

# Salt Stress Phenotypes in *Listeria monocytogenes* Vary by Genetic Lineage and Temperature

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## Abstract

*Listeria monocytogenes* can survive and grow under wide-ranging environmental stress conditions encountered both in foods and in the host. The ability of certain *L. monocytogenes* subtypes to thrive under stress conditions present in specific niches was hypothesized to reflect genetic characteristics and phenotypic capabilities conserved among strains within a subtype. To quantify variations in salt stress phenotypes among 40 strains selected to represent the diversity of the three major *L. monocytogenes* genetic lineages and to determine if salt stress phenotypes were associated with genetic relatedness, we measured growth under salt stress at both 7°C and 37°C. At 7°C, in brain–heart infusion with 6% NaCl, average growth rates among the lineages were similar. A comparison of doubling times after exposure to salt stress at 7°C or 37°C indicated that growth at 7°C provided crossprotection to subsequent salt stress for strains in lineages I and II. At 37°C, in brain–heart infusion with 6% NaCl, lineage I and III strains grew significantly faster ( $p < 0.0001$ ) than lineage II strains. Under salt stress at 37°C, differences in growth parameters were significantly ( $p < 0.005$ ) associated with genetic relatedness of the strains. Compatible solute uptake is part of the *L. monocytogenes* salt stress response, but growth differences between the lineages were not related to differences in transcript levels of osmolyte transporter-encoding genes *betL*, *gbuA*, *oppA*, and *opuCA*. The combination of phylogenetic and phenotypic data suggests that *L. monocytogenes* lineage I and III strains, which are most commonly associated with human and animal disease, may be better adapted to osmotic stress at 37°C, conditions that are present in the host gastrointestinal tract.

## Introduction

THE OPPORTUNISTIC FOODBORNE PATHOGEN *Listeria monocytogenes* is a food safety and public health concern, as it causes life-threatening infections in animals and at-risk human populations, including pregnant women, neonates, and the elderly. *L. monocytogenes* is commonly present in farm and food-processing environments, and is resistant to diverse environmental conditions, including low pH, high salt, and low temperatures (Swaminathan *et al.*, 2007). The ability to survive and/or proliferate under these stresses contributes to persistence of *L. monocytogenes* in both foods and food-processing environments, elevating the risk of transmission of this pathogen through foods to animal and human hosts.

Certain subtypes of *L. monocytogenes* are significantly associated with isolation from specific environmental niches (Gray *et al.*, 2004). Molecular subtyping methods have differentiated *L. monocytogenes* isolates into three genetic lineages (Rasmussen *et al.*, 1995; Wiedmann *et al.*, 1997): lineage I isolates are commonly associated with sporadic and epidemic cases of human listeriosis, lineage II isolates are most commonly isolated from foods and the environment, and lineage III isolates are typically associated with cases of animal listeriosis (Gray *et al.*, 2004; Ward *et al.*, 2004; Roberts *et al.*, 2006; Sauders *et al.*, 2006). Epidemics of invasive listeriosis in North America predominantly have been caused by lineage I strains. In general, lineage I strains are more virulent, based on *in vitro* assay results (Nightingale *et al.*, 2006), and have lower predicted infectious doses (Chen *et al.*, 2006) than lineage II strains. Variation in virulence (Wiedmann *et al.*, 1997) and in growth capabilities under adverse conditions (De Jesus and Whiting, 2003; Lianou *et al.*, 2006; van der Veen *et al.*, 2008) have been observed among different subtypes of *L. monocytogenes*, which may, in part, explain the predominance of some subtypes in human disease.

Osmotic stress is among the adverse conditions that *L. monocytogenes* may experience in foods and in the host gastrointestinal tract. Growth and survival of *L. monocytogenes* under high osmolarity is attributed mainly to its ability to accumulate compatible solutes, particularly glycine betaine (Ko *et al.*, 1994; Smith, 1996; Sleator *et al.*, 1999). Mechanisms that contribute to osmotic stress resistance, such as the glycine-betaine and carnitine compatible solute transporters, also play a role in allowing *L. monocytogenes* to grow at low temperatures (Sleator *et al.*, 2003b). In addition to the overlap in response between osmotic stress and cold stress, exposure

of *L. monocytogenes* to osmotic stress at 37°C also increases its resistance to subsequent exposure to bile salts (Begley *et al.*, 2002), and increases expression of virulence genes (Sue *et al.*, 2004). These observations support the hypothesis that osmotic stress encountered in the gastrointestinal tract serves as a signal to facilitate subsequent *L. monocytogenes* infection of the host (Sleator *et al.*, 2007).

We hypothesized that differences in the ability to adapt to osmotic stress may contribute to differences in survival and transmission of *L. monocytogenes*. For example, subtypes that are commonly isolated from foods, such as lineage II strains, may have a relative fitness advantage under cold and osmotic stress, whereas subtypes commonly isolated from clinical cases, such as lineage I and III strains, may have a survival advantage under conditions present in the gastrointestinal tract. Variations in osmotic stress phenotypes at different growth temperatures have not been quantified previously; it is unclear whether osmotic stress phenotypes vary with respect to the genetic background of the strain. Therefore, to identify specific characteristics that may allow niche adaptations among the three genetic lineages of *L. monocytogenes*, we selected a diverse set of 40 strains and measured the growth parameters of these strains in a broth medium at either 7°C or 37°C, with and without the addition of 6% NaCl. We combined growth and gene expression data with multilocus sequence typing (MLST) data to identify associations between phenotype and genetic relatedness of the strains. We observed significant interlineage differences in growth parameters after transfer to a high-salt medium at 37°C. Further, these differences in growth parameters are significantly associated with genetic relatedness among the strains, indicating that the ability to adapt to salt stress at host body temperatures is linked to the strains' evolutionary history.

## Materials and Methods

### *L. monocytogenes* isolates and growth conditions

The 40 strains that were selected to represent the genetic diversity of *L. monocytogenes* (Table 1) were stored at -80°C in brain-heart infusion (BHI) (Becton, Dickinson, and Company, Sparks, MD) broth with 15% glycerol. In preparation for the experiments, isolates were streaked to BHI agar from frozen stocks and incubated for 24 h at 37°C. A single colony was transferred to 5 mL BHI, followed by incubation at 37°C, with shaking at 230 rpm, for 20 h. For measurement of strain growth parameters under salt stress, isolates were grown at either 37°C or 7°C in BHI broth with and without 6% NaCl. Specifically, 750 µL of the 20 h culture was either (i) transferred to 75 mL BHI broth at 37°C, followed by incubation at 37°C for 3 h, without shaking, or (ii) transferred to 75 mL BHI broth at 7°C, followed by incubation at 7°C for 50 h, without shaking. Both culture conditions yielded midexponential-phase cultures. After growth to midexponential phase, cultures in BHI at 37°C were transferred 1:1000 into either (i) 75 mL BHI broth or (ii) 75 mL BHI + 6% NaCl and incubated at 37°C, without shaking. After growth to midexponential phase, cultures in BHI broth at 7°C were transferred 1:1000 into either (i) 75 mL 7°C BHI broth or (ii) 75 mL 7°C BHI broth + 6% NaCl and incubated at 7°C, without shaking. Cultures at 37°C were sampled every 90 min for 12 h (BHI) or 27 h (BHI + 6% NaCl) and cultures at 7°C were sampled every day for 7–9 days (BHI) or 14–16 days (BHI + 6% NaCl).

Samples were diluted in phosphate-buffered saline (pH 7.4) and plated onto BHI agar with an Autoplate 4000 (Spiral Biotech, Bethesda, MD); plates were incubated at 37°C for 24 h before enumerating colonies with a Q-Count (Spiral Biotech). Growth was monitored for two independent replicates of each strain in BHI and BHI + 6% NaCl at both temperatures. A level of 6% NaCl was selected to represent the water-phase salt content that *L. monocytogenes* experiences in foods, particularly those foods that are considered to be of high or moderate risk per serving for listeriosis, including deli meat, smoked seafood, and soft unripened cheeses (FDA, 2003). The water-phase salt content of these foods ranges from 2% to 8% for smoked seafood (Hwang *et al.*, 2009) and from 2.5% to 6.6% for soft cheeses (Ryser, 2007).

### Growth parameter data analysis

Growth parameters for each strain in BHI and BHI + 6% NaCl at 7°C or 37°C were estimated using the Baranyi model (Baranyi and Roberts, 1994) implemented in the NLStools v. 0.0-4 package in R v. 2.5.1. CFU/mL values for each strain at every time point were log-transformed and used to estimate the lag phase duration and maximum specific growth rate. The Shapiro-Wilk test for normality was used to determine if growth rates or lag phase durations for each growth temperature and NaCl combination fit a normal distribution. When the data were not normally distributed, significant differences between lineages were determined using the Kruskal-Wallis test in SAS v. 9.1 (SAS Institute, Cary, NC). For normally distributed data sets, analysis of variance was implemented using the mixed procedure in SAS with a linear model that included lineage, replicate, and date of assay, with date as a random effect and lineage and replicate as fixed effects. The Tukey multiple correction procedure was applied to all analysis of variance results. Adjusted *p*-values of <0.05 were considered significant. To determine differences in long-term adaptation to salt stress over temperature, a doubling time ratio was calculated from the doubling time in BHI without additional salt and the doubling time in BHI + 6% NaCl. A similar calculation to determine differences in the short-term response to salt stress by temperature could not be done, as there was no measurable lag phase duration in cultures grown in BHI without additional salt. Growth parameters for each strain under each growth condition are available at <http://foodscience.cornell.edu/cals/foodsci/research/labs/wiedmann/links/bergholz2010.cfm>.

### MLST and data analysis

All strains in this study were phylogenetically characterized using a 10-gene MLST scheme, which includes sequences from *ldh*, *prs*, *sigB*, *polC*, *rarA*, *pbpA*, *addB*, *lmo0490*, *lmo1555*, and *lmo2763* (den Bakker *et al.*, accepted). A species-level phylogeny was constructed using ClonalFrame version 1.1 (Didelot and Falush, 2007), using five independent runs of 200,000 pre-burn-in and 200,000 post-burn-in iterations. The convergence of the Markov Chain Monte Carlo simulations in the different runs was judged satisfactory based on the Gelman-Rubin test (Gelman and Rubin, 1992) as implemented in the ClonalFrame GUI. The maximum clade credibility tree and the posterior probabilities of the individual clades found in this tree were calculated using TreeAnnotator (<http://tree.bio.ed.ac.uk/software/>). A 95% majority consensus tree was

TABLE 1. *LISTERIA MONOCYTOGENES* STRAINS USED IN THIS STUDY

FSL designation	Serotype	Ribotype	Source	Reference
<i>Lineage I strains</i>				
F6-366	4b	1044A	Human, 1998–99 hot dog outbreak	Nelson <i>et al.</i> (2004)
J1-049	3c	1042C	Human, sporadic	Fugett <i>et al.</i> (2006)
J1-108	4b	1038B	Human, 1981 coleslaw outbreak	Fugett <i>et al.</i> (2006)
J1-110	4b	1038B	Food, 1985 cheese outbreak	Fugett <i>et al.</i> (2006)
J1-116	4b	1042B	Food, 1989 pate outbreak	Fugett <i>et al.</i> (2006)
J1-129	4ab	1042	Human, 1989 pate outbreak	Gilbert <i>et al.</i> (1993)
J1-169	3b	1052A	Human, sporadic	Fugett <i>et al.</i> (2006)
J1-175	1/2b	1042A	Water	
J1-194	1/2b	1042B	Human, sporadic	Sauders <i>et al.</i> (2003)
J1-220	4b	1042	Human, 1979 vegetable outbreak	Ho <i>et al.</i> (1986)
J1-225	4b	1042B	Human, 1983 milk outbreak	Fugett <i>et al.</i> (2006)
J2-064	1/2b	1052A	Animal	Fugett <i>et al.</i> (2006)
N1-017	4b	1042C	Food, brined trout	Norton <i>et al.</i> (2001)
R2-501	4b	1042B	Human, 2000 cheese outbreak	Fugett <i>et al.</i> (2006)
R2-503	1/2b	1051B	Human, 1994 milk outbreak	Fugett <i>et al.</i> (2006)
R2-763	4b	1044A	Human, 2002 turkey deli outbreak	Fugett <i>et al.</i> (2006)
<i>Lineage II strains</i>				
C1-115	3a	1039C	Human, sporadic	Fugett <i>et al.</i> (2006)
F2-032	1/2a	1045B	Food	Sauders <i>et al.</i> (2004)
F2-141	1/2a	1053A	Human, sporadic	Sauders <i>et al.</i> (2004)
F2-237	1/2a	1062D	Food, fish	Sauders <i>et al.</i> (2004)
F2-515	1/2a	1062A	Food, turkey	Sauders <i>et al.</i> (2004)
F2-539	1/2a	1039C	Human, healthy pregnant	Nelson <i>et al.</i> (2004)
G2-003	1/2c	1056A	Human	Vicente <i>et al.</i> (1985)
J1-022	1/2c	1039C	Human, sporadic	Cai <i>et al.</i> (2002)
J1-101	1/2a	1053A	Human, 1989 hotdog outbreak	Fugett <i>et al.</i> (2006)
J1-125	1/2c	1039C	Human, sporadic	Nightingale <i>et al.</i> (2007)
J2-003	1/2a	1039C	Animal	Cai <i>et al.</i> (2002)
J2-054	1/2a	1045B	Animal, sheep	Fugett <i>et al.</i> (2006)
R2-499	1/2a	1053A	Human, 2000 turkey deli outbreak	Fugett <i>et al.</i> (2006)
X1-001	1/2a	1030A	Human, skin lesion	Bishop and Hinrichs (1987)
<i>Lineage III strains</i>				
<i>IIIA</i>				
J1-031	4a	1059A	Human, sporadic	Fugett <i>et al.</i> (2006)
J1-168	4a	18606	Human	Fugett <i>et al.</i> (2006)
J2-071	4c	1061A	Animal	Pohl <i>et al.</i> (2006)
<i>IIIB<sup>a</sup></i>				
J1-158	4b	10142	Animal, goat	Fugett <i>et al.</i> (2006)
J1-208	4a	10142	Animal	Roberts <i>et al.</i> (2006)
W1-111	4c	18036	Unknown	Fugett <i>et al.</i> (2006)
W1-112	4a	1033A	Unknown	Fugett <i>et al.</i> (2006)
<i>IIIC</i>				
F2-208	4a	10148	Human, sporadic	Gray <i>et al.</i> (2004)
F2-270	4a	18007A	Human, sporadic	Gray <i>et al.</i> (2004)
W1-110	4c	1055A	Unknown	Fugett <i>et al.</i> (2006)

<sup>a</sup>Lineage IIIB is also known as lineage IV (Ward *et al.*, 2008).

constructed in the ClonalFrame GUI from the trees from the posterior sampling of the five runs.

To determine if genetic differences were related to phenotypic differences, pairwise distance matrices were created from the phenotypic and MLST gene sequence data. Phenotypic distance matrices were created by calculating the Euclidean distance between all pairs of strains for each phenotypic parameter (growth rate and lag phase duration) for each growth temperature and NaCl combination. The genetic pairwise distance matrix was created in PAUP\* version 4.010b (Wilgenbusch and Swofford, 2003) based on the uncorrected percentage difference between the concatenated nucleotide sequences of the 10 MLST genes between all pairs of strains.

The Mantel test implemented in the vegan package v1.15-1 in R v. 2.5.1 was used to identify significant correlations between each phenotypic distance matrix and the genetic distance matrix. A total of six comparisons were conducted with 1000 Monte Carlo permutations to generate *p*-values and a Bonferroni correction was applied to the *p*-values. A corrected *p*-value of <0.05 was considered significant.

#### Quantitative reverse transcription–polymerase chain reaction

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was used to quantitatively examine relationships

between transcript levels for genes encoding osmolyte uptake transporters and assignment of strain to lineage. A subset of four strains per lineage was selected to represent the average growth rate for each lineage in 37°C BHI + 6% NaCl. Selected strains included lineage I strains F6-366, J1-175, J1-194, and R2-503; lineage II strains F2-515, J1-101, J2-003, and R2-499; and lineage III strains F2-270, J1-031, J1-208, and J2-071. Strains were grown to midlog phase ( $\sim 10^7$  CFU/mL) in BHI + 6% NaCl at 37°C as described above. At midlog phase, 20 mL of culture was mixed with 40 mL of RNA Protect (Qiagen, Valencia, CA) and centrifuged at 10,000 rpm, 4°C, for 20 min. The supernatant was decanted, and cell pellets were stored at  $-80^\circ\text{C}$  overnight before RNA extraction with the RiboPure Bacteria RNA extraction kit (Ambion, Austin, TX). RNA was extracted from three independent cultures of each strain and treated with RiboPure DNase (Ambion) following the manufacturer's protocol. RNA quality was assessed by determining the ratio of absorbance at 260/230 nm on a Nanodrop 1000 (Ambion) and evaluated on a Bioanalyzer (Agilent, Santa Clara, CA). For the set of RNA samples, the 260/230 ranged from 1.98 to 2.45, and the RNA integrity numbers from the Bioanalyzer ranged from 7.9 to 10.0.

TaqMan primers and probes targeting *betL*, *gbuA*, *opuCA*, *oppA*, and *rpoB* were designed to amplify these genes across the diversity of *L. monocytogenes* strains. When available, gene sequences for the strains used in this assay were obtained from GenBank and the *L. monocytogenes* database at the Broad Institute ([www.broad.mit.edu/annotation/genome/listeria\\_group/MultiHome.html](http://www.broad.mit.edu/annotation/genome/listeria_group/MultiHome.html)) and used to design primers with Primer Express v. 1.0 (Applied Biosystems, Foster City, CA) (Table 2). For strains with no available sequence information (F2-270, J1-031, J1208, and J2-003), the coding region of each gene was amplified and sequenced. These sequences are available at [www.pathogentracker.net](http://www.pathogentracker.net). PCR efficiency of the primer and probe sets was determined for each strain using 10-fold serial dilutions of genomic DNA. For a specific primer and probe set to be utilized in this study, amplification of a target gene was required to be >85% efficient for all strains tested.

cDNA was synthesized from 1  $\mu\text{g}$  total RNA using the TaqMan Reverse Transcription kit (Applied Biosystems). Reverse transcription reactions contained 1 $\times$ TaqMan buffer, 5.5 mM magnesium chloride, 500  $\mu\text{M}$  each dNTP, 2.5  $\mu\text{M}$  random hexamers, 4 U RNase inhibitor, and 12.5 U MultiScribe Reverse Transcriptase and were carried out under the following conditions: 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C. Reactions containing all components except reverse transcriptase were prepared for all RNA samples to determine background levels of DNA. Tenfold serial dilutions of cDNA were used as the input for qPCR assays. qPCRs contained 1 $\times$ Universal TaqMan Mastermix (Applied Biosystems), 900 nmol each primer, and 250 nmol TaqMan probe and were run on the ABI Prism 7000 (Applied Biosystems) under the following conditions: 40 cycles at 95°C for 15 sec and 60°C for 1 min.  $C_t$  and reaction efficiencies were determined using ABI SDS v1.0 software. qPCRs were performed in duplicate for each cDNA sample tested.

Relative expression was determined using the method described by Pfaffl (2001), and the  $C_t$  values for *rpoB* were used for normalization within samples. Results from all samples were then compared to the transcript levels of the first replicate of strain with the slowest growth rate, FSL J1-101.

TABLE 2. TAQMAN QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION PRIMERS AND PROBES USED IN THIS STUDY

Target gene	Forward primer (5' $\rightarrow$ 3')	Probe (5' $\rightarrow$ 3')	Reverse primer (5' $\rightarrow$ 3')
<i>rpoB</i>	CCGGAGTCAACGGTAAACA	TTATCTCCCGTATTTTACC	CAGGTGTTCCGTCTGGCATA
<i>betL</i>	GCACCTGATGCCCTGAT	TTGACTCATGGACTATTT	GAAAGCCACCAAGCCCAAT
<i>gbuA</i>	GAAAAGCATGAGTATGGTCTTCCA	AACITGGTTTATTTCCG	TTTGTCCATTCCTTGAATTTCAAG
<i>oppA</i>	GAAGATGC AAAATGGTCAAAACG	CCTGTAACITGCAAATGACTAT	TGCACGACGCCATGAGTAAA
<i>opuCA</i>	ACATCGATAAAGGAGAATTTGTGT	TCGTTTTCCCAACACCA	GCCGGTTAATCATCTTCATTTGT

Comparison to a single replicate (rather than the average of all FSL J1-101 replicates) was required to allow for calculation of average and standard deviation of transcript levels for J1-101 as well as all other strains and to allow for use of amplification efficiency data (which can differ between replicates) in our calculations. The average  $\log_2$  expression and standard deviation from three independent RNA samples are reported for each strain tested.

To determine if genetic differences among strains were related to gene expression differences among strains, the Mantel test was implemented as described above. Genetic distance matrices were created for each of the compatible solute transporter genes based on nucleotide differences in the coding region of each gene. Gene expression distance matrices were created by calculating the Euclidean distance between all pairs of strains for each gene. A  $p$ -value of  $<0.05$  was considered significant.

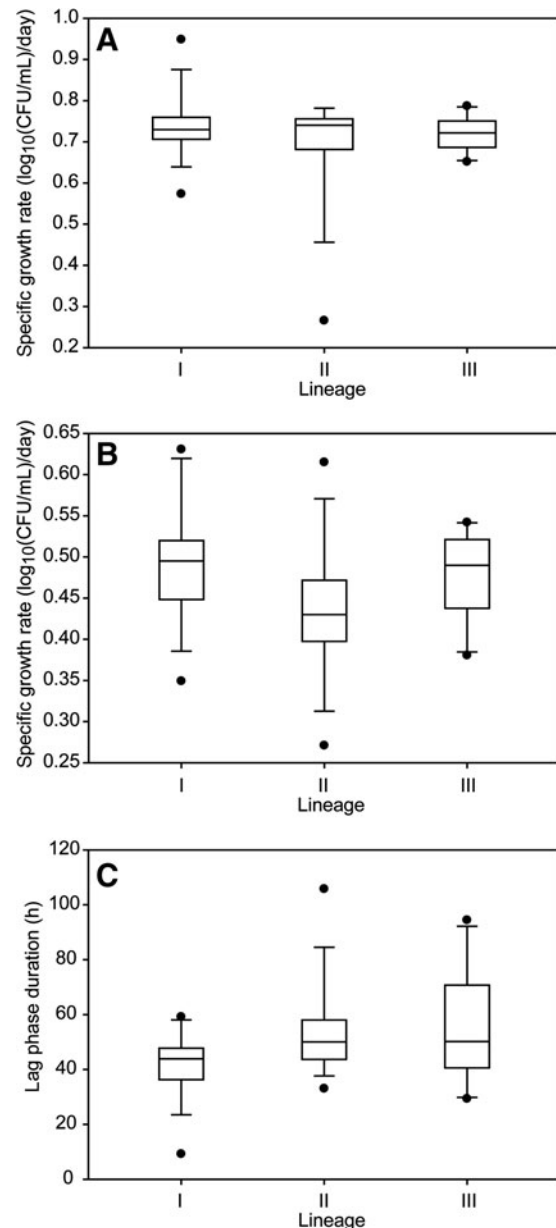
#### Molecular evolution of osmolyte uptake transporter genes

To analyze the molecular evolution of the osmolyte uptake genes, phylogenetic, recombination, and positive selection analyses were performed on *betL*, *opuCA*, *gluA*, and *oppA* sequences of the subset of strains used in the qRT-PCR experiments. Sequences were aligned manually in MacClade 4.08 (<http://macclade.org/macclade.html>) and maximum likelihood trees of the individual genes were inferred using PAUP\* version 4.010b using the nucleotide substitution model as inferred by the likelihood ratio test ( $p < 0.05$ ) as implemented in Modeltest 3.7 (Posada and Crandall, 1998). The presence of intragenic recombination was tested using the Sawyer test implemented in Geneconv software (Sawyer, 1989) with the default settings; the number of recombination events was inferred from the output as previously described (Nightingale *et al.*, 2005). The pairwise homoplasy index statistic as implemented in pairwise homoplasy index-pack software (Brueen *et al.*, 2006) was also used to infer past recombination events. Evidence for positive selection was assessed with PAML 4.1 (Yang, 2007) using the overall test as previously described (Nightingale *et al.*, 2005).

## Results

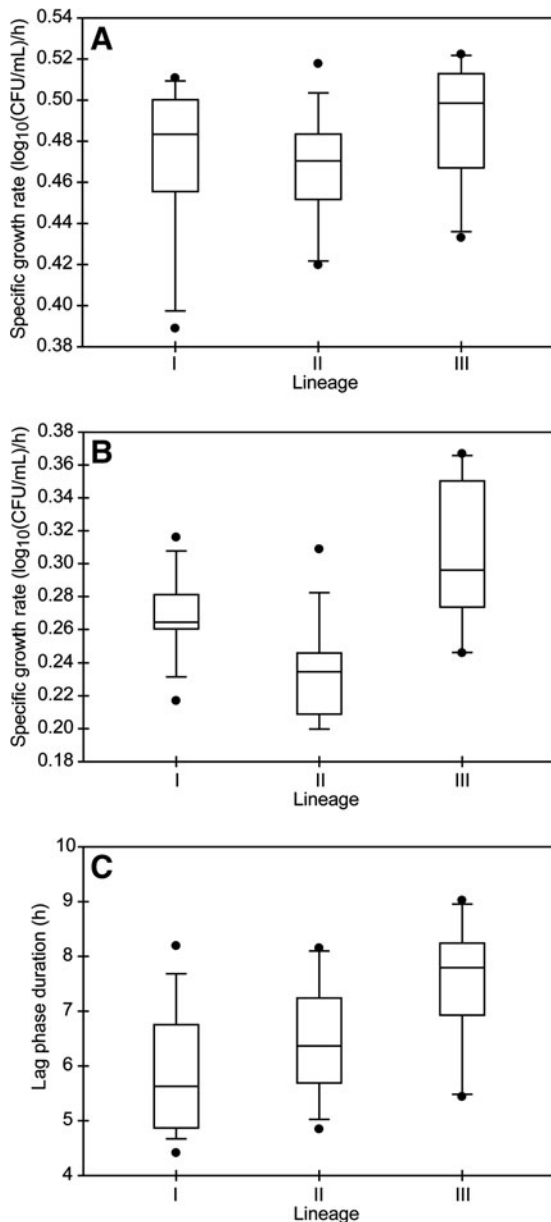
### Growth parameters at 7°C are similar across lineages

Average growth rates in BHI at 7°C were not significantly different ( $p = 0.705$ , Kruskal-Wallis test) across the three genetic lineages of *L. monocytogenes* (Fig. 1A). The average growth rates were  $0.74 \pm 0.11 \log_{10}(\text{CFU/mL})/\text{day}$  for lineage I,  $0.72 \pm 0.12 \log_{10}(\text{CFU/mL})/\text{day}$  for lineage II, and  $0.78 \pm 0.11 \log_{10}(\text{CFU/mL})/\text{day}$  for lineage III strains. While strains grew more slowly in BHI + 6% NaCl at 7°C than in BHI alone, growth rates remained similar across lineages in BHI + 6% NaCl. Significant differences in average growth rate were not observed among the lineages ( $p = 0.06$ ) (Fig. 1B). The average growth rate in BHI + 6% NaCl at 7°C was  $0.49 \pm 0.08 \log_{10}(\text{CFU/mL})/\text{day}$  for lineage I,  $0.44 \pm 0.09 \log_{10}(\text{CFU/mL})/\text{day}$  for lineage II, and  $0.48 \pm 0.06 \log_{10}(\text{CFU/mL})/\text{day}$  for lineage III strains. Strain-to-strain variation within a lineage accounted for 55% of the total variation, which was much greater than the variation between lineages



**FIG. 1.** Boxplots of the distribution of growth parameters for strains representing each genetic lineage of *Listeria monocytogenes* at 7°C. (A) Growth rate in brain–heart infusion (BHI), (B) growth rate in BHI + 6% NaCl, and (C) lag phase duration in BHI + 6% NaCl. The horizontal bar indicates the median for each lineage; I, lineage I; II, lineage II; III, lineage III. Boxes represent the 25th to 75th percentile of the values; whiskers represent the 10th and 90th percentiles. Values outside the 10th to 90th percentiles are represented by filled circles.

(4%). In BHI at 7°C, there was no detectable lag phase, as cultures were transferred to this medium from mid-exponential-phase growth in BHI at 7°C. In BHI + 6% NaCl at 7°C, the average lag phase duration was  $41.4 \pm 14.5$  h for lineage I,  $53.5 \pm 20.4$  h for lineage II, and  $54.4 \pm 22.8$  h for lineage III. The average lag phase duration was not significantly different ( $p = 0.061$ ) among lineages (Fig. 1C).



**FIG. 2.** Boxplots of the distribution of growth parameters for strains representing each genetic lineage of *L. monocytogenes* at 37°C. (A) Growth rate in BHI, (B) growth rate in BHI + 6% NaCl, and (C) lag phase duration in BHI + 6% NaCl. The horizontal bar indicates the median for each lineage; I, lineage I; II, lineage II; III, lineage III. Boxes represent the 25th to 75th percentile of the values; whiskers represent the 10th and 90th percentiles. Values outside the 10th to 90th percentiles are represented by filled circles.

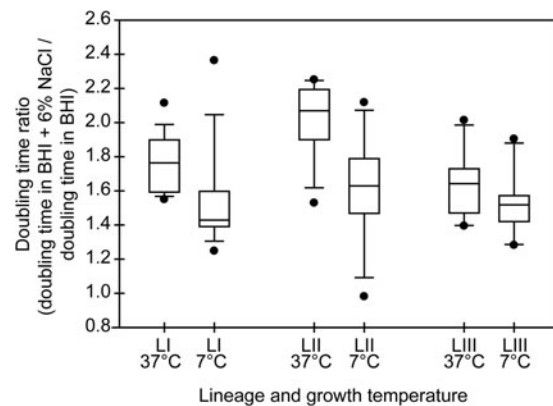
#### Growth parameters at 37°C in BHI + 6% NaCl differ among lineages

While growth rates in BHI at 37°C were similar across lineages, distinct differences in growth parameters among lineages were observed after transfer to BHI + 6% NaCl at 37°C of cultures that had been growth to midexponential growth in BHI at 37°C. The average growth rates in BHI at 37°C were  $0.47 \pm 0.04 \log_{10}(\text{CFU}/\text{mL})/\text{h}$  for lineage I,  $0.46 \pm 0.04 \log_{10}(\text{CFU}/\text{mL})/\text{h}$  for lineage II, and  $0.49 \pm 0.03$

$\log_{10}(\text{CFU}/\text{mL})/\text{h}$  for lineage III strains (Fig. 2A). Growth rates in BHI at 37°C were not normally distributed ( $p = 0.035$ ). The Kruskal–Wallis test detected no significant differences in growth rates among lineages ( $p = 0.093$ ). After transfer to BHI + 6% NaCl, the average growth rate for lineage I strains [ $0.27 \pm 0.03 \log_{10}(\text{CFU}/\text{mL})/\text{h}$ ] was significantly higher (adjusted  $p < 0.0001$ ) than the average growth rate for lineage II strains [ $0.23 \pm 0.03 \log_{10}(\text{CFU}/\text{mL})/\text{h}$ ] and significantly lower (adjusted  $p < 0.001$ ) than the average growth rate for lineage III strains [ $0.30 \pm 0.04 \log_{10}(\text{CFU}/\text{mL})/\text{h}$ ] (Fig. 2B). The average growth rate for lineage II strains was significantly lower (adjusted  $p < 0.0001$ ) than the average growth rate for lineage III strains. Variances in growth rates among the lineages were similar; strain-to-strain variation within a lineage accounted for 43% of the total variance, which was smaller than the variation among lineages (53%). In BHI at 37°C, there was no detectable lag phase, as cultures were transferred to this medium from midexponential-phase growth in BHI at 37°C. Transfer of midexponential-phase cells from BHI at 37°C to BHI + 6% NaCl yielded an average lag phase duration for lineage III strains ( $7.5 \pm 1.1 \text{ h}$ ) that was significantly longer than for lineage I strains ( $5.9 \pm 1.1 \text{ h}$ , adjusted  $p < 0.0001$ ) or for lineage II strains ( $6.5 \pm 1.1 \text{ h}$ , adjusted  $p = 0.001$ ) (Fig. 2C).

#### Growth at 7°C provides preadaptation to salt stress compared to growth at 37°C

We hypothesized that growth of *L. monocytogenes* at 7°C may enhance its subsequent adaptation to salt stress. This hypothesis is supported by previous reports of cold growth induction of expression of genes encoding compatible solute transporters, which are also used to combat osmotic stress (Smith, 1996; Sleator *et al.*, 1999; Bayles and Wilkinson, 2000). To test this hypothesis, we calculated the doubling time ratio, which is the doubling time in BHI + 6% NaCl divided by the doubling time in BHI, for each strain at each growth



**FIG. 3.** Boxplots of the distribution of doubling time ratios at 7°C and 37°C for strains representing each genetic lineage of *L. monocytogenes*. Doubling time ratios for each strain were calculated by dividing the doubling time in BHI + 6% NaCl by the doubling time in BHI. The horizontal bar indicates the median for each lineage; LI, lineage I; LII, lineage II; LIII, lineage III. Boxes represent the 25th to 75th percentile of the values; whiskers represent the 10th and 90th percentiles. Values outside the 10th to 90th percentiles are represented by filled circles.



temperature. If incubation temperature has no role in the long-term adaptation to salt stress, we would expect to see similar changes in doubling time due to the salt shift at both growth temperatures across the lineages. For lineage I strains, the average doubling time ratio at 37°C was  $1.76 \pm 0.18$ , indicating that the average doubling time in BHI + 6% NaCl was 1.76 times greater than the average doubling time in BHI without additional NaCl. The lineage I average doubling time ratio at 37°C was significantly higher ( $p = 0.014$ , Kruskal–Wallis test) than at 7°C, where the doubling time ratio was  $1.54 \pm 0.32$  (Fig. 3). For lineage II strains, the average doubling time ratio at 37°C was  $2.02 \pm 0.22$ , which was significantly higher ( $p = 0.005$ , Kruskal–Wallis test) than at 7°C, where the doubling time ratio was  $1.62 \pm 0.33$ . For lineage III strains, the average doubling time ratio at 37°C was  $1.64 \pm 0.18$  which was similar ( $p = 0.13$ ) to that at 7°C,  $1.52 \pm 0.21$ .

#### Associations between genetic relatedness and salt stress phenotype

The MLST data show that *L. monocytogenes* strains comprise three clades: lineage I, lineage II, and lineage III (Fig. 4). The lineage III clade includes IIIA and IIIC strains, with IIIB strains, which have been designated lineage IV by others (Ward *et al.*, 2008), on a separate branch. The MLST phylogenetic structure for these strains is similar to that described previously for *L. monocytogenes* (den Bakker *et al.*, 2008; Ragon *et al.*, 2008). To utilize all the nucleotide data rather than classifying strains into groups based on branching points, we used pairwise genetic distance as a measure of genetic similarity, which was compared to the pairwise differences for each growth parameter. Using the Mantel test, significant associations between genetic distance and phenotype differences were not found for growth in BHI at 37°C, BHI at 7°C, or BHI + 6% NaCl at 7°C. A significant association between genetic distance and phenotype differences was observed for

growth in BHI + 6% NaCl at 37°C (growth rate  $p < 0.001$ , lag phase duration  $p = 0.005$ ). These results indicate that strains that are closely related are more likely to have similar growth parameters in BHI + 6% NaCl at 37°C (Fig. 4) than strains that are genetically distant.

#### Gene expression and molecular evolution of osmolyte uptake transporters

To explore potential differences underlying the average difference in growth rates among lineages in BHI + 6% NaCl at 37°C, qRT-PCR was used to measure transcript levels of four osmolyte uptake genes (*opuCA*, *oppA*, *betL*, and *gbuA*) for four strains with growth rates similar to the average for each lineage. We observed considerable variation in transcript levels among strains (Table 3), but significant differences between the lineages were not detected for any of the four genes tested. Transcript levels of the four genes did not correlate with growth rate in BHI + 6% NaCl at 37°C. Comparative evaluation of DNA sequence data yielded no evidence for positive selection in the sequences for the four osmolyte uptake genes (Table 4); however, both recombination detection methods found significant evidence for recombination for *betL* and *opuCA* (Table 4).

Phylogenetic analyses of the DNA sequences for the individual osmolyte uptake genes generated topologies for each gene tree (Fig. 5) that appear to differ from that generated by the 10-gene MLST data (Fig. 4); however, except for *betL*, these differences did not receive enough bootstrap support (>70%) to be considered significant. DNA sequence data for *betL* yielded a polyphyletic lineage III (strains FSL J1-031 and FSL J2-071 group with lineage I instead of with the other lineage III strains) (Fig. 5A), thus differing significantly from the MLST-generated phylogeny. When the genetic distance for each osmolyte transporter gene was compared to the pairwise difference in transcript levels,

TABLE 3. RELATIVE EXPRESSION OF OSMOLYTE TRANSPORTER GENES DURING EXPONENTIAL GROWTH IN BRAIN–HEART INFUSION + 6% NaCl AT 37°C

Strain	Average $\log_2$ relative expression <sup>a</sup> for			
	<i>betL</i>	<i>gbuA</i>	<i>oppA</i>	<i>opuCA</i>
<i>Lineage I strains</i>				
FSL F6-366	$1.42 \pm 0.09$	$0.12 \pm 0.19$	$-0.63 \pm 0.36$	$-0.74 \pm 0.14$
FSL J1-175	$0.91 \pm 0.14$	$-0.09 \pm 0.14$	$-0.67 \pm 0.14$	$-0.49 \pm 0.25$
FSL J1-194	$0.87 \pm 0.49$	$0.12 \pm 0.26$	$-0.64 \pm 0.24$	$-0.22 \pm 0.39$
FSL R2-503	$0.92 \pm 0.21$	$0.44 \pm 0.37$	$-0.71 \pm 0.10$	$-0.63 \pm 0.31$
average	$1.03 \pm 0.33$	$0.15 \pm 0.29$	$-0.66 \pm 0.20$	$-0.52 \pm 0.32$
<i>Lineage II strains</i>				
FSL F2-515	$-0.23 \pm 0.10$	$0.24 \pm 0.14$	$-0.32 \pm 0.24$	$0.12 \pm 0.23$
FSL J1-101	$0.07 \pm 0.11$	$-0.54 \pm 0.66$	$-0.11 \pm 0.24$	$-0.79 \pm 0.79$
FSL J2-003	$0.16 \pm 0.36$	$0.04 \pm 0.46$	$-0.41 \pm 0.25$	$-0.24 \pm 0.37$
FSL R2-499	$0.83 \pm 0.26$	$0.36 \pm 0.27$	$0.19 \pm 0.44$	$-0.67 \pm 0.40$
average	$0.21 \pm 0.45$	$0.02 \pm 0.52$	$-0.16 \pm 0.35$	$-0.40 \pm 0.57$
<i>Lineage III strains</i>				
FSL F2-270	$2.98 \pm 0.12$	$1.52 \pm 0.09$	$1.29 \pm 0.17$	$1.75 \pm 0.36$
FSL J1-031	$1.47 \pm 0.02$	$-0.28 \pm 0.18$	$-0.88 \pm 0.07$	$-0.32 \pm 0.38$
FSL J1-208	$-0.08 \pm 0.34$	$-0.59 \pm 0.18$	$-1.15 \pm 1.31$	$-3.25 \pm 0.52$
FSL J2-071	$1.00 \pm 0.22$	$0.09 \pm 0.11$	$-0.78 \pm 0.12$	$-0.55 \pm 0.52$
Average	$1.34 \pm 1.16$	$0.18 \pm 0.85$	$-0.38 \pm 1.16$	$-0.59 \pm 1.90$

<sup>a</sup> $\log_2$  expression values for each strain are relative to replicate 1 of strain FSL J1-101.



TABLE 4. MOLECULAR EVOLUTION PARAMETERS FOR OSMOLYTE TRANSPORTER GENES

Gene	Test for positive selection				Test for recombination			
	Ln L for M1a <sup>a</sup>	Ln L for M2a <sup>b</sup>	Likelihood ratio <sup>c</sup>	p-Value	DNA substitution model <sup>d</sup>	PHI p-value <sup>e</sup>	Geneconv simulated p-value <sup>f</sup>	No. of fragments (no. of events) <sup>g</sup>
<i>betL</i>	-3238.6	-3238.6	0.000	1.000	HKY+I+G	$2.77 \times 10^{-27}$	<0.00001	20 (4)
<i>gbuA</i>	-2037.9	-2037.9	0.002	0.999	TrN+I+G	$3.68 \times 10^{-3}$	NS	NA
<i>oppA</i>	-2399.6	-2399.4	0.417	0.812	TrN	NS	0.006	5 (2)
<i>opuC</i>	-2213.1	-2213.1	0.003	0.998	TrN+G	$7.22 \times 10^{-6}$	<0.00001	32 (4)

<sup>a</sup>Log likelihood estimate for model 1a.

<sup>b</sup>Log likelihood estimate for model 2a.

<sup>c</sup>Likelihood ratio test, calculated as  $2[\text{Ln L (M1a)} - \text{Ln L (M2a)}]$ .

<sup>d</sup>As described by Posada and Crandall (1998).

<sup>e</sup>p-Value for the PHI test for recombination.

<sup>f</sup>Simulated p-values for the Geneconv test for recombination are based on 10,000 permutations and are corrected for multiple comparisons.

<sup>g</sup>Number of fragments where evidence of recombination was detected, number of events are groups of fragments linked to the same 5' or 3' breakpoints and classified as a single recombination event.

G, Gamma distribution; HKY, Hasegawa-Kishino-Yano model; I, proportion of invariable sites; NA, not applicable; NS, not significant; PHI, pairwise homoplasy index; TrN, Tamura-Nei model.

we found a significant association between transcript levels and genetic relatedness for *opuCA* ( $p = 0.019$ ) and *oppA* ( $p = 0.002$ ) (Fig. 5B, D). While a statistical association between transcript levels and genetic similarity exists for these two osmolyte transporters, differences in transcript levels for these genes do not explain the observed differences in growth rate among the lineages.

## Discussion

*L. monocytogenes* strains group into at least three phylogenetic lineages that vary in virulence potential and stress resistance (De Jesus and Whiting, 2003; Chen *et al.*, 2006; Nightingale *et al.*, 2006; Barmpalia-Davis *et al.*, 2008). The relative abilities of certain *L. monocytogenes* subtypes to persist in specific ecological niches are likely to reflect phenotypic characteristics that are shared within a subtype (Boerlin and Piffaretti, 1991; Gray *et al.*, 2004; Ward *et al.*, 2004). To quantify variation in salt stress phenotypes among the major genetic *L. monocytogenes* lineages and to determine if salt stress phenotypes are associated with genetic relatedness, we measured the ability of strains to grow under salt stress at two different temperatures. While adaptation to salt stress at 7°C did not differ among the lineages, lineage I and II strains that were initially grown at 7°C appeared to transition to growth in a high-salt medium more effectively than strains that were initially grown at 37°C and then transferred to high-salt conditions at 37°C. Under salt stress at 37°C, lineage II strains had a significantly slower average growth rate than lineage I and III strains. This difference in growth was not related to differences in transcript levels for four osmolyte uptake transporters. Differences in growth parameters at 37°C in the high-salt medium were significantly associated with genetic relatedness of the strains.

### Lineage-dependent differences in ability to grow under salt stress at 37°C

When analyzed by lineage, strains grown at 37°C differed in their abilities to adapt to exposure to a high-salt medium.

Specifically, lineage II strains had an impaired ability to adapt to salt stress compared to both lineage I and lineage III strains. On average, lineage II strains grown to midexponential phase at 37°C and then exposed to 6% NaCl had 24% and 15% longer doubling times than lineage III and lineage I strains, respectively. Differences in growth rates among the lineages were not due to inherent differences in growth capabilities, as there were no differences in average growth rates at 37°C in BHI alone. As foodborne pathogens are subjected to osmotic stress in the host gastrointestinal tract, our data suggest that lineage II strains may be less effective at propagating under these conditions than lineage I and III strains.

It is well established that significant phenotypic variation exists among strains of *L. monocytogenes*, including ability to grow at different temperatures (Barbosa *et al.*, 1994), or at different pH,  $a_w$ , and temperatures (Begot *et al.*, 1997), or to resist heat treatments (De Jesus and Whiting, 2003). Lineage-specific differences under host-related stresses have been observed; in one study, lineage II isolates had significantly higher inactivation rates in a dynamic gastric system than lineage I and III isolates (Barmpalia-Davis *et al.*, 2008). Taken together with our data, these results suggest that lineage II strains are at a disadvantage under mammalian host-like stress conditions at 37°C compared to lineage I and lineage III strains.

While differences in phenotypic traits have been linked to lineage classification, variability in phenotype within a lineage may also be large (De Jesus and Whiting, 2003). Differing levels of genetic diversity can also exist within lineages (den Bakker *et al.*, 2008). If phenotypic variability results from differences in gene content, or gene expression and regulation, closely related strains would be expected to have phenotypic similarities. Such relationships have been shown for the amphibian pathogen *Batrachochytrium dendrobatidis*, where genetic similarity is significantly associated with protein expression and sporangium morphology (Fisher *et al.*, 2009). A similar relationship exists in *Saccharomyces cerevisiae*, where similarity in stress resistance among strains is related to genetic similarity of the strains (Kvitek *et al.*, 2008). Our data show that differences in growth parameters under salt stress

at 37°C are significantly associated with genetic relatedness of the strains, indicating that closely related strains have a similar salt stress phenotype at 37°C. Therefore, differences in ability to adapt to salt stress reflect the evolutionary history of the strains.

*Differences in transcript levels of osmolyte uptake transporter genes do not account for growth differences under salt stress at 37°C among the lineages*

Given the significant differences in growth at 37°C in the high-salt medium among the different lineages, we explored possible underlying molecular mechanisms that might contribute to the relative adaptation capabilities among the lineages. In *L. monocytogenes*, osmolyte uptake transporters are considered to be the main contributors to managing osmotic stress, with additional roles in cryotolerance and virulence (Sleator *et al.*, 2003b). Specifically, the glycine betaine transporters BetL and Gbu as well as the carnitine transporter OpuC are used by *L. monocytogenes* to combat osmotic stress (Sleator *et al.*, 2003b). The oligopeptide permease, Opp, as well as the Gbu and OpuC transporters are also involved in cryotolerance (Borezee *et al.*, 2000; Sleator *et al.*, 2003b). Conceivably, differences in expression (at the gene or protein level) and differences in uptake efficiency of these osmolyte uptake systems could contribute to the growth differences observed among lineages. While transcript levels for these four osmolyte uptake transporters varied across strains, we did not detect significant differences in gene expression among the lineages. While differences in transporter protein expression and activity have been observed between strains of *L. monocytogenes* (Dykes and Moorhead, 2000; Sleator *et al.*, 2003a), our approach focused only on differences at the level of transcription. While we observed variation in transcript levels for the osmolyte transporter encoding genes, the differences in growth between the lineages could not be explained by differences in gene expression of these osmolyte uptake transporters. The faster growth rates of lineage I and III strains in high salt may be due to other factors, for example, differences in membrane fatty acid modifications or expression of surface proteins, two components of the salt stress response recently identified in *Bacillus subtilis* (Hahne *et al.*, 2010).

While variation in transcript levels was significantly associated with genetic relatedness for two of the osmolyte uptake transporter genes (*opuC* and *oppA*), variation in gene expression was not related to sequence similarity for *gbuA* or *betL*. Interestingly, analysis of *betL* DNA sequences showed evidence of a number of recombination events, similar to results from analysis of some virulence-associated genes, such as *inlA* (den Bakker *et al.*, 2008). We speculate that the lack of an association between transcript levels and nucleotide similarity for *betL* may be partially due to recombination. For example, recombination events can disrupt linkages between regulatory elements and gene sequences.

*Growth at 7°C provides crossprotection to subsequent salt stress*

We observed that the ability to adapt to salt stress at 7°C was similar across lineages, and that differences in growth parameters among the strains under these conditions were

not associated with genotype. Nufer *et al.* also reported no correlation between growth at 4°C and REP and ERIC PCR genotypes of six *L. monocytogenes* strains (Nufer *et al.*, 2007). Our results provide strong phenotypic evidence supporting a regulatory overlap between the *L. monocytogenes* responses to osmotic and to low temperature stresses. Proteins such as the glycine betaine transporter Gbu (Ko and Smith, 1999), the carnitine transporter OpuC (Fraser *et al.*, 2000), the alternative sigma factor SigL (Raimann *et al.*, 2009), and the cold-shock protein CspD (Schmid *et al.*, 2009) are involved in the osmotic stress response, and are also important for growth of *L. monocytogenes* at low temperatures (Angelidis and Smith, 2003; Raimann *et al.*, 2009; Schmid *et al.*, 2009). Because of these known overlaps in stress response, exposure to low temperature may lead to crossprotection against subsequent osmotic stress. Our data demonstrate that growth at 7°C enhances the long-term adaptation to salt stress, as the increase in doubling time due to the shift to a high-salt medium was significantly smaller for lineage I and II strains at 7°C than at 37°C. The crossprotective effect of cold on salt tolerance was affected by the genetic background of the strains, as the increase in doubling time due to salt shift was similar at both 7°C and 37°C for lineage III strains. While no differences exist among lineages in the ability to adapt to salt stress at 7°C, growth at 7°C appears to provide some crossprotection to salt stress for lineage I and II strains. Consistent with our data, others have shown that the inoculum state (e.g., different stress exposures of the inoculum) can provide preadaptation to subsequent stress conditions that result in improved growth rates. For example, Tiganis *et al.* (2009) have found that *L. monocytogenes* grown at pH 6.0 showed increased growth rates under osmotic stress as compared to bacteria when grown at pH 7.2 before osmotic stress exposure, and others have shown that stationary-phase *L. monocytogenes* exhibit higher growth rates under temperature, pH, and osmotic stress than exponential-phase cells (Viallette *et al.*, 2003). In addition to the well-documented effect of inoculum state on lag phase duration (Uyttendaele *et al.*, 2004; Geornaras *et al.*, 2006), a few initial studies have also shown an effect of inoculum state on *L. monocytogenes* growth rates, and further work is required to fully explore these events, including the impact of genetic background on preadaptation effects.

## Conclusions

In a comparison of growth parameters among lineages of *L. monocytogenes*, we have shown that the ability of these lineages to adapt to salt stress is dependent on growth temperature before salt exposure. All three lineages had a similar ability to manage salt stress after growth at 7°C. However, when grown at 37°C, lineage II strains did not adapt to subsequent salt stress at 37°C as well as lineage I and III strains. Therefore, lineage I and III strains have an advantage under osmotic stress at host body temperature, which may contribute to successful survival and spread in the host. The significant association between growth rate under salt stress at 37°C and genetic relatedness supports the idea that the ability to adapt to salt stress is linked to evolutionary history of the strains. Ultimately, the approaches applied and observations generated from this study will provide a useful framework for identifying and elucidating novel molecular

mechanisms that contribute to differences in osmotic stress response among *L. monocytogenes* strains.

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### Disclosure Statement

No competing financial interests exist.

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