acetyltransferase	2.3	1.8
Energy metabolism: glycolysis/gluconeogenesis			
LMOf2365_2428	Enolase	2.1	2.9
LMOf2365_2429	Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent	2.3	3.9
LMOf2365_2430	Triosephosphate isomerase	3.3	1.5
LMOf2365_2431	Phosphoglycerate kinase	2.2	2.2
LMOf2365_2432	Glyceraldehyde-3-phosphate dehydrogenase, type I	2.4	19.0
LMOf2365_2528	Putative fructose-bisphosphate aldolase	3.2	3.4
Energy metabolism: amino acids and amines			
LMOf2365_1169	Ethanolamine utilization protein	2.4	8.9
Energy metabolism: anaerobic			
LMOf2365_0221	L-lactate dehydrogenase	2.6	4.9
Cellular processes: adaptations to atypical conditions			
LMOf2365_1381	Cold-shock domain family protein	2.9	1.8
LMOf2365_1908	Cold-shock domain family protein	2.7	1.9

Table 2 continued

Category/gene	Function[a]	Fold change[b]	
		Microarray[c]	RT-PCR[d]
Cellular processes: pathogenesis			
LMOf2365_0057	**Putative accessory gene regulator protein B**	**2.5**	**8.3**
LMOf2365_0058	**Putative accessory gene regulator protein D**	**3.1**	**4.4**
Cellular processes: toxin production and resistance			
LMOf2365_1997	Putative tellurite resistance protein	2.1	2.7
DNA metabolism: chromosome-associated proteins			
LMOf2365_1963	DNA-binding protein HU	2.3	6.3
DNA metabolism: DNA replication, recombination, and repair			
LMOf2365_0054	Single-strand binding protein	2.1	2.5
Regulatory functions: DNA interactions, protein interactions			
LMOf2365_1042	Putative histidine kinase	2.5	2.7
LMOf2365_1043	DNA-binding response regulator	2.4	4.3
LMOf2365_1770	DNA-binding response regulator	2.2	5.5
LMOf2365_1878	transcriptional regulator, MarR family	2.5	3.9
Transcription: DNA-dependent RNA polymerase			
LMOf2365_2579	DNA-directed RNA polymerase, alpha subunit	2.2	4.9
Biosynthesis of cofactors, prosthetic groups, and carriers: heme, porphyrin, and cobalamin			
LMOf2365_1709	Glutamate-1-semialdehyde-2,1-aminomutase 2	2.1	4.4
Protein synthesis: tRNA aminoacylation			
LMOf2365_1539	Histidyl-tRNA synthetase	3.3	4.3
Transport and binding proteins: cations and iron-carrying compounds			
LMOf2365_1877	Manganese ABC transporter, ATP-binding protein	2.1	24.3
Transport and binding proteins: anions			
LMOf2365_2282	Phosphate transporter family protein	2.1	2.4
Transport and binding and signal transduction proteins			
LMOf2365_0030	Phosphotransferase system, beta-glucoside-specific, IIABC component	4.6	24.3
LMOf2365_1175	Glycerol uptake facilitator protein	2.0	17.8
LMOf2365_1659	Putative ABC transporter, permease protein	2.2	1.6
LMOf2365_2147	**ABC transporter, ATP-binding protein**	**2.6**	**5.7**
Cell envelope: other			
LMOf2365_0056	Putative lipoprotein	3.1	4.4
LMOf2365_2240	Putative membrane protein	2.7	4.8
LMOf2365_2457	Putative membrane protein	5.1	3.9
LMOf2365_2610	Putative lipoprotein	2.4	1.7
Protein synthesis: ribosomal proteins: synthesis and modification			
LMOf2365_0053	Ribosomal protein S6	2.3	1.9
LMOf2365_0261	Ribosomal protein L1	2.6	2.0
LMOf2365_0262	Ribosomal protein L10	2.6	2.1
LMOf2365_1559	Ribosomal protein L27	3.1	3.9
LMOf2365_1561	Ribosomal protein L21	4.3	2.4
LMOf2365_1814	Ribosomal protein L19	2.1	6.3
LMOf2365_1824	Ribosomal protein S16	2.5	3.2
LMOf2365_1967	Putative ribosomal protein S1	2.5	2.6
LMOf2365_2521	Ribosomal protein L31	2.6	2.2
LMOf2365_2580	Ribosomal protein S11	2.1	2.7
LMOf2365_2581	Ribosomal protein S13	2.6	4.4
LMOf2365_2582	Ribosomal protein L36	3.8	5.7

Table 2 continued

Category/gene	Function[a]	Fold change[b]	
		Microarray[c]	RT-PCR[d]
LMOf2365_2586	Ribosomal protein L15	3.1	2.5
LMOf2365_2587	Ribosomal protein L30	3.8	3.4
LMOf2365_2588	Ribosomal protein S5	2.4	4.9
LMOf2365_2589	Ribosomal protein L18	2.9	4.1
LMOf2365_2590	Ribosomal protein L6	2.5	3.4
LMOf2365_2591	Ribosomal protein S8	2.3	5.1
LMOf2365_2593	Ribosomal protein L5	3.1	3.2
LMOf2365_2594	Ribosomal protein L24	2.4	2.9
LMOf2365_2595	Ribosomal protein L14	2.7	3.9
LMOf2365_2596	Ribosomal protein S17	2.1	2.2
LMOf2365_2597	Ribosomal protein L29	3.2	2.5
LMOf2365_2598	Ribosomal protein L16	2.2	1.4
LMOf2365_2599	Ribosomal protein S3	2.2	3.5
LMOf2365_2600	Ribosomal protein L22	3.5	2.6
LMOf2365_2601	Ribosomal protein S19	3.2	2.5
LMOf2365_2602	Ribosomal protein L2	2.8	2.7
LMOf2365_2603	Ribosomal protein L23	3.1	3.0
LMOf2365_2605	Ribosomal protein L3	2.7	4.6
LMOf2365_2606	Ribosomal protein S10	2.4	3.0
LMOf2365_2634	Ribosomal protein S7	4.4	12.1
LMOf2365_2635	Ribosomal protein S12	3.8	14.4
Protein fate: degradation of proteins, peptides, and glycopeptides			
LMOf2365_0980	Putative peptidase	2.8	2.1
Protein fate: protein and peptide secretion and trafficking			
LMOf2365_2585	Preprotein translocase, SecY subunit	3.0	4.6
Protein fate: protein folding and stabilization			
LMOf2365_1284	Trigger factor	2.2	3.4
LMOf2365_2252	Protein export protein	2.3	3.9
Protein synthesis: translation factors			
LMOf2365_2632	Translation elongation factor Tu	2.8	3.9
LMOf2365_2633	Translation elongation factor G	3.1	6.7
Genes encoding hypothetical proteins			
LMOf2365_0204	Conserved hypothetical protein	2.2	13.5
LMOf2365_0475	Hypothetical protein	2.7	3.7
LMOf2365_0840	Lipoprotein, putative	2.3	1.5
LMOf2365_1040	Conserved hypothetical protein	2.2	2.8
LMOf2365_1041	Conserved hypothetical protein	2.5	3.7
LMOf2365_1350	Conserved hypothetical protein	3.2	3.2
LMOf2365_1560	Conserved hypothetical protein	3.0	3.0
LMOf2365_1670	Conserved hypothetical protein	2.1	1.5
LMOf2365_1686	Conserved hypothetical protein	2.2	3.0
LMOf2365_1916	Conserved hypothetical protein	2.6	2.1
LMOf2365_2063	Conserved hypothetical protein TIGR00044	2.0	4.1
LMOf2365_2460	Conserved hypothetical protein	4.5	2.0
Unknown function			
LMOf2365_0982	LemA protein	2.2	3.2
LMOf2365_1044	TrkA domain protein	2.4	2.1

Table 2 continued

Category/gene	Function[a]	Fold change[b]	
		Microarray[c]	RT-PCR[d]
LMOf2365_1505	Iojap-related protein	2.4	2.5
LMOf2365_1899	DedA family protein	2.3	2,702
LMOf2365_2458	PspC domain protein, truncated	3.7	1.4
LMOf2365_2459	PspC domain protein	4.8	1.7
LMOf2365_2611	Pyridine nucleotide-disulfide oxidoreductase family protein	2.1	8.3

Genes that were also induced in the *ctsR* mutant 2-1 under pressure treatment [28] are in *boldface*

Only the genes that met the stringent criteria for being upregulated in *L. monocytogenes* Scott A *ctsR* mutant 2-1 (i.e., change of more than twofold; $p < 0.01$) are listed here

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

[b] Change indicates the transcript ratios between *L. monocytogenes* Scott A *ctsR* mutant 2-1 and the wild-type (*L. monocytogenes* Scott A) grown under nisin treatment (20 μg/ml, 24 h) as determined by microarray and qRT-PCR

[c] Numbers are average values from four independent experiments

[d] Numbers are average values from three independent experiments

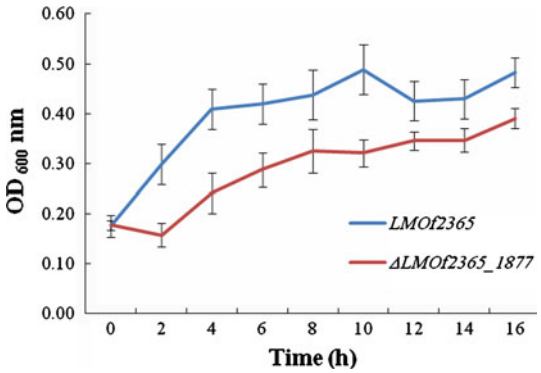

Fig. 2 Growth curves of *L. monocytogenes* F2365 and Δ*LMOf2365_1877* at 37 °C in nisin (250 μg/ml in BHI). Cell growth was measured spectrophotometrically by monitoring the OD_{600} at 2-h intervals for 16 h at 37 °C. Data shown here are the averages of three independent experiments with standard deviations

The *LMOf2365_1877* deletion mutant (Δ*LMOf2365_1877*) displayed a nisin-sensitive phenotype

Microarray and qRT-PCR assays identified many genes that were differentially expressed by the mutant. To study whether these differentially expressed genes were influenced by nisin treatment, a deletion mutant was constructed. *LMOf2365_1877* was chosen since it was over expressed in the *ctsR* mutant 2-1 under nisin treatment. We were unable to obtain a *LMOf2365_1877* deletion mutant in the *ctsR* mutant 2-1 background; therefore, a single deletion mutant was constructed in *L. monocytogenes* F2365 strain. Compared to the wild-type strain, the *LMOf2365_1877* deletion mutant (Δ*LMOf2365_1877*) had increased sensitivity to nisin (Fig. 2). The fact that Δ*LMOf2365_1877* was sensitive to nisin indicates that this gene might be involved in nisin resistance. Consistent with our data, deletion of a different ABC transporter (*anrB*) in *L. monocytogenes* also showed sensitivity to nisin [9] although *anrB* has a different chromosomal location and shows little homology with *LMOf2365_1877*.

Discussion

The lantibiotic nisin has been employed commercially for over 50 years in the food industry to combat *L. monocytogenes*. The *L. monocytogenes* Scott A pressure tolerant *ctsR* mutant 2-1 is nisin-sensitive. In this manuscript, genes that show changes in expression in the *ctsR* mutant 2-1 under nisin treatment were identified using microarray assays and verified using qRT-PCR. Genes that showed either a twofold increase or decrease in expression are listed in Tables 2 and 3. An understanding of the role of these genes and the interaction of nisin with the *ctsR* product will further our understanding of the nisin-resistance mechanism in *L. monocytogenes*. The genes that showed more than a twofold increase were varied, ranging from ribosomal and translational factors to energy metabolism and transport proteins. Some of the results were predictable, for instance, the increase seen in cold-shock domain family proteins, but others were unexpected, such as those involved in energy metabolism. In all, there were 113 genes that showed more than a twofold increase in expression, while only four were found to show more than a twofold decrease in expression. Because *ctsR* is a negative regulator, this difference was not surprising.

One group of genes that showed differential expression encoded for transport and binding proteins. Within this group, an ATP-binding protein of an ABC transporter

Table 3 Genes down-regulated in *L. monocytogenes* strain Scott A *ctsR* mutant 2-1 under nisin treatment as identified by microarray and qRT-PCR analysis

Category/gene	Function[a]	Fold change[b]	
		Microarray[c]	RT-PCR[d]
Cellular processes: adaptations to atypical conditions			
LMOf2365_1602	Universal stress protein family	−2.5	−250
Regulatory functions: DNA interactions			
LMOf2365_2233	Transcriptional regulator, MarR family	−2.5	−2.5
Cellular processes: pathogenesis			
LMOf2365_0213	Listeriolysin O	−3.3	−10.0
Genes encoding hypothetical proteins			
LMOf2365_2819	Conserved hypothetical protein	−2.0	−125

Only the genes that met the stringent criteria for being down-regulated in *L. monocytogenes* Scott A *ctsR* mutant 2-1 (i.e., change of twofold; $p < 0.01$) are listed here

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

[b] Change indicates the transcript ratios between *L. monocytogenes* Scott A *ctsR* mutant 2-1 and the wild-type (*L. monocytogenes* Scott A) grown under nisin treatment (20 μg/ml, 24 h) as determined by microarray and real-time PCR; negative values indicate transcript levels that are lower in *L. monocytogenes* Scott A *ctsR* mutant 2-1 than in the wild type (e.g., a value of _3.2 indicates a 3.2-fold-lower transcript level in *L. monocytogenes* Scott A *ctsR* mutant 2-1 than in the wild type)

[c] Numbers are average values from four independent experiments

[d] Numbers are average values from three independent experiments

(LMOf2365_2147), a putative permease protein of an ABC transporter (LMOf2365_1659), and an ATP-binding protein of a manganese ABC transporter (*LMOf2365_1877*) all showed increased expression in the *ctsR* mutant 2-1 (2.6, 2.2, and 2.1-fold, respectively). ABC transporters are normally exporters or importers for *L. monocytogenes*, and often, these transporters are involved in signaling systems, such as those involved in antimicrobial sensitivities and biofilm formation in *L. monocytogenes* [9, 37]. Some ABC transporters are known to be involved in signaling for genes that are involved in changing the charges of proteins found on bacterial cell walls, therefore changing the overall charge of the cell wall [38]. It is believed that one way in which *L. monocytogenes* is able to resist nisin is by changing the charge of its cell wall. Studies have found that in resistant strains of *L. monocytogenes*, changes to teichoic acid allows the bacteria to regulate the charge of the cell wall, and this may actually be one of the most important mechanisms leading to nisin resistance [3]. In the current study, there was an increase in expression of genes encoding for certain ABC transporters, which may lead to changes in the cell wall of the bacteria. Whether these changes result in the sensitivity to nisin that is seen in the *ctsR* 2-1 mutant are unknown.

Listeria monocytogenes strain F2365 that carries a deletion for *LMOf2365_1877* was constructed and tested under nisin treatment. As predicted, this mutant displayed increased sensitivity to nisin compared to the wild type. Similarly, deletion of another ABC transporter (anrB) in *L. monocytogenes* edg strain also showed nisin sensitivity [9]. Another gene of interest in which we observed an increase of expression was a putative tellurite-resistance protein (*LMOf2365_1997*). It has been demonstrated through the use of a *telA* mutant that TelA is important for resistance of *L. monocytogenes* for a number of antibiotics that are active against the cell wall, including nisin [8]. Again, here we see that although this putative tellurite resistance protein is upwardly expressed in the *ctsR* 2-1 mutant, there was no observable increase in resistance to nisin. The mutant remains sensitive to the lantibiotic. Tellurite is a rare element in the environment and it is unknown what role it has in the resistance to nisin.

Another interesting set of genes in which we observed up-regulation are those involved in energy metabolism and propanediol utilization. Similar to *Salmonella enterica*, *L. monocytogenes* is able to utilize 1, 2-propanediol as a sole carbon source [16, 34]. The *pdu* genes of *L. monocytogenes* are believed to be involved in persistence of the bacteria outside of the human host [14]. These genes are up-regulated in the *ctsR* 2-1 mutant and may play a role in the persistence of the bacteria under nisin treatments. Further studies investigating the interplay between nisin and propanediol utilization are warranted.

Of the four genes that showed a decrease in expression in the *ctsR* 2-1 mutant, the one that is most interesting encodes for listeriolysin O (*LMOf2365_0213*). It showed a 3.3-fold decrease compared to the wild type. Listeriolysin O (LLO) is a pore-forming toxin produced by *L. monocytogenes* that belongs to the cholesterol-dependent cytolysin family [19].

LLO is a virulence factor that is known to be necessary to allow *L. monocytogenes* to escape from host vacuoles, but its exact mechanism is unclear. In *L. monocytogenes*, reduced virulence was associated with mutations in the *ctsR* gene [20, 21, 24]. It is interesting that LLO is controlled in part by *ctsR* regulation, but its connection to nisin resistance remains unclear.

The genes affected by pressure treatment [28] in the *ctsR* mutant 2-1 were compared to the genes affected by nisin treatment. Three genes (*LMOf2365_0057*, *0058, 2147*) were found to be induced in both the nisin and the pressure treatments. *LMOf2365_0057* and *LMOf2365_0058* encode for putative accessory gene regulator protein B and D, respectively, whereas *LMOf2365_2147* encodes for an ATP-binding protein of one of the ABC transporters. These three genes may be associated with general stress responses.

The tailing effect (a small portion of the bacterial population can be relatively resistant after a certain applied pressure) is a major challenge to the food industry. A majority of the pressure-resistant mutants contained mutations in the *ctsR* gene [25, 36], indicating the involvement of this gene in high-pressure treatment. Nisin is the only bacteriocin that can be used as a food preservative. Since the *ctsR* mutant 2-1 is both pressure tolerant- and nisin-sensitive, and a majority of pressure-resistant mutants contained mutations in the *ctsR* gene [25, 36], combining pressure and nisin treatments together can effectively eliminate the survival of these mutants and prevent tailing effect. Actually, pressure treatments combined with nisin have been successfully used to kill bacteria in food [17, 26]. For example, a combination of high pressure treatment with nisin has been shown to inhibit the growth of *L. monocytogenes* [22], therefore, preventing the tailing effect. The fact that our pressure-resistant *ctsR* mutant 2-1 has sensitivity to nisin provided a possible molecular explanation for the above observation. Since nisin acts on bacterial cytoplasmic membranes, and high-pressure treatment also causes damage to the membranes [23], it is hypothesized that the observed synergy between nisin and high hydrostatic pressure results in cumulative damage to the membranes.

The results obtained in this study are intriguing and show that although seemingly not connected, nisin treatment can affect many far reaching genes that seem to be managed, in some fashion, by ctsR involvement. Further functional studies to elucidate these connections would be helpful in bettering our understanding of how *L. monocytogenes* survives under nisin treatment and would help design intervention strategies in food processing.

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