

Increased Thermal and Osmotic Stress Resistance in *Listeria monocytogenes* 568 Grown in the Presence of Trehalose Due to Inactivation of the Phosphotrehalase-Encoding Gene *treA*[∇]

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The food-borne pathogen *Listeria monocytogenes* is a problem for food processors and consumers alike, as the organism is resistant to harsh environmental conditions and inimical barriers implemented to prevent the survival and/or growth of harmful bacteria. One mechanism by which listeriae mediate survival is through the accumulation of compatible solutes, such as proline, betaine and carnitine. In other bacteria, including *Escherichia coli*, the synthesis and accumulation of another compatible solute, trehalose, are known to aid in the survival of stressed cells. The objective of this research was to investigate trehalose metabolism in *L. monocytogenes*, where the sugar is thought to be transferred across the cytoplasmic membrane via a specific phosphoenolpyruvate phosphotransferase system and phosphorylation to trehalose-6-phosphate (T6P). The latter is subsequently broken down into glucose and glucose-6-phosphate by α,α -(1,1) phosphotrehalase, the putative product of the *treA* gene. Here we report on an isogenic *treA* mutant of *L. monocytogenes* 568 (568: Δ TreA) which, relative to the wild-type strain, displays increased tolerances to multiple stressors, including heat, high osmolarity, and desiccation. This is the first study to examine the putative trehalose operon in *L. monocytogenes*, and we demonstrate that *lmo1254* (*treA*) in *L. monocytogenes* 568 indeed encodes a phosphotrehalase required for the hydrolysis of T6P. Disruption of the *treA* gene results in the accumulation of T6P which is subsequently dephosphorylated to trehalose in the cytosol, thereby contributing to the stress hardiness observed in the *treA* mutant. This study highlights the importance of compatible solutes for microbial survival in adverse environments.

The disaccharide trehalose (α -D-glucopyranosyl-1,1- α -D-glucopyranoside) is widely distributed in nature and found in shellfish, insects, plants, mammals, bacteria, and fungi (2). It is a nonreducing sugar and compatible solute with unique physicochemical properties, which include acting as a molecular chaperone to prevent misfolding of proteins and stabilizing membranes through hydrogen bonding with phospholipids (46, 57). Trehalose also aids in the survival of many organisms during exposure to adverse conditions caused by temperature extremes, desiccation, high osmolarity, and oxidative stress (15).

Although, trehalose metabolism has been documented for Gram-negative bacteria such as *Enterobacter sakazakii* (*Cronobacter*) (8), *Pseudomonas fluorescens* (35), *Rhizobium japonicum* (52), and *Salmonella enterica* serovar Typhimurium (9), only that of *Escherichia coli* has received a thorough analysis (28, 32, 45). When subjected to high osmolarities, *E. coli* synthesizes large amounts of trehalose to act as a compatible solute. Perhaps the most interesting phenomenon surrounding this accumulation is the simultaneous high-osmolarity-dependent synthesis and degradation of the disaccharide (28). Spatial separation of these metabolic processes allows for their

functional coexistence; the *de novo* synthesis is localized in the cytoplasm, whereas hydrolysis of trehalose is carried out in the periplasm (28). When the osmolarity of the external environment is low, *E. coli* uses exogenous trehalose as a carbon and energy source. This catabolic process involves the importation of the disaccharide via a trehalose-specific phosphotransferase system (PTS) resulting in its simultaneous phosphorylation to trehalose-6-phosphate (T6P), which in turn is cleaved by trehalose-6-phosphate hydrolase (α,α -phosphotrehalase) (45). Similar PTS uptake systems for trehalose catabolism are found in Gram-positive bacteria such as *Bacillus subtilis* which do not appear to accumulate the sugar for starvation or stress relief. Instead, trehalose is used solely as a carbon/energy source (47).

Listeria monocytogenes is an important Gram-positive food-borne pathogen that has received much attention due to its virulence and ability to survive and grow in foods and the food-processing environment. Information regarding trehalose metabolism in *Listeria* spp. is currently limited to inference based on the published genomic sequence of a putative trehalose operon (1, 21) and possible similarities to related Gram-positive bacteria (7, 14, 26, 47, 48). Also, growth studies have shown that generally *Listeria* spp. can utilize the disaccharide as their sole source of carbon and that utilization is repressed by the presence of glucose in the growth medium (19, 42, 49).

L. monocytogenes strain 568 is an environmental serotype 1/2a strain originally isolated from a shrimp-processing facility (25). In a previous study we reported on *L. monocytogenes* 568 transposon insertion mutants that displayed enhanced thermo-

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TABLE 1. Bacterial strains and plasmids used in this study

Species and strain or plasmid	Description	Reference or source
Strains		
<i>L. monocytogenes</i>		
568	Wild type; serotype 1/2a; from shrimp-processing plant	25
32F11	Tn917 mutant of 568; Erm ^r	18
568:ΔTreA	568 deletion mutant; TreA ⁻	This study
ΔTreA:pAM:TREOP	pAM401:TreA complement of 568ΔT6P; Crm ^r Tet ^s TreA ⁺	This study
568/pAULAΔTreA	568 carrying pAULA-ΔTreA with <i>treA</i> deletion insert	This study
<i>E. coli</i>		
TCTOP:ΔTreA	TOP10 carrying pCR2.1 TOPO with 568 <i>treA</i> insert with deletion	This study
TCTOP:TREOP	TOP10 carrying pCR2.1 TOPO with intact 568 Tre operon	This study
DH5α	Cloning host; F ⁻ <i>endA1 glnV44 thi-1 recA1 gyrA96 deoR nupG φ80lacZ ΔM15 Δ(lacZYA-argF)U169 hsdR17 (r_K⁻ m_K⁺) λ⁻</i>	24
XL10-GOLD Kan ^r	Cloning host; <i>endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac hte Δ(mcrA)I83 Δ(mcrCB-hsdSMR-mrr)173 ietR F⁺[proAB lacI^qZ ΔM15 Tn10(Tet^r) Amy Tn5(Kan^r)]</i>	Stratagene
XL10-GOLD	XL10-GOLD carrying pCVV1 expression vector	This study
TCE-pAULA:TreA	DH5α host carrying pAUL-A with <i>treA</i> deletion insert	This study
TOP10	F ⁻ <i>mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str^r) endA1 λ⁻</i>	Invitrogen
Plasmids		
pAUL-A	Gram-positive and Gram-negative shuttle vector; <i>oriTS</i> , LacZa', Erm ^r	10
pAM401	Gram-positive and Gram-negative shuttle vector; Crm ^r Tet ^r	60
pCR:ΔTreA	pCR2.1 TOPO with 568 <i>treA</i> insert with deletion	This study
pAULA:ΔTreA	pAUL-A with truncated 568 <i>treA</i> gene; Erm ^r	This study
pAM401:TREOP	pAM401 with intact 568 <i>treA</i> gene; Crm ^r , Tet ^s <i>treA</i> ⁺	This study
pCVV1	pCYB1-derived expression vector carrying <i>B. subtilis</i> <i>treA</i> ; Tac promoter, Amp ^r	56
pCR2.1 TOPO	ColE1 Amp ^r Kan ^r <i>lacZα</i>	Invitrogen

tolerance (18). In one of these mutants, the location of the Tn917 insertion was determined to be in the putative *treA* gene (*lmo1254*), the gene thought to encode phosphotrehalase, a phospho-(1,1)-glucosidase (21). The objective of the current study was to elucidate the reason for the observed thermal-resistant phenotype. Therefore, a *treA* gene deletion mutant of *L. monocytogenes* 568 was created to assess the impact of this gene on the biochemistry and physiology of the organism. Furthermore, the importance of the *treA* gene to a multitude of other food-relevant environmental stressors was examined. To the best of our knowledge, this is the first report involving experimental analysis of any of the three genes of the putative trehalose operon in *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. For long-term storage, all strains and transformants were kept at -80°C in broth media supplemented with glycerol (15%, wt/vol): brain heart infusion (BHI) for *L. monocytogenes* or Luria-Bertani (LB) for *E. coli*. Reconstitution of frozen stocks was done by streaking the cultures onto BHI or LB agar followed by incubation at 37°C. Recovery of survivors following stress treatments was carried out on tryptic soy agar supplemented with 0.6% yeast extract (TSA-YE) and 1% sodium pyruvate (4, 11). Survival studies for oxidative stress, acid stress, tolerance to ethanol, and osmotic stress were performed in BHI broth augmented according to the methods listed below. Trehalose utilization experiments were carried out in modified Welshimer's broth (MWB) (42). Antibiotic concentrations in selective media were as follows: for cloning *treA* into *E. coli* using TOPO pCR2.1, ampicillin (Amp), 100 μg/ml; for selection of *E. coli* carrying pAUL-A with the *treA* deletion insert (pAULA:ΔTreA), erythromycin (Erm), 250 μg/ml; for selection of pAULA:ΔTreA-transformed *L. monocytogenes* 568, Erm, 5 μg/ml; for complementation experiments with *E. coli* carrying pAM401, chloramphenicol (Crm), 50 μg/ml; and for selection of ΔTreA:pAM:TREOP-transformed *L. monocytogenes*, Crm,

10 μg/ml. All media and antibiotics were purchased from Oxoid Canada Inc. (Mississauga, ON, Canada) and Sigma-Aldrich Chemical Company (Oakville, ON, Canada), respectively.

DNA extractions, purification, and manipulations. Large-scale plasmid DNA extractions of the vectors pAUL-A and pAM401 were carried out using the Qiagen Maxi-Prep kit (Qiagen Inc., Mississauga, ON, Canada) according to the manufacturer's instructions. Small-scale genomic DNA extractions were performed using the UltraClean microbial DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA). DNA-modifying enzymes (XbaI restriction endonuclease, T4 ligase, and calf intestinal alkaline phosphatase [CIAP]) were provided by New England BioLabs (Pickering, ON, Canada). DNA TOPO cloning kits were supplied by Invitrogen (Burlington, ON, Canada). KlenTaq and *Taq* DNA polymerases were supplied by Sigma-Aldrich.

Construction of a chromosomal ΔTreA deletion mutant. A 462-bp deletion in the center of the *treA* gene, encoding phosphotrehalase, in *L. monocytogenes* 568 was created by allelic replacement by the gene splicing and overlap extension (SOE) method (29). Briefly, genomic DNA from an overnight culture of *L. monocytogenes* 568 was used as the template for two separate PCRs. Primers SOE-T6P-A (5'-ACGGTCTAGAACTGATTCTCTGGCGCTCAT-3') and SOE-T6P-B (5'-GGATTGATAAAGCGTGGGAAGCGCAACGATTTCATT TTAT-3') were used to generate a 591-bp amplicon at the 5' end of the *treA* gene using KlenTaq polymerase. The second reaction produced a 491-bp amplicon at the 3' end of the gene and employed primers SOE-T6P-C (5'-TTCCACGCTTATCAATCC-3'), and SOE-T6P-D (5'-CTGCTCTAGAGGGGATATTCCC GGTATCAC-3'). SOE-T6P-C was complementary to the 5' tail of SOE-T6P-B. Sequences for the restriction endonuclease XbaI (underlined regions) were introduced into primers SOE-T6P-A and SOE-T6P-D to facilitate cloning. The PCR products were cleaned with the EZ-10 spin column PCR purification kit (Bio Basic, Inc., Markham, ON, Canada). Amplicons from each of the PCRs were diluted 1,000-fold, mixed in a 1:1 ratio, and used (1 μl) as the template for a new PCR using primers SOE-T6P-A and SOE-T6P-D. The 1,061-bp amplicon was run out on a 1% Tris-acetate-EDTA (TAE) agarose gel, excised, purified (GeneClean gel purification kit; Q-Biogene, CA), and TA cloned into *E. coli* TOP10 using the TOPO TA cloning kit. The presence of the insert was confirmed by digestion of the extracted plasmid with XbaI and fractionation on a TAE agarose gel. The Δ*treA* fragment was excised, purified as before, and then

ligated into the dephosphorylated temperature-sensitive shuttle vector pAUL-A with T4 DNA ligase. The resulting plasmid, designated pAULA:Δ*treA*, was electroporated into *E. coli* DH5α (3, 36) and transformants selected on LB agar containing 250 μg/ml Erm.

Electroporation into *L. monocytogenes* 568 and selection of its isogenic *treA* deletion mutant. A maxiprep of the pAULA:Δ*treA* plasmid was prepared, and the DNA was electroporated into competent *L. monocytogenes* 568 cells (22). Selection of transformed *Listeria* was performed on BHI agar containing Erm (5 μg/ml) after 3 to 4 days at 28°C. The presence of the pAULA:Δ*treA* plasmid was confirmed by plasmid extraction and restriction digestion of the DNA with XbaI. For the integration of pAULA:Δ*treA* into the chromosome of *L. monocytogenes* 568, a single colony of a confirmed plasmid-carrying transformant was patched onto BHI agar with 5 μg/ml Erm, followed by incubation at 42°C for 2 days. The colony was transferred to a fresh plate and incubated under the same conditions. A total of three transfers/incubations were conducted. Merodiploid intermediates were confirmed by the presence of two amplicons (wild-type allele, 1,523 bp; deleted allele, 1,061 bp) by PCR using primers SOE-T6P-A and SOE-T6P-D. Spontaneous excision of the integrated plasmid via intramolecular homologous recombination of the deleted allele was achieved by 3 subsequent overnight incubations in BHI at 28°C in the absence of Erm followed by a final overnight incubation at 42°C in BHI. The culture was diluted in 0.1% peptone water, spiral plated onto BHI agar, and incubated at 35°C for 48 h. One hundred colonies were patched onto BHI and then replica plated onto MWB containing 0.5% trehalose as the sole source of carbon. The plates were incubated at 37°C for 48 h, and colonies displaying poor growth on the MWB-trehalose medium were subjected to PCR using primers SOE-T6P-A and SOE-T6P-D to select recombinants displaying a single amplicon of 1,061 bp. The in-frame deletion was confirmed by DNA sequencing, and the deletion mutant was named *L. monocytogenes* 568:Δ*treA*.

Complementation of *L. monocytogenes* 568:Δ*treA*. Phosphotrehalase activity was restored in *L. monocytogenes* 568:Δ*treA* by transforming the mutant with the shuttle vector pAM401 carrying an intact copy of the *treA* gene. Briefly, a full-length amplicon for the *treA* and *treB* genes, including the putative promoter region upstream of *treB*, was amplified from *L. monocytogenes* 568 using the primers TreOp-F (5'-CCTTCTAGAGGTCACCGTTGGAT AAA TTCC-3') and TreOp-R (5'-AATCTAGA ACCGCAATGTCCAATAATTT-3'). The underlined bases denote XbaI restriction sites to facilitate cloning. The amplicon was TA cloned into TOPO pCR2.1, and transformants were selected on LB with Amp (100 μg/ml). The plasmid carrying the insert was isolated, purified, and then digested with XbaI. The insert was gel purified and ligated to pAM401 linearized with XbaI and dephosphorylated with CIAP. The resulting construct, pAM401:TREOP, was electroporated into competent cells of the deletion mutant *L. monocytogenes* 568:Δ*treA* as described above. Also, pAM401 containing no insert was electroporated into the wild-type *L. monocytogenes* 568 to create *L. monocytogenes* 568/pAM401. This strain was used as a control in later experiments to account for possible fitness losses due to the presence of the plasmid in the complemented mutant. The transformants were selected on BHI containing Crm (10 μg/ml) and verified by PCR using the TreOp-F and TreOp-R primers. Restoration of *treA* activity was confirmed by growth on MWB-trehalose and measurements of phosphotrehalase activity (see below).

Preparation of crude cell extracts for enzyme assays. Extracts from the mutant and wild-type *L. monocytogenes* 568 were prepared according to the method of Helfert et al. (26) with modifications. The cultures were grown in 50 ml of BHI with 1% trehalose for 16 h at 30°C and 200 rpm in an orbital shaker. The cells were harvested by centrifugation (6000 × *g*, 15 min, 0°C). Cell pellets were washed twice by resuspending them in 40 ml of bis-Tris sonication buffer (50 mM bis-Tris [pH 7.0], 10 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and repeating the centrifugation. The pellets were then resuspended in 5 ml of sonication buffer and sonicated on ice at 60% power for 20 s with a 30-s rest between pulses (the total pulse time was 5 min) with a Virsonic 600 cell disrupter (Virtis, Gardiner, NY). Cellular debris was removed by centrifugation (10,000 × *g*, 30 min, 0°C). The quantity of total protein in the supernatant was determined using an Ettan 2D-Quant kit (Amersham Biosciences, Inc., Baie d'Urfé, PQ, Canada).

Assay for α,α-(1,1)-phosphotrehalase activity. The phosphotrehalase activity in crude cell extracts of the mutant and parent strains was measured as the amount of glucose liberated from the hydrolysis of T6P using a commercial glucose hexokinase (HK) kit (Sigma-Aldrich). A 500-μl aliquot of crude cell extract was incubated with 10 mM T6P for 2 h at 37°C. Control samples contained no added T6P. Following incubation, sample aliquots (200 μl) were mixed with 1.0 ml of glucose assay reagent. Negative control samples were combined with 1.0 ml sterile double-distilled water (ddH₂O). Results were reported as liberated glucose per mg cell protein.

Effect of glucose on expression of *treA*. In order to assay the repression of *treA* activity by glucose, *L. monocytogenes* 568 was grown overnight at 37°C in MWB containing 10 mM trehalose. The overnight culture (10 μl) was used to seed 50 ml of fresh MWB containing 10 mM trehalose and 0, 1, 5, and 10 mM glucose. The cultures were incubated at 37°C at 200 rpm for 20 h and then harvested by centrifugation. The *treA* activity was measured as described above.

Extraction of intracellular listerial trehalose-6-phosphate. The intracellular accumulation of T6P was determined in *L. monocytogenes* 568 and its *treA* mutant after growth (18 h, 30°C, 50 ml) in 5 different media: tryptic soy broth (TSB), TSB-YE, TSB with 1% trehalose (TSB-Tre), BHI, and MWB with 1% glucose. Cells were harvested by centrifugation (6,000 × *g* for 10 min at 0°C), resuspended in 1 ml of PBS (pH 7.0), and sonicated as before. Subsequently, an equal volume of ice-cold 2 M HClO₄ was added along with glass beads, and tubes were shaken for 10 min. The cellular debris was removed by centrifugation at 20,000 × *g* (30 min, 0°C), and the samples were then neutralized with 3 M K₂CO₃ before being passed through LC-NH₂ solid-phase ion-exchange columns (Supelco, Bellefonte, PA). Elution of T6P was carried out with 2 ml of 2.5 M ammonium hydroxide (pH 12.5). The ammonia was removed by freeze-drying followed by redissolving the sample in 50 mM bis-Tris buffer (pH 7.0).

Determination of T6P levels in cell extracts. Intracellular T6P in *L. monocytogenes* 568 and 568:Δ*treA* was determined using the method of Van Vaecck et al. (56). The inducible expression vector pCVV1 containing the intact *treA* gene from *Bacillus subtilis* (a gift from C. Van Vaecck and J. M. Thevelein, Katholieke Universiteit, Leuven, Flanders, Belgium) was electroporated into competent *E. coli* XL10-Gold (Stratagene, La Jolla, CA). Transformants were recovered on LB containing 100 μg/ml Amp. Isolation and purification of the overexpressed enzyme was carried out following the methods of Gotsche and Dahl (23) and Van Vaecck et al. (56) with modifications. In brief, 10 ml of an overnight culture of *E. coli* XL10-Gold carrying pCVV1 was used to seed 1 liter of LB broth containing 0.5 mM IPTG (isopropyl-β-D-thiogalactoside) and Amp (100 μg/ml). The culture was grown for 5 h at 37°C and 200 rpm. The cells were harvested at 6,000 × *g* for 10 min at 4°C. Washing and lysis of the pellets by sonication were carried out as described above, followed by removal of cellular debris by ultracentrifugation at 40,000 × *g* for 30 min at 4°C (Beckman SW40 rotor). Proteins in the supernatant were precipitated by stepwise addition of ammonium sulfate to 80% saturation. The solution was stirred overnight at 4°C, and the precipitate was collected by centrifugation (15,000 × *g*, 4°C, 20 min). The pellet was dissolved in 5 ml of extraction buffer and then dialyzed against several changes of bis-Tris buffer. Proteins were first fractionated by passage through a DEAE Sephadex A-50 anion-exchange column and elution in 5-ml fractions with a NaCl gradient. The presence of the 64-kDa phosphotrehalase enzyme in the fractions was determined on a 12% SDS-polyacrylamide gel by the method of Laemmli (33). These fractions were pooled, precipitated with ammonium sulfate, centrifuged, and redissolved in bis-Tris buffer. For size fractionation, the sample was loaded on a Superdex 75 gel filtration column equilibrated with bis-Tris buffer. Elution of 2-ml fractions was carried out with the same buffer. After analysis on a 12% SDS-polyacrylamide gel, fractions with a prominent 64-kDa band were pooled and analyzed for enzyme activity using pure T6P as a substrate (see above). In addition, the activity of the purified enzyme was quantified using *p*-nitrophenyl-α-D-glucopyranoside (PNPG) as a substrate, where 1 unit of phosphotrehalase will hydrolyze 1 nmol of PNPG/min at 37°C. The specific activity was calculated based on units/mg of protein as determined with the Ettan 2D Quant protein kit.

For the TP6 assay, various quantities of the intracellular cell extracts were mixed with 2.0 U of purified phosphotrehalase, and the total reaction volume was brought to 200 μl. The mixture was incubated at 37°C for 2 h. The reactions were run in duplicate. The amount of glucose liberated in the reaction was measured using the glucose HK assay.

Extraction and assay of trehalose from bacterial cultures. Overnight TSB cultures were grown at 30°C and used to inoculate fresh 100-ml cultures in TSB, BHI, and TSB-Tre. The cultures were grown for 16 h at 30°C at 200 rpm and harvested by centrifugation (6,000 × *g*, 15 min, 2°C). The pellets were washed twice in phosphate-buffered saline (PBS) (pH 7.0), followed by mixing with 4 ml of sterile ultrapure water tempered to 2°C and sonication on ice. Cellular debris was removed by centrifugation (10,000 × *g* for 20 min) and saved for protein analysis. Precipitation of proteins in the supernatant was achieved by addition of acetic acid to pH 2.0, incubation on ice for 1 h, and centrifugation as before. The pH of each extract was adjusted to 6.0 with KOH (1 M), followed by storage at -80°C until analysis. The pellet from the acid precipitation was combined with the original pellet and solubilized in Laemmli buffer (33). The amount of extracted protein in the pellet was determined using the Ettan 2D-Quant kit according to the manufacturer's instructions (Amersham Biosciences).

Since trehalose consists of two glucose molecules, the glucose HK assay was

used to measure the amount of glucose liberated following the hydrolysis of trehalose with purified porcine kidney trehalase (Sigma-Aldrich). Two hundred microliters of the deproteinized cell extracts was incubated with 0.05 U of trehalase at 37°C for 8 h. In order to compensate for endogenous glucose in the samples, controls with no trehalase were incubated along with each trehalase-treated sample. Also, standards containing pure trehalose were analyzed. Following incubation, the glucose content in all samples were determined by the glucose HK method, and intracellular the trehalose content $\{0.5 \times ([\text{glucose}_{\text{trehalase}}] - [\text{glucose}_{\text{no trehalase}}])\}$ was reported as $\mu\text{g}/\text{mg}$ cell protein.

Assessment of stress tolerance. *L. monocytogenes* 568: ΔTreA and wild-type *L. monocytogenes* 568 were subjected to several stress conditions. For heat treatments, overnight cultures in BHI, TSB, TSB-YE, and TSB-Tre were grown at 30°C. Aliquots (10 μl) from each culture were used to inoculate 5 ml of the same medium, followed by incubation for 16 h at 30°C. For cold-temperature growth curves, the cells were first cultured in BHI for 5 days at 4°C. For the remaining stress studies, the cells were cultured in BHI and then grown in 5 ml of BHI with 1% trehalose for 16 h at 30°C. Harvest of cultures was by centrifugation at $6,000 \times g$ for 10 min at room temperature followed by washing once with 0.1% peptone water and resuspension in the appropriate test medium (see below).

Heat treatments. The cell pellets were washed twice in 0.1% peptone water and then diluted in the original medium to obtain initial concentrations of 10^7 to 10^8 CFU/ml. Samples (100 μl) from each treatment were distributed into 16 thin-walled 0.2-ml PCR tubes. The tubes were placed in a Biometra Tgradient thermocycler (Whatman Biometra, Göttingen, Germany) and heat treated at 52°C for 105 min. Duplicate samples were removed at 15-min intervals and cooled in an ice-water bath for 1 min. The samples were diluted in sterile 0.1% peptone water and spiral plated onto TSA-YE containing 1% sodium pyruvate. CFU were enumerated following 72 h of incubation at 35°C, and the log values were plotted against time to obtain thermal death time curves. The entire experiment was repeated 3 times.

Cold growth. Duplicate tubes containing 5 ml of BHI tempered to 4°C were inoculated with 10 μl of the 5-day culture. The tubes were incubated at 4°C without shaking, and aliquots were removed periodically for absorbance measurements and colony enumerations. Absorbance measurements were taken at 600 nm, and cell enumerations were performed by spiral plating on TSA-YE (48 h at 35°C). The experiment was repeated twice.

Resistance to freeze-thaw cycles. The cell pellets were washed in sterile phosphate-buffered saline (PBS) (pH 7.0) then resuspended in 10 ml of PBS in 50-ml polypropylene centrifuge tubes to obtain cell densities of 10^7 to 10^8 CFU/ml. The initial cell numbers were determined by plating on TSA-YE. The tubes were frozen to -80°C in an ultra-low-temperature freezer. After 2 h, the suspensions were thawed in a water bath (37°C) and an aliquot was diluted and spiral plated. After 30 min at 37°C, the tubes were placed in the freezer, and they were thawed again after 24 h to determine the viable CFU. The freeze-thaw cycles were continued for 9 days, with samples being taken every 24 h. Colony enumerations were performed after 72 h at 35°C. The experiment was repeated 3 times.

Resistance to desiccation. The washed pellets from each growth medium were resuspended in 0.1% peptone water to cell densities of ca. 10^8 CFU/ml. Ten microliters of each suspension (i.e., 10^6 CFU) was aseptically transferred to 3 wells of a 12-well flat-bottomed tissue culture plate, followed by drying for 2 h in a biosafety cabinet (Baker Co., Sanford, ME) with lids removed. Subsequently, the plates were stored in a vacuum jar containing 10- to 20-mesh Drierite desiccant (WA Hammond Drierite Co., Xenia, OH) at ambient temperature (20°C) for 14 days. A Hobo Pro v2 temperature/relative humidity (RH) data logger was also placed inside the jar to record the RH. Following the desiccation period, BHI (5 ml) was added to the wells of each culture plate, which were placed in an incubator at 30°C and monitored for growth (A_{600}). Absorbance readings were taken until the cells reached stationary phase. CFU were determined by extrapolating values from a standard curve relating CFU to absorbance at 600 nm (not shown), and the Microsoft Excel add-in program DMFit (version 2.1; <http://www.ifr.ac.uk/safety/dmfit/>) was used to determine lag times and growth rates. Three replicates of this experiment were carried out.

Survival in acid, ethanol, hydrogen peroxide, and sodium chloride. The pellets were resuspended to 10^7 to 10^8 CFU/ml in BHI adjusted to achieve one of the following stress conditions: pH 3.5, adjusted with HCl; 18% (vol/vol) ethanol; 0.1% (vol/vol) H_2O_2 ; and 20% (wt/vol) NaCl. For each sample treatment, a 100- μl aliquot was removed immediately following mixing, diluted in peptone water, and spiral plated on TSA-YE. Samples were removed and spiral plated at specified time intervals for each sample treatment. All plates were incubated at 35°C for 72 h before colony enumerations. These experiments were replicated at least twice.

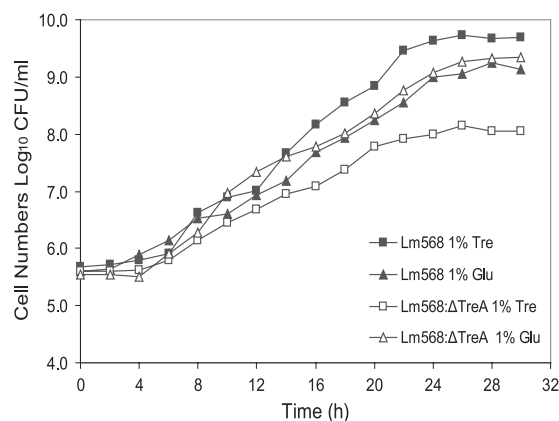


FIG. 1. Growth curves for *L. monocytogenes* 568 and its *treA* mutant 568: ΔTreA in modified Welshimer's broth (42) at 37°C and 150 rpm. The values displayed for each data point are the means \pm standard deviations ($n - 1$) for four samples (two replicates from two independent experiments).

Statistical analysis. Stress test data were subjected to analysis of variance (ANOVA) using SYSTAT software, and pairwise comparisons were assessed by the Tukey method at the 95% confidence level. Critical parameters for analysis of thermal death curves were obtained using the Pruitt-Kamau model (equations 1 and 2) (43). Survivor curves were modeled based on a modification of the Fermi distribution model of Whiting (59) for curves not exhibiting a tailing phenomenon:

$$\log(N/N_0) = \log\{(1 + e^{-\beta \times \text{tcm}})[1 + e^{-\beta(t - \text{tcm})}]\} \quad (1)$$

where N is the number of survivors at any time (t), N_0 is the initial population at $t = 0$, β is the maximum specific death rate, and tcm is the lag period. For comparison of the overall thermal resistance for the wild type and its mutant, a logistic decimal reduction time (D_L) was calculated as previously described by Pruitt and Kamau (43):

$$D_L = \text{tcm} + [\ln(9 + 10e^{-\beta})]/\beta \quad (2)$$

Where appropriate, Student's t test ($\alpha = 0.05$) was used for direct comparison of the means obtained for trehalose or T6P accumulation in the mutant versus the wild type.

RESULTS

Growth kinetics of the deletion mutant. DNA sequencing demonstrated that the *L. monocytogenes* 568: ΔTreA mutant contained a 462-bp in-frame deletion in the central region of the putative *treA* gene. The colony morphology of the deletion mutant was not different from that of the wild-type *L. monocytogenes* 568 when grown on TSA-YE or BHI agar. The growth kinetics in BHI, TSB (data not shown), and MWB with glucose (Fig. 1) were also similar. Interestingly, *L. monocytogenes* 568: ΔTreA grew in MWB with 1.0% trehalose as the sole carbon source, although the growth rate was significantly ($P < 0.05$) decreased and a lower maximum cell density was obtained (Fig. 1). To confirm that the mutant was indeed using trehalose and not another constituent in the medium, both the wild-type and the mutant were inoculated into MWB with no added carbon source. Neither culture grew after 48 h at 37°C. Also investigated was the possibility that the apparent utilization of trehalose by the mutant was due to natural degradation of the disaccharide into glucose during incubation at 37°C. However, no glucose could be detected in the sterile MWB with 1% trehalose following 48 h at 37°C using either the

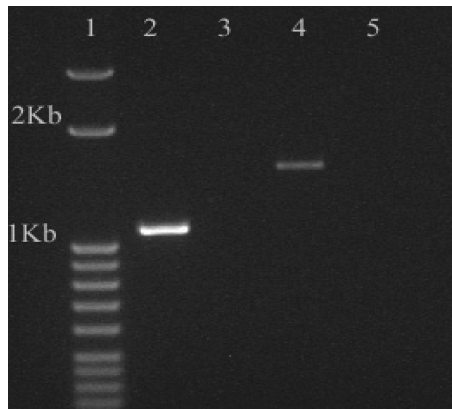


FIG. 2. Transcriptional analysis by RT-PCR of the *treA* genes in *Listeria monocytogenes* 568 and mutant 568:Δ*TreA*. Lane 1, DNA ladder; lane 2, *L. monocytogenes* 568:Δ*TreA* amplicon from cDNA; lane 3, control for *L. monocytogenes* 568:Δ*TreA* with no RT added to the reaction mixture; lane 4, *L. monocytogenes* 568 amplicon from cDNA; lane 5, control for *L. monocytogenes* 568 with no RT added to the reaction mixture.

glucose HK assay or high-pressure liquid chromatography (HPLC) analysis (data not shown).

Enzymatic activity of crude cell extracts. According to the published genome map of *L. monocytogenes* EGD-e (GenBank accession number AL591824), the only putative transport mechanism for the utilization of trehalose is through the enzyme IIBC trehalose-specific PTS (*treB*) and phosphotrehalase (*treA*). In order to verify the absence of trehalase activity, the glucose hexokinase assay was used to determine whether glucose is liberated from pure trehalose during incubation for 4 h at 37°C with crude cell proteins extracted from the mutant and the wild type. In neither case were increases in glucose detected in comparison to the control extracts without trehalose.

Reverse transcription-PCR (RT-PCR) demonstrated that a truncated phosphotrehalase transcript was produced by the deletion mutant (Fig. 2); however, the enzyme was not active, as free glucose was not produced from T6P during incubation of crude cell extracts from the mutant for 1, 4, and 8 h at 37°C. Conversely, extracts from the wild-type strain grown in MWB (with trehalose) contained high levels of phosphotrehalase activity, since 10 μl of cell extract liberated large quantities of glucose from T6P after only 1 h at 37°C (Fig. 3).

Repression of *treA* by glucose. We examined the effect of the presence of glucose on the *treA* activity in early-stationary-phase cells of *L. monocytogenes* 568. Crude extracts from cells grown in different concentrations of glucose demonstrated that the transcription of *treA* or enzyme activity was repressed by the presence of glucose (Fig. 3). Cells grown in the presence of 10 mM trehalose as the sole carbon source exhibited the highest level of phosphotrehalase activity, with more than 80 μg of glucose/mg cell protein being liberated from T6P after 1 h at 37°C. The addition of glucose (5 and 10 mM) to MWB cultures resulted in only 29 and 12 μg of glucose/mg protein being released from T6P, respectively. At 1 mM glucose, catabolite repression was not as strict, since crude cell extracts liberated 71 μg of glucose/mg cell protein.

Accumulation of intracellular trehalose-6-phosphate. *L. monocytogenes* 568 and 568:Δ*TreA* were assessed for intracellular

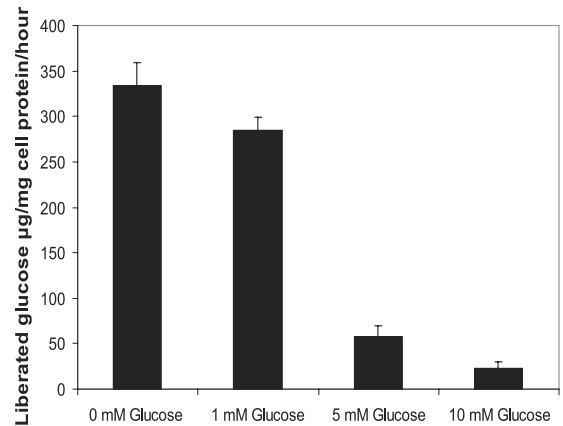


FIG. 3. Repression of phosphotrehalase activity in *Listeria monocytogenes* 568 by the presence of glucose in modified Welshimer's broth (MWB) supplemented with 10 mM trehalose and various concentrations of glucose. The results show the means ± standard deviations for four samples (two replicates from two independent experiments).

levels of T6P after growth in different substrates. The growth medium affected the concentration of T6P in both the wild-type and the mutant. This could be correlated to the amount of trehalose present in the medium (Fig. 4). As such, neither strain displayed detectable levels of T6P after growth in MWB with glucose as the carbon source. However, where low levels of trehalose were detected in TSB (72 mg/liter), the phosphorylated sugar was present in cell extracts in small amounts after growth in this medium: 2.4 and 3.2 μg/mg cell protein for *L. monocytogenes* 568 and 568:Δ*TreA*, respectively. Supplementing TSB with yeast extract (0.6%, wt/vol) resulted in a significant increase ($P < 0.001$) in the intracellular pool of T6P for the mutant (8.2 μg/mg cell protein) but not for the wild-type strain (2.5 μg/mg cell protein), in comparison to growth in the unmodified medium. Yeast extract was found to contain substantial levels of trehalose, as a 0.6% (wt/vol) solution con-

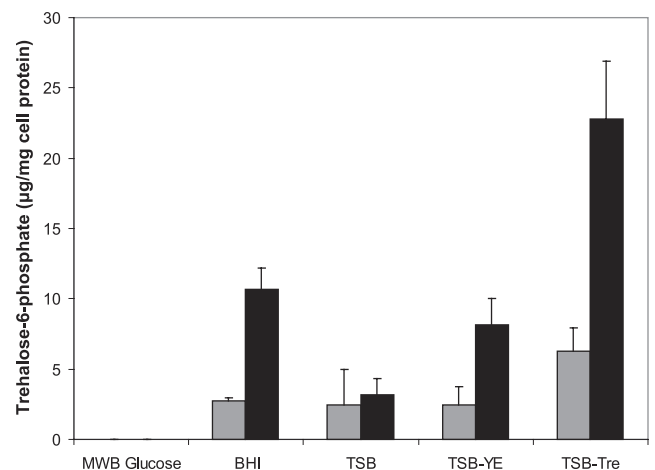


FIG. 4. Intracellular accumulation of trehalose-6-phosphate by *L. monocytogenes* 568 (gray bars) and *L. monocytogenes* 568:Δ*TreA* (black bars) after growth in five different media (MWB plus Glu, TSB, TSB-YE, TSB-Tre, and BHI). The results show the means ± standard deviations for four samples (two replicates from two independent experiments).

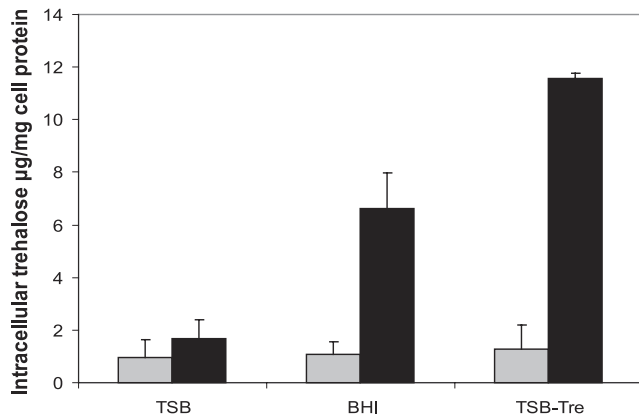


FIG. 5. Intracellular trehalose contents in *Listeria monocytogenes* 568 (gray bars) and its *treA* mutant *L. monocytogenes* 568:ΔTreA (black bars) after growth in either TSB, BHI, or TSB-Tre (supplemented with 1% trehalose) for 16 h at 37°C. The results show the means \pm standard deviations for four samples (two replicates from two independent experiments).

tained approximately 730 mg/liter. The addition of 1% trehalose to TSB resulted in more than twice as much T6P in the wild type (6.3 μ g/mg cell protein) as for cells grown in TSB without trehalose, while growth in BHI did not significantly ($P > 0.05$) raise T6P levels (2.7 μ g/mg cell protein), although BHI was found to naturally contain approximately 300 mg/liter of trehalose. In contrast, *L. monocytogenes* 568:ΔTreA showed a gradient of increasing quantities of T6P depending on the type of medium. Although T6P levels for cells grown in TSB were not significantly ($P > 0.05$) greater than those for the wild type, the addition of yeast extract and 1% trehalose increased intracellular T6P from levels of 3.2 to 8.2 and 22.8 μ g/mg cell protein, respectively. Moreover, free trehalose in BHI was effectively transported into the *L. monocytogenes* 568:ΔTreA mutant, since cells grown in this medium accumulated the second-highest levels of T6P (10.7 μ g/mg cell protein).

Quantification of trehalose in cell extracts and phosphatase activity. Intracellular trehalose was detected in all cell extracts regardless of treatment or strain (Fig. 5). Trehalose levels for the wild type did not depend ($P > 0.05$) on the trehalose content in the growth medium, as concentrations ranged from 0.95 to 1.3 μ g/mg cell protein. Levels of trehalose in *L. monocytogenes* 568:ΔTreA extracts from the same treatment were consistently significantly greater ($P < 0.05$) than those for *L. monocytogenes* 568; e.g., mutant cells grown in TSB contained twice the trehalose levels found in cells of *L. monocytogenes* 568 grown in the same medium. In BHI (300 mg/liter trehalose), mutant trehalose concentrations increased significantly ($P < 0.05$) to approximately 6.6 μ g/mg protein, and the level was even higher (11.6 μ g/mg protein) when the mutant was grown in TSB-Tre (10,070 mg/liter trehalose). Current knowledge suggests that trehalose is imported into *Listeria* only via the PTS system as T6P. Therefore, the formation of trehalose in the mutant due to dephosphorylation of T6P was investigated by incubating crude extracts from *L. monocytogenes* 568:ΔTreA grown in MWB plus 1% glucose with T6P (10 mM) for 4 h at 37°C. After removal of phosphorylated sugars using ion exchange as before, sample trehalose was digested with por-

cine trehalase for 2 h to release 0.89 ± 0.21 μ g glucose/mg protein. Levels of glucose in control extracts not treated with trehalase remained unchanged. This indicates that the crude cell extracts contain phosphatase activity capable of cleaving the phosphate group from T6P.

Heat tolerance as affected by growth medium. All thermal death curves from the heat treatment (52°C) of early stationary-phase *L. monocytogenes* 568 and 568:ΔTreA cells from four different broth media displayed a lag period prior to entering a log-linear region of maximum inactivation (not shown). The results for the model parameters are given in Table 2.

The D_L (ca. 30 min) for the wild-type strain in BHI was significantly longer ($P < 0.05$) than D_L values obtained in TSB or TSB-YE (22.6 and 22.1 min, respectively). However, there was no significant difference ($P > 0.05$) between *L. monocytogenes* 568 cells grown and heated in BHI or TSB-Tre. This indicated that BHI (trehalose content, 310 mg/liter) in general induces a higher level of heat resistance in *L. monocytogenes* 568 than the other media used in this study. Growth in TSB (trehalose content, 70 mg/liter) resulted in similar thermal resistance for the mutant and the parent strain, as D_L values were not significantly different ($P > 0.05$) from each other (Table 2). Supplementation of TSB with either 0.6% yeast extract (trehalose content, 730 mg/liter) or 1% trehalose (10,070 mg/liter) resulted in significant increases ($P < 0.05$) in heat resistance for the mutant, with TSB-Tre cultures showing the highest level of thermotolerance ($D_L = 33.3$ min) among the TSB-based media (Table 2). This progression in thermal resistance correlated with increases in both intracellular T6P accumulated from the uptake of trehalose (Fig. 4) and high intracellular trehalose levels (Fig. 5). Interestingly, *L. monocytogenes* 568:ΔTreA cells grown in BHI were by far the most thermal-resistant cells observed in this study ($D_L = 44.1$ min) despite the fact that lower T6P and/or trehalose levels were found in these cells than in cells grown in TSB-Tre (Fig. 4 and 5) or TSB-YE. This indicates that other factors in BHI apart from an endogenous content of trehalose increased the ther-

TABLE 2. Thermal inactivation kinetics parameters for *Listeria monocytogenes* strains 568 and 568:ΔTreA at 52°C following growth on different media^a

Strain and growth medium (trehalose content, mg/liter)	β^b	ASE ^c (β)	tcm ^d	ASE (tcm)	Logistic D_L value ^e (min)
<i>568</i>					
TSB (70)	0.235	0.009	12.86	1.70	22.57
TSB-YE (730)	0.228	0.010	12.09	1.63	22.11
TSB-Tre (10,070)	0.202	0.019	15.62	3.26	26.35
BHI (310)	0.196	0.013	18.29	2.27	29.95
<i>568:ΔTreA</i>					
TSB (70)	0.203	0.010	11.80	2.27	23.05
TSB-YE (730)	0.176*	0.012	14.31	3.13	27.14
TSB-Tre (10,070)	0.161*	0.005	19.21*	1.43	33.31*
BHI (310)	0.152*	0.004	29.03*	1.10	44.09*

^a The inactivation kinetics was modeled using the modified Fermi distribution function (43). *, significantly different from value for the wild-type 568 for the same treatment ($P < 0.05$).

^b β , inactivation rate parameter for the log-linear region of the death curve.

^c ASE, asymptotic standard error.

^d tcm, lag parameter prior to the region of maximum inactivation rate.

^e D_L , time for the initial log decline when a lag phase exists (equation 2).

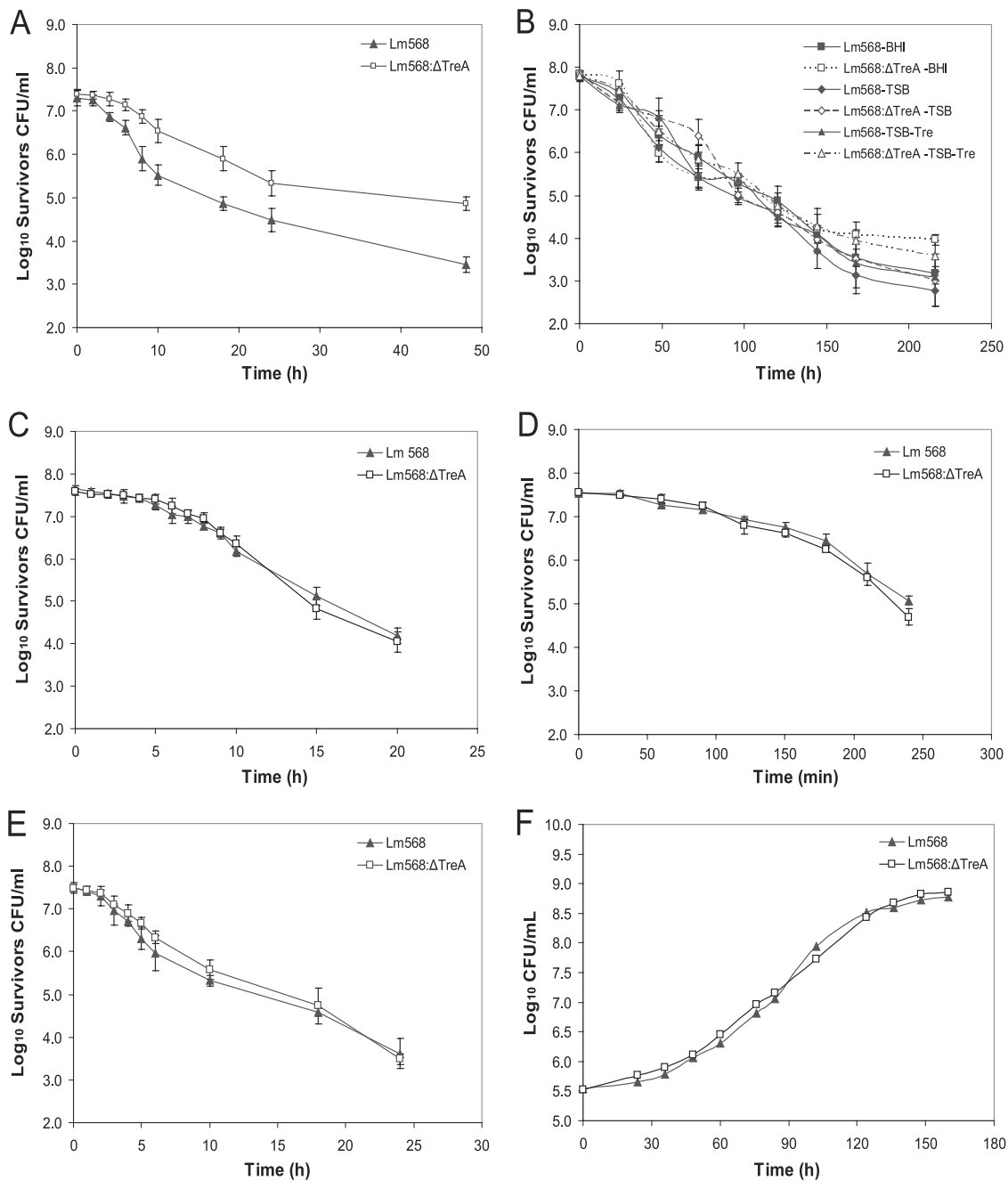


FIG. 6. Effects of various stressors on the survival or growth of *Listeria monocytogenes* 568 and mutant *L. monocytogenes* 568:ΔTreA. Cells were subjected to high osmolarity (20% NaCl in BHI) (A), repeated freeze-thaw cycling (−80 and 37°C every 24 h in BHI, TSB, and TSB-Tre) (B), ethanol (18%, vol/vol) (C), hydrogen peroxide (0.1%, vol/vol) (D), high acidity (pH 3.5) (E), and low temperature (4°C) (F). The results are the mean populations for three independent experiments with corresponding standard errors.

mal resistance of *L. monocytogenes* 568:ΔTreA. The enhanced thermal resistance of the mutant seen in BHI and TSB-Tre appears to be due mainly to an enhancement of the lag period (tcm values) (Table 2).

Tolerance to other environmental stresses. In addition to heat stress, several other stress factors were examined to evaluate the phosphotrehalase mutant’s stress hardiness. The *L. monocytogenes* 568:ΔTreA mutant demonstrated an increased capacity to survive high osmolarity (20% NaCl), repeated

freeze-thaw cycles, and desiccation. After 6 h in BHI with 20% NaCl, survivor counts for the phosphotrehalase mutant were significantly higher ($P < 0.05$) than those for *L. monocytogenes* 568 at all subsequent sampling times, resulting in 1.5 log more *L. monocytogenes* 568:ΔTreA survivors than wild-type survivors after 48 h (Fig. 6A). Less dramatic but noteworthy was the result of the exposure to repeated freeze-thaw cycles. Although, the mutant generally did not survive significantly ($P > 0.05$) better than the wild type for the majority of sampling

TABLE 3. Desiccation tolerances of *Listeria monocytogenes* strain 568 and its phosphotrehalase mutant 568: Δ TreA^a

Treatment	568		568: Δ TreA	
	Lag time (h)	Specific growth rate (h ⁻¹)	Lag time (h)	Specific growth rate (h ⁻¹)
Untreated	4.36 \pm 0.64	0.882 \pm 0.039	4.56 \pm 0.41	0.855 \pm 0.029
Desiccated	27.57 \pm 0.90*	0.861 \pm 0.018	23.24 \pm 0.75*	0.878 \pm 0.022

^a Cells ($\sim 10^6$ CFU) were air dried for 2 h in the bottom of 12-well tissue culture plates and then desiccated for 14 days at 16% RH and 20°C. Following desiccation, BHI was added to the wells and growth of untreated (control) and desiccated cells at 30°C monitored by measuring absorbance at 30-min intervals. Analysis of growth curves was done using DMFit (version 2.1; <http://www.ifr.ac.uk/safety/dmfit/>). *, significant difference ($P < 0.05$) between the means from triplicate experiments as determined by Student's *t* test.

times, the number of *L. monocytogenes* 568: Δ TreA survivors in BHI were 0.8 log greater than that of *L. monocytogenes* 568 survivors in the same medium (Fig. 6B). Moreover, *L. monocytogenes* 568: Δ TreA survived desiccation (RH of 16%) better than *L. monocytogenes* 568, as desiccated 568: Δ TreA cells exhibited a significantly ($P < 0.05$) shorter lag time (23.2 h) than *L. monocytogenes* 568 cells (27.6 h) prior to the onset of exponential growth following the treatment (Table 3). On the other hand, there were no significant differences ($P > 0.05$) between the survival and growth of the wild type and *L. monocytogenes* 568: Δ TreA when they were subjected to the other stress factors (ethanol, H₂O₂, low pH, and low temperature) examined in this study (Fig. 6C to F).

Complementation of 568: Δ T6P. In order to confirm that the deletion in the *treA* gene was responsible for the observed phenotype, *L. monocytogenes* 568: Δ TreA was back complemented with a vector containing the intact *treA* gene. As the promoter for the *tre* operon resides upstream from the *treB* gene (encoding the trehalose-specific PTS enzyme IIBC), it was decided to clone the entire *treA/treB* region into the XbaI site of the Gram-positive shuttle vector pAM401 to create pAM:TREOP. The construct was electroporated into mutant *L. monocytogenes* 568: Δ TreA and the resultant complemented strain named Δ TreA:pAM:TREOP. The phosphotrehalase activity in Δ TreA:pAM:TREOP was restored, as crude cell extracts liberated nearly 50 μ g of glucose/mg cell protein from 10 mM T6P after 1 h at 37°C. This indicated an overexpression of *treA*, since extracts from *L. monocytogenes* 568 liberated less than half this amount (22.5 μ g/mg cell protein) of glucose under the same conditions (Fig. 7). Moreover, the thermal death profile for the complemented mutant cells demonstrated a marked reduction in thermotolerance relative to mutant *L. monocytogenes* 568: Δ TreA (Fig. 8). The calculated D_L values for *L. monocytogenes* 568 and Δ TreA:pAM:TREOP were 30.5 and 23.1 min, respectively. However, the D_L value of 45.9 min for mutant *L. monocytogenes* 568: Δ TreA was significantly greater ($P < 0.05$) (Table 4). It should be noted that the resulting phenotype was the result of the activation of the *treA* gene and not due to the presence of the pAM401 vector, since the phosphotrehalase activity and heat resistance of *L. monocytogenes* 568/pAM401 (D_L value = 28.5 min) did not vary significantly ($P > 0.05$) from those of wild-type *L. monocytogenes* 568.

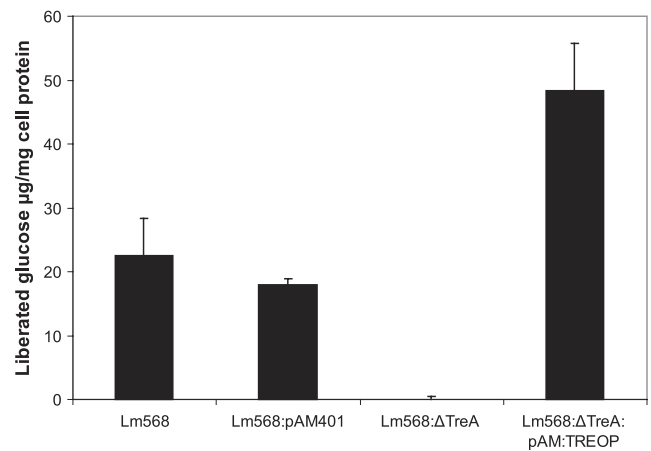


FIG. 7. Phosphotrehalase activity in crude cell extracts from *L. monocytogenes* 568 (wild type), *L. monocytogenes* 568/pAM401 (wild type with pAM401 shuttle vector), *L. monocytogenes* 568: Δ TreA (*treA* deletion mutant of *L. monocytogenes* 568) and its trans complement *L. monocytogenes* 568: Δ TreA:pAM:TREOP (the entire *tre* operon carried on the pAM401 shuttle vector) as measured by the glucose hexokinase assay. The results show the means \pm standard deviations ($n - 1$) for four samples (two replicates from two independent trials).

DISCUSSION

In this study, we have demonstrated that a 462-bp deletion in the *treA* gene (*lmo1254* for strain EGD-e) of *L. monocytogenes* strain 568 results in a thermo- and osmotolerant phenotype when the cells are grown in complex media containing trehalose. To our knowledge this represents the first study investigating the putative trehalose operon of *Listeria*. Based on DNA sequence, the predicted protein encoded by *treA* in *L. monocytogenes* EGD-e is highly similar to α, α -(1,1)-phosphotrehalase in *Bacillus subtilis* (12, 23, 47, 48) and to T6P hydro-

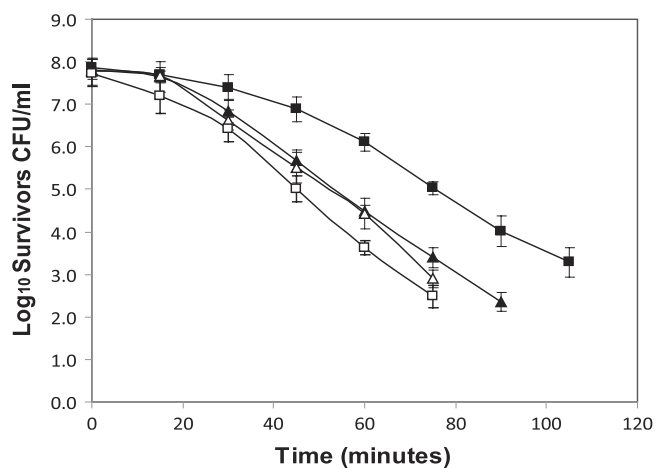


FIG. 8. Heat resistance of *L. monocytogenes* 568, the *treA* mutant, and the back-complemented *treA* mutant. Wild-type strain *L. monocytogenes* 568 (▲), *L. monocytogenes* 568/pAM401 (△), deletion mutant *L. monocytogenes* 568: Δ TreA (■), and pAM401:TREOP-transformed *treA* mutant Δ TreA:pAM:TREOP (□), were grown for 16 h at 37°C in BHI broth and subjected to a heat treatment of 52°C. The plot represents the mean survivors from three independent heat trials \pm standard errors.

TABLE 4. Thermal inactivation kinetics parameters at 52°C for *Listeria monocytogenes* 568, 568:Δ*treA*, complemented mutant Δ*treA*:pAM:TREOP, and 568/pAM401^a

Strain	β^b	ASE ^c (β)	tcm ^d	ASE (tcm)	Logistic D_L value ^e (min)
568	0.175	0.006	17.47	1.76	30.54
568:Δ <i>treA</i>	0.141*	0.005	29.64*	1.79	45.88*
Δ <i>treA</i> :pAM:TREOP	0.186	0.008	10.79*	2.12	23.08
568/pAM401	0.174	0.012	15.35	3.66	28.49

^a The inactivation kinetics was modeled using the modified Fermi distribution function (43). *, significantly different from value for the wild-type 568 for the same treatment ($P < 0.05$).

^b β , inactivation rate parameter for the log-linear region of the death curve.

^c ASE, asymptotic standard error.

^d tcm, lag parameter prior to the region of maximum inactivation rate.

^e D_L , time for the initial log decline when a lag phase exists (equation 2).

lase, the product of the *treC* gene, of *E. coli* (45). The predicted trehalose operon in *Listeria* was also shown to be similar in organization to the catabolic operons of other closely related bacteria such as *Lactococcus lactis* and *Lactobacillus plantarum* (1). In addition to *treA*, this operon also includes *treB*, which encodes a trehalose-specific IIBC transport protein complex required for the phosphorylation of trehalose via the phosphoenolpyruvate PTS (PEP-PTS) (47). The operon is regulated by the product of *treR*, a protein highly similar to the GntR-like family of regulators. The deletion of this gene in *Bacillus subtilis* strain 168 results in the constitutive expression of the operon (48).

In *B. subtilis*, T6P is formed during the translocation of trehalose across the cytoplasmic membrane via the PEP-PTS, and this in turn is hydrolyzed by phosphotrehalase to glucose and glucose-6-phosphate. We have demonstrated that the product of *treA* is indeed required for the cleavage of T6P in *L. monocytogenes* 568. Crude cell lysates from the wild-type strain efficiently hydrolyzed pure T6P, while *L. monocytogenes* 568:Δ*treA* lysate was devoid of this activity. According to the genomic map of *L. monocytogenes* strain EGD-e, there does not appear to be another putative mechanism for the passage of trehalose into the cytoplasm. Also, *L. monocytogenes* appears not to produce exogenous trehalases, as free glucose was not detected in MWB following growth of *L. monocytogenes* 568 with trehalose as the sole carbon source. Therefore, trehalose must first pass through a trehalose-specific or semispecific channel in the cytoplasmic membrane, requiring phosphorylation by a trehalose-specific enzyme IIBC complex (40) followed by cleavage of T6P by phosphotrehalase in the cytosol.

Depending on the trehalose concentrations in the growth media, our *treA* mutant accumulated high levels of T6P. In *B. subtilis*, trehalose is the external molecular inducer of the operon, whereby, in its absence, the TreR repressor turns down the transcription of *treA* (48). Moreover, T6P acts as the internal inducer for *treA*, where the phosphorylated sugar interferes with the repressor/*tre*-operator interaction, thus allowing for the transcription of the *treA* gene to proceed. Additionally, a *cis*-acting catabolite responsive element (CRE) has been identified in the promoter region of the operon just upstream of the open reading frame (ORF) for *treB* (37). Catabolites such as glucose, fructose, or mannitol repress the uptake of

trehalose (12, 19, 26). Here, glucose was shown to act as a repressor in a concentration-dependent manner, as decreasing phosphotrehalase activities were observed in extracts from *L. monocytogenes* 568 cells cultured in MWB with trehalose and increasing levels of glucose.

Studies conducted with yeasts have indicated that a buildup of T6P is toxic and results in increased sensitivity to environmental stresses (13, 17, 20, 55, 56). Duong et al. (14) also found that a *Lactobacillus acidophilus treB* (phosphotrehalase) deletion mutant was intolerant to stresses normally endured by the wild-type strain. In our study, elevated concentrations of intracellular T6P in *L. monocytogenes* 568:Δ*treA* came with a significant increase in the thermo- and osmotolerance relative to those of the parent *L. monocytogenes* 568. As the phosphorylated version of the disaccharide does not provide the same protection as trehalose (30, 39), this cannot explain the increase in stress hardiness. However, the increase in T6P came together with an accumulation of trehalose, reaching levels corresponding to ca. 50% of the T6P levels. Moreover, as no trehalase activity was observed in these extracts, trehalose levels remained stable. To explain the presence of trehalose in the mutant, dephosphorylation of T6P in crude mutant cell extracts was demonstrated in repeated experiments. This dephosphorylation of T6P and conversion into the compatible solute, trehalose, may be through the action of nonspecific phosphatases (15), which could explain why high levels of T6P are not causing the stress sensitivity. The same benefit may not be seen in phosphotrehalase mutants of other microorganisms such as *E. coli*, which possesses a separate mechanism for trehalose synthesis/degradation, including a trehalase that would directly cleave the trehalose molecule, thus preventing its simultaneous accumulation (53). Also, the facts that *L. monocytogenes* 568:Δ*treA* grew at a highly reduced rate in MWB with trehalose as the sole carbon source and that a truncated transcript from *treA* was detected may suggest some residual enzyme activity below the sensitivity of our assay.

When exposed to environmental stresses, intracellular trehalose can provide protection to cells of both bacteria and yeasts. In *Saccharomyces cerevisiae*, biosynthesis of the disaccharide is initiated by the same stimuli that induce a heat shock response (16, 17, 30, 38). In *E. coli*, the putative stationary-phase sigma factor σ^S controls the expression of *otsA* and *otsB*, the genes encoding T6P synthase and T6P phosphatase, respectively. However, this is only for stationary-phase-associated thermotolerance, as trehalose biosynthesis is not needed for the development of adaptive thermotolerance in logarithmic-phase cells (27). Back complementation with *treA* (and *treB*) removed the intracellular trehalose and decreased the thermotolerance at 52°C in comparison to that of *L. monocytogenes* 568:Δ*treA*; however, the Δ*treA*:pAM:TREOP complemented strain was also slightly more heat sensitive than wild-type *L. monocytogenes* 568. This may be explained by the fact that the *trans*-complemented mutant overexpresses phosphotrehalase activity. Normally, the *treA* gene is induced by the presence of T6P reaching a threshold level. Therefore, in the presence of extracellular trehalose, a basal intracellular T6P level exists. However, if *treA* is constitutively overexpressed, accumulation of T6P would cease and therefore no dephosphorylation of T6P could occur in Δ*treA*:pAM:TREOP; hence, trehalose would be absent in the cytoplasm. The mech-

anism by which trehalose confers thermotolerance is thought to be through its interaction with cellular proteins, preventing their denaturation and aggregation. Therefore, trehalose may act as a molecular chaperone working synergistically with heat shock proteins at elevated temperatures (31, 57).

In addition to having elevated heat resistance, the *treA* mutant was also more resistant than *L. monocytogenes* 568 to high osmolarity and desiccation. A 1.5-log increase in survivors was observed for the mutant over *L. monocytogenes* 568 after 48 h in the presence of 20% NaCl. Since desiccation can be viewed as an extreme case of osmotic stress (8, 41), further evidence for increased resistance to osmotic stress and membrane protection is provided by the finding that *L. monocytogenes* 568:Δ*TreA* had shorter recovery times than *L. monocytogenes* 568 when reconstituted in BHI after desiccation. This observation was presumably due to the presence of more surviving and/or undamaged cells. *Listeria* spp. normally deal with osmotic stress by accumulating the compatible solutes glycine betaine, carnitine, and proline (51). However, other bacteria display an osmotically induced accumulation of trehalose (44). For example, *E. coli* will accumulate trehalose under high osmolarity if the preferred osmolyte, glycine betaine, is not available (53). The preference for glycine betaine over trehalose under desiccation is interesting, since in *E. coli* it gives no survival advantage, yet accumulated endogenous trehalose provides significant protection for these cells (58). This indicates that for *E. coli* the role of these compatible solutes differs during conditions of drying and those of milder osmotic stress.

In other microorganisms, such as *E. coli* and *Saccharomyces cerevisiae*, intracellular trehalose accumulation also gives protection against high alcohol concentrations, oxidative stress, and cold temperatures (6, 31, 50). However, enhanced survivability was not observed when *L. monocytogenes* 568:Δ*TreA* was subjected to these stressors. *L. monocytogenes* 568 appears to have a naturally high resistance to ethanol and H₂O₂, relative to other *L. monocytogenes* strains (34). Therefore, the margin of protective capacity may be too narrow to expect increased survivability for the mutant. Also, since listeriae are known psychrotrophs (5, 54), it is not surprising that the accumulation of trehalose by the mutant, did not affect its growth profile at 4°C. However, in the case of mesophiles, such as *E. coli*, the accumulation of trehalose would be highly advantageous for survival at 4°C (31).

To conclude, this is the first study reporting on any of the genes of the putative trehalose operon in *L. monocytogenes*. We have demonstrated that *lmo1254* in *L. monocytogenes* 568 indeed encodes a phosphotrehalase that is required for the hydrolysis of T6P. Disruption of the *treA* gene results in the accumulation of T6P which is subsequently dephosphorylated, resulting in a buildup of the compatible solute trehalose. Although listeriae do not have a natural mechanism for the synthesis of trehalose as seen in other bacteria (e.g., *E. coli*), we have shown that the subsequent accumulation of intracellular trehalose in an *L. monocytogenes* strain 568 *treA* mutant leads to a phenotype of enhanced resistance against heat, high osmolarity, desiccation, and freeze-thaw cycling stresses. Therefore, in the artificially induced mutant, trehalose provides benefits similar to those observed in bacteria naturally synthesizing this molecule as a protectant against harsh conditions. This mutant may be used as a model system to gain a

better understanding of the protective capacity of organic osmolytes in *Listeria monocytogenes* and other closely related bacteria.

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