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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*\(^7\)

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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 *imo1254* (*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 hardness 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 foodborne 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-
tolerance (18). In one of these mutants, the location of the Tn17 insertion was determined to be in the putative treA gene (lm01254), 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 thermoresistant 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 trehalase 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 plasmids 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 using the UltraClean microbial DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA). DNA-modifying enzymes (XbaI restriction endonuclease, Taq polymerase, and KlenTaq polymerase) were obtained from New England Biolabs (Pickering, ON, Canada). DNA TOPO cloning kits were supplied by Invitrogen (Burlington, ON, Canada). QIAGEN Maxi-Prep kits were obtained from Qiagen Inc., Mississauga, ON, Canada. 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, Taq polymerase, and KlenTaq polymerase) were obtained from New England Biolabs (Pickering, ON, Canada). DNA TOPO cloning kits were supplied by Invitrogen (Burlington, ON, Canada). QIAGEN Maxi-Prep kits were obtained from Qiagen Inc., Mississauga, ON, Canada.

**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′-ACCGTCTAGAAGCTTTGCGGCGCT-3′), SOE-T6P-B (5′-GGAGTTAAAAAGGGTAGGCAGGCTCCA-3′), and SOE-T6P-D (5′-CTGCTCTAGAGGGGATTOCC GGTATAC-3′), SOE-T6P-C was complementary to the 5′ tail of SOE-T6P-B. Sequences for the deletion restriction XbaI (underlined regions) were introduced into primers SOE-T6P-A and SOE-T6P-D to facilitate cloning. The PCR products were cleaved 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; QiBiogene, 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 ΔtreA fragment was excised, purified as before, and then

**TABLE 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Species and strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong> L. monocytogenes</td>
<td>568 Wild type; serotype 1/2a; from shrimp-processing plant</td>
<td>25</td>
</tr>
<tr>
<td>32F1</td>
<td>Tn917 mutant of 568; Erm'</td>
<td>18</td>
</tr>
<tr>
<td>568:ΔTreA</td>
<td>568 deletion mutant; TreA</td>
<td>This study</td>
</tr>
<tr>
<td>ΔTreA:pAM:TreOP</td>
<td>pAM401:TreA complement of 568ΔT6P; Crm' Tet' TreA+</td>
<td>This study</td>
</tr>
<tr>
<td>568:paulaΔTreA</td>
<td>568 carrying paulaΔTreA with treA deletion insert</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli</td>
<td>TCTOP:ΔTreA</td>
<td>TOP10 carrying pCR2.1 TOPO with 568 treA insert with deletion</td>
</tr>
<tr>
<td>TCTOP:TreOP</td>
<td>TOP10 carrying pCR2.1 TOPO with intact 568 Tre operon</td>
<td>This study</td>
</tr>
<tr>
<td>DH5α</td>
<td>Cloning host; F− endA1 glnV44 thi−1 recA1 gyrA96 relA1 lac hte</td>
<td>24</td>
</tr>
<tr>
<td>Δ(lacZYA-argF)U169 hsdR17 mcrA</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>XL10-GOLD Kan'</td>
<td>Cloning host; endA1 glnV44 recA1 thi−1 g496 deoR1 gyrA96 deoR1 g680lacZ ΔM15</td>
<td>Stratagene</td>
</tr>
<tr>
<td>XLCCV1</td>
<td>XL10-GOLD carrying pCVV1 expression vector</td>
<td>This study</td>
</tr>
<tr>
<td>TCE-paulaA:TreA</td>
<td>DH5α host carrying pAUL-A with treA deletion insert</td>
<td>This study</td>
</tr>
<tr>
<td>TOP10</td>
<td>F− mcrA Δ(mrr-hsdRMS-mcrBC) ΔlacZ474 deoR1 lacYI ara−1 rpsL13 lacZ57 galU galK rpsL13 (Strr) endA1 Δ</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td>paulaA</td>
<td>Gram-positive and Gram-negative shuttle vector; oriTS, LacZa-, Erm'</td>
</tr>
<tr>
<td>pAM401</td>
<td>Gram-positive and Gram-negative shuttle vector; Crm' Tet'</td>
<td>60</td>
</tr>
<tr>
<td>pCR:ΔTreA</td>
<td>pCR2.1 TOPO with 568 treA insert with deletion</td>
<td>This study</td>
</tr>
<tr>
<td>paulaA:ΔTreA</td>
<td>paulaA with truncated 568 treA gene; Erm'</td>
<td>This study</td>
</tr>
<tr>
<td>pAM401:TreOP</td>
<td>pAM401 with intact 568 treA gene; Crm', Tet' treA+</td>
<td>This study</td>
</tr>
<tr>
<td>pCVV1</td>
<td>pCYB1-derived expression vector carrying B. subtilis treA; Tac promoter, Amp'</td>
<td>56</td>
</tr>
<tr>
<td>pCR2.1 TOPO</td>
<td>ColE1 Amp' Kan' lacZa</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

All media and antibiotics were purchased from Oxoid Canada Inc. (Mississauga, ON, Canada) and Sigma-Aldrich Chemical Company (Oakville, ON, Canada), respectively.
ligated into the dephosphorylated temperature-sensitive shuttle vector pUAA-L with T4 DNA ligase. The resulting plasmid, designated pUAA:LtreA, 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 tre deletion mutant. A mixture of the pUAA:LtreA 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 pUAA:LtreA plasmid was confirmed by plasmid extraction and restriction digestion of the DNA with XbaI. For the integration of pUAA:LtreA 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. Mlodzik 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-TO-6A and SOE-TO-6D.

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 (5 μg/ml Erm) and incubated at 35°C for 24 h. One hundred colonies were patched onto BHI agar 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 MBW-trehalose medium were subjected to PCR using primers SOE-TO-6A and SOE-TO-6D to select recombinants displaying a single amplicon of 1,061 bp. The in-frame deletion was confirmed by sequencing, and the deletion mutant was named L. monocytogenes 568:ΔtreA.

Complementation of L. monocytogenes 568:ΔtreA. Phosphotrehalase activity was restored in L. monocytogenes 568:ΔtreA 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′-CCGGTACCATATAAGGAAAATCCTG-3′) and TreOp-R (5′-AATCTAGAACCGGCAATGTCCAATAATT-3′). The underlined bases denote XbaI restriction sites to facilitate cloning. The amplicon was TA cloned into TOPO CR2.1, 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 CIP. The resulting construct, pAM401:TreOP, was electroporated into competent cells of the deletion mutant L. monocytogenes 568:ΔtreA 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. Resistant colonies were confirmed on MBW-trehalose medium and measured for 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 Hellett 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 BHI-trehalose buffer (50 mM treBis-[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 Vishonic 600 cell disruptor (Virtis, Gardiner, NY). Cellular debris was removed by centrifugation (10,000 × g, 30 min, 0°C). The supernatant was confirmed on MBW-trehalose medium and stored at −80°C until use. Prior to analysis, the supernatant was brought to 1000 μl by the addition 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 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 bi-Tris buffer. Proteins were first fractionated by passage through a DEAE Sephadex A-50 anion-exchange column and elution in 5-ml fractions with an increasing gradient of ammonium hydroxide (pH 12.5). The protein was removed by freeze-drying following by redissolving the sample in 50 mM b-Tris buffer (pH 7.0).

Determination of T6P levels in cell extracts. Intracellular T6P in L. monocytogenes 568 and 568:ΔtreA was determined using the method of Van Vaeck et al. (35). The intracellular T6P content of L. monocytogenes 568:ΔtreA, which was isolated from Bacillus subtilis (a gift from C. Van Vaeck and J. M. Thewelein, 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 Goto and Dahl (22) and Van Vaeck et al. (56) with minor modifications. Briefly, the cloning vector 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 ultra-centrifugation 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 bi-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 bi-Tris buffer. For size fractionation, the sample was loaded on a Superdex 75 gel filtration column equilibrated with bi-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 (4 mg/ml).

In addition, the enzyme activity was also tested on 10 mg/ml p-nitrophenyl-β-D-glucosaminide (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 TPB 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 then 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 (6000 × 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 acidic 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 18 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 × [g(glucone)/g(trehalose)]) was reported as µg/ml 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, TSA, TSBl-YE, and TSBL-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 phenomenon:

\[ \log(N/N_0) = \log\left(1 + e^{-\frac{t - t_{cm}}{N}}\left(1 + e^{(t - t_{cm})/\mu}\right)\right) \]

where \(N\) is the number of survivors at any time (t), \(N_0\) is the initial population at \(t = 0\), \(\mu\) 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 (DL\(\alpha\)) was calculated as previously described by Pruitt and Kamau (43):

\[ DL\alpha = t_{cm} + \left[\ln(9 + 10^{-\beta})\right] \beta \]

Where appropriate, Student’s t test (α = 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, TSBl (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
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 mM glucose/mg cell protein was liberated 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:ΔTreA were assessed for intracellular 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:ΔTreA, 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-
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 μg/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 μg/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 μg/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 μg/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 μg/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 μg/mg protein, and the level was even higher (11.6 μg/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 porcine trehalase for 2 h to release 0.89 ± 0.21 μg 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 D₅ (ca. 30 min) for the wild-type strain in BHI was significantly longer (P < 0.05) than D₅ 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 D₅ 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 thermostolerance (D₅ = 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 (D₅ = 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 thermal resistance for the wild-type strain in BHI was significantly longer (P < 0.05) than D₅ 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 D₅ 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 thermostolerance (D₅ = 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 (D₅ = 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 thermokinetic parameters for the model parameters are given in Table 2.

![Table 2. Thermal inactivation kinetics parameters for Listeria monocytogenes strains 568 and 568:ΔTreA at 52°C following growth on different media.](attachment:image)

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

<table>
<thead>
<tr>
<th>Strain and growth medium (trehalose content, mg/liter)</th>
<th>b₀</th>
<th>ASE (β)</th>
<th>tcm</th>
<th>ASE (tcn)</th>
<th>Logistic D₅ value (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSB (70)</td>
<td>0.235</td>
<td>0.009</td>
<td>12.86</td>
<td>1.70</td>
<td>22.57</td>
</tr>
<tr>
<td>TSB-YE (730)</td>
<td>0.228</td>
<td>0.010</td>
<td>12.09</td>
<td>1.63</td>
<td>22.11</td>
</tr>
<tr>
<td>TSB-Tre (10,070)</td>
<td>0.202</td>
<td>0.019</td>
<td>15.62</td>
<td>3.26</td>
<td>26.35</td>
</tr>
<tr>
<td>BHI (310)</td>
<td>0.196</td>
<td>0.013</td>
<td>18.29</td>
<td>2.27</td>
<td>29.95</td>
</tr>
<tr>
<td>568:ΔTreA</td>
<td>b₀</td>
<td>ASE (β)</td>
<td>tcm</td>
<td>ASE (tcn)</td>
<td>Logistic D₅ value (min)</td>
</tr>
<tr>
<td>-------------------------------------------------------</td>
<td>----</td>
<td>--------</td>
<td>-----</td>
<td>----------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>TSB (70)</td>
<td>0.203</td>
<td>0.010</td>
<td>11.80</td>
<td>2.27</td>
<td>23.05</td>
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<tr>
<td>TSB-YE (730)</td>
<td>0.176</td>
<td>0.012</td>
<td>14.31</td>
<td>3.13</td>
<td>27.14</td>
</tr>
<tr>
<td>TSB-Tre (10,070)</td>
<td>0.161</td>
<td>0.005</td>
<td>19.21</td>
<td>1.43</td>
<td>33.31</td>
</tr>
<tr>
<td>BHI (310)</td>
<td>0.152</td>
<td>0.004</td>
<td>29.03</td>
<td>1.01</td>
<td>41.04</td>
</tr>
</tbody>
</table>

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 b₀, inactivation rate parameter for the log-linear region of the death curve.

ASE, asymptotic standard error.

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

d D₅, time for the initial log decline when a lag phase exists (equation 2).
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

![Graphs](image1.png)  
**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.
times, the number of *L. monocytogenes* 568:ΔTreA 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:ΔTreA survived desiccation (RH of 16%) better than *L. monocytogenes* 568, as desiccated 568:ΔTreA 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:ΔTreA when they were subjected to the other stress factors (ethanol, H$_2$O$_2$, low pH, and low temperature) examined in this study (Fig. 6C to F).

**Complementation of 568:ΔT6P.** In order to confirm that the deletion in the treA gene was responsible for the observed phenotype, *L. monocytogenes* 568:ΔTreA 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 IIIBC), 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:ΔTreA and the result complemented strain named ΔTreA:pAM:TREOP. The phosphotrehalase activity in ΔTreA:pAM:TREOP was restored, as crude cell extracts liberated nearly 50 μg 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 μg/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:ΔTreA (Fig. 8). The calculated $D_2$ values for *L. monocytogenes* 568 and ΔTreA:pAM:TREOP were 30.5 and 23.1 min, respectively. However, the $D_2$ value of 45.9 min for mutant *L. monocytogenes* 568:ΔTreA 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 ($D_2$ value = 28.5 min) did not vary significantly ($P > 0.05$) from those of wild-type *L. monocytogenes* 568.

**TABLE 3. Desiccation tolerances of *Listeria monocytogenes* strain 568 and its phosphotrehalase mutant 568:ΔTreA**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>568</th>
<th>568:ΔTreA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lag time (h)</td>
<td>Specific growth rate (h$^{-1}$)</td>
</tr>
<tr>
<td>Untreated</td>
<td>4.36 ± 0.64</td>
<td>0.882 ± 0.039</td>
</tr>
<tr>
<td>Desiccated</td>
<td>27.57 ± 0.90*</td>
<td>0.861 ± 0.018</td>
</tr>
</tbody>
</table>

* Cells (~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 37°C monitored by measuring absorbance at 30-min intervals.

During growth curves was done using DMFit (version 2.1; http://www.ifr.ac.uk/safety/dmfit/).

**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:ΔTreA (treA deletion mutant of *L. monocytogenes* 568) and its trans complement *L. monocytogenes* 568:ΔTreA:pAM:TREOP (the entire tre operon carried on the pAM401 shuttle vector) as measured by the glucose hexokinase assay. The results show the means ± standard deviations (n = 4) 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 trehalase 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-
lase, the product of the treC gene, of *E. coli* (45). The predicted trehalase 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: ΔTreA 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 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: ΔTreA 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 hardness. 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: ΔTreA 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:ΔTreA; however, the ΔTreA: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 ΔTreA:pAM:TREOP; hence, trehalose would be absent in the cytoplasm.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>β^b^</th>
<th>ASE^c^ (β)</th>
<th>τcm^d^</th>
<th>ASE^c^ (τcm)</th>
<th>Logistic D_{10} value^e^ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>568</td>
<td>0.175</td>
<td>0.006</td>
<td>17.47</td>
<td>1.76</td>
<td>30.54</td>
</tr>
<tr>
<td>568:ΔTreA</td>
<td>0.141*</td>
<td>0.005</td>
<td>29.64*</td>
<td>1.79</td>
<td>45.88*</td>
</tr>
<tr>
<td>ΔTreA:pAM:TREOP</td>
<td>0.186</td>
<td>0.008</td>
<td>10.79*</td>
<td>2.12</td>
<td>23.08</td>
</tr>
<tr>
<td>568:pAM401</td>
<td>0.174</td>
<td>0.012</td>
<td>15.35</td>
<td>3.66</td>
<td>28.49</td>
</tr>
</tbody>
</table>

^a^ The inactivation kinetics was modeled using the modified Fermi distribution function (43). *^b^* significantly different from value for the wild-type 568 for the same treatment (P < 0.05).  
^c^ ASE, asymptotic standard error.  
^d^ τcm, lag parameter prior to the region of maximum inactivation rate.  
^e^ D_{10}, time for the initial log decline when a lag phase exists (equation 2).
anism by which trehalose confers thermostolerance 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.

**ACKNOWLEDGMENTS**

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