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Stress Response and Adaptation of *Listeria monocytogenes* 08-5923 Exposed to a Sublethal Dose of Carnocyclin A

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Carnocyclin A (CCLA) is an antimicrobial peptide produced by *Carnobacterium maltaromaticum* ATCC PTA-5313, which can be used to control the growth of *Listeria monocytogenes* in ready-to-eat meat products. The aim of this research was to elucidate the cellular responses of *L. monocytogenes* 08-5923 exposed to a sublethal dose of CCLA. Microarray, quantitative reverse transcription-PCR, tandem mass spectrometry, and electron microscopy were used to investigate the alteration in gene expression, protein production, and morphological changes in cells of *Listeria* following treatment with CCLA. The genes involved in metabolism (*baiE*, *trn*, and *pykA*), cell wall synthesis (*murZ* and *dacB2*), and cell division (*clpE* and *divIVA*) were upregulated following a 15-min exposure to CCLA as a result of stress responses. Genes involved in cell division, cell wall synthesis, flagellar synthesis, and metabolism were downregulated after 4 h as a result of adaptation. Analysis of total soluble proteins confirmed the downregulation of *pykA* and *gnd* after 4 h of exposure to CCLA. The absence of flagella was observed in *L. monocytogenes* following 30 h of exposure to CCLA. A sublethal dose of CCLA induced adaptation in *L. monocytogenes* 08-5923 by inhibition of expression of genes and proteins critical for synthesis of cell wall structures and maintaining metabolic functions. Both the mannose- and cellobiose-specific phosphotransferase systems could be targets for CCLA.

Listeria monocytogenes has been responsible for numerous foodborne illness outbreaks as a result of consumption of contaminated ready-to-eat (RTE) meat products (1). A listeriosis outbreak from a single manufacturer in Canada in 2008 resulted in 22 deaths and 57 confirmed positive cases (http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/listeria-monocytogenes-eng.php).

To control the growth of *L. monocytogenes*, bacteriocin-containing antimicrobials such as Micocin (Griffith Laboratories, Canada) can be used in RTE meat products. One active compound in Micocin, carnocyclin A (CCLA), is a 5.9-kDa cyclic, class IIc bacteriocin (2) that has strong antilisterial activity. CCLA forms ion channels in the membrane and results in dissipation of membrane potential, which leads to cell death (3). Neither the stress response of *Listeria* to CCLA nor the development of resistance in *Listeria* have been elucidated. Previous research focused on class IIa bacteriocins, which cause cell surface alterations in *Listeria* (4) and, in particular, result in cell lysis by targeting the mannose-specific phosphotransferase (PTS) system (5, 6). The disruption of the mannose-specific PTS system plays a role in bacteriocin resistance (7, 8). Alternative sigma factors, such as σ^B (sigB) (9–11) and σ^{54} (rpoN) (12), are also involved in resistance to class IIa bacteriocins. These alternative sigma factors are also involved in other environmental stresses, such as cold shock, acid, and osmotolerance in *Listeria* (13, 14).

It has been postulated that CCLA can trigger alteration in the expression levels of genes that are also regulated by σ^B/σ^{54}. The differential expression of genes and proteins and the morphology of *L. monocytogenes* 08-5923, one of the two strains that were involved in the 2008 listeriosis outbreak in Canada (GenBank accession number NC_013768) (15), in the presence and absence of CCLA was analyzed.

MATERIALS AND METHODS

Bacterial strains and cultures. *L. monocytogenes* 08-5923 was grown in tryptic soy broth (TSB; Becton, Dickinson, Ontario, Canada) at 21°C for 24 h prior to use. *Carnobacterium maltaromaticum* ATCC PTA-5313 (formerly *Carnobacterium maltaromaticum* UAL307) was grown in all-purpose Tween (APT; BD-Canada) broth at 21°C.

CCLA isolation and purification. CCLA was isolated from an overnight culture of *C. maltaromaticum* ATCC PTA-5313 grown at 21°C and purified according to previous methods (2).

RNA isolation. Twenty-five ml of log-phase *L. monocytogenes* 08-5923 (optical density at 600 nm [OD_{600}] of 0.2) was grown in TSB or in TSB with 7 μg/ml CCLA (1/10 the MIC on *L. monocytogenes* 08-5923) for 4 h at 21°C (final OD_{600}, 0.5) to obtain cells that were at the mid-log phase of growth. RNAprotect bacterial reagent (Qiagen Inc., Ontario, Canada) was added to the cell culture according to the manufacturer's instructions. Total RNA was isolated using the RNeasy minikit (Qiagen Inc.) and treated with DNase I (New England BioLabs Ltd., Ontario, Canada) on column according to the manufacturers' protocol. RNA quantity was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Ontario, Canada), and the samples were stored at −80°C. RNA quality and quantity were assessed on an Agilent 2100 bioanalyzer (Agilent Technologies Inc., Ontario, Canada) using an Agilent Nano 6000 assay, and an RNA with an integrity number (RIN) of >7.0 was used for subsequent microarray experiments. For quantitative reverse transcriptase real-time PCR (qRT-PCR), total RNA was isolated as described above from cells in lag phase (15 min), mid-log phase (4 h), and early stationary phase (30 h).

Gene expression microarrays and data analysis. The *L. monocytogenes* EGD-e 385 K gene expression microarray from Roche NimbleGen, including all of the 2,857 annotated open reading frames (ORFs) of the genome, was used. The cDNA was synthesized from RNA extracted from three independent biological repetitions (SOP#M007 and SOP#M008; J. Craig Venter Institute [http://pfgrc.jcvi.org/index.php/microarray

Received 31 January 2014 Accepted 11 April 2014
Published ahead of print 18 April 2014
Editor: M. W. Griffiths
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doi:10.1128/AEM.00350-14

TABLE 1 Primers used in qRT-PCR for confirmation of the genes found differentially expressed in microarray experiments

Gene	Primer sequence (5′–3′)	
	Forward	Reverse
ftsE	TATGCGATGGAAGTGGTTGA	CTCACCACCGGAAAGTTCAT
ftsX	AATGGTTGGATGACCTTTGC	CGTTGCAAGCTTGTTCATGT
rpoB	GAAATCTGGGTTCGTCGTGT	GCTACGTTTGGACGTTGGTT
fliF	TCTACATGAACACGCCCAAA	AAAATGTTGCCGCTTTTGTC
clpE	AGCAAACTTTGGGTCGAATG	GTTCACGGTTTGCTTGGTTT
baiE	ACAAGCAAAGGCAGACATCC	CAATAAAGCCATGCCCAGTT
deoR	CAGGCCTTGAGCAAAATGAT	ATAATACGCGCGATTGGAAG
hisC	TGCGAAACGTTTTGAACAAG	CAGCACCAGAACGCGTAATA
flgD	AAATGGCGCAACTTTCCTTA	CGCCGTTTAGTGAAACACCT
yitT	TGGATTTTCCAGCGAATACC	ATCACTACCGCCAGTTGTCC
tgl	GACAAGCCGAGTTTCAAAGC	CGGCATCTGTGACAGTTTGT
lmo0867	CAATCGGCTATCTCGCTTTT	TGCGATAATTGCCATCGTTA
ywzB	ACTTTTTGGGCACTTCAAGC	CCTAACACAATCGAAATAATCACAA
lmo1796	CACCCGGAAGACGTTGTTAT	ACGAATCGCTTTCGCAATAC
ftsW	GGGATCGCTAGTCTGATTGC	TACCAAGCATCATCGACAGC
lmo2311	GCTCAATGCGTTTATGACGA	CGCTGGCAAATAATCTCGTT
trn	CCGAAAAGAGTCGCGATAAT	TGCTGCATAGGTGGATGCTA
hrcA	GGGATATTCCCGATGGTCTT	TCTTGTGGGCCCTAGGAGTA
abc	AATGAGAGCGCCAATTATGG	TGCGCGATACCTTCTACAAA
fmt	AGAAGCCGACCTGCTTGTAA	GTCAAGCAGCGCGTAGTGTA
lmo2317	CCCTTTTCAACCGTGATGTT	GCTCACGAACCCTTTCCATA
mfs	GGGGAAAAAGCTGGAATTGT	TTGCAATCGTTGGCATGTAT
malP	CAATTGGATGGAGCGAAAAT	CGCCATTACGGTGAATTTCT
mptC	ATTCCAGCTGCAGCACTTTT	CAACGGCAACTACCATTCCT
mbl	GCAATTCGTGAAGTTGCTGA	ACCGGAAGGCTCAAAAATCT
gidA	GGCGTGTGTAAAGGCGTTAT	GGTTATTTGGACCGCTTGAA
rodA	CTTCCGCTTGGTCTTGTAGC	CAATGAGCGCAATCGAACTA
divIVA	TCCGTGAGCGTTTACGTATG	CGCAAGTTCTGTCGCATCTA
murZ	GCGATTTATTTACGGGCAGA	TTGCACGAACAGCTGCTAAC
fliM	GTGTGACATCACCGAACGAC	TCTTCCACCGAGAAAAATGG
motB	TTCATGGAAGCGATCATTCA	CGCACCATGATTTCTACACG
mptAB	ACGTTTCGGCAATACCAAAG	ACAGAGTGAGCCATGGAACC
murE	TTATGGATCTCCGACCCAAG	ACGATACATCGTGCCAATCA
pdp	AGTGAAAATCGCACCGAAAG	GCAATAATTCCGAGGCATGT
agrB	CTCCGGCAGACACAGAAAGT	TGCGAATGGTATTAGCAACG
bglK	CCGAAAAATGGCTTGGTAAA	CCGCGAACTAATTCACCATT
cheA	CGACGTTCCGAATTGAGATT	CGTGAACGTGTTGAATGTCC
celC	TTGCAGCTATTCGTGACACC	CATACCAGATCCGCCGTAGT
dacB2	ACCAAAAAGCGAGAGAAGCA	GGCATTTTCTTGCAACCATT
celA	ATTTCGAGGAAGCAGAAGCA	TTCCACTTTTTCACCGGAAG
celB	TGGATATCGTGATGCTTGGA	CGGCTTTCCCGTTTAACATA

TABLE 2 Primers used in qRT-PCR for confirmation of the downregulation of proteins

Gene name	Primer sequence (5′–3′)	
	Forward	Reverse
alaS	GGTAGCTGCTGGAAACGAAG	TTTCGCTCTCGATTGTTCCT
adhE	CAAGCTGGCTTCAAAGTTCC	ACGATCGAATGCTTCTGCTT
pflA	CCCACTTAACCCAGAAGCAG	TACCACCGTTGATAGCACCA
pnpA	TATTCACTCGTGGCCAAACA	GCCAGTTTCCCCAACACTAA
pykA	AAGTGCTGCAGTTGTTGTGG	TTGCATCTTTAGCACCAACG
gnd	GGAAGAGAATGCGGACAAAA	ATCTGTAGCATCGCCAGCTT
fri	GGCGAACAAATGGATGAAGT	GCTTCTTCTACGCTCGCATT
rpsK	TACTCGTAAACGCCGTGTGA	TAGGGAACCTGCACTTGACC
rpsH	AGTAAGCCAGGTTTGCGTGT	TTTAGCACGGGCTTCTTTGT
rplU	TGAAACAGGTGGGAAACAAA	TAGCGGAATCTCCACCTACG
rplL	AATTTGGCGTAACTGCTGCT	ACCAGTGATTTCACGAACCA
hup	GCAGCGAAAGCAGTAGAAGC	GTACGAGGGTTACGGCCTTT
rpsT	TTGATGAAGCAGCTGCAAAC	CGAGCAGCATTGTTTTTGTG
csp	GAAAAAGGCTTCGGTTTCATC	ATTCAACGCTTTGACCTTCG

/protocols]) and labeled with Cy3 monoreactive dye (GE Healthcare Life Sciences, Ontario, Canada). Hybridization of the labeled cDNA probes using the NimbleGen hybridization station and washing were carried out according to the NimbleGen protocol. Microarray raw data were preprocessed and normalized by NimbleScan (version 2.6; Roche NimbleGen).

GeneSifter software (trial version; Geospiza, Inc.) (16) was used for the analysis of normalized results. The statistical analyses were the averages from three independent samples (CCLA-treated *L. monocytogenes* 08-5923 versus untreated) with the cutoff being a 2-fold change in expression level and P values of <0.05 to determine differentially expressed (DE) genes. Genes that showed 2-fold or greater changes in expression levels were selected for subsequent qRT-PCR.

Total cytosoluble protein identification. *L. monocytogenes* 08-5923 was grown in TSB or in TSB with CCLA at 21°C for 4 h. Cells were pelleted by centrifugation at 10,600 × *g* for 1 min and washed once with 1× SigmaFAST protease inhibitor cocktail tablet (Sigma-Aldrich Ltd., Ontario, Canada) reconstituted in double-distilled water (ddH_2O). Cell pellets were resuspended in the fresh cocktail, mixed 1:1 (vol/vol) with 0.1-mm Zirconia-silica beads (BioSpec Inc., OK), and lysed by bead beating for 45 s a total of 3 times. The cell envelope was removed by centrifugation at 10,600 × *g* for 2 min. The concentration of the total soluble protein was determined by Bio-Rad protein assay by following the manufacturer's instructions (Bio-Rad Laboratories Ltd., Ontario, Canada).

The total soluble proteins were separated by SDS-PAGE with 16% polyacrylamide (17). Five μg of proteins from the untreated and treated *Listeria* samples were loaded onto each lane and subjected to electrophoresis at 130 V for 80 min. The gel was fixed (45 min in a solution containing 50% methanol and 10% acetic acid), and protein bands were visualized by EZBlue gel staining (Sigma-Aldrich Ltd.). The density of protein bands was measured by AlphaEase FluorChem densitometry (Alpha Innotech; Fisher Scientific) to identify differentially expressed proteins.

The differentially expressed proteins in treated and untreated *L. monocytogenes* 08-5923 samples were analyzed by the Mass Spectrometry Facility, Department of Chemistry, University of Alberta. Protein bands that were differentially expressed were excised from the SDS-PAGE gel, digested in gel by trypsin (Sigma-Aldrich Ltd.), and analyzed by ultraperformance liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer (UPLC-ESI-Q-TOF-MS) (Q-TOF Premier; Waters, MA). Proteins were identified from mass spectrometry data using the Mascot search engine (Matrix Science).

qRT-PCR and data analysis. qRT-PCR experiments were performed to validate the differential expression of genes at different times of exposure to CCLA. The experimental design included three biological replicates and two technical replicates within each biological replicate. Genes that were ≥2-fold up- or downregulated ($P < 0.05$) from the microarray experiments, as well as genes encoding the differentially expressed proteins, were quantified using SYBR green (Invitrogen, Ontario, Canada). Primers (Tables 1 and 2) were designed (using Primer3 [http://frodo.wi.mit.edu/primer3/]) and tested for specificity with genomic DNA prior to the analysis. Total RNA was isolated and reverse transcribed into cDNA using SuperScript III reverse transcriptase (Promega, WI) according to the manufacturer's protocol. Quantifications of the transcripts were carried out (7500 Fast real-time PCR system; Applied Biosystems, CA) using a QuantiFast SYBR green PCR kit (Qiagen Inc.). Relative quantification values were obtained using a comparative threshold cycle method ($\Delta\Delta C_T$) (18). The housekeeping gene *rpoB* was selected as the reference. The C_T slope method was used to validate the internal control (*rpoB*). *rpoB* was amplified from 10-fold serial dilutions of genomic DNA prepared from *L. monocytogenes*. The C_T slope was linear, and the qRT-PCR efficiency of *rpoB* was 100.9%. A one-tailed unpaired *t* test was performed for determination of the significance ($P < 0.05$) in differential expression of genes.

Cell morphology examination. Culture of *L. monocytogenes* 08-5923 was fixed with 10% (vol/vol) formaldehyde and pelleted by centrifugation at 11,000 × *g* for 15 s. The cell pellet was resuspended in 0.1 M phosphate buffer containing 2.5% glutaraldehyde and 2% paraformaldehyde,

TABLE 3 Identification of differentially expressed genes in CCLA-treated *L. monocytogenes* 08-5923[a]

Gene name and function	Gene annotation	Product	Fold change
Cell division			
clpE	*lmo0997*	ATP-dependent protease	+3.69
ftsW	*lmo1071*	Cell division protein FtsW	−2.14
divIVA	*lmo2020*	Cell division initiation protein	−2.01
rodA	*lmo2428*	Similar to cell division protein RodA	−2.64
ftsX	*lmo2506*	Cell division transport system permease protein	−2.08
ftsE	*lmo2507*	Cell division transport system ATP-binding protein	−2.32
mbl	*lmo2525*	Rod shape-determining protein MreB	−2.03
Cell wall synthesis			
pdp	*lmo0835*	Peptidoglycan binding protein	−2.21
murE	*lmo2038*	UDP-N-acetylmuramoylalanyl-D-glutamate-2,6-diaminopimelate ligase	−2.02
murZ	*lmo2552*	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	−2.41
dacB2	*lmo1855*	D-Alanyl-D-alanine carboxypeptidase	−2.41
Membrane functions			
abc	*lmo0767*	ABC transporter, permease protein	+2.13
mfs	*lmo1250*	Major facilitator superfamily	+2.05
ywzB	*lmo2527*	Similar to *B. subtilis* YwzB protein	+2.37
mptAB	*lmo0096*	PTS system, mannose-specific IIA and IIB component	+2.14
mptC	*lmo0097*	PTS system, mannose-specific IIC component	−2.29
celA	*lmo2765*	PTS system, cellobiose-specific IIA component	+2.35
celB	*lmo2762*	PTS system, cellobiose-specific IIB component	+2.10
celC	*lmo2763*	PTS system, cellobiose-specific IIC component	+2.81
Gene regulation			
deoR	*lmo2107*	Similar to *deoR* family transcriptional regulator	+2.28
hrcA	*lmo1475*	Heat-inducible transcription repressor	+2.43
agrB	*lmo0048*	Similar to *Staphylococcus* two-component sensor histidine kinase AgrB	+3.18
Nucleotide and protein synthesis			
fmt	*lmo1823*	Methionyl-tRNA formyltransferase	−2.07
hisC	*lmo1925*	Histidinol-phosphate aminotransferase	−2.26
gidA	*lmo2810*	tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA	−2.61
Metabolism			
tgl	*lmo0717*	Transglycosylase	−2.01
baiE	*lmo0754*	Bile acid 7-alpha dehydratase	−2.74
trn	*lmo1903*	Thioredoxin	−2.21
yitT	*lmo1909*	YitT family protein	−2.05
malP	*lmo2121*	Maltose phosphorylase	+2.01
bglK	*lmo2764*	Beta-glucoside kinase	+2.85
Motility			
flgD	*lmo0696*	Flagellar basal body rod modification protein	−2.70
fliF	*lmo0713*	Flagellar MS-ring protein	−2.20
motB	*lmo0686*	Chemotaxis protein	−2.23
fliM	*lmo0699*	Flagellar motor switch protein	−2.26
cheA	*lmo0692*	Two-component sensor histidine kinase	−2.82
Unknown			
lmo0867	*lmo0867*	Hypothetical protein	−2.39
lmo1796	*lmo1796*	Hypothetical protein	−2.33
lmo2311	*lmo2311*	Hypothetical protein	+2.25
lmo2317	*lmo2317*	Hypothetical protein	+2.07

[a] Identification of differentially expressed genes in CCLA-treated (4 h) *L. monocytogenes* 08-5923 is represented as fold change ($n = 3$; $P < 0.05$). +, upregulation; −, downregulation.

stained with 2% sodium phosphotungstate (PTA), and examined using a transmission electron microscope (TEM; Philips Morgagni 268; FEI, OR) operating at 80 kV.

Microarray data accession number. The complete microarray data set generated in this study is deposited for public access in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE56558.

RESULTS

Functional classification of DE genes. cDNA microarray experiments were performed to compare the expression levels of genes in untreated and treated (CCLA for 4 h) *L. monocytogenes*. Global differential gene expression changes in response to CCLA were determined using a high-density NimbleGen microarray for *L.*

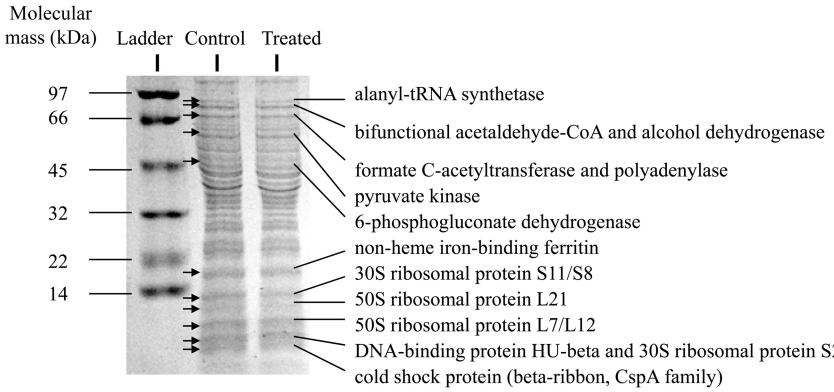

FIG 1 Differential production of proteins in *L. monocytogenes* 08-5923; treated (CCLA for 4 h) and control samples are shown on an SDS-PAGE gel. Proteins expressed differentially are indicated by arrows. CoA, coenzyme A.

monocytogenes of 2,857 genes. Forty genes were found to be differentially expressed with a minimum threshold of a 2-fold difference ($P < 0.05$) (Table 3). These genes were grouped into several functional categories, including cell division, cell wall synthesis, motility, transport, and translation (Table 3).

Differential expression of total soluble proteins. To gain further insight, the protein expression patterns of whole-cell lysate from *L. monocytogenes* with and without exposure to CCLA (4 h) were compared using SDS-PAGE. Out of 11 protein bands that were visibly different in density (Fig. 1) between the lysate from the CCLA-treated and untreated cells, there were 14 proteins identified by UPLC-ESI-Q-TOF-MS. The proteins were involved in glycolysis (PykA), pyruvate metabolism (AdhE and PflA), stress response (Csp protein), DNA replication (Hup), translation (Rps and Rpl proteins), and purine/pyrimidine biosynthesis (PnpA) (Table 4). The downregulation of PykA in treated *L. monocytogenes* confirmed the observed downregulation of the gene encoding this protein as shown in Fig. 2G.

Confirmation of DE genes in *L. monocytogenes*. To validate results from microarray experiments and to investigate the gene expression changes over time, a total of 54 genes (40 DE genes from microarray experiments and 14 DE genes from SDS-PAGE) (Fig. 2) were selected for qRT-PCR, and gene expression levels were quantified following treatment with CCLA after 15 min (lag phase), 4 h (log phase), and 30 h (early stationary phase). For a number of the genes, expression was significantly ($P < 0.05$) upregulated after 15 min of exposure to CCLA and downregulated after 4 h and 30 h.

The upregulation of genes encoding proteins involved in metabolism and membrane functions was observed upon 15 min of exposure to a sublethal dose of CCLA. Genes encoding proteins involved in metabolism, including bile acid 7-alpha dehydratase (BaiE), thioredoxin (Trn), and pyruvate kinase (PykA), were significantly upregulated ($P < 0.05$). The genes involved in cell wall synthesis (*murZ* and *dacB2*) were upregulated ($P < 0.05$). Genes involved in cell division, including *clpE* and *divIVA*, were upregulated, while *malP* was downregulated in cells exposed to CCLA for 15 min. After 4 h of exposure to CCLA, genes involved in cell division (*clpE*, *ftsW*, *divIVA*, *rodA*, *ftsX*, and *ftsE*), cell wall synthesis (*mbl*, *murE*, and *murZ*), flagellar synthesis (*flgD*, *motB*, and *cheA*), and metabolism (*pykA* and *gnd*) were significantly downregulated. Upregulation of *lmo2311*, which is regulated by σ^{54} (19), was observed. After the cells were exposed to CCLA for 30 h, more genes were downregulated compared to those after 4 h of exposure. These genes were involved in cell division, cell wall synthesis, membrane function, transcription regulation, metabolism,

TABLE 4 Identification of differentially expressed proteins by MS

Protein name	Product	Accession no.	Calculated pI	Nominal molecular mass (Da)	Differential expression
AlaS	Alanyl-tRNA synthetase	YP_003416804	5.13	92,938	Down
AdhE	Bifunctional acetaldehyde-coenzyme A/alcohol dehydrogenase	YP_003416936	6.48	95,135	Down
PflA	Formate C-acetyltransferase	CAK20840	5.41	83,950	Down
PnpA	Polyadenylase	YP_003416626	5.23	79,780	Down
PykA	Pyruvate kinase	YP_003416871	5.39	62,673	Down
Gnd	6-Phosphogluconate dehydrogenase	YP_003416672	5.11	52,497	Down
Fri	Nonheme iron-binding ferritin	YP_003416184	4.86	18,036	Down
RpsK	30S ribosomal protein S11	YP_003415461	11.40	13,834	Down
RpsH	30S ribosomal protein S8	YP_003415450	9.48	14,635	Down
RplU	50S ribosomal protein L21	YP_003416842	9.57	11,207	Down
RplL	50S ribosomal protein L7/L12	YP_003415499	4.54	12,462	Down
Hup	DNA-binding protein HU-beta	YP_003417289	9.65	9,876	Down
RpsT	30S ribosomal protein S20	YP_003416777	10.70	9,163	Down
Csp	Cold shock protein, CspA family	ADB71297	4.45	7,261	Down

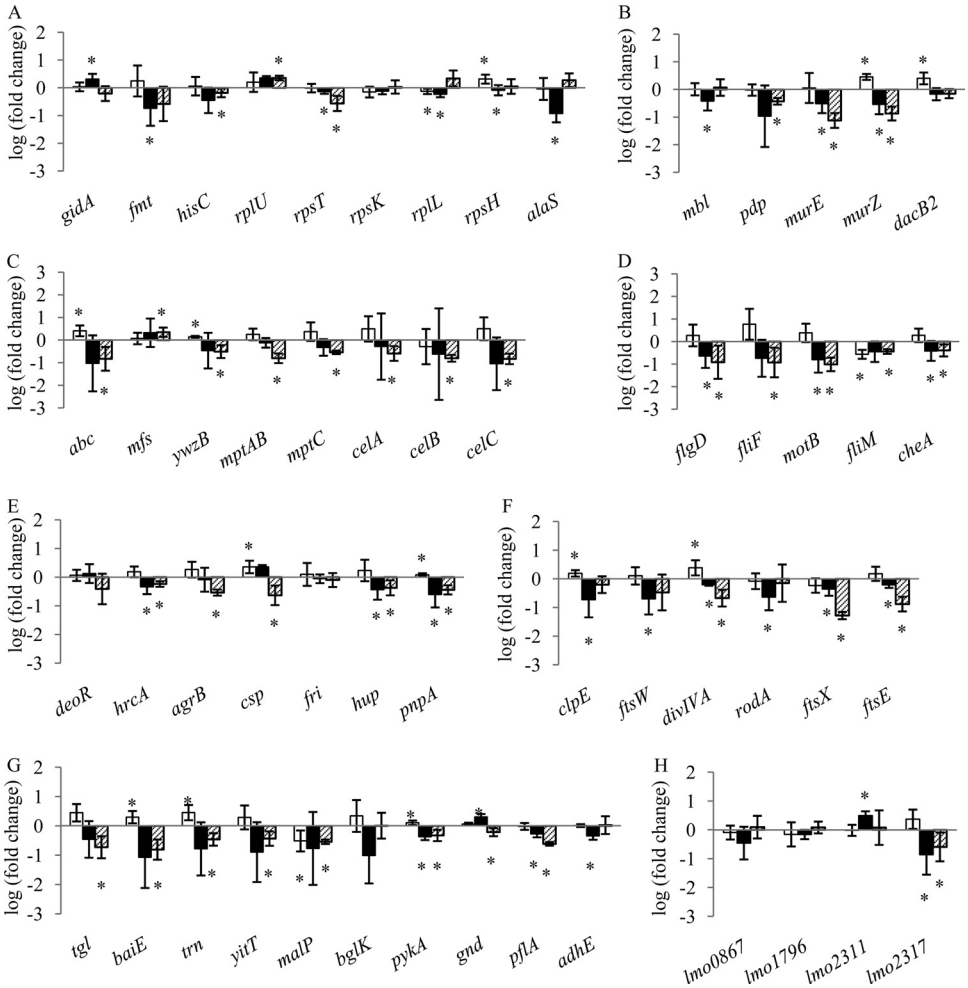

FIG 2 qRT-PCR measurement of DE genes in *L. monocytogenes* 08-5923 exposed to CCLA for 15 min (□), 4 h (■), and 30 h (▨). Untreated samples were used as a reference. Genes were grouped according to their biological function. (A) Nucleotide/protein synthesis; (B) cell wall synthesis; (C) membrane functions; (D) motility; (E) transcription regulation; (F) cell division; (G) metabolism; (H) unknown functions. Note the different *y* axes for panels C and D. Data are expressed as log values (fold change). Means ± standard deviations ($n = 3$) are given. An asterisk indicates genes significantly up- or downregulated at $P < 0.05$.

and motility (Fig. 2). Both the mannose-specific PTS genes (*mpt* genes) and the cellobiose-specific PTS genes, such as *celA*, *celB*, and *celC*, were downregulated ($P < 0.05$).

Cell morphology. The morphologies of untreated and treated (CCLA for 4 h or 30 h) *L. monocytogenes* 08-5923 samples were compared using TEM to examine whether exposure to CCLA could result in visible damage to the cell structure. No morphological changes were observed after 4 h of treatment of *L. monocytogenes* 08-5923 with CCLA (Fig. 3). However, after 30 h the cells had no flagella attached to the cell surface (Fig. 3). This corresponds to the downregulation of motility genes observed previously.

DISCUSSION

Commercial preservatives that are used to control the growth of *L. monocytogenes* in RTE meats include Micocin, which contains carnocyclin A along with two class IIa bacteriocins, carnocyclin BM1 and piscicolin 126. Although the stress responses of *L. monocytogenes* to class IIa bacteriocins have been characterized, the response to class IIc cyclic bacteriocins has not been studied to date. Our study described the cellular alteration and adaptation of *L. monocytogenes* when exposed to a sublethal dose of a class IIc bacteriocin, which provides insight into the general stress response and resistance mechanisms to bacteriocins.

Treatment of *L. monocytogenes* 08-5923 with CCLA resulted in

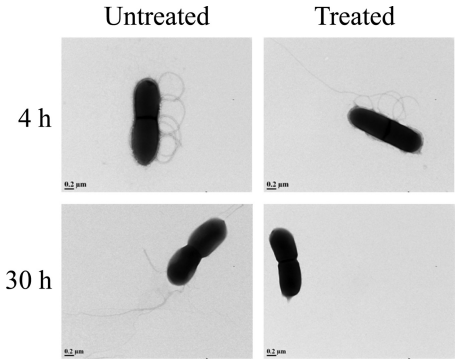

FIG 3 Transmission electron micrographs of cells of *L. monocytogenes* 08-5923 in the presence (treated, 4 h and 30 h) and absence (untreated) of CCLA (28,000× magnification).

differential expression of genes that are known to be involved in cell division, cell wall synthesis, membrane function, transcriptional regulation, nucleotide and protein synthesis, metabolism, and cell motility. The PTS function, cell wall synthesis, motility, and cell division genes were affected the most. The downregulation of these genes was not because of inhibition of cell growth, as at each time point the OD_{600} values of treated and untreated cells were comparable. No filamentation of cells was observed. This indicates that the downregulation of these genes was due to the adaptation to CCLA.

The upregulation of σ^{54}-regulated *mptAB* and *cel* detected by cDNA microarray after 4 h of exposure to CCLA indicates an immediate adaptive response of *Listeria* cells, as the mannose-specific (7) and cellobiose-specific PTS systems are receptors for bacteriocins (20), and the upregulation of mannose-specific PTS systems has been observed in bacteriocin-sensitive cells (21, 22). Gong et al. (3) demonstrated that CCLA forms ion-selective channels in lipid bilayers, which indicates that CCLA interaction with membranes is not receptor mediated. However, our microarray results suggested that both the mannose-specific and cellobiose-specific PTS systems are receptors for CCLA. The downregulation of the mannose-specific and cellobiose-specific PTS systems observed by qRT-PCR analysis after 30 h of exposure to CCLA demonstrates that *Listeria* limits the expression of genes to minimize the production of the PTS targets to CCLA. This downregulation of the PTS targets could play a role in adaptation to CCLA, as these targets have been suggested to play a role in resistance to class II bacteriocins (6, 23, 24).

The expression of genes involved in cell wall synthesis (*mur*) followed the same pattern as that of the PTS systems after 30 h of exposure to CCLA. Although the function of Mur proteins in the antimicrobial stress response of *Listeria* has not been described in the literature, Mur is the target of antibiotics in *Staphylococcus aureus* (25).

Absence of flagella in *Listeria* cells exposed to CCLA for 30 h could be a result of inhibition of flagellar synthesis, because the basal body of the flagella may not develop properly as genes encoding various components of flagella, including the MS ring (*fliF*), motor switch (*fliM*), motor rotation (*motB*), and rod synthesis (*flgD*), were downregulated. In addition, *cheA*, a chemotaxis gene responsible for virulence (26), was downregulated. Flagellar motility is also important for biofilm formation of *L. monocytogenes* (27). Others have found that stress from antimicrobials can inhibit the synthesis of flagella in *E. coli* (28), but the impact of bacteriocins on flagellar synthesis in *L. monocytogenes* has not been described in the literature. Lack of flagella can inhibit adhesion of *Listeria* (29) and may affect its ability to form biofilms.

The cell division gene *ftsE* was upregulated when *L. monocytogenes* was exposed to CCLA for 15 min. High pressure for 15 min also upregulates *ftsE* in *Listeria* (30). After 4 h of exposure to CCLA, *ftsE* was downregulated, along with *ftsX* and *ftsW*. Gene *ftsE* in *L. monocytogenes* is regulated by σ^B (31), which is a general stress response regulator. Various stresses, such as liquid smoke (32), cold (33), antibiotic (31, 34), and heat (35), also downregulate *fts* genes in *Listeria*. The up- or downregulation of *fts* genes may depend on the type of stress and time of exposure.

In summary, a sublethal dose of carnocyclin A targeted the PTS systems of *Listeria* and cells began to repair injury by upregulation of genes involved in cell wall synthesis. After 4 h, cells gradually became desensitized to CCLA and started to decrease activities such as cell division, cell wall synthesis, and motility. After 30 h, cells adapted to CCLA through a reduction of several metabolic processes and motility functions. Adaptation to bacteriocins could affect the sensitivity to antimicrobials that are used to control the growth of *L. monocytogenes*.

ACKNOWLEDGMENTS

This project was supported by funding from the Alberta Livestock and Meat Agency and Alberta Innovates BioSolutions.

We thank Jing Zheng of the Mass Spectrometry Facility, Department of Chemistry, University of Alberta, for mass spectrometry analysis and Arlene Oatway of the Advanced Microscopy Facility, Department of Biological Sciences, for technical support in TEM analysis.

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