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A novel gene, *lstC*, of *Listeria monocytogenes* is implicated in high salt tolerance

Laurel S. Burall, Alexandra C. Simpson, Luoth Chou, Pongpan Laksanalamai, Atin R. Datta*

Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, Laurel, MD 20708, USA

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ABSTRACT

Listeria monocytogenes, causative agent of human listeriosis, has been isolated from a wide variety of foods including deli meats, soft cheeses, cantaloupes, sprouts and canned mushrooms. Standard control measures for restricting microbial growth such as refrigeration and high salt are often inadequate as *L. monocytogenes* grows quite well in these environments. In an effort to better understand the genetic and physiological basis by which *L. monocytogenes* circumvents these controls, a transposon library of *L. monocytogenes* was screened for changes in their ability to grow in 7% NaCl and/ or at 5 °C. This work identified a transposon insertion upstream of an operon, here named *lstABC*, that led to a reduction in growth in 7% NaCl. In-frame deletion studies identified *lstC* which codes for a GNAT-acetyltransferase being responsible for the phenotype. Transcriptomic and RT-PCR analyses identified nine genes that were upregulated in the presence of high salt in the Δ*lstC* mutant. Further analysis of *lstC* and the genes affected by Δ*lstC* is needed to understand LstC's role in salt tolerance.

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1. Introduction

Listeria monocytogenes is a foodborne pathogen that causes invasive listeriosis and gastroenteritis. Invasive listeriosis typically affects the elderly, pregnant women and immune-compromised individuals and causes meningitis, encephalitis, miscarriage and stillbirth (Datta, 2003). These infections are associated with a high mortality rate of approximately 20% making *L. monocytogenes* responsible for many of the foodborne fatalities, despite a relatively low incidence of disease (Silk et al., 2012). Among pregnant Hispanic women, nearly a third of these infections lead to fetal loss or neonatal death (Silk et al., 2012). Additionally, these infections have a high percentage of hospitalizations and protracted recoveries due to the neurological damage associated with them (Havelaar et al., 2012). *L. monocytogenes* has also been shown to cause gastroenteritis characterized by nausea, abdominal cramping and diarrhea, though the prevalence of these infections is unknown as gastroenteritis cases are not routinely screened for *L. monocytogenes* (Barbuddhe and Chakraborty, 2009; Norton and Braden, 2007).

Listeria contamination is a problem in foods considered ready-to-eat as they are not subjected to a final kill step prior to consumption. These ready-to-eat (RTE) foods include soft cheeses and deli meats, as well as fresh produce including salad greens, sprouts and cantaloupe. RTE foods with long shelf-life, high water activity and neutral pH are at greater risk for becoming a vehicle of foodborne listeriosis because of the high growth of *L. monocytogenes*. The standard practices for controlling microbial growth in such foods, e.g. refrigeration and high salt, are not adequate as *Listeria* survive and grow relatively well under such conditions (Lado and Yousef, 2007). *Listeria* has been isolated from various foods containing high levels of NaCl and several studies have indicated extended survival of *Listeria* in high salt concentrations. However, under experimental conditions, *Listeria* growth appears to be limited to 7–9% of NaCl (Lado and Yousef, 2007). The ability to survive and grow in foods containing high salt is further complicated by the presence of naturally occurring compatible solutes, also known as osmoprotectants, e.g. glycine betaine, carnitine and glycerol in many foods. Although it is known that high salt reduces microbial growth by reducing water activity, changing electrochemical potential across cell membrane, a thorough understanding of the genetics and underlying adaptation mechanisms is required for development of effective control measures.

Several studies have identified genes that affect *Listeria*'s ability to grow at low temperature and high salt environments through modification of compatible solute transport, membrane lipid composition alteration and changes in ribosomal protein

* Corresponding author. Tel.: +1 240 402 3422; fax: +1 301 210 7975.
E-mail address: atin.datta@fda.hhs.gov (A.R. Datta).

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(Laksanalamai et al., 2010). Previous studies have already shown the applicability of transposon mutagenesis to aid in the identification of genes associated with *Listeria*'s ability to grow in what would normally be considered restrictive conditions (Burall et al., 2012; Chassaing and Auvray, 2007; Ells et al., 2009; Madeo et al., 2012). Previous work in our laboratory identified a role for *iap* in salt and cold tolerance and verified a previously established role for the *gbu* operon in cold and salt tolerance (Angelidis and Smith, 2003a, 2003b; Burall et al., 2012; Ko and Smith, 1999). In this study, we applied the same techniques used before to screen for novel genes implicated in the cold and salt tolerance phenotypes of *L. monocytogenes*. This work identifies and characterizes the phenotype and expression alterations associated with the deletion of a previously unknown gene, encoding a putative acetyltransferase, with a role in salt tolerance.

2. Materials & methods

2.1. Strains, media and reagents

L. monocytogenes LS402 is a serotype 4b strain that was isolated during the Italian corn salad gastroenteritis outbreak (Aureli et al., 2000). *L. monocytogenes* LS411 is a serotype 4b food strain from the 1985 Los Angeles Jalisco cheese invasive listeriosis outbreak (Linnan et al., 1988). These two strains served as parent strains for the various mutants generated in this study. A full list of *L. monocytogenes* strains used in this study is provided in Table 1. *E. coli* HB101 was used to maintain pLTV3 and its culture conditions are described elsewhere (Burall et al., 2012; Camilli et al., 1990). As previously described (Burall et al., 2012), *L. monocytogenes* strains were grown in brain heart infusion (BHI) broth or BHI agar. Salt growth was assessed in BHI broth or BHI agar supplemented with 7% NaCl. Antibiotics used for isolation, characterization and maintenance of the mutants and antibiotic usage during spectrophotometric screens are described elsewhere (Burall et al., 2012). *E. coli* TOP10 (Invitrogen, Carlsbad, CA) was used for maintenance of pKSV7 (Smith and Youngman, 1992) with chloramphenicol (10 ug/mL) used for maintenance and selection of it and its derived constructs. Antibiotics were obtained from Sigma–Aldrich (St. Louis, MO). All media were obtained from BD (Franklin Lakes, NJ). Restriction enzymes and DNA ligase were purchased from New England Biolabs (Ipswich, MA), and used as per the manufacturer's protocols.

Table 1
Listeria monocytogenes strains used in this study.

Strain	Genetic background	Source
LS402	Wildtype, 1997 Italian Corn Salad Outbreak, 4b	Martin Wiedmann
LS411	Wildtype, 1985 Jalisco Cheese Outbreak, 4b	Martin Wiedmann
LS401	Wildtype, EGDe, serotype 1/2a	Lab collection
LS661	Wildtype, 2010 Chicken Salad Outbreak, 1/2a	Lab collection
LS667	Wildtype, 2011 Cantaloupe Outbreak, serotype 1/2b	Lab collection
LS654	LS402 Tn917:*LMOf2365_2170-lstA* intergenic	This study
LS592	LS402 Δ*lstA*	This study
LS593	LS411 Δ*lstA*	This study
LS652	LS402 Δ*lstABC*	This study
LS653	LS411 Δ*lstABC*	This study
LS725	LS402 Δ*lstC*	This study
LS723	LS411 Δ*lstC*	This study
LS832	LS401 Δ*lstC*	This study
LS824	LS667 Δ*lstC*	This study
LS950	LS661 Δ*lstC*	This study
LS951	LS402 pIMK2	This study
LS952	LS725 pIMK2	This study
LS953	LS725 pIMK2-*lstC*	This study

2.2. Generation of transposon mutants

Competent LS402 was electroporated with pLTV3 as described previously (Burall et al., 2012; Park and Stewart, 1990). Transformants were selected on BHI agar containing tetracycline (12.5 μg/mL), erythromycin (1 μg/mL) and lincomycin (25 μg/mL). The presence of pLTV3 in the transformants was confirmed by isolation of the plasmid from *Listeria* using the QiaPrep miniprep plasmid isolation kit (Qiagen, Valencia, CA) with the addition of 10 μg/mL lysozyme (Sigma–Aldrich, St. Louis, MO) in P1 buffer and incubation of the resuspended cells for 10 min at room temperature before proceeding with the manufacturer's protocol. The plasmid recovered from *L. monocytogenes* was verified via restriction digest with *Bam*HI. A library of LS402 Tn917 mutants was then generated using the procedures described previously (Burall et al., 2012). The library, consisting of approximately 5200 $Tet^{S}Ery^{R}Lin^{R}$ isolates, was assembled in 96-well microtiter plates into groups of 60 isolates each for screening purposes.

2.3. Screening of transposon mutants

The ability of the mutants to grow in three different conditions was examined in both BHI broth or agar cultures. Growth was assessed using a Spectramax M2 (Molecular Devices, Downingtown, PA) and a Growth Curves Bioscreen-C (Growth Curves USA, Piscataway, NJ) measuring absorbance at 600 nm for BHI broth cultures or visual assessment for BHI agar cultures. The growth of each mutant was assessed individually but analyzed against the averaged results of all the prospective mutants. The three conditions were unmodified BHI broth at 37 °C (control), unmodified BHI broth at 5 °C (cold), and BHI broth supplemented with 7% NaCl at 37 °C (salt). A starter plate containing 60 isolates, each in its own respective well, was used to inoculate all of the trial conditions as described elsewhere (Burall et al., 2012). Growth on agar plates was assessed daily while growth in the microtiter plates was assessed using absorbance at 600 nm at intervals previously used (Burall et al., 2012). Mutants identified as having altered growth in either of the stress conditions with no alteration in non-stress conditions were further tested (secondary screen) for their altered phenotypes. These secondary screens allowed the use of replicate cultures of each mutant to verify reproducibility. Putative cold-altered mutants were assessed using the Bioscreen-C set at 5 °C from 24 h to 120 h post inoculation. A third screen was performed in quadruplicate without antibiotics on any putative mutants still showing altered growth during secondary screens, using LS402 as the control strain. Cold-growth was measured for at least eight days to allow entry into stationary phase to determine maximum growth levels. The absorbance measurements were used to determine exponential growth rate, maximum absorbance and, where possible, the length of lag and exponential phases. Any outliers, defined as ±1 standard deviation from the average, were selected for further screening. In the third screening, individual strains were compared to the averaged results for LS402 and outliers were defined as before. Growth data was also plotted to visually compare alterations more readily observed in a growth curve which allowed more nuanced analysis of the growth, for example a variable growth rate that was not observable in a single averaged number.

2.4. Analysis of growth phenotypes

Candidate Tn917 mutants, consistently showing altered growth either in 7% salt or at 5 °C, and subsequent deletion mutants were assessed via colony forming units (CFU)/mL in both the cold and salt conditions to confirm the changes in their growth phenotype.