EFFECTS OF GROWTH TEMPERATURE ON THE REGULATION OF HOST CELL INVASION AND ACID STRESS RESPONSE IN LISTERIA MONOCYTOGENES

by Reid Aaron Ivy

EFFECTS OF GROWTH TEMPERATURE ON THE REGULATION OF HOST CELL INVASION AND ACID STRESS RESPONSE IN *LISTERIA MONOCYTOGENES*

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Listeria monocytogenes is a foodborne bacterial pathogen that has been isolated from various natural and urban environments during different seasons and has been shown to persist in food processing environments. *L. monocytogenes* has the ability to grow at refrigeration temperatures and is therefore a danger to makers and consumers of ready-to-eat (RTE) food products, as these foods are not subjected to a heating step prior to consumption. During transmission from the environment through foods to humans, *L. monocytogenes* must adapt to a range of environmental conditions including varying temperature, preservative stress, gastric stress, and host immunological defenses. Mechanisms of environmental adaptation in *L. monocytogenes* include two-component response regulators (RRs), alternative sigma factors, and other positive (e.g., PrfA) and negative (e.g., CtsR) transcriptional regulators. We characterized the effects of *L. monocytogenes* growth temperature on (i) the contributions to *in vitro* host cell invasion of 14 RRs, alternative sigma factor σ^B, virulence regulator PrfA and surface associated proteins InlA and FlaA and (ii) the response to sudden acid shock. Overall, invasion was higher for *L. monocytogenes* grown at 30°C compared to 37°C. RR mutants Δ*cheY* and Δ*degU* were invasion-deficient when grown at 30°C, but not 37°C. We observed 37°C-growth-dependent

functional synergisms between σ^B and PrfA in contributing to host cell invasion and contributory synergisms between FlaA and InlA at both temperatures. Bacteria grown at 37°C were more resistant to HCl acid shock (pH 3.5) than cells grown at 7°C. For cells grown at 37°C, the transcriptional response to acid treatment involved the induction of genes with prior implication in acid response and intracellular survival, whereas the response of 7°C-grown cells involved the induction of two large operons of bacteriophage genes, which may explain the increased survival of 37°C grown cells. Our data show that growth temperature affects regulation of host cell invasion and the response to acid shock, which are both vital stages in *L. monocytogenes* transmission. Therefore, growth temperature should be considered an important variable in modeling *L. monocytogenes* stress survival and virulence.

BIOGRAPHICAL SKETCH

Reid A. Ivy was born in Hot Springs, AR in 1980 and lived in Benton, AR until he graduated from Benton High School in 1999. He attended the University of Arkansas Fayetteville (UAF) as an undergraduate from 1999-2003. During his undergraduate training he completed an internship at Tyson Foods, Inc, and served as an undergraduate researcher in the UAF Food Safety Lab. He earned a B.S. in Microbiology in 2003, and less than three weeks later, started the M.S. program in Cell and Molecular Biology under Dr. Michael Johnson in the Department of Food Science, where he learned the fundamentals of microbiology research and gained an appreciation for the importance of food safety research. After completing his M.S. degree in 2005, Reid accepted a job as a technician in Dr. Martin Wiedmann's lab at Cornell University and was admitted to the Ph.D program in Food Science and Technology in the fall of 2006. During his time as a student, Reid gave oral presentations at the American Society for Microbiology (ASM) General Meeting in Toronto, ON and The International Association for Food Processors Annual Meeting in Columbus, OH and participated in the 2008 Kadner Institute in Boulder, CO. He was awarded an ASM student travel grant in 2007 and an Institute for Food Technologists Foundation graduate scholarship in 2009. He has participated in extracurricular activities within the Food Science Department, including Food Science Club, IFTSA College Bowl, 4H Career Explorations and in the spring of 2009 founded the Cheese Club at Cornell with three other graduate students.

This dissertation is dedicated to my Mother and Father. Without their sacrifices, the work presented here would not have been possible

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LIST OF ABBREVIATIONS

ABHI	Acidified BHI
AGF	Artificial gastric fluid
BHI	Brain heart infusion
CDC	Centers for Disease Control
cDNA	Complementary DNA
CFU	Colony forming units
FSL	Food Safety Lab
GSEA	Gene set enrichment analysis
HK	Histidine kinase
HMM	Hidden Markov model
LB	Luria-Bertani
L. monocytogenes	*Listeria monocytogenes*
MOPS	3-morpholinopropane-1-sulfonic acid
PFU	Plaque forming units
PBS	Phosphate-buffered saline
RR	Response regulator
TCS	Two component regulatory system

CHAPTER ONE

Introduction

L. monocytogenes is a gram-positive, foodborne pathogen. Symptoms of listeriosis include gastroenteritis, meningitis, and spontaneous abortion. Listeriosis has a mortality rate of 20-30% (Bortolussi, 2008) and has been estimated to cause approximately one-third of all deaths attributed to known foodborne pathogens every year in the U.S. (Mead *et al.*, 1999). Though the number of culture-confirmed cases of listeriosis have declined since 1997 (CDC, 2010), recent estimates have put the annual cost of listeriosis in the U.S. at $2.3 billion (Ivanek *et al.*, 2004). Therefore, a reduction in the number of listeriosis cases would have a significant economic and social benefit, and an improved understanding of *L. monocytogenes* transmission is vital to achieving this goal.

Foodborne *L. monocytogenes* pathogenesis begins with the organism's attachment and entry into intestinal epithelial cells, which is facilitated by the interaction of internalin A (InlA) expressed on the bacterial surface with E-cadherin expressed on the host epithelial surface. Inside the host vacuole, the hemolysin Listeriolysin O (LLO), aided by metalloprotease (Mpl), destabilizes the host cell vacuole, allowing the organism to escape into the host cell cytosol. Bacterial proliferation occurs in the host cytosol while the *L. monocytogenes* ActA protein facilitates the assembly of comet-like tails made of host cell actin, which propel the bacterium into neighboring cells. Phospholipases, encoded by *plcA* and *plcB* contribute to lysis of the second double-membrane vacuole, which allows the entire intracellular proliferation process to repeat. Subsequent systematic dissemination may occur, resulting in central nervous system disorders such as encephalitis and

meningoencephalitis (Vazquez-Boland *et al.*, 2001). *L. monocytogenes* has the ability to cross the fetal/placental barrier (Disson *et al.*, 2008); therefore, perinatal listeriosis may result in spontaneous abortion or stillbirth.

L. monocytogenes is transmitted from natural environmental reservoirs, including soil, water, or fecal matter (Sauders *et al.*, 2006), through food to humans. The organism persists in food processing environments (Lappi *et al.*, 2004; Orsi *et al.*, 2008) where it may be transferred to food products. In foods such as ready-to-eat deli meat, the organism may be exposed to low temperatures and growth inhibitors, which impart stresses such as acid stress (e.g., sodium lactate) and/or osmotic stress (e.g., NaCl). Upon ingestion, *L. monocytogenes* likely experiences an up-shift in environmental temperature, along with a battery of environmental stresses including, low pH of the gastric environment, osmotic stress from the lower intestinal environment, and finally low pH in the host cell vacuole or in macrophages. In each of the stages of transmission, the organism must adapt to varying environmental temperatures and stresses.

Adaptive mechanisms in *L. monocytogenes* include two-component regulatory systems, the master virulence regulator PrfA (Scortti *et al.*, 2007), alternative sigma factors such as σ^B (Wiedmann *et al.*, 1998), and repressors such as CtsR (Nair *et al.*, 2000) and HrcA (Hu *et al.*, 2007a). A two-component regulatory system (TCS) consists of an often-membrane-associated sensory histidine kinase (HK) that receives a signal from the extracellular environment via membrane-bound receptors. Receptor stimulation induces HK autophosphorylation at a histidine residue. Transfer of the phosphoryl group from the HK to an associated response regulator (RR) results in the activation of the RR. The active conformation of the RR exposes a DNA binding domain or some cases, an RNA, protein, or ligand binding domain (Stock *et al.*, 2000; Galperin *et al.*, 2001). Sixteen putative RRs have been identified in *L. monocytogenes*

(Kallipolitis and Ingmer, 2001; Glaser *et al.*, 2001), and some have been implicated in response to environmental stress and virulence. For example, RRs KdpE (Brondsted *et al.*, 2003) and LisR (Cotter *et al.*, 1999; Sleator and Hill, 2005) have been shown to be involved in osmotolerance. LisR has also been shown to play a role in adaptation to acid stress (Cotter *et al.*, 1999) and osmotic stress (Sleator and Hill, 2005). RR VirR has been shown to contribute to the invasion of intestinal epithelial cell line, Caco-2 (Mandin *et al.*, 2005), as have motility RRs CheY (Dons *et al.*, 2004) and DegU (Knudsen *et al.*, 2004).

Alternative sigma factors, when activated, can modulate the simultaneous transcription of many genes. The involvement of alternative sigma factors in stress response and pathogenesis is conserved across many bacterial pathogens (Kazmierczak *et al.*, 2005). Alternative sigma factors σ^B, σ^H, σ^C, and σ^L have been identified in *L. monocytogenes*. σ^B directly regulates over 100 genes (Raengpradub *et al.*, 2008) (Kazmierczak *et al.*, 2003) and is involved in osmotolerance (Becker *et al.*, 1998), acid stress, and oxidative stress (Ferreira *et al.*, 2001) in *L. monocytogenes*. In addition to regulating stress response genes, σ^B contributes to *L. monocytogenes* virulence by regulating *prfA* transcription (Schwab *et al.*, 2005) and the *inlAB* operon (Kazmierczak *et al.*, 2003; Kim *et al.*, 2005), which encodes surface-expressed invasion proteins, InlA and internalin B (InlB).

σ^H is homologous to a stationary phase sigma factor in *Bacillus subtilis* (Britton *et al.*, 2002). Though the σ^H regulon has not been characterized in *L. monocytogenes*, σ^H expression has been shown to increase after acid stress (Phan-Thanh and Mahouin, 1999), and *sigH* transcript levels have been shown to increase after exposure stress in other organisms (Kim *et al.*, 2008; Ehira *et al.*, 2009). σ^L (RpoN) has been shown to regulate the transcription of 77 genes (Arous *et al.*, 2004) and be involved in cold, organic acid and osmotic stress in *L. monocytogenes*

(Raimann *et al.*, 2009). σ^L, along with σ^B, has been shown to contribute to resistance to antimicrobial peptides (Palmer *et al.*, 2009).

In addition to sigma factors, negative regulators CtsR (Nair *et al.*, 2000) and HrcA (Hu *et al.*, 2007a) have been shown to regulate stress response genes in *L. monocytogenes*. Specifically, CtsR regulates Class III heat shock genes such as those encoding the Clp chaperone complexes (Nair *et al.*, 2000), and HrcA regulates Class I genes such as *dnaK* and *groEL* (Hu *et al.*, 2007b). In addition to regulating heat shock response in *L. monocytogenes*, CtsR- (Olesen *et al.*, 2009) and HrcA- regulated (Hanawa *et al.*, 1999) genes have been implicated in acid stress in *L. monocytogenes*. Both of these regulators have been shown to participate in regulatory networks with σ^B (Hu *et al.*, 2007a; Hu *et al.*, 2007b)

Activation of regulatory networks may lead to the induction of stress responses that are protective against subsequent stresses or contribute to virulence-associated phenotypes. For example, exposure to a variety sublethal stresses such as ethanol and moderately low pH has been shown to increase the resistance to subsequent acid stress (Lou and Yousef, 1997; Skandamis *et al.*, 2008). Low pH is an inherent host defense that foodborne pathogens encounter in the stomach and therefore, increased resistance, could potentially lead to larger bacterial loads reaching the intestinal epithelial cell barrier and resulting in increased risk of listeriosis for the consumer. Furthermore, exposure to sodium lactate (Conte *et al.*, 2000; Garner *et al.*, 2006) or NaCl (Garner *et al.*, 2006), both common preservatives of ready-to-eat foods, has been shown to increase the invasion of human intestinal epithelial cells *in vitro* (Garner *et al.*, 2006). Therefore adaptation to environments encountered before ingestion may trigger adaptive mechanisms and lead to increased resistance to the host defenses.

L. monocytogenes persists and grows at a wide range of environmental temperatures. The organism has been isolated from natural or urban environments during spring, summer, and autumn seasons (Sauders *et al.*, 2006) representing a range of environmental temperatures, and its ability to grow at refrigeration temperatures (i.e., 0.4°C) is well documented (Walker *et al.*, 1990). Concordantly, recent North American outbreaks of listeriosis have been linked to ready-to-eat refrigerated products such as deli-meat (Taillefer *et al.*, 2010) and frankfurters (Mead *et al.*, 2006). Therefore, in these instances of foodborne listeriosis, exposure to low temperatures before ingestion is likely.

Environmental temperature affects *L. monocytogenes* virulence gene regulation. The expression of PrfA-regulated core virulence genes, which are organized into *Listeria* pathogenicity island I (LIPI-1), is optimal at internal body temperature of 37°C (Leimeister-Wachter *et al.*, 1992). Translation of *prfA* mRNA is modulated by a thermosensor that blocks ribosomal binding at lower temperatures (Johansson *et al.*, 2002), though some surface expressed proteins involved in host cell invasion, such as certain internalins (McGann *et al.*, 2007) and flagella (Peel *et al.*, 1988; O'Neil and Marquis, 2006), are expressed at higher levels during growth at temperatures lower than 37°C.

L. monocytogenes motility is repressed by MogR at 37°C (Shen and Higgins, 2006). However, motility factors FlaA (O'Neil and Marquis, 2006) and RRs CheY [which regulates chemotaxis (Dons *et al.*, 1994)] and DegU [which regulates transcription of flagellar assembly and motility genes (Williams *et al.*, 2005)] have been shown to be required for invasion of Caco-2 cells (which are human intestinal epithelial cells) for *L. monocytogenes* grown at ≤ 30°C, indicating that growth at temperatures lower than mammalian body temperature (i.e., 37°C) may affect the contribution of virulence factors in *L. monocytogenes*.

In natural environments or in a food product, *L. monocytogenes* is likely adapted to temperatures that are different than those encountered in the human body. For example, *L. monocytogenes* would rarely encounter temperatures as high as 37°C in the natural environment. To determine how growth at outside-of-host temperatures affects stress survival and virulence of *L. monocytogenes*, we investigated how growth at temperatures lower than 37°C affects its (i) regulation of host cell invasion, including contributions of environmentally-responsive regulators mechanisms and the transcriptional regulation and synergistic interactions of known invasion proteins and (ii) its response to simulated gastric stress (i.e. sudden acid shock at 37°C). This information will lead to an improved understanding of how exposure to natural and food environments affects *L. monocytogenes* stress survival and virulence characteristics.

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CHAPTER TWO

Growth Temperature-dependent Contributions of Response Regulators, σ^B, PrfA, and Motility Factors to *Listeria monocytogenes* Invasion of Caco-2 Cells

ABSTRACT

Foodborne pathogens encounter rapidly changing environmental conditions during transmission, including exposure to temperatures below 37°C. The goal of this study was to develop a better understanding of the effects of growth temperatures and temperature shifts on regulation of invasion phenotypes and invasion-associated genes in *Listeria monocytogenes*. We specifically characterized the effects of *L. monocytogenes* growth at different temperatures (30°C versus 37°C) on (i) the contributions to Caco-2 invasion of different regulators [including σ^B, PrfA, and 14 Response Regulators (RR)] and invasion proteins (i.e., InlA and FlaA), and on (ii) *gadA*, *plcA*, *inlA* and *flaA* transcript levels and their regulation. Overall, Caco-2 invasion efficiency was higher for *L. monocytogenes* grown at 30°C as compared to bacteria grown at 37°C ($p = 0.0051$ for the effect of temperature on invasion efficiency; ANOVA); the increased invasion efficiency of the parent strain 10403S (serotype 1/2a) observed after growth at 30°C persisted for 2.5 h exposure to 37°C. For *L. monocytogenes* grown at 30°C, the motility RRs DegU and CheY and σ^B, but not PrfA, significantly contributed to Caco-2 invasion efficiency. For *L. monocytogenes* grown at 37°C, none of the 14 RRs tested significantly contributed to Caco-2 invasion, while σ^B and PrfA contributed synergistically to invasion efficiency. At both growth temperatures there was significant synergism between the contributions to invasion of FlaA and InlA; this synergism was more pronounced after growth at 30°C as compared to growth at 37°C. Our data show that growth

temperature affects invasion efficiency and regulation of virulence-associated genes in *L. monocytogenes*. These data support increasing evidence that a number of environmental conditions can modulate virulence associated phenotypes of foodborne bacterial pathogens, including *L. monocytogenes*.

INTRODUCTION

Listeria monocytogenes is a Gram-positive, non-spore forming rod that is capable of causing disease in humans and animals. *L. monocytogenes* can enter intestinal epithelia via an internalization process initiated by the interaction of InlA, expressed on the surface of invading *L. monocytogenes*, and E-cadherin, expressed on the epithelial cell surface. Although the organism is widespread in nature, 99% of *L. monocytogenes* infections are foodborne (Mead *et al.*, 1999). *L. monocytogenes* therefore encounters a variety of different environments and associated stress conditions during transmission from the environment through foods to humans, including a wide range of temperature, pH, and osmotic stress conditions.

The alternative sigma factor, σ^B, the pleiotropic transcriptional regulator PrfA, and two-component regulatory systems (TCS) have all been shown to regulate key processes important for *L. monocytogenes* stress response and virulence (Kallipolitis and Ingmer, 2001; Kazmierczak *et al.*, 2003; Williams *et al.*, 2005a; Scortti *et al.*, 2007). In addition to a large stress response regulon, σ^B specifically regulates the transcription of genes involved in responses to stresses encountered during passage through the gastrointestinal system such as osmotic stress and acid stress. σ^B also regulates transcription of genes encoding different internalins that are involved in entry into host cells (Raffelsbauer *et al.*, 1998; Kazmierczak *et al.*, 2003; Kim *et al.*, 2005; McGann *et al.*, 2007b). PrfA regulates the *L. monocytogenes* core virulence genes *plcA*, *plcB*, *hly*, *mpl*, and *actA,* which are important for escape from the vacuole

and cell-to-cell spread (Scortti *et al.*, 2007) and also co-regulates, with σ^B, other virulence genes, including *inlA* (McGann *et al.*, 2008). Among the 16 putative or confirmed TCS in *L. monocytogenes*, a number of them have been shown to regulate bacterial adaptation (Autret *et al.*, 2003; Brondsted *et al.*, 2003; Kallipolitis *et al.*, 2003; Dons *et al.*, 2004; Mandin *et al.*, 2005; Sleator and Hill, 2005; Williams *et al.*, 2005b; Larsen *et al.*, 2006), including VirR, DegU and CheY, which have been implicated in regulating mechanisms contributing to host cell invasion (Knudsen *et al.*, 2004; Dons *et al.*, 2004; Mandin *et al.*, 2005).

Growth temperature has been shown to have a profound effect on activities of key *L. monocytogenes* regulators (Leimeister-Wachter *et al.*, 1992; Liu *et al.*, 2002; Dons *et al.*, 2004; Chan *et al.*, 2007b; McGann *et al.*, 2007a; van der Veen *et al.*, 2007). For example, expression levels of the genes comprising the PrfA-regulated *Listeria* pathogenicity island I (LIPI-I), which are important in escape from the host cell vacuole and cell-to-cell spread, are maximal at 37°C due to a *prfA* mRNA thermosensor that represses PrfA translation at lower temperatures (Johansson *et al.*, 2002). Also, certain σ^B-dependent genes have been shown to be involved in adaptation to cold temperatures (Becker *et al.*, 2000; Chan *et al.*, 2007a), and σ^B-dependent internalin genes including *inlC2* and *inlD* have been shown to be expressed at higher levels at temperatures ≤ 30°C compared to 37°C (McGann *et al.*, 2007a). Finally *L. monocytogenes* motility, which has been shown to contribute to host cell invasion (Dons *et al.*, 2004) and increased virulence in a mouse model (O'Neil and Marquis, 2006), is temperature-dependent. At 37°C, the transcription of *flaA* (the gene encoding the flagellin structural protein) and other motility-associated genes is repressed by MogR (Grundling *et al.*, 2004; Shen and Higgins, 2006), though the stringency of this repression may vary among different strains of *L. monocytogenes*. For example, repression of motility genes and associated motility phenotypes has been

shown to be less stringent in *L. monocytogenes* 10403S compared to other strains (Way *et al.*, 2004; Grundling *et al.*, 2004).

While *L. monocytogenes* will have likely been adapted to a temperature below 37°C before ingestion, many studies on *L. monocytogenes* host cell invasion have been done only on *L. monocytogenes* cells grown at 37°C and have only determined the effects of single gene mutations on invasion (Cotter *et al.*, 1999; Kallipolitis and Ingmer, 2001; Autret *et al.*, 2003; Williams *et al.*, 2005a). Therefore, a more comprehensive evaluation of the effect of growth temperature on the contributions of RRs, transcriptional regulators, and motility genes as well as functional synergisms between select genes to host cell invasion is necessary in order to better understand how adaptation to environments outside a mammalian host affects *L. monocytogenes* virulence. The objective of this study was, thus, to characterize the effects of growth temperature (30°C vs. 37°C) on (i) the contributions of σ^B and PrfA, 14 RRs, and invasion proteins InlA and FlaA to Caco-2 invasion, and on (ii) transcript levels of *inlA*, *flaA*, *gadA*, and *plcA*. Growth temperatures of 30°C and 37°C were chosen because *L. monocytogenes* grown at 30°C and 37°C show very similar lag phase durations and growth rates, while *L. monocytogenes* grown at lower temperatures [e.g. 22.5°C; (Pal *et al.*, 2008)] show considerably longer lag phase durations and slower growth rates. As Caco-2 invasion efficiency has been shown to vary with bacterial growth phase (Garner *et al.*, 2006a), it is critical to use bacteria grown with similar growth parameters and synchronized to similar growth phases to ensure that differences in invasion efficiency represent a temperature effect rather than differences in growth phase. In addition, *L. monocytogenes* grown at 30°C have previously been shown to display differential expression of key virulence associated characteristics, including (i) down-regulation of PrfA activity (Johansson *et al.*, 2002) and (ii) upregulation of motility (O'Neil and Marquis, 2006). Consistent with our choice of

30°C as a growth temperature that allows for appropriate comparison between *L. monocytogenes* grown at typical mammalian body temperature and bacteria grown under environmental conditions, others (Toledo-Arana *et al.*, 2009) have recently used 30°C as a growth temperature representing the saprophytic stage of the life of *L. monocytogenes*. Despite these key reasons for using growth temperatures of 30°C and 37°C to study temperature dependent phenotypes of *L. monocytogenes*, we appreciate that these temperatures represent an experimental model that does not necessarily reflect natural transmission where foodborne bacteria are more likely exposed to refrigeration or room temperatures or heat shock conditions prior to ingestion.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Bacterial strains used in this study are listed in Table 2.2.1. For invasion assays, *L. monocytogenes* 10403S (Bishop and Hinrichs, 1987) and associated mutant strains were grown to early stationary phase as previously described (McGann *et al.*, 2007a). Briefly, a 12 to 18 h culture grown at 37°C with aeration (i.e., shaking at 220 rpm) in brain heart infusion broth (BHI), was diluted 1:100 into 5 ml of fresh BHI and grown at 37°C with aeration to OD_{600} = 0.4. This culture was diluted 1:100 into another 5 ml of fresh BHI and grown at 30°C or 37°C with aeration to early stationary phase (defined as growth to OD_{600} = 1.0, followed by an additional 3 h incubation). For temperature shift experiments, a 1 ml aliquot of the early stationary phase culture was centrifuged at 12,000 rpm for 10 min, resuspended in Phosphate Buffered Saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 1.8 mM KH_2PO_4; pH 7.2) and incubated statically at 30°C or 37°C for 0, 2.5 h, or 5 h.

Mutant Construction. Internal, in-frame, non-polar deletion mutant alleles in 14 RR genes (Table 2.2.1) were created using splicing overlap extension (SOE) PCR (see

Table S2.1 for primers) and cloned into the temperature-sensitive suicide shuttle vector pKSV7 (Smith and Youngman, 1992). as previously detailed by our group (Wiedmann *et al.*, 1998). Mutant alleles were introduced into *L. monocytogenes* 10403S using standard allelic exchange mutagenesis procedures (Camilli *et al.*, 1993). Mutant strains were confirmed with sequencing. *lmo0287* is likely to be essential as null mutants in this gene could not be constructed here, consistent with similar studies by others (Kallipolitis and Ingmer, 2001; Williams *et al.*, 2005a).

Caco-2 Invasion Assays. The Caco-2 cell-line (ATCC HTB-37) was maintained at 37°C (4-6% CO_2 and 85% humidity) in Caco-2 medium, which was Dulbecco's minimal essential medium (DMEM) with Earle's salts, 1% sodium pyruvate, 20% fetal bovine serum, 1.0% non-essential amino acids, 1.5 g/L sodium bicarbonate, and, when appropriate, penicillin G and streptomycin (each at 100 μg/ml) (all reagents were obtained from Gibco, a subsidiary of Invitrogen, Carlsbad, CA). All invasion assays were performed at 37°C as previously described (Garner *et al.*, 2006b) with minor modifications. Briefly, 48 h prior to the assay, Caco-2 cells were seeded (from a culture passaged no more than 60 times) into 24 well tissue culture plates (Corning Inc., Corning, NY) at a density of 5×10^4 cells/well in Caco-2 medium without antibiotics. For infection, approximately 2×10^7 CFU *L. monocytogenes* were added to each well [representing a multiplicity of infection (MOI) of approx. 200]. All inocula were enumerated on BHI agar plates. Thirty minutes post infection, the Caco-2 monolayers were washed three times with PBS to remove any unassociated *L. monocytogenes*, and the medium was replaced with fresh Caco-2 medium. Forty-five min after infection, the medium was replaced with Caco-2 medium plus 150 μg/ml gentamycin to kill any extracellular *L. monocytogenes*. At 90 min post infection, Caco-2 cells were washed 3 times with PBS and lysed with ice-cold distilled water.

Table 2.1. Strains used in this study

Strain designation[a]	Genotype	Description of Gene Product	Reference[b]
X1-001	Parent strain	Strain 10403S; serotype 1/2a	Bishop and Hinrichs, 1987
K4-006	Δ*inlA*	Internalin A, required for internalization into selected host cells	Bakardjiev *et al.,* 2004
H6-199	Δ*flaA*	Flagellum structural protein	O'Neil and Marquis, 2006
B4-007	Δ*lisRK*[c]	LisRK, contributes to log-phase acid resistance	This study
B2-078	Δ*agrA*	Response regulator (RR), contributes to protein secretion	This study
B2-080	Δ*resD*	RR, contributes to virulence gene repression in presence of select carbohydrates	This study
B2-086	Δ*lmo1022*	Putative RR, no known role	This study
B2-096	Δ*lmo1060*	Putative RR, no known role	This study
C5-017	Δ*lmo2010*	Putative RR, no known role	This study
C5-019	Δ*lmo2583*	Putative RR, no known role	This study
B2-100	Δ*phoP*	RR, similar to *B. subtilis* PhoP	This study
C5-041	Δ*virR*	Novel RR involved in invasion of Caco-2 cells	This study
C5-036	Δ*lmo1507*	Putative RR, no known role	This study
C5-033	Δ*degU*	RR, regulates expression of motility genes	This study
B2-104	Δ*kdpE*	RR, contributes to growth at high osmolarity and low temperature	This study
B2-105	Δ*cheY*	RR, contributes to chemotaxis	This study
B2-102	Δ*cesR*	RR, contributes to ethanol and β-lactam tolerance	This study
A1-254	Δ*sigB*	Alternative sigma factor σ^B, regulates general stress response	Wiedmann *et al.*, 1998
B2-046	Δ*prfA*	PrfA, regulation of virulence genes	Cheng and Portnoy, 2003
B2-068	Δ*sigB* Δ*prfA*		McGann *et al.*, 2007b
I1-001	Δ*inlA* Δ*flaA*		Marquis Lab

[a]All strain designation carry the prefix FSL

[b]References where a given mutant was previously described

[c]This mutant includes an internal deletion of the *lisR* gene, which also removed a portion of the ribosome binding site of *lisK* sensory kinase gene

Intracellular *L. monocytogenes* were enumerated by plating the appropriate dilutions of the Caco-2 lysate on BHI agar, using a spiral plater (Spiral Biotech; Norwood, MA). At least three independent trials of the invasion assays were performed with duplicate wells tested for each treatment in each replicate.

Quantitative reverse transcriptase-PCR (qRT-PCR). Transcript levels of *inlA*, *flaA*, *plcA*, *gadA*, *rpoB*, *sigB*, and *prfA* were quantified for select strains grown to early stationary phase at 30°C or 37°C using TaqMan probes and primers and the ABI Prism 7000 Sequence Detection System as previously described (Sue *et al.*, 2003; Chaturongakul and Boor, 2006; McGann *et al.*, 2007a) with one exception: copy numbers for each gene were normalized to *rpoB* levels. Primers and probes for *inlA*, *plcA*, *gadA*, *sigB*, *rpoB*, and *prfA* have been previously described (Sue *et al.*, 2004; Kim *et al.*, 2005; Kazmierczak *et al.*, 2006). *flaA* Taqman primers (*flaA*-F: 5'-TCGTAAAAATAACGAAGGCATGAC-3'; flaA-R: AGAACTGTTAATACGTTTACCAGATGCT-3') and the *flaA* MGB probe (FAM-5'-CAAGCGCAAGAAC-3'NFQ) were designed using Primer Express 1.0 (Applied Biosystems).

Statistical Analyses. All statistical analyses were performed in JMP 7.0 (SAS Institute Inc.). Invasion efficiencies were initially analyzed using a one-way analysis of variance (ANOVA; $\alpha = 0.05$). "Strain" and, where appropriate, "date of experiment" were included as variables in the model. For each ANOVA, data were log-transformed to ensure that the data set satisfied ANOVA assumptions of normality of residuals and equality of variances. To determine whether a particular mutant strain differed from the parent strain (e.g., in invasion efficiency), a post hoc Dunnett's many to one test was used (Shun *et al.*, 2003). To compare invasion efficiencies for a given

strain exposed to multiple conditions (i.e., as shown in Table 2.2) or among strains (in cases where a strain with a double mutation was included in the comparison), a post hoc Tukey honestly significantly different (HSD) test was used. For qRT-PCR, normalized log copy numbers for a given gene were compared among strains by using one-way ANOVA, followed by Tukey HSD. To measure whether two gene deletions showed an effect on invasion efficiency or transcript level that is more than additive (which indicates synergism), the parent strain, single mutants and double mutants were assigned unique allelic states by coding dummy variables (e.g., "gene1" and "gene2") for each allelic state with 1 meaning the gene is present and 0 meaning the gene is absent. A two-way ANOVA was performed to determine the effect of each allelic state on invasion efficiency or transcript level in these analyses. "Date of experiment," "gene1," "gene2," and "gene1*gene2" were included as effects in the two-way ANOVA model. A significant effect of gene1*gene2 in the model (i.e., $p < 0.05$) indicates a more than additive effect of the double mutation (i.e., deletion of both *gene1* and *gene2* in the same genetic background has a greater effect than the sum of the effects of deleting *gene1* alone and *gene2* alone).

RESULTS

Contributions of RRs to Caco-2 invasion. Overall ANOVA analysis showed a highly significant effect of temperature on invasion efficiency ($p = 0.0051$), and all RR mutants (except Δ*degU* and Δ*cheY*) and 10403S had numerically higher mean Caco-2 invasion efficiencies after growth to early stationary phase in BHI at 30°C as compared to growth at 37°C. For several strains, including 10403S, Δ*resD*, Δ*lmo1022*, Δ*phoP*, Δ*cesR*, Δ*kdpE*, this difference was statistically significant ($p < 0.05$; t-test; Figure 2.1). For the *L. monocytogenes* parent strain and the 14 RR mutants grown at 30°C, ANOVA showed a significant effect of strain on Caco-2

Table 2.2. Caco-2 invasion efficiencies of *L. monocytogenes* 10403S and select mutants after exposure to varying temperature treatments

Growth temperature	Temperature of PBS hold (2.5 h)	Caco-2 Invasion Efficiency (calculated as [CFU Recovered/CFU Infected] x 100) (std. dev.) for[a]				
		10403S	Δ*cheY*	Δ*degU*	Δ*flaA*	Δ*inlA*
30°C	None	0.67 (0.17)^{A}	0.018 (0.007)^{B,*}	0.015 (0.008)^{B,*}	0.014 (0.010)^{B,*}	0.024 (0.010)^{A,*}
30°C	30°C	0.64 (0.19)^{A}	0.013 (0.003)^{B,*}	0.010 (0.005)^{B,*}	0.010 (0.006)^{B,*}	0.023 (0.007)^{A,*}
30°C	37°C	0.36 (0.10)^{A}	0.006 (0.002)^{B,*}	0.007 (0.002)^{B,*}	0.007 (0.004)^{B,*}	0.014 (0.005)^{A,*}
37°C	None	0.14 (0.03)^{B}	0.14 (0.06)^{A}	0.20 (0.05)^{A}	0.14 (0.06)^{A}	0.0008 (0.0003)^{B,*}
37°C	37°C	0.11 (0.04)^{B}	0.12 (0.05)^{A}	0.14 (0.10)^{A}	0.09 (0.06)^{A}	0.0007 (0.0007)^{B,*}
37°C	30°C	0.11 (0.03)^{B}	0.13 (0.05)^{A}	0.17 (0.08)^{A}	0.14 (0.07)^{A}	0.0007 (0.0005)^{B,*}

[a]Within a given column values with identical letters (A or B) are not significantly different ($p \geq 0.05$; Tukey HSD test). Within a given row, invasion efficiencies that are lower for a given mutant, as compared to the parent strain exposed to the same condition ($p \leq 0.05$; ANOVA; Dunnett's test) are marked with an asterisk (*). Data represent the mean and standard deviation of three biological replicates.

invasion efficiency ($p < 0.0001$). Specifically, Δ*degU* and Δ*cheY* both had approximately 50-fold lower mean invasion efficiencies compared to the parent strain ($p < 0.0001$ for each; Dunett's test) when grown at 30°C (Figure 2.1). These results confirm the findings of other studies that have shown a role for CheY in host cell invasion (Dons *et al.*, 2004). Unlike all other strains tested, the Δ*cheY* and Δ*degU* strains showed numerically higher invasion efficiency after growth at 37°C compared to 30°C (Figure 2.1).

For *L. monocytogenes* parent strain 10403S and the 14 RR mutants grown to early stationary phase at 37°C, the effect of the factor "strain" on Caco-2 invasion efficiencies was not statistically significant ($p = 0.3900$; ANOVA). Therefore, the appropriate conservative statistical test (i.e., ANOVA) found no statistically significant reduction in Caco-2 invasion for any of the 14 RR mutants grown at 37°C. As *L. monocytogenes* motility has been reported in other *L. monocytogenes* strains to be regulated by DegU and CheY at temperatures ≤ 30°C (Dons *et al.*, 2004; Knudsen *et al.*, 2004; Williams *et al.*, 2005a; Mauder *et al.*, 2008), we conducted swarming assays to evaluate motility of the *L. monocytogenes* 10403S, Δ*degU*, Δ*cheY*, and Δ*flaA* strains. While 10403S was clearly motile when grown at 30°C, Δ*degU*, Δ*cheY*, and Δ*flaA* showed reduced swarming (6%, 6%, and 1%, respectively, of the swarming area for 10403S grown at 30°C; see Supplemental Figure S2.1). When grown at 37°C, the parent strain showed limited and considerably reduced swarming compared to 30°C (Figure S2.1), and the three mutant strains showed no detectable swarming at this temperature. The finding that 10403S exhibited some swarming at 37°C is consistent with previous reports that *L. monocytogenes* 10403S (the parent strain used here) shows limited *flaA* expression even at 37°C (Way *et al.*, 2004; Grundling *et al.*, 2004).

Figure 2.1. Caco-2 invasion efficiencies of *L. monocytogenes* 10403S and 14 mutant strains with deletions in genes encoding response regulators. Bacteria were grown to early stationary phase (growth to $O.D._{600}$ = 1.0, followed by 3 h) with aeration (i.e., shaking at 220 rpm) at 30°C or 37°C. Invasion assays were performed at 37°C. Data represent the mean of at least three biological replicates. Error bars represent standard deviation. A Δ*inlA* strain was included as a control. Overall ANOVA analyses of the 14 response regulator mutants and the parent strain showed a significant effect of temperature ($p = 0.0051$); all strains (except Δ*cheY* and Δ*degU*) showed numerically higher invasion efficiencies when grown at 30°C. Strains showing statistically higher invasion after growth at 30°C compared to growth at 37°C ($p < 0.05$; t-test) are denoted with a (^). For 10403S and response regulator mutants, ANOVA showed a significant effect of strain on invasion efficiency for bacteria grown at 30°C ($p < 0.0001$). For bacteria grown at 37°C this effect wasn't significant ($p = 0.3900$). Among response regulator mutants grown at 30°C, Δ*cheY* and Δ*degU* showed significantly lower invasion efficiencies as compared to the parent strain ($p < 0.0001$; Dunnett's; denoted by *).

30° C
37° C
% invading bacteria
10
1
0.1
0.01
0.001
0.0001
*
*
ΔinlA
ΔagrA
> ΔresD
> Δlmo1022
Δlmo1060
> ΔphoP
> ΔcesR
> ΔkdpE
ΔcheY
ΔlisR
Δlmo2010
Δlmo2583
ΔdegU
Δlmo1507
ΔvirR
> 10403S

Effects of temperature shifts and holds on growth temperature-dependent phenotypes. Based on the data reported above, we evaluated whether the increased invasion efficiency of 10403S at 30°C is maintained after a shift to 37°C. While *L. monocytogenes* 10403S grown at 30°C and held (in PBS) at 37°C for 2.5 h (30°C→37°C) showed numerically (about 2-fold) reduced invasion efficiency compared to bacteria either before the shift or bacteria shifted to 30°C for 2.5 h (Table 2.2), the invasion efficiency of the "30°C→37°C" treatment group was not significantly different from the control groups grown and held at 30°C (30°C→30°C in Table 2.2) ($p > 0.05$; Tukey HSD) but was significantly higher compared to control groups grown and held at 37°C (37°C→37°C) ($p < 0.05$; Tukey HSD). These results suggest that increased invasion efficiency of *L. monocytogenes* grown at 30°C is largely maintained during a 2.5 h hold at 37°C in PBS. When *L. monocytogenes* 10403S was grown at 30°C, and shifted to and held at 37°C for 5 h (in PBS), the invasion efficiency was reduced (0.15% invasion efficiency) and virtually identical to that of *L. monocytogenes* grown at 37°C (0.14% invasion efficiency) with no significant difference ($p > 0.05$; Tukey HSD). This indicates that prolonged exposure of *L. monocytogenes* grown at 30°C to 37°C (in PBS) reduces invasion efficiencies to values typical for bacteria grown at 37°C. Growth and de novo protein synthesis (which are unlikely to occur in PBS) thus does not seem to be required for reduced invasiveness observed at 37°C.

When the *L. monocytogenes* parent strain grown at 37°C was switched to 30°C and held at this temperature for 2.5 h, bacteria showed no changes in invasion efficiency and maintained lower invasion efficiency typical for bacteria grown at 37°C ($p < 0.05$; Tukey HSD; Table 2.2), suggesting that the increased invasion phenotype cannot be induced by simply switching the organism to 30°C but requires growth at 30°C. Controls included in these experiments showed that exposure to PBS for 2.5 h

had no effect on invasion efficiency; bacteria grown at 30°C and exposed to PBS at 30°C for 2.5 h as well as bacteria grown at 37°C and exposed to PBS at 37°C for 2.5 h did not differ in their invasion efficiencies from bacteria grown at 30°C or 37°C, respectively, without subsequent exposure to PBS ($p > 0.05$; Tukey HSD; Table 2.2).

As *L. monocytogenes* Δ*degU*, Δ*cheY,* and Δ*flaA* showed lower invasion compared to the parent strain after growth to early stationary phase at 30°C, we also tested whether this invasion defect was maintained after a shift from 30°C to 37°C, followed by a 2.5 h hold at this temperature (in PBS). All three strains (Δ*degU*, Δ*cheY,* and Δ*flaA*) maintained lower invasion efficiencies than the parent strain even after bacteria grown at 30°C were held for 2.5 h at 37°C (Table 2.2). Invasion efficiencies for these strains grown at 30°C and shifted to 37°C for 2.5 h were not significantly different ($p > 0.05$; Table 2.2) from invasion efficiencies for (i) strains grown at 30°C (prior to shift) or (ii) strains grown at 30°C and subsequently held at 30°C in PBS for 2.5 h (Table 2.2). The invasion efficiencies for Δ*degU*, Δ*cheY,* and Δ*flaA* grown at 30°C and held for 2.5 h at 37°C also continued to be significantly lower compared to the invasion efficiencies for the same strains grown at 37°C ($p < 0.05$; Tukey HSD); invasion efficiencies for these three strains in the "30°C→37°C" treatment group were approximately 20 to 30-fold lower compared to the same strains grown at 37°C (Table 2.2). These three mutant strains thus maintained their invasion deficient phenotype even after 2.5 h exposure to 37°C. To test the effect of prolonged exposure to 37°C on the reduced invasion efficiency of motility-deficient mutant strains, invasion efficiency of the Δ*flaA* strain was also evaluated for bacteria grown at 30°C and shifted to 37°C with a hold in PBS at 37°C for 5 h. Even after a hold at 37°C for 5h, the Δ*flaA* strain maintained reduced invasion efficiency (0.006%) compared to the invasion efficiency of either the parent strain grown at 37°C (0.14%) or Δ*flaA* grown at 37°C (0.14%; both comparisons had $p > 0.05$; Tukey HSD), suggesting that

increased invasion efficiency at 37°C, of a Δ*flaA* mutant, requires growth at 37°C and probably *de novo* protein synthesis (which is unlikely to occur in PBS). When the Δ*degU*, Δ*cheY,* and Δ*flaA* strains were grown at 37°C and shifted to 30°C with a hold at 30°C for 2.5 h, all three strains maintained the higher invasion efficiency as they displayed when grown at 37°C (Table 2.2).

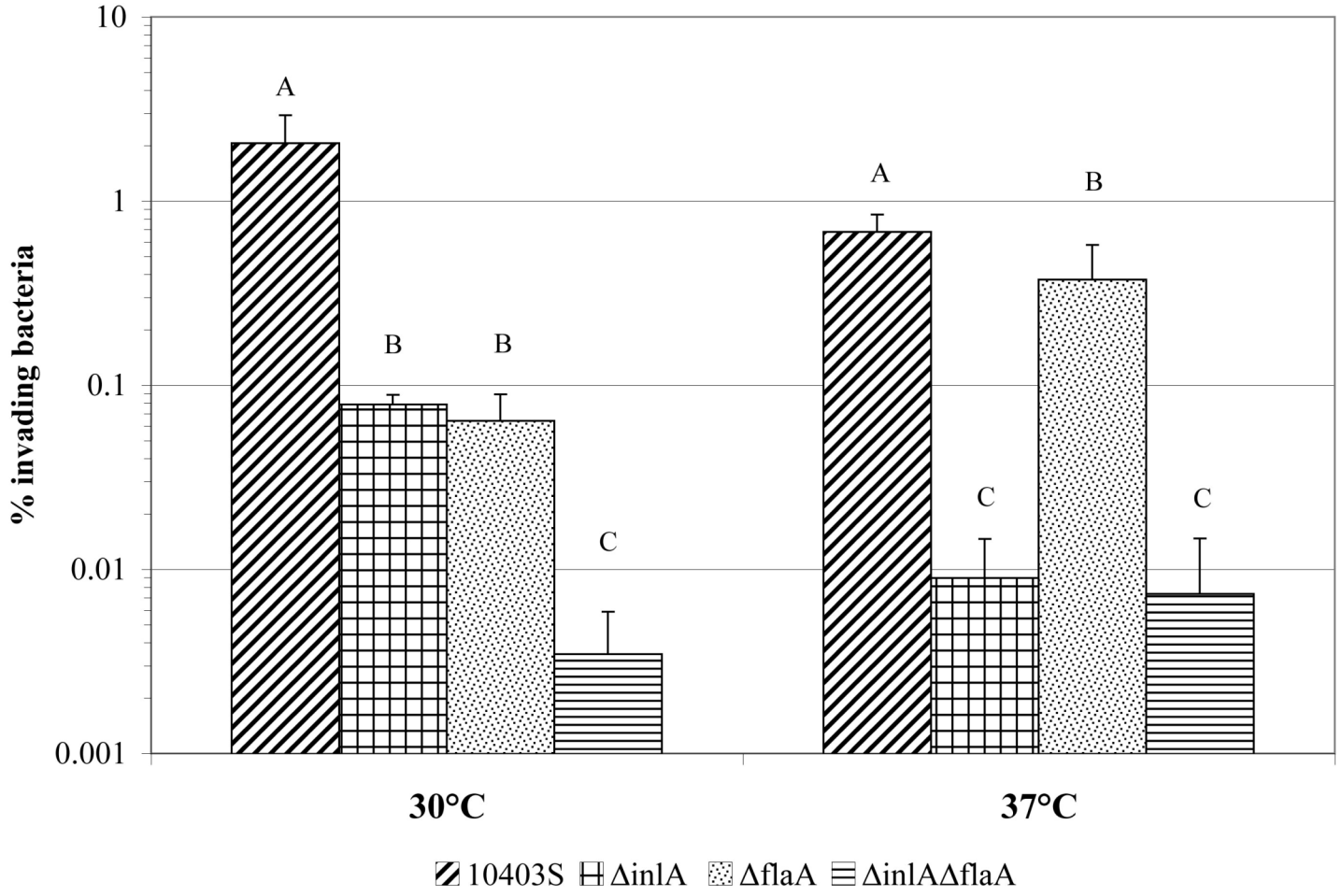
Temperature-dependent contributions of InlA and FlaA to Caco-2 invasion. In initial experiments, the *L. monocytogenes* Δ*inlA* strain showed about 4.4-fold higher invasion efficiency when grown at 30°C compared to 37°C (Figure 2.1). In subsequent experiments, when grown at 37°C, the Δ*inlA* strain showed, on average, greater than 100-fold lower invasion efficiency compared to either the parent strain or the Δ*degU*, Δ*cheY,* or Δ*flaA* strains grown at 37°C (Table 2.2). However, when grown at 30°C, the Δ*inlA* strain showed, only a 26-fold lower average invasion efficiency compared to the parent strain (Table 2.2). These experiments also confirmed a significantly higher invasion efficiency for the Δ*inlA* grown at 30°C as compared to 37°C ($p < 0.05$; Tukey HSD; Table 2.2). Temperature shifts (i.e., 30°C→37°C, 2.5 h or 37°C→30°C, 2.5 h) did not significantly affect these statistical differences (Table 2.2). These results indicate an InlA-independent increase in Caco-2 invasion for *L. monocytogenes* grown at 30°C (as compared to 37°C), which is maintained after a shift to 37°C.

To further investigate the contributions of InlA and flagellin to Caco-2 invasion after growth at 30°C and 37°C and to specifically determine whether there is synergism between InlA and flagellin in facilitating invasion, 10403S, Δ*flaA*, Δ*inlA* and Δ*inlA*Δ*flaA* were grown at 30°C or 37°C and tested for their Caco-2 invasion efficiencies (Figure 2.2). After growth at 30°C, Δ*flaA* showed approximately 30-fold lower invasion efficiency than the parent strain (Figure 2.2). At both 30°C and 37°C, the invasion efficiency of the Δ*inlA*Δ*flaA* strain was lower ($p < 0.05$; Tukey HSD)

than that of the parent strain (0.007% vs. 0.680% for 37°C and 0.003% vs. 2.064% for 30°C, respectively). Furthermore, two-way ANOVA analysis showed a significant "flaA*inlA" interaction effect on Caco-2 invasion for *L. monocytogenes* grown at both 30°C ($p = 0.0015$) and 37°C ($p = 0.0269$). Interestingly, the F-ratio of the interaction effect for bacteria grown at 30°C ($F = 20.15$) was higher than that of bacteria grown at 37°C ($F = 6.97$), suggesting a greater contribution of the interaction effect to the observed variance at 30°C as compared to 37°C. These statistical findings indicate that the effects of deleting *inlA* and *flaA* on Caco-2 invasion are more than additive, indicating a synergism between InlA and FlaA in facilitating Caco-2 invasion for bacteria grown at either 30°C or 37°C.

Temperature-dependent contributions of transcriptional regulators σ^B and PrfA to Caco-2 invasion. To determine growth temperature effects on the contributions of σ^B and PrfA to Caco-2 invasion, Δ*prfA*, Δ*sigB*, and Δ*sigB*Δ*prfA* strains grown to early stationary phase at 30°C or 37°C were used for Caco-2 invasion assays (performed at 37°C). After growth at 30°C, the invasion efficiency of Δ*prfA* was 2.737%, which was not significantly different ($p > 0.05$; Tukey HSD) than the parent strain (2.064% invasion efficiency); the Δ*sigB* (0.376% invasion) and Δ*sigB*Δ*prfA* (0.231% invasion) strains were significantly less invasive ($p < 0.05$; Tukey HSD) than the parent strain (Figure 2.3). These results indicate that only σ^B, not PrfA is involved in invasion when *L. monocytogenes* is grown at 30°C. When the bacteria were grown at 37°C, Δ*prfA* (0.322%), Δ*sigB* (0.060%), and Δ*sigB*Δ*prfA* (0.018%) had significantly lower invasion efficiencies ($p < 0.05$; Tukey HSD) than the parent strain (0.680%) (Figure 2.3); the Δ*sigB*Δ*prfA* strain also showed lower invasion efficiency than the parent strain and the Δ*prfA* strain ($p < 0.05$; Tukey HSD). A two-way ANOVA also showed a

Figure 2.2. Caco-2 invasion efficiencies of *L. monocytogenes* 10403S, Δ*inlA*, Δ*flaA*, and Δ*inlA*Δ*flaA* strains grown to early stationary phase with aeration (i.e., shaking at 220 rpm) at either 30°C or 37°C. Data represent the mean of four biological replicates. Error bars represent standard deviations. Strains with different letters had significantly different invasion efficiencies at a given growth temperature ($p < 0.05$, post hoc Tukey HSD; lower invasion for the Δ*flaA* strain as compared to the parent strain (for growth at 37°C) was only borderline significant ($p = 0.0279$; Tukey HSD). Two-way ANOVA was performed within each temperature to determine effects of single and double mutations on invasion efficiency (see Table 3 for p-values).


10
1
0.1
0.01
0.001
% invading bacteria
A
B
B
C
A
C
B
C
30°C
37°C
10403S
ΔinlA
ΔflaA
ΔinlAΔflaA

significant "sigB*prfA" interaction effect, indicating a synergism between σ^B and PrfA in regulating Caco-2 invasion for *L. monocytogenes* grown at 37°C (Table 2.3).

Temperature-dependent regulation of genes involved in Caco-2 invasion. To determine the effects of growth temperature on σ^B and PrfA-dependent regulation of *L. monocytogenes* genes involved in invasion, we determined transcript levels for *inlA*, *flaA*, *plcA*, *gadA, sigB, prfA* and *rpoB* in 10403S, Δ*sigB*, Δ*prfA*, and Δ*sigB*Δ*prfA* grown to early stationary phase at 30°C or 37°C. Neither *prfA* nor *sigB* transcript levels differed between the parent strain grown at 30°C and 37°C ($p > 0.05$, t-test). The transcript levels for the σ^B -dependent *gadA* and the PrfA-dependent *plcA* (measured as indicators of σ^B and PrfA activity, respectively) (Figure 2.4) also did not differ significantly between bacteria grown at 30°C and 37°C ($p > 0.05$; t-test) suggesting no differences in PrfA and σ^B activity between *L. monocytogenes* 10403S grown to early stationary phase in BHI at these two temperatures.

For bacteria grown to early stationary phase at 30°C, *inlA* transcript levels were 10 times lower in the Δ*sigB* strain than the parent strain ($p < 0.05$; ANOVA; Tukey HSD; Figure 2.4), while there was no significant effect of the *prfA* deletion on *inlA* transcript levels ($p = 0.1282$; two-way ANOVA) (Table S2.2). These data indicate that *inlA* transcription is σ^B-dependent, but PrfA-independent under these conditions. For *L. monocytogenes* grown at 37°C, *inlA* transcript levels were significantly lower in Δ*sigB* compared to the parent strain ($p < 0.05$ ANOVA; Tukey HSD Figure 2.4), while *inlA* transcript levels were not significantly different in Δ*prfA* compared to the parent strain ($p > 0.05$; ANOVA; Tukey HSD; Figure 2.4). However, the sigB*prfA interaction effect on *inlA* transcript levels was borderline significant ($p = 0.0799$; two-way ANOVA; Table S2.2), suggesting contributions of both σ^B and PrfA to regulation of *inlA* transcription in *L. monocytogenes* grown at 37°C. In the *L. monocytogenes* parent

Figure 2.3. Caco-2 invasion efficiencies of *L. monocytogenes* 10403S, Δ*prfA*, Δ*sigB*, and Δ*sigB*Δ*prfA* strains grown to early stationary phase with aeration (i.e., shaking at 220 rpm) at either 30°C or 37°C. Data for the parent strain (10403S) are the same as those shown in Figure 3.2. Data represent the mean of four biological replicates. Error bars represent standard deviation. Strains with different letters have significantly different invasion efficiencies at a given growth temperature ($p < 0.05$, *post hoc* Tukey HSD). Two-way ANOVA was performed within each temperature to determine effects of single and double mutations on invasion efficiency (see Table 3 for p-values).

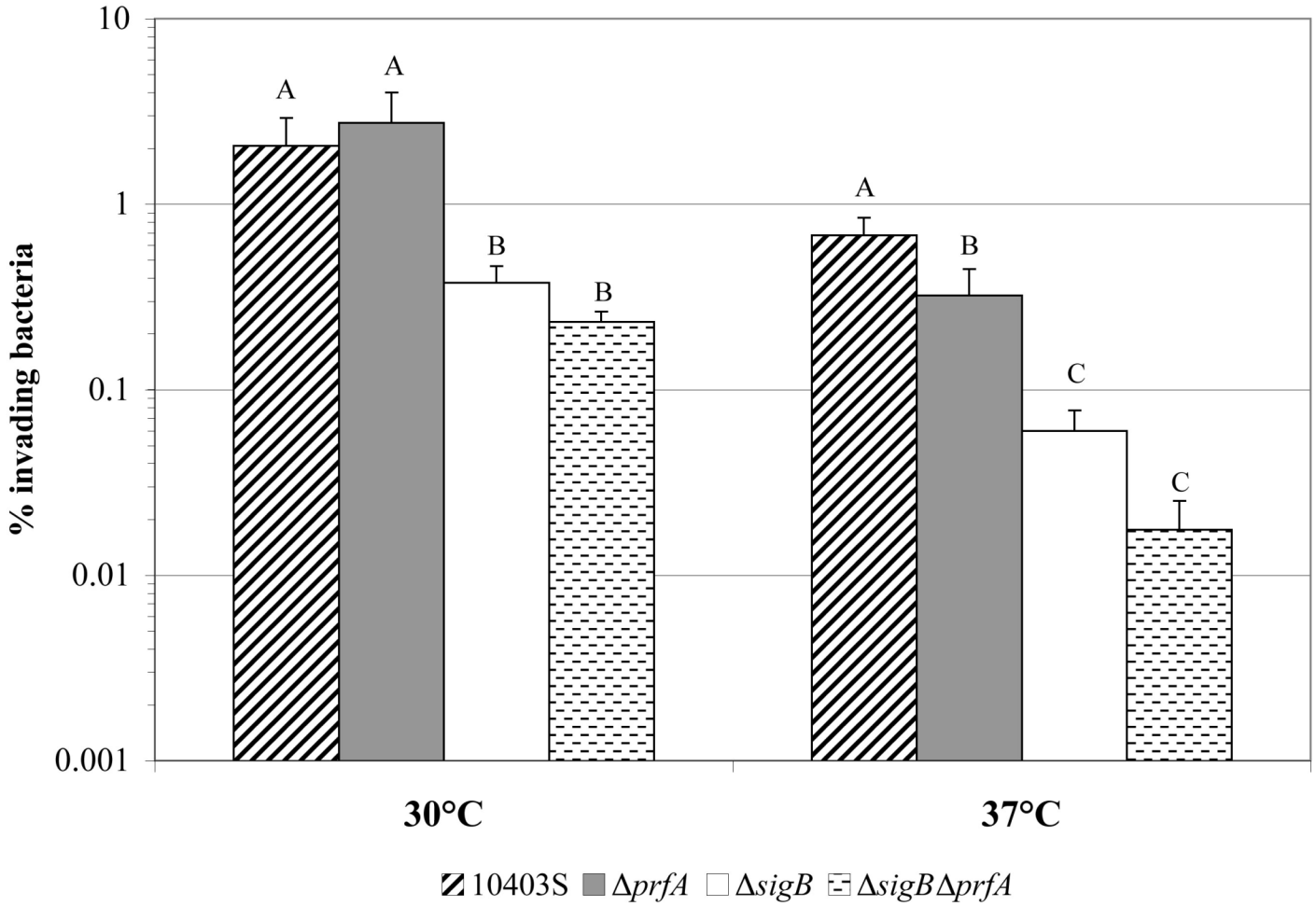
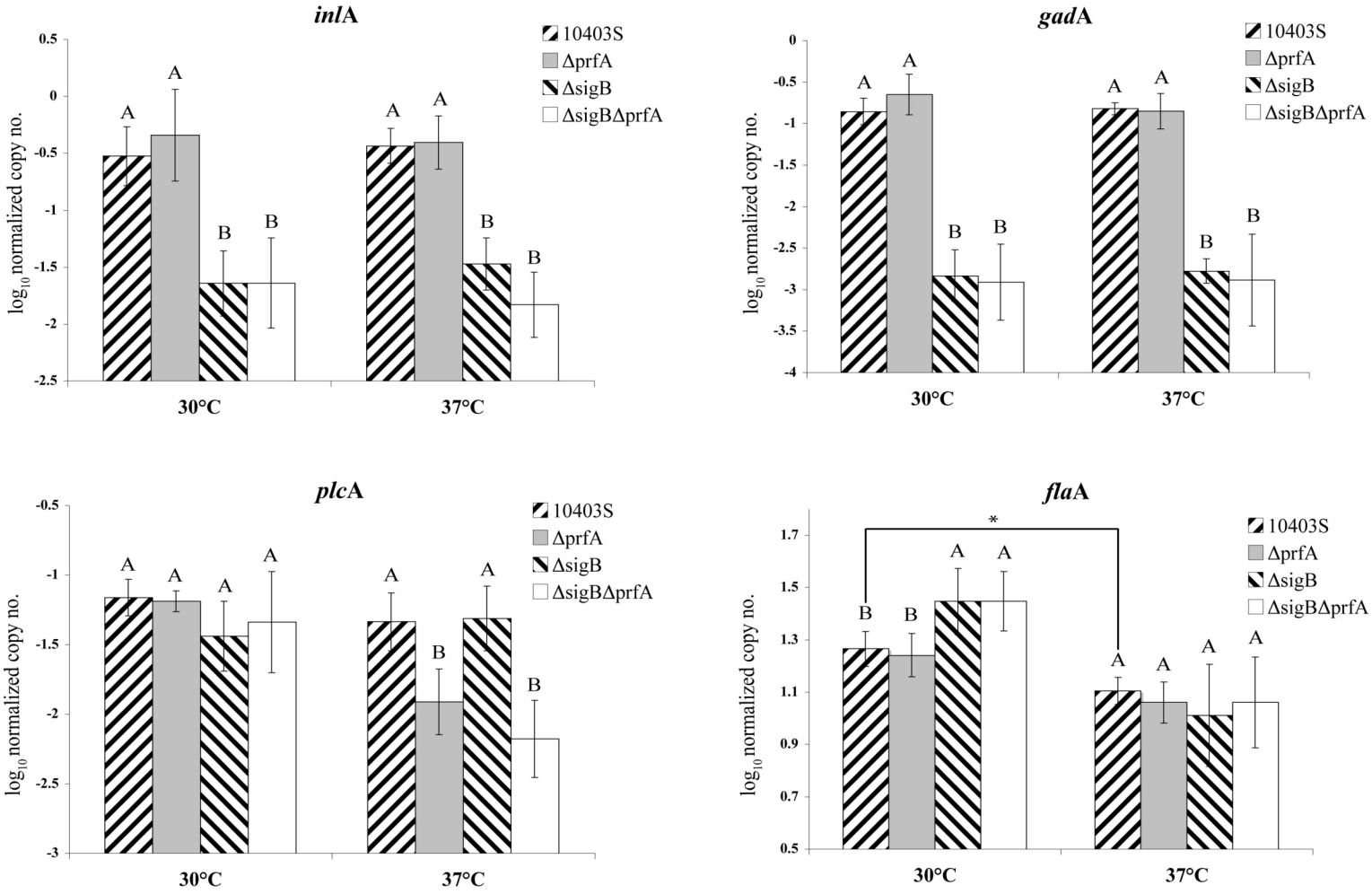

10
1
0.1
0.01
0.001
% invading bacteria
A
A
B
B
A
B
C
C
30°C
37°C
10403S
ΔprfA
ΔsigB
ΔsigBΔprfA

Table 2.3. Effects of various gene deletions on *Listeria monocytogenes* Caco-2 invasion efficiency

Deletion Variable[a]	p-values from two-way ANOVA for *L. mono.* grown at	
	30°C	37°C
sigB	0.0001***	<0.0001***
prfA	0.4401	0.0014**
sigB*prfA	0.226	0.0057**
inlA	0.001***	<0.0001***
flaA	0.0009***	0.0256*
inlA*flaA	0.0015**	0.0269*

[a] The variables listed in this column represent either single-gene deletions (e.g., "sigB") or interactions between two gene deletions (e.g. "sigB*prfA"). The p-values for the single-gene deletions measure the individual effect of deleting each respective gene. The "gene*gene" variable measures synergistic deletion effects by comparing the effect of deleting both genes to the effect of deleting either one gene or the other; significant values are marked with (***) p-value ≤ 0.001, (**) p-value ≤ 0.01, or (*) p-value ≤ 0.05. The actual data used for these analyses are presented in Figure 2.2 (*inlA* and *flaA* data) and Figure 2.3 (*sigB* and *prfA* data).

Figure 2.4. Normalized transcript levels of four genes in *L. monocytogenes* 10403S, Δ*sigB*, Δ*prfA* and Δ*sigB*Δ*prfA* grown to early stationary phase with aeration (i.e., shaking at 220 rpm) at 30°C or 37°C. Data presented as $\log_{10}$ [target gene mRNA level/rpoB mRNA level]. Bars represent the average of four biological replicates and error bars represent standard deviation. Strains with different letters (e.g. A,B) had significantly different transcript levels ($p < 0.05$, *post hoc* Tukey HSD). Two-way ANOVA was performed for each gene at each temperature to determine effects of single and double mutations on transcript levels (see Table S2.2 for p-value summary). For each gene, a t-test was used to compare transcript levels in 10403S between 30°C and 37°C. Only *flaA* levels were significantly different between temperatures ($p = 0.0014$; t-test; indicated by *).


*inl*A
*gad*A
*plc*A
*fla*A
10403S
ΔprfA
ΔsigB
ΔsigBΔprfA
log10 normalized copy no.
30°C
37°C
A
B
*

strain, *flaA* transcript levels were higher after growth at 30°C [1.26; standard deviation (SD) = 0.07] as compared to 37°C (1.104; SD = 0.05) ($p = 0.0014$, t-test; data shown in Figure 2.4).

Although the difference is small, these results indicate at least some temperature-dependent regulation of *flaA* transcription in 10403S. *flaA* transcript levels were not significantly different between Δ*prfA* and the parent strain for either growth temperature ($p > 0.05$; Tukey HSD; Figure 2.4). In *L. monocytogenes* grown at 37°C, *flaA* transcript levels were not different between the Δ*sigB* strain and the parent strain ($p > 0.05$; Tukey HSD; Figure 2.4). However, at 30°C *flaA* transcription was significantly higher in Δ*sigB* and Δ*sigB*Δ*prfA* compared to the parent strain ($p < 0.05$; Tukey HSD; Figure 2.4) and two-way ANOVA showed a significant "sigB effect" on *flaA* transcript levels ($p = 0.0003$; Table S2.2). These results are consistent with other studies that suggest a role of σ^B in down-regulating chemotaxis genes (Raengpradub *et al.*, 2008; Toledo-Arana *et al.*, 2009).

DISCUSSION

***L. monocytogenes* grown at 30°C shows a higher Caco-2 invasion efficiency as compared to bacteria grown at 37°C.** Our data showed that, with the exception of mutants with deletions of motility-related genes, all *L. monocytogenes* strains showed higher Caco-2 invasion efficiency when grown at 30°C as compared to 37°C. This finding supports previous work (Dons *et al.*, 2004) reporting that a different *L. monocytogenes* strain (12067) showed about 10-fold increased association with Caco-2 cells and 2-fold increased invasion of Caco-2 cells for bacteria grown at 24°C (where 12067 was motile) compared to 37°C (where this strain was not motile); this previous study did not include a statistical evaluation of these differences in invasion efficiencies (Dons *et al.*, 2004). As most *L. monocytogenes*, including strain 10403S,

show increased flagellar motility when grown at 30°C, a trait that contributes to Caco-2 cell invasion (Dons *et al.*, 2004) and intestinal colonization in mice (O'Neil and Marquis, 2006), expression of motility genes at ≤ 30°C may explain the increased Caco-2 invasion of *L. monocytogenes* grown at ≤ 30°C compared to bacteria grown at 37°C.

Interestingly, other bacterial pathogens have also been shown to differ in their invasiveness and virulence depending on growth temperature (Konkel and Tilly, 2000). While in a number of bacterial pathogens virulence genes have been found to be more highly expressed at 37°C as compared to lower temperatures (Maurelli, 1989), some pathogens have been shown to express invasion factors at higher levels when grown at temperatures below 37°C. For example, *Yersinia pseudotuberculosis invA*, which encodes an invasin required for host cell invasion, appears to be expressed at higher levels in bacteria grown at 28°C as compared to 37°C (based on visual examination of Western blot data) (Isberg *et al.*, 1988). *Yersinia enterocolitica* has also been shown to express a motility phenotype when grown below 37°C, while motility, which may play a role in initiation of host cell invasion, is down-regulated in bacteria grown at 37°C (Young *et al.*, 2000). Therefore, adaptation to environments outside the host may increase the virulence potential of *L. monocytogenes* and other bacterial pathogens.

Enteric pathogens transmitted to humans from food and environmental sources often experience a sudden change in environmental temperature when they are ingested. Our results show that invasion phenotypes of the parent strain and the motility mutants grown at 30°C persisted for 2.5 hrs after the bacteria were switched to 37°C. As 70 to 90% of human stomach contents are emptied after 2 h (Bennink *et al.*, 1999), our findings, in combination with other studies (Dons *et al.*, 2004; O'Neil and Marquis, 2006), suggest that *L. monocytogenes* grown at temperatures that permit

motility [i.e., 12°C - 30°C (Di Bonaventura *et al.*, 2008)] may have increased invasion potential that could be maintained during gastric passage. As regulation of motility appears to differ considerably between *L. monocytogenes* strains (Grundling *et al.*, 2004; Way *et al.*, 2004), further studies will need to use different strains to validate our findings on growth temperature dependence of Caco-2 invasion efficiency.

In addition to growth temperature, other studies (Garner *et al.*, 2006a; Andersen *et al.*, 2007) have shown that exposure to other environmental conditions (e.g., organic acids, anaerobic conditions) appear to affect the virulence potential of *L. monocytogenes*. For example *L. monocytogenes* grown in an oxygen-limited environment displayed 100-fold increased invasion efficiency in Caco-2 cells as well as increased virulence in a guinea pig model, including 10 to 100-fold higher *L. monocytogenes* fecal shedding levels, compared to numbers for bacteria grown under aerobic conditions (Andersen *et al.*, 2007). Furthermore, *L. monocytogenes* grown in the presence of sodium lactate or NaCl showed an about 10-fold higher invasion efficiency compared to bacteria grown without these compounds (Garner *et al.*, 2006a). Overall, growth temperatures as well as other pre-invasion environmental conditions (e.g., anaerobiosis) thus appear to affect the regulation of genes with roles in host attachment and invasion across different environmentally transmitted pathogens, including *L. monocytogenes*.

CheY and DegU significantly contribute to invasion in *L. monocytogenes* grown ≤ 30°C. Our data, in conjunction with other reports [e.g., (Dons *et al.*, 2004)], clearly show that DegU and CheY play a temperature-dependent role in enhancing invasion of host cells across different *L. monocytogenes* strain backgrounds. For example, Δ*cheA*, Δ*cheY*, and Δ*cheYA* mutants in an *L. monocytogenes* 12067 background all showed about 100-fold reduced Caco-2 cell invasion as well as reduced motility as

compared to their parent strain after growth at 24°C (Dons *et al.*, 2004), while Williams *et al.* (2005a) found that *L. monocytogenes* EGD Δ*degU* and Δ*cheY* strains, grown at 37°C, did not show reduced invasion efficiency of Cos-1 fibroblast cells. Previous studies have shown increased swarming and flagellar motility of *L. monocytogenes* grown at 24°C compared to *L. monocytogenes* grown at 37°C (Knudsen *et al.*, 2004; Dons *et al.*, 2004; Shen and Higgins, 2006), due to repression of *L. monocytogenes* motility genes at 37°C (Grundling *et al.*, 2004; Shen and Higgins, 2006). Therefore, contributions of motility genes to virulence phenotypes are generally apparent only when *L. monocytogenes* is grown at temperatures where the organism is typically motile (i.e., ≤ 30°) (Dons *et al.*, 2004; Knudsen *et al.*, 2004; Williams *et al.*, 2005b; O'Neil and Marquis, 2006). While DegU has also been shown to contribute to motility in *L. monocytogenes* grown at 24°C and to virulence in a mouse model (Williams *et al.*, 2005b), we are not aware of any previous studies reporting a specific effect of a *degU* null mutation on invasion of human intestinal epithelial cells.

While we found no contributions of other RRs, besides DegU and CheY, to invasion of Caco-2 cells, some studies have shown contributions of other RRs to virulence phenotypes in *L. monocytogenes* strains other than 10403S and in other invasion models. For example, Williams *et al.* (2005a) reported that RRs Lmo1507 and LisR are involved in invasion of Cos-1 cells. A deletion of *lisK* (encoding the LisR-associated sensory kinase) in *L. monocytogenes* LO28 was also found to affect virulence in mice (Cotter *et al.*, 1999), further supporting contributions of LisRK to *L. monocytogenes* virulence in some models. Similarly, *L. monocytogenes* EGD with a deletion of the RR VirR, grown at 37°C, has also been reported to be deficient in Caco-2 cell invasion (Mandin *et al.*, 2005), even though the *L. monocytogenes* 10403S Δ*virR* strain used in our current study did not show evidence for reduced invasion,

again possibly reflecting strain differences. Overall, different TCS thus appear to contribute to *L. monocytogenes* virulence and virulence associated characteristics, even though contributions appear to differ based on growth conditions, strain backgrounds, and assays used.

While InlA and FlaA show significant synergism in their contributions to Caco-2 invasion, synergism is less pronounced after growth at 37°C. While flagellin has been identified as a critical part of the motility machinery involved in Caco-2 invasion in *L. monocytogenes* grown at temperatures that allow motility gene expression (i.e., ≤ 30°C) (Dons *et al.*, 2004; O'Neil and Marquis, 2006), we found flagellin and InlA contribute synergistically to Caco-2 cell invasion in *L. monocytogenes* 10403S grown at 30°C and 37°C. While studies in other *L. monocytogenes* strains (e.g. 12067, EDGe), have shown that contributions of motility factors to host cell invasion typically are only apparent in *L. monocytogenes* grown at 30°C or less (Dons *et al.*, 2004; Shen and Higgins, 2006), some studies (Way *et al.*, 2004; Grundling *et al.*, 2004) have shown increased flagellar motility at 37°C for strain 10403S as compared to other strains. For example, *flaA* repression by MogR was shown to be less stringent in 10403S compared to EGDe (Grundling *et al.*, 2004) and 10403S activated a flagellum-dependent innate immune response even after growth at 37°C (Way *et al.*, 2004). Critical contributions of InlA to invasion of human intestinal epithelial cells (Dramsi *et al.*, 1993; Lingnau *et al.*, 1995) and virulence after oral infection (Lecuit *et al.*, 1999; Garner *et al.*, 2006b) have been well established for host species carrying the E-cadherin allotype that allows for InlA binding (e.g., humans, guinea pigs). Although we observed a synergism between the contributions of FlaA and InlA to Caco-2 invasion in *L. monocytogenes grown* at both 30°C and 37°C, we also found that the Δ*inlA* strain shows increased invasion efficiency when grown at 30°C

compared to 37°C suggesting InlA-independent contributions of flagellar motility to Caco-2 cell invasion. This observation further supports the importance of flagellar motility in *L. monocytogenes* virulence.

σ^B, but not PrfA, contributes to invasion of bacteria grown at 30°C, whereas σ^B and PrfA show synergistic contributions to invasion if bacteria are grown at 37°C. Our data showed that (i) σ^B contributes to Caco-2 invasion for both *L. monocytogenes* grown at 30°C and 37°C and (ii) σ^B positively regulates *inlA* transcription at both of these temperatures. The observation that σ^B contributes to Caco-2 invasion in bacteria grown at both temperatures is consistent with a number of studies that have shown that σ^B is critical for invasion of intestinal cells *in vitro* (Kim *et al.*, 2004; Garner *et al.*, 2006b) and in a guinea pig model of listeriosis (Garner *et al.*, 2006b). While McGann *et al.* (2007a) found higher transcript levels in *L. monocytogenes* grown at 30°C, as compared to bacteria grown at 37°C, for certain σ^B-dependent internalin genes (i.e., *inlC2*, *inlD*, *lmo331*, *lmo0610*) and *opuCA*, which has been shown to be regulated by both σ^A and σ^B (Cetin *et al.*, 2004; Chan *et al.*, 2007a), the same study also found that *inlA* transcript levels were similar in *L. monocytogenes* grown at 30°C and 37°C (McGann *et al.*, 2007a). Another study also reported that genes found to be σ^B-dependent in the intestine were generally not differentially expressed at 30°C and 37°C (Toledo-Arana *et al.*, 2009). Overall, these data suggest that σ^B activity and σ^B-dependent regulation of invasion is similar in *L. monocytogenes* grown at 30°C and 37°C, even though some σ^B-dependent genes (e.g., *opuCA*, *inlC2D*) may be regulated by additional temperature-dependent mechanisms (McGann *et al.*, 2007a; Chan *et al.*, 2007a). We did find though that σ^B-dependent negative regulation of *flaA* transcript levels, which was previously described by two studies (Raengpradub *et al.*, 2008; Toledo-Arana *et al.*, 2009), was only apparent here

in bacteria grown at 30°C, even though this regulation has previously been reported in bacteria exposed to salt stress at 37°C (Raengpradub *et al.*, 2008). Temperature effects on σ^B-dependent regulation of some genes may thus be dependent on other environmental conditions (e.g., osmotic stress).

Contributions of PrfA to Caco-2 cell invasion in *L. monocytogenes* grown at 37°C, but not in bacteria grown at 30°C, are consistent with previous findings of low PrfA activity in *L. monocytogenes* grown at temperatures less than 37°C (Leimeister-Wachter *et al.*, 1992) as well as data showing that PrfA-dependent *inlA* expression is significantly higher at 37°C compared to 25°C (Dramsi *et al.*, 1993). Interestingly, in our study here, *prfA* transcript levels and PrfA activity (as measured by *plcA* transcription levels) were not significantly different in the parent strain between 30°C and 37°C growth conditions; this observation may reflect low baseline PrfA activity in *L. monocytogenes* grown at 37°C in BHI (i.e., the conditions used here) as low levels of easily catabolized sugar and/or presence of other compounds (e.g., charcoal) seem to be required to induce PrfA activity at 37°C (Ripio *et al.*, 1996; Milenbachs *et al.*, 1997; Gilbreth *et al.*, 2004). While, PrfA activity and PrfA-dependent phenotypes are thus clearly temperature-dependent, with maximum PrfA activity in bacteria grown under certain conditions at 37°C, it is increasingly clear that transcriptional patterns and phenotypic characteristics of *L. monocytogenes* are governed by complex, environmental condition-dependent interactions between multiple regulators.

Synergisms between PrfA and σ^B were confirmed here through formal statistical analyses that showed (i) a statistically significant interaction effect between *sigB* and *prfA* deletions on Caco-2 invasion in bacteria grown at 37°C, but not in bacteria grown at 30°C and (ii) a borderline significant interaction effect between *sigB* and *prfA* deletions on *inlA* transcript levels in bacteria grown at 37°C, but not at 30°C. While this type of temperature-dependent synergism has not previously been

described, contributions of both σ^B and PrfA to Caco-2 cell invasion and co-regulation of *L. monocytogenes* virulence genes, including *inlA*, have been reported previously (Lingnau *et al.*, 1995; Kazmierczak *et al.*, 2003; Sue *et al.*, 2004; Kim *et al.*, 2005; McGann *et al.*, 2007b). The observation that *flaA* also appears to be down-regulated by σ^B, possibly with an antisense RNA type mechanism (Toledo-Arana *et al.*, 2009), further supports a temperature-dependent regulatory network involving PrfA and σ^B that affects multiple effector proteins contributing to *L. monocytogenes* invasion and virulence, even though possible (temperature-dependent) contributions of PrfA itself to transcription of *flaA* and other motility regulated genes (Michel *et al.*, 1998; Milohanic *et al.*, 2003) will require further confirmation. For example, while others (Ripio *et al.*, 1997) previously reported (based on visual examination of an RNA slot blot) negative regulation of *flaA* transcription by PrfA* (i.e., a PrfA protein that is constitutively active) in *L. monocytogenes* grown at 20°C, we did not find any effect of the *prfA* deletion on *flaA* transcript levels.

CONCLUSIONS

Overall, our data show that *L. monocytogenes* uses a number of regulatory mechanisms to modulate virulence gene expression, particularly expression of genes important for invasion, under different temperatures. Specifically, modulation of gene expression in *L. monocytogenes* grown at temperatures less than 37°C (mimicking environmental conditions prior to host infection) appears to increase the invasiveness of this pathogen, priming it for subsequent infection of a mammalian host. Importantly, our data also suggest an initial specific model for regulation of key invasion associated genes at transition of *L. monocytogenes* from environment to host. During growth at temperatures below mammalian body temperatures, CheY and DegU activity induce a motility phenotype, and although PrfA-dependent virulence gene

expression is minimal, InlA expression is assured by σ^B-dependent *inlA* transcription. After introduction into the host environment, passage through the gastrointestinal system can further activate σ^B (e.g., through acid and osmotic stress), "priming" the cell for intestinal cell invasion through increased *inlA* transcription (Sue *et al.*, 2004). Once *L. monocytogenes* enters the intracellular environments, PrfA-dependent gene expression becomes critical for intracellular survival and spread (Freitag *et al.*, 1993) with σ^B taking on a modulating role by downregulating expression of genes encoding cytolysins, which may cause excessive host cell damage (Ollinger *et al.*, 2008). Concurrently, motility appears to be downregulated in *L. monocytogenes* grown at 37°C, including through σ^B-dependent mechanisms (Raengpradub *et al.*, 2008; Toledo-Arana *et al.*, 2009), possibly facilitating evasion of Toll-like receptor-mediated host responses (Hayashi *et al.*, 2001; Torres *et al.*, 2004).

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CHAPTER 3

Listeria monocytogenes Grown at 7°C Shows Reduced Acid Survival and an Altered Transcriptional Response to Acid Shock Compared to *L. monocytogenes* grown at 37°C.

ABSTRACT

Survival of the foodborne pathogen *Listeria monocytogenes* in acidic environments (e.g., stomach and low pH foods) is vital to its transmission. *L. monocytogenes* grows at temperatures as low as -0.4°C, and refrigerated, ready-to-eat foods have been sources of *L. monocytogenes* outbreaks. The purpose of this study was to determine whether growth at a low temperature (i.e., 7°C) affects survival in artificial gastric fluid (AGF) or acidified BHI (ABHI) at 37°C over time. A full genome microarray was used to determine changes in *L. monocytogenes* 10403S gene expression after exposure to ABHI for 5 or 15 min. *L. monocytogenes* cells grown at 7°C were less resistant to AGF or ABHI than bacteria grown at higher temperatures (i.e., 30°C or 37°C). For *L. monocytogenes* grown at 7°C, stationary phase cells were more resistant to ABHI than log phase cells, indicating both temperature and growth phase effects on acid survival. After 5 or 15 min exposure to ABHI, there were more genes differentially transcribed in 37°C-grown cells (201 genes) than in 7°C-grown cells (73 genes). In general, *L. monocytogenes* grown at 37°C shows growth phase-dependent acid response involving regulators previously implicated in maintaining pH homeostasis and/or adaptation to the intracellular environment (i.e., σ^B, σ^H, CtsR, and HrcA). For *L. monocytogenes* grown at 7°C to log-phase, the σ^B regulon was positively enriched after acid treatment, whereas for bacteria grown at 7°C to

stationary phase, none of the stress regulons tested were enriched and two large operons encoding bacteriophage-like proteins were induced, suggesting that a primary response to acid stress of 7°C-grown stationary phase 10403S is lysogenic prophage induction. Overall, our data show that *L. monocytogenes* grown at 37°C is more resistant to acid stress than 7°C-grown cells, possibly due to an adaptive transcriptional response that is largely absent in 7°C-grown cells. Our results suggest that temperatures commonly encountered during food storage and distribution affect the ability of *L. monocytogenes* to survive gastric passage and ultimately cause disease.

INTRODUCTION

Listeria monocytogenes is a gram-positive, non-sporeforming foodborne pathogen that, when consumed in a food product, has the potential to cause symptoms ranging from gastroenteritis to severe neurological disorders or spontaneous abortion. Post-processing contamination of a food product with *L. monocytogenes* is of particular concern for certain ready-to-eat products as *L. monocytogenes* can to grow at low temperatures (e.g., -0.4°C) (Walker *et al.*, 1990). Therefore, even if the organism is introduced into the product in low numbers, it can proliferate during distribution and storage. A 1999 survey conducted by Audits International (available online at www.foodrisk.org/exclusives/audits/downloads/Audits-FDA_temp_study.pdf) showed that the average home refrigerator temperature is closer to 7°C than the recommended 4°C. This higher temperature may allow considerable growth of *L. monocytogenes* in a food product and thus increase the level of *L. monocytogenes* contamination at the time of consumption.

Once consumed, *L. monocytogenes* must survive gastric passage to reach the intestinal epithelial cell barrier, where it can invade and subsequently spread

systemically. Human stomach pH ranges from 1-3 (Schubert, 2009); therefore, *L. monocytogenes* will likely rely on acid stress response mechanisms to survive exposure to this environment. The adaptation of *L. monocytogenes* to acidic environments involves mechanisms that maintain intracellular pH homeostasis by directing H^+ ions out of the cell (e.g., F_0F_1 ATPases, or cation/H^+ antiporters), consumption of internal H^+ through decarboxylation reactions (e.g., glutamate, lysine decarboxylases), or generation of ammonium ions (e.g., amino acid deiminases) as well as proteins involved in macromolecule repair, such as heat shock proteins (Ryan *et al.*, 2008). The negative regulators HrcA (heat regulation at CIRCE) and CtsR (class three stress gene repressor), which regulate Class I and Class III heat shock genes, respectively, have been implicated in acid stress adaptation in *L. monocytogenes* and other organisms. For example, deleting HrcA-regulated *dnaK* impairs acid stress survival in *L. monocytogenes* (Hanawa *et al.*, 1999). Genes encoding Clp chaperone-proteases (e.g., *clpC*, *clpE*, and *clpP*), which help rid the bacterial cell of aggregating proteins occurring during environmental stress (Kress *et al.*, 2009), are examples of genes regulated by CtsR in *L. monocytogenes* (Nair *et al.*, 2000), and increased transcript levels of *clpP* and *clpE* transcript levels have been observed after acid treatment (Olesen *et al.*, 2009). σ^H is an alternative sigma factor that regulates the transcription of genes encoding chaperonins, including *groEL*, *clpB*, and *dnaK* in *Corynebacterium glutamicum* (Ehira *et al.*, 2009) and is induced after acid shock in *Streptomyces coelicolor* (Kim *et al.*, 2008). σ^H has also been shown to be expressed at higher levels in *L. monocytogenes* after acid stress (Phan-Thanh and Mahouin, 1999), indicating that it may be involved in the acid response in *L. monocytogenes* and other gram-positive bacteria. The alternative sigma factor σ^B regulates stress response genes in *Staphylococcus aureus* (Bischoff *et al.*, 2004), *Bacillus subtilis* (Hecker *et al.*, 2007), and *L. monocytogenes*, including genes

involved in acid stress response (Kazmierczak *et al.*, 2003). Furthermore, σ^B has been shown to be involved in stationary phase acid survival in *L. monocytogenes* (Wiedmann *et al.*, 1998; Ferreira *et al.*, 2001).

The adaptation of *L. monocytogenes* to certain environments affects its response to acidic environments. For example response to certain sub-lethal treatments of ethanol and acid has been shown to increase the acid resistance of *L. monocytogenes* (Lou and Yousef, 1997). Adaptation to a low temperature (i.e., 10°C) was shown to reduce *L. monocytogenes* survival at pH 2.5 compared to cells grown at 30°C (Patchett *et al.*, 1996). However, *L. monocytogenes* adapted to refrigeration temperatures will likely experience an even larger up-shift in temperature during ingestion and passage through the gastric environment will likely be accompanied by a sudden and drastic down-shift in environmental pH, which may impose an added stress on the organism. In addition to prior adaptation to stress, growth rate and growth phase also affect acid resistance in *L. monocytogenes* (Patchett *et al.*, 1996; Samelis *et al.*, 2003).

The objective of this study was to compare the response to a sudden drop in pH for *L. monocytogenes* grown at 7°C to that of cells grown at 37°C. *L. monocytogenes* 10403S was grown to log or stationary phase at 7°C, 30°C, or 37°C and exposed to pH 3.5 at 37°C either in artificial gastric fluid or in acidified BHI and survival was monitored over time. Then, using a full genome microarray, changes in gene transcription were determined, after exposure to acidified BHI for 5 and 15 min, for *L. monocytogenes* grown to log or stationary phase at 7°C or 37°C. This study will contribute to the understanding of how adaptation to food-relevant environments, specifically refrigeration temperatures, affects the ability of *L. monocytogenes* to survive sudden drops in environmental pH such as passage through the stomach.

MATERIALS AND METHODS

Stains and growth conditions. *L. monocytogenes* strains 10403S (FSL X1-001; serotype 1/2a; lineage II), FSL J1-194 (serotype 1/2b; lineage I), and Mack (FSL F6-367; serotype 1/2a lineage II) were streaked from frozen stocks (stored at -80°C) onto BHI agar and stored at 4°C for working stocks. For acid survival and microarray studies, one colony from the working stock was inoculated into 5 ml BHI broth and grown at 37°C overnight (12-18 h). The overnight culture was diluted 1:100 into a fresh of 5 ml BHI and grown with aeration (i.e., 220 rpm) at 37°C to $O.D._{600}$ = 0.4. A 1:100 dilution of this culture was made into 50 ml of the final growth medium in a 500 ml, side-arm Nephelo flask (Belco, Vineland, NJ) and this culture was grown at the appropriate temperature with aeration (i.e., 220 rpm) to log phase, defined as $O.D._{600}$ = 0.4 for 37°C and 7°C, or early stationary phase, defined as $O.D._{600}$ = 1.0 followed by an additional 3 h incubation for 30°C and 37°C or 120 h for 7°C.

Artificial gastric fluid. To assess the effect of growth temperature on *L. monocytogenes* survival in artificial gastric fluid, 10403S and J1-194 were grown to stationary phase at 7°C, 30°C, or 37°C in BHI. Synthetic gastric fluid survival assay was conducted according to Garner *et al.*, 2007 (Garner *et al.*, 2006) with slight modifications. Briefly, 1 ml of culture was added to 4 ml 1.25X artificial gastric fluid for a final concentration of 1X (8.3g/L proteose peptone, 3.5g D-glucose, 2.05g NaCl, 0.6g KH_2PO_4, 0.147g $CaCl_2 \cdot 2H_20$, 0.37g KCl, adjusted to pH 2.5 with HCl) and a final pH of approx. 3.5. The suspensions were incubated at 37°C for 0, 0.5, 1, or 2 h. Cultures were enumerated at each time-point by plating appropriate dilutions on BHI with a spiral plater (Advanced instruments, Norwood, MA).

Acidified BHI. To assess the effect of growth temperature on the ability of *L. monocytogenes* to survive exposure to acidified BHI over time, 10403S was grown to log phase or early stationary phase at 7°C or 37°C in BHI buffered with 100 mM MOPS (adjusted to pH 7.2 with NaOH). Five ml of culture was adjusted to pH 3.5 with 12 N HCl. The suspensions were incubated at 37°C in a water bath for the appropriate amount of time. Viable bacteria were enumerated by spiral plating appropriate dilutions on BHI.

RNA extraction and microarray. To determine the effect of growth temperature on the *L. monocytogenes* transcriptional response to acid stress, 10403S was grown to log or stationary phase at 7°C or 37°C. A 5 ml aliquot of each culture was adjusted to a final pH of 3.5 by addition of 12 N HCl then incubated at 37°C in a water bath without aeration for 5 or 15 min. Samples (4.5 ml) were taken from (i) the untreated culture, (ii) 5 min acid-treated, and (iii) 15 min acid-treated cultures. RNA was stabilized by adding 0.5 ml of a 10% acidified phenol in ethanol solution to each sample for a final concentration of 1% phenol (Bhagwat *et al.*, 2003). The bacterial suspensions were immediately pelleted by centrifugation at 1800*g* for 10 min. RNA extraction was immediately performed using TRI Reagent® (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol with slight modifications. Briefly, cell pellets were resuspended in 5 ml TRI Reagent®, combined with 3 cc of 0.1 mm zirconium beads and homogenized by beating in a Mini-Beadbeater-8 (BioSpec Products Inc., Bartlesville, OK) for 4 min. Tubes were centrifuged at 3200*g* for 10 min. 1-Bromo-3-chloropropane (0.5 ml) was added to each supernatant, and samples were allowed to phase-separate at room temperature for 10 min. Samples were then centrifuged at 10,000*g* for 10 min. The aqueous phase was removed and combined with 2.5 ml ice-cold isopropanol, allowed to incubate at room temperature for 10 min, and centrifuged for 20 min at 14,000*g*. RNA pellets were washed by centrifuging 5

min at 4000*g* in 70% and 100% ethanol, resuspended in nuclease-free water and stored at -80°C, pending DNase treatment. DNase treatment was carried out as previously reported (Raengpradub *et al.*, 2008). Following DNase treatment, samples were directly purified using the RNeasy minElute cleanup kit (Qiagen, Germantown, Maryland), resuspended in nuclease free water, and stored at -80°C.

Microarray design, cDNA labeling, competitive hybridization and scanning. The microarray used here has been described in detail by (Raengpradub *et al.*, 2008). Briefly, the array consists of 70-mer oligonucleotides representing 2,857 ORFs from *L. monocytogenes* EGD-e, which represents the same lineage, serotype and ribotype as *L. monocytogenes* 10403S (only 45 probes on the array have an EGD-e/10403S cross-hybridization identity of < 90). Arrays were printed at the Cornell University Life Sciences Core Laboratories Center (Ithaca, NY). cDNA labeling, cleanup, hybridization and slide scanning were performed as described previously (Raengpradub *et al.*, 2008) except that in this study, cDNA was synthesized from 6 μg of total RNA. For *L. monocytogenes* grown to log or stationary phase, competitive hybridizations were performed between total cDNA from non-acid-treated cells grown to 7°C and non-acid-treated cells grown to 37°C to determine baseline differences in gene transcription between growth temperatures. To determine changes in gene transcription after acid treatment, separate competitive hybridizations were performed between cDNA from untreated cells (grown at 7°C or 37°C to log or stationary phase) and (i) cells acid treated for 5 min or (ii) cells acid treated for 15 min. Four biological replications were completed for the entire experiment. Dye swapping (i.e., for each competitive hybridization experiment, each sample was labeled with Cy3 in two replications and Cy5 in two replications) was carried out to minimize dye incorporation bias.

***Listeria* bacteriophage enumeration.** To enumerate plaque-forming substances in *L. monocytogenes* supernatants, 10403S was grown at 7°C to stationary phase. Five ml aliquots were exposed to acidified BHI, as described above, for 15 min at 37°C, neutralized to pH 7-7.2 with NaOH and placed at 7°C or 37°C for 2 or 4 h. Untreated control samples were placed at 7°C or 37°C (corresponding to growth temperature) in acidified BHI. At the appropriate time point, *L. monocytogenes* cultures were enumerated by plating the appropriate dilution on BHI, and 0.5 ml of the culture was filtered through a 0.22 μM syringe filter. Ten-fold serial dilutions of the filtrate were prepared and 100 μl of the appropriate dilution was mixed with 300 μl of a 1:10 dilution of an overnight (12-18 h at 37°C) culture of *L. monocytogenes* Mack diluted 1:10 in LB MOPS broth and mixed with 4 ml of LB-MOPS agar (10 g/L tryptone, 5 g/L yeast extract, 10.5 g/L MOPS free acid + Glucose (1 g/L) + Salts (1.47 g/L $CaCl_2$, 1.02 g/L $MgCl_2$). The entire mixture was poured over a plate of LB-MOPS + Glu + Salts agar (1.5%). Plates were incubated upright at room temp (approx 25°C) for 24-48 h. Plaques were enumerated and results were recorded as plaque forming units (PFU/ml). We chose *L. monocytogenes* Mack as the host strain because it has been shown to be a host for *Listeria* prophage A118 (Hodgson, 2000).

Transmission electron microscopy. High titer phage stocks were prepared by twice passing isolated plaques using the phage isolation protocols described above. A drop of the 0.2 μM filtered phage stock (3.5×10^9 PFU/ml) was placed onto a small piece of the carbon and allowed to dry for a minute. One drop of staining solution (2% uranyl acetate; pH 4.2) was transferred onto the carbon film containing the phage sample. Excess stain was removed with filter paper and the grid was allowed to air dry

before imaging using the FEI Tecnai 12 (Cornell Center for Materials Research, electron microscopy and optical facility, Ithaca, NY).

Statistical analysis. For gastric fluid survival experiments, separate analyses of variance (ANOVA) of percent survival by temperature and by strain were performed for each time point. To determine genes that were significantly differentially expressed after 5 or 15 min acid shock (i.e. pH 3.5) in cells grown at 7°C or 37°C to log or stationary phase, microarray results for each condition were analyzed according to Raengpradub *et al.* (2008) (Raengpradub *et al.*, 2008). Briefly, TIFF images from array scans were analyzed using GenePix Pro 6.0 software. The LIMMA (version 2.16.2) package for R (version 2.8) was used to determine differentially expressed genes. Data were background corrected using "normexp" and normalized using "printtiploess." To determine genes that were differentially expressed after acid addition for each temperature and growth phase condition, the "modelMatrix" design for comparing more than one condition to a reference sample was employed with the untreated sample as the reference sample. The "duplicateCorrelation" function was used to account for duplicate spots and a linear model was fit to the normalized data using "lmfit" followed by empirical Bayes smoothing using the "eBayes" function to calculate moderated t-statistics and B-statistics. p-values were adjusted by controlling for false discovery rate using the Benjamini-Hochberg method.

In microarray experiments, to determine differentially expressed genes between 37°C untreated cultures and 7°C untreated cultures, genes were considered differentially expressed if the absolute value of the fold change between the two temperatures was ≥ 2.0 with and adjusted p-value ≤ 0.05. These criteria were used previously in determining the growth-phase dependent *L. monocytogenes* 4°C regulon (Chan *et al.*, 2007), though in the previous study, cultures were grown at 4°C or 37°C

without aeration whereas, in this study here, *L. monocytogenes* were cultured at 7°C or 37°C with aeration (i.e., shaking at 220 rpm). Because we exposed the cultures to acid for relatively short times (i.e., 5 or 15 min), we used a less conservative definition of differential expression for the acid treatment experiments in order to increase the sensitivity to acid-induced changes in gene transcription. Specifically, for acid stress experiments genes were considered differentially expressed after acid treatment if the absolute value of the fold change after acid treatment was ≥ 1.5 with an adjusted p-value ≤ 0.05.

To determine differences in phage titer between incubation temperatures and between acid-treated and non-acid-treated cultures, matched pairs analyses, which takes into account differences in initial phage titer for each replicate, were performed for each time after acid treatment.

GSEA and HMM. To compare differences in the transcript levels of gene sets (e.g., sets of genes having the same biological function or sets of genes in the same regulon) between two conditions (e.g. between growth temperatures or before and after acid treatment) gene set enrichment analysis (GSEA) (Subramanian *et al.*, 2005) was performed. GSEA was conducted as described previously (Chan *et al.*, 2007). Briefly, a ranked list of every probe M value ($\log_2$ of fold change between growth temperature or after acid treatment) obtained from the LIMMA analyses were compared to lists of gene names categorized by biological function (based on The Institute for Genomic Research Comprehensive Microbial Resource: http://cmr.tigr.org) or lists of select transcriptional regulons. Definitions of σ^B, CodY, and PrfA regulons have been described previously (Chan *et al.*, 2007). σ^H and σ^L regulons are based on genes found to be positively regulated (i.e. showing lower transcript levels in an isogenic deletion mutant) by these sigma factors (Chaturongakul

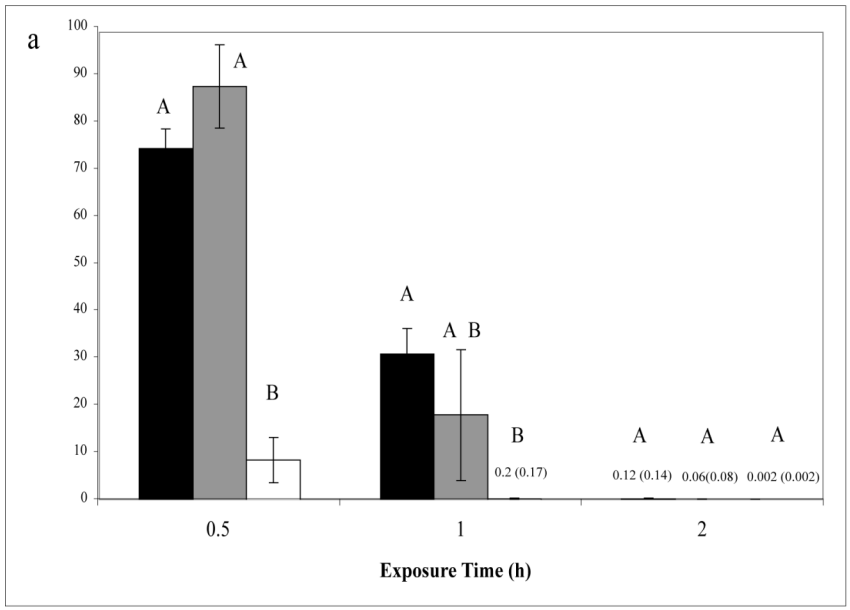
et al., submitted for publication). HrcA and CtsR regulons are genes that were reported to be negatively regulated (i.e., showing higher transcript levels in isogenic deletion mutants) by these two proteins in Hu *et al*. (2007a) and Hu *et al*. (2007b) respectively. For each growth temperature/growth phase combination (e.g., cells grown to log phase at 7°C), separate GSEA analyses were performed for cultures exposed to 5 and 15 min acid treatment. Gene sets that were significantly ($q \leq 0.05$) enriched for genes with positive fold changes at a given temperature or after acid treatment were said to be positively enriched whereas gene sets that were significantly enriched among negative fold changes were said to be negatively enriched.

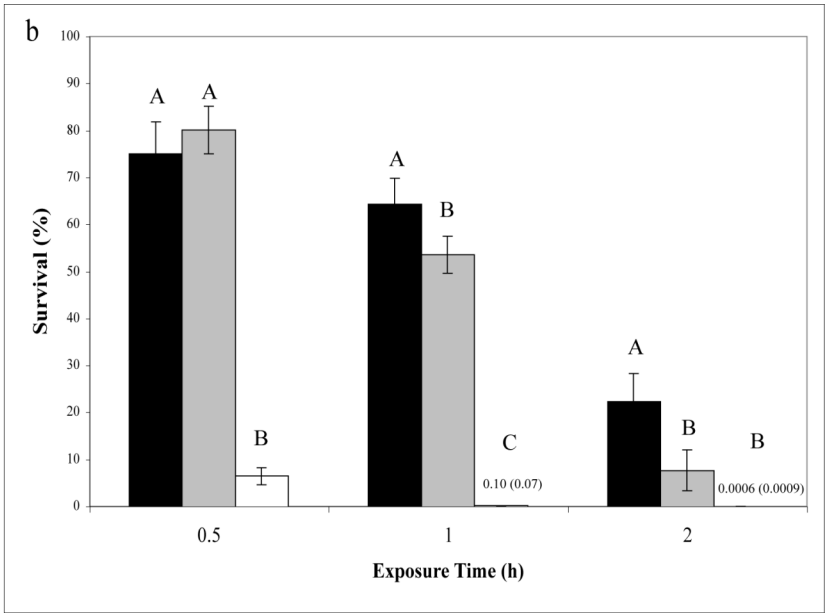
A database of Hidden Markov Model (HMM) search results from previous studies was searched to identify putative binding sites upstream of genes found to be differentially expressed after acid treatment. Searches to identify putative binding sites for σ^B (Raengpradub *et al.*, 2008), HrcA (Hu *et al.*, 2007a), CtsR (Hu *et al.*, 2007b), σ^H, and σ^L (Chaturongakul et al., submitted for publication) have been previously reported. Criteria used for filtering results for significant hits varied in these reports. For example, Raengpradub *et al*. (2008) reported a putative promoter to be significant if it was 20-300 bp upstream of an ORF and had an E-value of ≤ 0.01, Hu *et al*. (2007b) reported a putative promoter to be significant if it was ≤ 300 bp upstream of an ORF with an E-value of ≤ 0.01, and Chaturongakul et al. (submitted for publication) reported a significant hit to be ≤ 750 bp upstream of an ORF with an E-value of ≤ 0.1. In our study, the same distance range and E-value criteria were used to filter the search results for every regulator. For this study here, putative binding sites were considered significant if they were 5 - 300 bp upstream of the predicted ORF with an E-value of ≤ 0.02.

RESULTS

Growth at 7°C increases susceptibility to artificial gastric fluid compared to growth at 30°C or 37°C. *L. monocytogenes* 10403S and J1-194 grown at 7°C, 30°C, or 37°C to stationary phase were exposed to artificial gastric fluid at 37°C for 0.5, 1 or 2h. 10403S grown at 7°C showed significantly lower survival after 0.5 and 1 h acid exposure ($p < 0.05$; Tukey HSD). For example, after 0.5 h exposure, 10403S grown at 7°C showed 8.5% survival compared to 88.5% and 75.1% survival for 30°C- and 37°C-grown bacteria (Figure 3.1a). After 2 h exposure, 10430S grown at 7°C showed 0.002% survival compared to 0.06% and 0.12% survival for 30°C- and 37°C-grown 10403S, respectively, though at this time point the effect of growth temperature on survival was not statistically significant ($p = 0.3645$; ANOVA). After 0.5 and 1 h exposure to artificial gastric fluid, J1-194 grown at 7°C showed significantly lower survival than J1-194 grown at 30°C or 37°C, and after 2 h exposure, J1-194 grown at 7°C showed significantly lower survival than J1-194 grown at 37°C ($p < 0.05$; Tukey HSD; Figure 3.1b). For example, after 0.5 h exposure, J1-194 grown at 7°C showed 6.4 % survival compared to 80.1% and 75.1% survival, for 30°C- and 37°C grown J1-194, respectively (Figure 3.1b). Finally, at 2 h exposure J1-194 grown at 7°C showed 0.00061 % survival compared to 7.6 % and 22.3 % for 30°C- and 37°C-grown J1-194, respectively. Overall, these results show that *L. monocytogenes* grown at 7°C are more susceptible to artificial gastric fluid than cells grown at higher temperatures. After 1 h and 2 h treatment, the lineage I strain, J1-194, grown at 30°C or 37°C showed significantly greater survival than the lineage II strain, 10403S, grown at the same temperature ($p < 0.05$; t-tests). Other studies have shown that different strains of *L. monocytogenes* differ in their ability to survive stress (Lianou *et al.*, 2006; Adrião *et al.*, 2008). The ability to respond to acid stress can, thus, vary among different strains of *L. monocytogenes*, though additional acid stress experiments using additional strains

Figure 3.1. *L. monocytogenes* survival after challenge with artificial gastric fluid. *L. monocytogenes* 10403S (panel a) and J1-194 (panel b) were grown to stationary phase at 37°C (black), 30°C (gray) or 7°C (white) in BHI before being exposed to artificial gastric fluid (pH approx 3.5) at 37°C for varying amounts of time. Capital letters represent statistical groupings of growth temperatures within exposure time (ANOVA; *post hoc* Tukey HSD; $p \leq 0.05$). Cells grown at 37°C were present in higher numbers than cells grown at 7°C at 0.5 and 1 hr post acid exposure. Bars represent the average of three biological replicates. Error bars represent standard deviation. Means (standard deviations) that are below visible range are displayed in lieu of bars.


a
100
90
80
70
60
50
40
30
20
10
0
A
A
B
A
A B
B
A
A
A
0.2 (0.17)
0.12 (0.14)
0.06(0.08)
0.002 (0.002)
0.5
1
2
Exposure Time (h)


b
Survival (%)
100
90
80
70
60
50
40
30
20
10
0
A
A
B
A
B
C
A
B
B
0.10 (0.07)
0.0006 (0.0009)
0.5
1
2
Exposure Time (h)

will need to be performed before certain strains or genetic lineages of *L. monocytogenes* can be classified as "acid resistant" or "acid sensitive."

***L. monocytogenes* grown at 37°C are more resistant to acidified BHI.** To assess the effects of growth temperature and growth phase on the sensitivity of *L. monocytogenes* 10403S to sudden inorganic acid stress (which will be referred to subsequently as acid treatment), cells were cultured to log or stationary phase at 37°C or 7°C in MOPS-buffered BHI. Then the medium was adjusted to pH 3.5 with HCl, and survival at 37°C was determined after 5, 15, 60, and 120 min of exposure. The growth medium was buffered to help ensure homogeneity of culture pH between temperatures and between growth phases (the average pH of 7°C cultures was 7.11 (SD = 0.01) and 6.92 (SD = 0.14) for log and stationary phase cells, respectively, and the average pH for 37°C cultures was 7.08 (SD = 0.06) and 6.89 (SD = 0.15) for log and stationary phase cells, respectively).

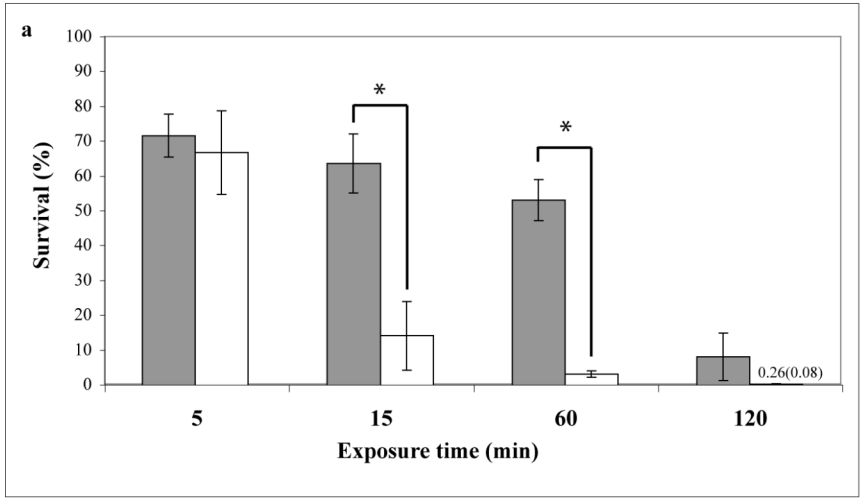
Survival of 7°C-grown log phase cells was significantly lower than 37°C-grown, log phase cells at 15 and 60 min ($p < 0.05$; t-tests; Fig 3.2a). The survival of *L. monocytogenes* cells grown to log phase at 7°C was 14.0% and 3.2% after 15 min and 60 min acid treatment, respectively compared to 63.4% and 53.0% for log phase cells grown at 37°C (Fig 3.2a). At 120 min, 37°C grown cells showed mean survival of 8.4% compared to 7°C grown cells, which showed 0.26% (Fig 3.2a), and the t-test p-value for this comparison was borderline significant ($p = 0.0928$). These results indicate that cells grown to log phase at 7°C are more susceptible to acid shock in BHI that those grown to log phase at 37°C.

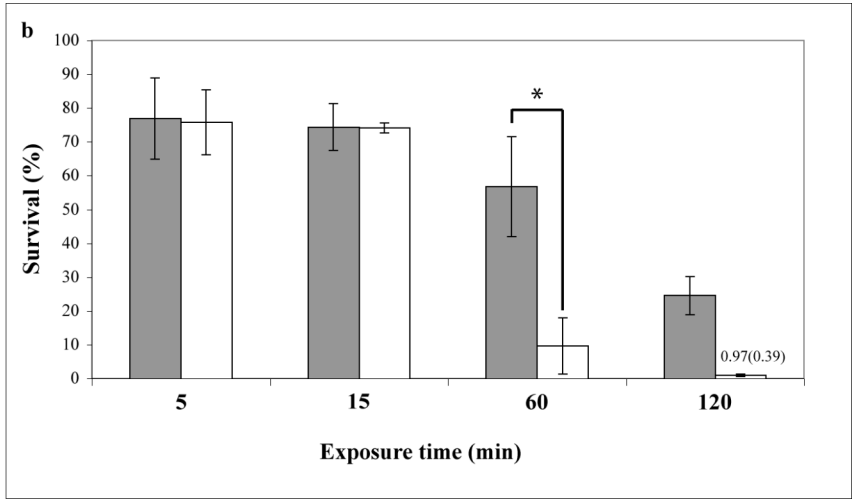
For *L. monocytogenes* grown to stationary phase and exposed to acid for 5 or 15 min, there was no effect of temperature on survival ($p = 0.9051$ for 5 min; $p = 0.9559$ for 15 min; t-test). However, stationary phase cells grown at 37°C showed 56.7% and 24.5%

survival at 60 and 120 min, respectively compared to 9.6% and 0.97%, respectively, for 7°C-grown cells (Figure 3.2b), and at 60 min, survival of 37°C-grown cells was significantly higher than 7°C-grown cells ($p = 0.0285$). These results indicate that stationary phase *L. monocytogenes* cells grown at 37°C to stationary phase are better able to survive exposure to pH = 3.5 for 60 min than stationary phase cells grown at 7°C. *L. monocytogenes* grown to stationary phase at 7°C were more resistant to acid treatment after 15 min than cells grown to log phase at 7°C (14.1% survival vs. 74.2% survival; $p = 0.0009$; t-test), indicating that in addition to growth temperature, growth phase also affects acid stress survival. These results are consistent with previous results showing that stationary phase cells or cells grown

Cold response genes are up-regulated at 7°C before acid treatment. Initial analyses showed 355 genes (209 in log phase, 163 in stationary phase) that had significantly higher transcript levels (LIMMA; fold-change ≥ 2.0; adj. p-value ≤ 0.05) at 7°C compared to 37°C (see Table S3.1 for a complete list of genes up-regulated at 7°C compared to 37°C). There were 17 genes that showed significantly higher transcript levels at 7°C in both log and stationary phase. There were 298 genes (198 in log phase, 124 in stationary phase) that showed lower transcript levels at 7°C compared to 37°C (see Table S3.2 for a complete list of genes down-regulated at 7°C), and there were 24 genes that showed lower transcript levels at 7°C in both log and stationary phase. There were 14 genes that showed higher transcript levels at 7°C in one growth phase and lower transcript levels at 7°C in the other growth phase (Tables S3.1 and S3.2). at slower growth rates are more resistant to acid stress compared to log-phase cells or cells growing at faster growth rates, respectively (Patchett *et al.*, 1996; Davis *et al.*, 1996; Samelis *et al.*, 2003). Overall, our results show that *L. monocytogenes* grown at lower temperatures are more susceptible to acid shock in an

Figure 3.2. *L. monocytogenes* survival in acidified BHI. *L. monocytogenes* 10403S was grown to log phase (a) or stationary phase (b) at 7°C (white bars) or 37°C (black bars) before the cultures were adjusted to pH 3.5 and incubated at 37°C for varying amounts of time. At both log and stationary phase, cells grown at 7°C showed faster reductions in viability than cells grown at 37°C. Time-points showing statistically higher survival in 37°C-grown cells are marked with a (*). Bars represent the average of three biological replicates. Error bars represent standard deviation. Means (standard deviations) that are below visible range are displayed in lieu of bars.


a
Survival (%)
100
90
80
70
60
50
40
30
20
10
0
*
*
0.26(0.08)
5
15
60
120
Exposure time (min)


b
Survival (%)
100
90
80
70
60
50
40
30
20
10
0
*
0.97(0.39)
5
15
60
120
Exposure time (min)

artificial gastric fluid model or in an acidified BHI system, and that, in general, cells in log phase are more susceptible to acid treatment than stationary phase cells.

In addition to single gene comparisons between 7°C and 37°C, GSEA was used to compare predefine gene sets between the two conditions. For *L. monocytogenes* grown to log phase, the category "cellular processes chemotaxis and motility" (Table S3.3) were positively enriched at 7°C compared to 37°C, as was the σ^L regulon (Table S3.4). Nine functional gene categories (Table S3.3) were negatively enriched at 7°C compared to 37°C (i.e., genes in these categories as a group showed lower transcript levels at 7°C compared to 37°C). The CodY, σ^B, σ^L, and HrcA regulons were also negatively enriched in 7°C-grown stationary phase cells (Table S3.4).

For *L. monocytogenes* grown to stationary phase the category "energy metabolism ATP-proton motive force interconversion" (Table S3.3) and the CodY-regulon (Table S3.4) were positively enriched at 7°C indicating that genes involved in proton transport and CodY may be involved in log phase growth at cold temperatures. In stationary phase, the σ^H, σ^L, and CtsR regulons were negatively enriched at 7°C compared to 37°C, indicating that these regulators may show reduced activities in stationary phase at cold temperatures.

A previous study defined the *L. monocytogenes* cold (i.e., 4°C) regulon in log and stationary phase (Chan *et al.*, 2007). Though Chan *et al*. (2007) determined differential gene transcription levels in *L. monocytogenes* grown at 4°C vs. 37°C grown without aeration, and though in our study *L. monocytogenes* were grown at 7°C or 37°C with aeration, our results largely confirmed their results. Similarly to Chan *et al*. (2007), we found lower transcript levels of PrfA-regulated virulence genes (i.e., *plcA*, *hlyA*, *actA*, and *plcB*) at 7°C, enrichment of CodY-dependent genes at 7°C in stationary phase, and enrichment of σ^L-dependent genes at 37°C in stationary phase

(Table 7). Furthermore, we found, in log phase at 7°C, up-regulation of lmo1450, which encodes an RNA helicase, down regulation of *groEL* which encodes a heat shock protein, higher transcription of bacteriophage genes at 7°C, and higher transcript levels of motility genes at 7°C which are all consistent with Chan *et al*. (2007). However, there were observed differences between our results and the results of Chan *et al*. (2007). For example, using the same criteria to define differential transcription (i.e. fold-change ≥ 2.0; adj. p-value ≤ 0.05), we found 209 and 163 genes to be expressed at higher levels at 7°C compared to 37°C in log and stationary phase, respectively whereas Chan *et al*. (2007) found 110 and 237 genes to be transcribed at higher levels at 4°C compared to 37°C in log and stationary phase, respectively. Differences in growth conditions between our study and Chan *et al*. (2007) may explain the observed differences in numbers of differentially transcribed genes between the two studies.

The early transcriptional response to acid shock differs between *L. monocytogenes* cells grown at 7°C and cells grown at 37°C. To determine how growth temperature affects transcriptional response to acid stress, a full genome microarray was used to determine changes gene transcription after exposure to pH 3.5 for 5 or 15 min for log or stationary phase cells grown at 7°C or 37°C in MOPS-buffered BHI. In general, there were more genes that showed higher transcript levels after acid shock (either 5 or 15 min) for *L. monocytogenes* grown at 37°C compared to cells grown at 7°C. Specifically, for cells grown at 37°C to log phase, 65 genes showed higher transcript levels after acid treatment compared to 16 genes for 7°C-grown log phase cells (Table 3.1). There were no genes that showed higher transcript levels after acid treatment in both 37°C-grown and 7°C-grown log phase cells (Table 3.1). In cells grown to stationary phase at 37°C, 13 genes showed higher transcript

levels after acid treatment compared to 33 for cells grown at 7°C to stationary phase (Table 3.1). Only one gene (lmo2293) showed higher transcript levels in both 7°C-grown and 37°C-grown stationary phase cells after acid treatment (Table 3.1). Three genes (lmo2290-lmo2291) showed higher transcript levels after acid exposure in both 7°C-grown stationary phase and 7°C-grown log phase cells (Table 3.1). For *L. monocytogenes* grown at 37°C to log phase, there were 85 genes that showed lower transcript levels after acid stress, compared to 13 genes that showed lower transcript levels after acid treatment for 7°C-grown log phase cells (Table 3.1). There were nine genes that showed lower transcript levels after acid stress in both 7°C-grown and 37°C-grown log phase cells (Table 3.1). For *L. monocytogenes* grown at 37°C to stationary phase, 49 genes showed lower transcript levels after acid stress, compared to 13 genes for cells grown at 7°C to stationary phase (Table 3.1). Only five genes showed lower transcript levels after acid stress in both 7°C-grown and 37°C-grown stationary phase *L. monocytogenes* (Table 3.1). There were two genes (lmo0883 and lmo2210) that showed lower transcript levels in both 7°C-grown log phase cells and 7°C-grown stationary phase cells, and eight genes that showed lower transcript levels after acid stress in both 37°C-grown log phase and 37°C-grown stationary phase cells (Table 3.1). In general there was little overlap between growth temperatures or growth phases in genes differentially transcribed after acid treatment, and in general, there were more genes differentially expressed in 37°C grown cells after acid treatment that in 7°C-grown cells (Table 3.1).

Genes differentially expressed after acid treatment in *L. monocytogenes* grown to log phase at 37°C. In *L. monocytogenes* grown to log phase at 37°C, there were 150 genes that showed differential expression after acid treatment for 5 or 15 min. (See

Table 3.1. Number and examples of genes differentially transcribed[a] after acid treatment[b] of *Listeria monocytogenes* grown to log or stationary phase at 7°C or 37°C

Growth temperature and growth phase prior to acid stress	Transcribed at higher levels after acid		Transcribed at lower levels after acid	
	Number	Examples	Number	Examples
7°C log phase only	13	lmo0066, lmo0320, lmo1151	6	lmo0299, lmo0735, lmo1114
7°C stationary phase only	28	*lmaA*, *lmaB*, *lmaD*	5	lmo0049, lmo0728, lmo2445
37°C log phase only	64	*clpC*, *grpE*, *clpP*	68	lmo0680, lmo1406, *minD*
37°C stationary phase only	12	*rplU*, lmo2362, *rplP*	38	lmo0770, *murC, ndk*
7°C log phase and 7°C stationary phase	3	lmo2290, lmo2291, lmo2292	1	lmo2210
37°C log phase and 37°C stationary phase	0		4	lmo0998, lmo1597, lmo1828
7°C log phase and 37°C log phase	0		7	lmo1639, lmo1749, lmo2408
7°C stationary phase and 37°C stationary phase	1	lmo2293	2	lmo1049, *atpI*
7°C log phase and 37°C stationary phase	0		0	
7°C stationary phase and 37°C log phase	1	lmo0903	2	lmo1828, lmo1926
7°C log phase and 7°C stationary phase and 37°C log phase	0		1	lmo0883
7°C log phase and 7°C stationary phase and 37°C stationary phase	0		0	
37°C log phase and 37°C stationary phase and 7°C log phase	0		1	lmo1639
37°C log phase and 37°C stationary phase and 7°C stationary phase	0		3	lmo0217, lmo1828, *deoD*
All conditions tested	0		0	
Total	122		138	

[a]Genes showing a fold change with an absolute value ≥ 1.5 and an adj. p value ≤ 0.05 (LIMMA) after acid treatment for 5 or 15 min.

[b]Acid treatment was BHI-MOPS adjusted to pH 3.5 with HCl followed by incubation at 37°C

Tables 3.2 and 3.3 for lists of select genes and Table S3.5 for a full list of genes differentially expressed after acid stress for 37°C-grown log phase cells).

(i) genes with higher transcript levels after acid treatment. For *L. monocytogenes* grown at 37°C to log phase, 65 genes were expressed at higher levels after acid treatment. There were 10 operons that had at least two genes showing higher transcript levels after acid treatment (Table 3.2). GSEA of fold changes after 5 min acid treatment revealed a significant positive enrichment for "protein fate: protein folding and stabilization" indicating that genes in this category may be involved in acid adaptation (Table 3.4). An example of a gene in this category that showed significantly higher transcript levels after acid treatment of 37°C-gown log-phase cells was heat shock gene *grpE* (adj. p-value < 0.01; LIMMA; Table 3.2). For cells grown to 37°C to log phase and acid treated for 15 min, genes encoding proteins in the category "purines, pyrimidines, nucleosides, and nucleotides: pyrimidine ribonucleotide biosynthesis" were enriched for positive fold-changes (Table 3.4), exemplified by *pyrD* and *pyrAB* (both encode enzymes involved in pyrimidine biosynthesis), which showed significantly higher transcript levels after acid treatment for 15 min (adj p-value <0.01; LIMMA). For log phase cells grown at 37°C, GSEA showed significant positive enrichment for σ^B -regulated genes and σ^H-regulated genes after both 5 and 15 min acid treatment (Table 3.5), indicating that the these two transcriptional regulators may be involved in the response of log-phase cells to sudden acid treatment. Examples of σ^B-regulated genes having fold-changes greater than 1.5 and an adjusted p-value ≤ 0.05 after 5 or 15 min acid treatment included oligopeptides transporter genes lmo0136 and lmo0137 (Table 3.2). Other σ^B-dependent genes that showed significantly higher transcript levels after acid treatment of 37°C-grown log phase cells include *hrcA*, which encodes the class I heat shock gene repressor protein. Class I gene *grpE*, which is cotranscribed with both showed significantly higher

Table 3.2. Operons differentially transcribed[a] after 5 or 15 min acid treatment[b] in *Listeria monocytogenes* grown at 37°C to log or stationary phase.

Gene Name	Gene Function[c]	Fold change[d] after acid treatment				Putative Binding Sites[e]
		Log phase		Stationary phase		
		5 min	15 min	5 min	15 min	
lmo0113	similar to protein gp35 from Bacteriophage A118	-	-	-	-1.81 *	-
lmo0114	similar to putative repressor C1 from lactococcal bacteriophage Tuc2009	-	-	-	-1.57 *	-
lmo0136	similar to oligopeptide ABC transporter, permease protein	2.27 **	2.10 **	-	-	-
lmo0137	similar to oligopeptide ABC transporter, permease protein	1.64 **	1.54 *	-	-	B
lmo0180	similar to sugar ABC transporter, permease protein	1.71 *	1.80 *	-	-	-
lmo0181	similar to sugar ABC transporter, sugar-binding protein	1.75 *	1.87 **	-	-	h
lmo0230	similar to *B. subtilis* YacH protein	1.53 *	1.56 *	-	-	B,i
lmo0231	similar to arginine kinase	-	1.60 **	-	-	-
lmo0232 (*clpC*)	endopeptidase Clp ATP-binding chain C	1.59 **	1.67 **	-	-	-
lmo0680	similar to flagella-associated protein flhA	-1.59 **	-1.87 ***	-	-	-
lmo0681	similar to flagellar biosynthesis protein FlhF	-1.54 *	-1.84 **	-	-	-
lmo0683	similar to chemotactic methyltransferase CheR	-2.16 **	-2.68 **	-	-	-
lmo0806	similar to transcription regulator	-	2.03 *	-	-	i
lmo0808	similar to spermidine/putrescine ABC transporter, permease	1.87 *	2.08 *	-	-	-
lmo0809	similar to spermidine/putrescine ABC transporter, permease	1.90 **	2.07 **	-	-	-
lmo0810	similar to spermidine/putrescine-binding protein	2.27 **	2.25 ***	-	-	-
lmo0811	similar to carbonic anhydrase	2.55 **	2.78 **	-	-	-
lmo0847	similar to Glutamine ABC transporter (binding and transport protein)	2.27 **	2.29 **	-	-	-

Table 3.1 (Continued)

Gene Name	Gene Function[c]	Fold change[d] after acid treatment				Putative Binding Sites[e]
		Log phase		Stationary phase		
		5 min	15 min	5 min	15 min	
lmo0848	similar to amino acid ABC transporter, ATP-binding protein	1.82 **	1.91 **	-	-	-
lmo1041	similar to molybdate ABC transporter binding protein	-	-	-	-1.66 *	-
lmo1046	molybdenum cofactor biosynthesis protein C	-	-	-	-1.58 *	-
lmo1089 (*tagD*)	highly similar to glycerol-3-phosphate cytidylyltransferase (gct), CDP-glycerol pyrophosphorylase (teichoic acid biosynthesis protein D)	-1.86 ***	-2.03 ***	-	-	-
lmo1090	similar to glycosyltransferases	-1.51 **	-1.64 ***	-	-	b, h
lmo1406 (*pflB*)	pyruvate formate-lyase	-4.03 *	-4.30 *	-	-	-
lmo1407 (*pflC*)	pyruvate-formate lyase activating enzyme	-2.86 *	-2.72 *	-	-	-
lmo1474 (*grpE*)	heat shock protein GrpE	1.84 **	1.71 **	-	-	-
lmo1475 (*hrcA*)	transcription repressor of class I heat-shock gene HrcA	1.83 **	1.76 **	-	-	B,I,III
lmo1541	similar to unknown protein	-	-	1.60 *	1.57 **	-
lmo1542 (*rplU*)	ribosomal protein L21	-	-	1.51 *	1.69 ***	-
lmo1556 (*hemC*)	highly similar to porphobilinogen deaminases (hydroxymethylbilane synthase)	-	-1.59 *	-	-	h
lmo1557 (*hemA*)	highly similar to glutamyl-tRNA reductase	-1.72 ***	-1.87 ***	-	-	-
lmo1589 (*argB*)	highly similar to N-acetylglutamate 5-phosphotransferase	-	-4.37 *	-	-	b

Table 3.1 (Continued)

Gene Name	Gene Function[c]	Fold change[d] after acid treatment				Putative Binding Sites[e]
		Log phase		Stationary phase		
		5 min	15 min	5 min	15 min	
lmo1590 (*argJ*)	highly similar to ornithine acetyltransferase and amino-acid acetyltransferases	-6.76 *	-4.84 *	-	-	-
lmo1591 (*argC*)	similar to N-acetylglutamate gamma-semialdehyde dehydrogenases	-25.44 *	-11.04 *	-	-	-
lmo1833 (*pyrD*)	highly similar to dihydroorotase dehydrogenase	-	1.57 **	-	-	h
lmo1835 (*pyrAB*)	highly similar to carbamoyl-phosphate synthetase (catalytic subunit)	1.83 *	1.91 **	-	-	-
lmo1856 (*deoD*)	purine nucleoside phosphorylase	-	-1.59 ***	-	-	-
lmo1857	similar to hypoyhetical protein	-	-1.70 ***	-	-	-
lmo1929 (*ndk*)	similar to nucleoside diphosphate kinase	-	-	-	-1.73 ***	-
lmo1932	heptaprenyl diphosphate synthase component I	-	-	-	-1.51 *	-
lmo2040 (*ftsL*)	similar to cell-division protein FtsL	-	-1.51 ***	-	-	-
lmo2041	similar to unknown proteins	-	-1.68 ***	-	-	-
lmo2114	similar to ABC transporter (ATP-binding protein)	2.28 **	2.25 **	-	-	-
lmo2115	similar to ABC transporter (permease)	2.23 **	2.16 **	-	-	-
lmo2260	similar to unknown proteins	1.87 *	1.78 *	-	-	-
lmo2261	similar to unknown proteins	1.63 *	-	-	-	-
lmo2293	Protein gp10 [Bacteriophage A118]	-2.34 *	-	-	-	-
lmo2295	Protein gp8 [Bacteriophage A118]	-2.88 *	-2.39 *	-	-	-

Table 3.1 (Continued)

Gene Name	Gene Function[c]	Fold change[d] after acid treatment				Putative Binding Sites[e]
		Log phase		Stationary phase		
		5 min	15 min	5 min	15 min	
lmo2296	similar to coat protein [Bacteriophage SPP1]	-2.32 *	-	-	-	-
lmo2362	similar to amino acid antiporter (acid resistance)	-2.97 **	-2.80 **	-	-	-
lmo2363	similar to glutamate decarboxylase	-2.59 *	-2.28 *	-	-	-
lmo2408	similar to repressor protein	-1.61 ***	-1.94 ***	-	-	-
lmo2409	unknown	-3.28 **	-4.21 ***	-	-	h
lmo2484	similar to *B. subtilis* YvlD protein	-	2.11 *	-	-	B
lmo2487	similar to *B. subtilis* YvlB protein	1.56 *	1.74 **	-	-	-
lmo2625 (*rplP*)	ribosomal protein L16	-	-	-	1.69 *	-
lmo2630 (*rplW*)	ribosomal protein L23	-	-	-	1.50 *	-
lmo2632 (*rplC*)	ribosomal protein L3	-	-	-	1.50 *	-
lmo2633 (*rpsJ*)	ribosomal protein S10	-	-	-	1.64 *	-

[a]Operons with at least two genes showing a fold change with an absolute value ≥ 1.5 after acid treatment and an adj. p value ≤ 0.05 after acid treatment

[b]Acid treatment was done at 37°C in BHI adjusted to pH 3.5 with HCl

[c]Gene functions were based on annotation provided by TIGR (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi).

[d]Superscripts are adjusted p values: "***" (< 0.001), "**" (< 0.01), "*" (≤ 0.05), "NS" (> 0.05)

[e]Putative binding sites for Sigma B (B,b), Sigma H (H,h), Sigma L (L, l), CtsR (III,iii), HrcA (I, i) are based on a recombing of the HMM results reported in Hu 2007a, Hu 2007b, Raengpradub 2008, and (Chatanurongakul 2010 submitted). Hits were considered

Table 3.3. Selected genes differentially transcribed[a] after 5 or 15 min. acid treatment[b] in *L. monocytogenes* grown at 37 °C to log or stationary phase

Name	Description of encoded protein[c]	Fold change after acid stress[d]				Putative Binding Sites[e]
		Log phase		Stationary phase		
		5 min	15 min	5 min	15 min	
lmo0130	similar to 5-nucleotidase, putative peptidoglycan bound protein (LPXTG motif)	1.63 **	1.65 **	-	-	-
lmo0135	similar to oligopeptide ABC transport system substrate-binding proteins	2.36 **	2.27 **	-	-	-
lmo0152	similar to oligopeptide ABC transporter-binding protein	2.15 **	2.36 **			-
lmo0189	highly similar to *B. subtilis* Veg protein	-	-	-	1.66 **	-
lmo0200 (*prfA*)	listeriolysin positive regulatory protein	1.72 *	1.80 **			B
lmo0217	similar to *B. subtilis* DivIC protein	-1.56 **	-1.96 ***	-2.37 *	-3.03 ***	-
lmo0238 (*cysE*)	similar to serine O-acetyltransferase	-1.52 *	-1.65 **	-	-	-
lmo0269	similar to transporter	-	-2.13 **	-	-	-
lmo0278	similar to sugar ABC transporter, ATP-binding protein	-	1.79 *	-	-	-
lmo0279	highly similar to anaerobic ribonucleoside-triphosphate reductase	-1.63 *	-1.64 *	-	-	-
lmo0354	similar to fatty-acid--CoA ligase	-2.15 **	-2.19 **	-	-	-
lmo0372	similar to beta-glucosidase	-	1.59 *	-	-	l

Table 3.3 (Continued)

Name	Description of encoded protein[c]	Fold change after acid stress[d]				Putative Binding Sites[e]
		Log phase		Stationary phase		
		5 min	15 min	5 min	15 min	
lmo0455	similar to unknown proteins	-1.54 *	-1.64 **	-	-	-
lmo0490	similar to shikimate 5-dehydrogenase	-2.67 **	-2.96 **	-	-	h
lmo0496	similar to B. subtilis YnzC protein	-	1.52 **	-	-	B, III
lmo0523	similar to B. subtilis YybC protein	-	-	-	6.34 *	-
lmo0524	similar to putative sulfate transporter	-	-	-	-1.72 *	-
lmo0533	similar to unknown proteins	-1.57 ***	-1.71 ***	-	-	-
lmo0573	conserved hypothetical protein	1.73 *	1.97 **	-	-	-
lmo0578	putative conserved membrane protein	-	-	-	-2.15 **	-
lmo0604	similar to B. subtilis YvlA protein	1.54 *	1.61 *	-	-	-
lmo0618	similar to protein kinase	-1.51 **	-1.56 **	-	-	-
lmo0648	similar to membrane proteins	-	-	-	-1.83 **	-
lmo0676	similar to flagellar biosynthesic protein FliP	-1.92 ***	-2.49 ***	-	-	-
lmo0721	putative fibronectin-binding protein	-1.56 **	-1.54 **	-	-	-
lmo0770	similar to transcriptional regulator (LacI family)	-	-	-	-1.68 *	-
lmo0773	similar to alcohol dehydrogenase	-1.79 ***	-1.81 ***	-	-	-
lmo0800	similar to B. subtilis YqkB protein	-	1.52 *	-	-	-

Table 3.3 (Continued)

Name	Description of encoded protein[c]	Fold change after acid stress[d]				Putative Binding Sites[e]
		Log phase		Stationary phase		
		5 min	15 min	5 min	15 min	
lmo0814	similar to oxidoreductases	-1.97 **	-1.78 *	-	-	i
lmo0883	similar to B. subtilis YbtB protein	-2.22 ***	-2.81 ***	-	-	b
lmo0912	similar to transporters (formate)	-3.98 **	-4.14 ***	-	-	i
lmo0960	similar to proteases	-2.37 ***	-2.10 **	-	-	-
lmo0997 (*clpE*)	ATP-dependent protease	2.01 **	1.83 *	-	-	iii
lmo1049	similar to molybdopterin biosynthesis protein MoeB	-	-	-	-1.75 **	-
lmo1066	similar to extragenic suppressor protein SuhB and to myo-inositol-1(or 4)-monophosphatase	-1.64 **	-1.69 **	-	-	-
lmo1138	similar to ATP-dependent Clp protease proteolytic component	1.63 **	1.61 **	-	-	iii
lmo1166	similar to NADPH-dependent butanol dehydrogenase	-	-	-	-1.84 **	-
lmo1227	similar to uracil-DNA glycosylase	-	-	-	-1.55 **	-
lmo1294 (*miaA*)	similar to tRNA isopentenylpyrophosphate transferase	-	-1.67 ***	-	-	H
lmo1348	similar to aminomethyltransferase	-	-	-	-2.62 *	-

Table 3.3 (Continued)

Name	Description of encoded protein[c]	Fold change after acid stress[d]				Putative Binding Sites[e]
		Log phase		Stationary phase		
		5 min	15 min	5 min	15 min	
lmo1360 (*folD*)	highly similar to methylenetetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase	-1.81 ***	-2.02 ***	-	-	-
lmo1397 (*cinA*)	similar to competence-damage inducible protein CinA	-	-	-	-1.54 *	-
lmo1441	similar to putative peptidoglycan acetylation protein	-	-1.86 **	-	-	h
lmo1544 (*minD*)	highly similar to cell division inhibitor (septum placement) protein MinD	-	-1.52 ***	-	-	h
lmo1604	2-cys peroxiredoxin	-	-	-	-1.51 *	-
lmo1605 (*murC*)	UDP-N-acetyl muramate-alanine ligases	-	-	-	-1.63 *	-
lmo1625	similar to putative transporters	-	-1.53 ***	-	-	-
lmo1634	similar to Alcohol-acetaldehyde dehydrogenase	-6.66 *	-6.24 *	-	-	-
lmo1639	similar to dna-3-methyladenine glycosidase	-1.54 **	-1.71 **	-2.06 *	-1.83 *	-
lmo1710	similar to putative flavodoxin	-	-1.60 ***	-	-	-
lmo1749	similar to shikimate kinase	-2.46 ***	-2.88 ***	-	-	-
lmo1750	similar to unknown protein	-1.66 *	-1.61 *	-	-	h
lmo1752	unknown	-	1.55 **	-	-	-
lmo1803	similar to FtsY of *E. coli* and SRP receptor alpha-subunit	1.67 **	1.70 **	-	-	-

Table 3.3 (Continued)

Name	Description of encoded protein[c]	Fold change after acid stress[d]				Putative Binding Sites[e]
		Log phase		Stationary phase		
		5 min	15 min	5 min	15 min	
lmo1867	similar to pyruvate phosphate dikinase	-	1.53 **	-	-	-
lmo1884	similar to xanthine permeases	1.54 *	1.53 *	-	-	I, h, l
lmo1917 (*pflA*)	similar to pyruvate formate-lyase	-4.54 *	-	-	-	b, h
lmo1926	similar to chorismate mutase	-1.93 **	-2.52 ***	-	-	-
lmo1939 (*cmk*)	similar to cytidylate kinase	-	-	-	-1.62 *	-
lmo1992	similar to alpha-acetolactate decarboxylase	1.68 ***	1.65 ***	-	-	-
lmo2006 (*alsS*)	similar to alpha-acetolactate synthase protein, AlsS	-	1.52 **	-	-	-
lmo2020 (*divIVA*)	similar to cell-division initiation protein (septum placement)	-	-	-	-1.93 **	-
lmo2057 (*ctaB*)	highly similar to heme A farnesyltransferase	1.60 **	1.61 ***	-	-	-
lmo2090 (*argG*)	similar to argininosuccinate synthase	-8.74 *	-10.59 *	-	-	-
lmo2100	similar to transcriptional regulator (GntR family) and to aminotransferase (MocR-like)	1.61 *	-2.79 **	-	-	-
lmo2139	similar to ABC transporter (ATP-binding protein)	-	-	-	-1.54 **	-
lmo2152	similar to thioredoxin	1.52 **	-	-	-	-
lmo2173	similar to sigma-54-dependent transcriptional activator	-3.11 *	-3.23 *	-	-	-

Table 3.3 (Continued)

Name	Description of encoded protein[c]	Fold change after acid stress[d]				Putative Binding Sites[e]
		Log phase		Stationary phase		
		5 min	15 min	5 min	15 min	
lmo2176	similar to transcriptional regulator (tetR family)	-	-	-1.68 *	-1.91 **	-
lmo2184	similar to ferrichrome ABC transporter (binding protein)	-	1.50 **	-	-	B
lmo2190 (*mecA*)	competence negative regulator mecA	-	1.52 **	-	-	-
lmo2200	similar to transcription regulator	1.51 *	-	-	-	-
lmo2238	similar to transport system permease protein	-3.46 *	-3.75 **	-	-	-
lmo2241	similar to transcriptional regulators (GntR family)	-	-1.60 **	-	-	-
lmo2250 (*arpJ*)	similar to amino acid ABC transporter, permease protein	-3.89 *	-3.53 *	-	-	iii, l
lmo2293	Protein gp10 [Bacteriophage A118]	-	-	-	4.28 *	h
lmo2304	Bacteriophage A118 gp65 protein	-	-	-	3.03 *	-
lmo2334	similar to transcriptional regulator	-	-	-	-1.84 *	-
lmo2352	similar to LysR family transcription regulator	-	1.51 *	-	-	-
lmo2362	similar to amino acid antiporter (acid resistance)	-	-	-	1.85 *	-
lmo2378	similar to proteins involved in resistance to cholate and to NA(+) and in pH homeostasis	-	-	-	-1.56 *	-

Table 3.3 (Continued)

Name	Description of encoded protein[c]	Fold change after acid stress[d]				Putative Binding Sites[e]
		Log phase		Stationary phase		
		5 min	15 min	5 min	15 min	
lmo2380	similar to proteins involved in resistance to cholate and to NA(+) and in pH homeostasis	-	1.51 *	-	-	iii
lmo2393	similar to *B. subtilis* YuzD protein	1.71 **	1.68 **	-	-	-
lmo2433	similar to acetylesterase	1.98 *	2.10 **	-	-	-
lmo2468 (*clpP*)	ATP-dependent Clp protease proteolytic subunit	1.58 ***	1.64 ***	-	-	III
lmo2536 (*atpI*)	highly similar to ATP synthase subunit i	-	-	-	-1.56 ***	-
lmo2569	similar to dipeptide ABC transporter (dipeptide-binding protein)	1.74 **	1.78 **			-
lmo2586	similar to formate dehydrogenase alpha chain	-	-	-1.57 NS	-1.79 *	-
lmo2591	surface protein (GW repeat) similar to N-acetylmuramidase	1.58 **	1.53 **			-
lmo2658	similar to spermidine/spermine N1-acetyl transferase			-	-1.65 **	-
lmo2690	similar to transcription regulator, TetR family	-	1.54 **			b
lmo2718 (*cydA*)	highly similar to cytochrome D ubiquinol oxidase subunit I	-1.59 *	-			-
lmo2720	similar to acetate-CoA ligase	-2.07 **	-1.79 **			-
lmo2741	similar to drug-efflux transporters			-1.79 NS	-2.62 **	-
lmo2769	similar to ABC transporter, ATP-binding protein	-2.55 **	-3.28 ***	-1.94 NS	-1.66 NS	iii

Table 3.3 (Continued)

Name	Description of encoded protein[c]	Fold change after acid stress[d]				Putative Binding Sites[e]
		Log phase		Stationary phase		
		5 min	15 min	5 min	15 min	
lmo2773	similar to transcription antiterminator	-	-	1.53 *	1.72 ***	-
lmo2827	similar to transcriptional regulator (MarR family)	1.50 *	-	-	-	-
lmo2829	similar to yeast protein Frm2p involved in fatty acid signaling	-	-	-	-	-
lmo2851	similar to AraC-type regulatory protein	1.73 *	-	-	-	I, III

[a]genes showing a fold change with an absolute value ≥ 1.5 after acid treatment and an adj. p value ≤ 0.05 after acid treatment

[b]Acid treatment was BHI-MOPS adjusted to pH 3.5 with HCl followed by incubation at 37°C

[c]gene functions were based on annotation provided by ListiList (http://genolist.pasteur.fr/ListiList).

[d]Superscripts are adjusted p values: "***" (< 0.001), "**" (< 0.01), "*" (≤ 0.05), "NS" (> 0.05)

[e]Putative binding sites for Sigma B (B,b), Sigma H (H,h), Sigma L (L, l), CtsR (III,iii), HrcA (I, i) are based on a recombing of the HMM results reported in Hu 2007a, Hu 2007b, Raengpradub 2008, and (Chatanurongakul 2010 submitted). Hits were considered significant if they had an E value ≤ 0.02 and ≤ 300 bp upstream of the respective coding sequence. Uppercase denotes genes for which regulation has been confirmed by other studies (i.e. differential transcription in an isogenic deletion mutant vs. wild-type).

transcript levels after acid treatment for 5 and 15 min acid treatment (Table 3.2). *dnaK* which is also in the same operon had a fold change of 1.6 after acid stress for 5 min. [the p-value for *dnaK* was borderline significant (p = 0.057)].This indicates that σ^B-dependent heat shock genes are likely involved in the response to acid treatment The regulon of class III heat shock gene repressor CtsR, which is involved in a regulatory network with σ^B (Hu *et al.*, 2007b), was positively enriched after acid stress for 5 or 15 min, respectively (Table 3.5). CtsR-regulated *clpC*, *clpE*, *lmo1138*, and *clpP* all showed fold changes greater than 1.5 at 5 or 15 min after acid stress (adj p < 0.05; LIMMA) (Tables 3.2 and 3.3).

(ii) genes with lower transcript levels after acid treatment. In *L. monocytogenes* grown to log phase at 37°C, there were 85 genes that showed significantly lower transcription levels after acid treatment. There were 10 operons that had at least 2 genes showing significantly lower transcript levels after acid treatment (Table 3.2). For log phase cells acid treated for 5 or 15 min, GSEA revealed negative enrichments in the gene categories "energy metabolism fermentation" and "amino acid biosynthesis: glutamate family" (Table 3.4) indicating that these gene categories are down-regulated after acid stress. Examples of genes in "glutamate family" category that showed significantly lower transcript levels after 5 and 15 min acid treatment acid stress are lmo1589 (*argB*), lmo1590 (*argJ*), and lmo1591 (*argC*) (Table 3.2). Amino acid metabolism genes have been shown to play a role in acid tolerance in *Salmonella* Typhimurium (Álvarez-Ordóñez *et al.*, 2010) and *Escherichia coli* (Richard *et al.*, 2003).

GSEA showed a negative enrichment of HrcA-regulated genes after acid stress for 5 or 15 min (Table 3.5). Though HrcA-regulated genes showed negative enrichment after acid stress, as mentioned above, HrcA-regulated *grpE* and *hrcA*

Table 3.4. *Listeria monocytogenes* gene biological function categories[a] showing positive or negative enrichment[b] after acid treatment at 37°C

Functional Category	Bacteria grown to log phase at				Bacteria grown to stationary phase at			
	7°C		37°C		7°C		37°C	
	Acid treated for		Acid treated for		Acid treated for		Acid treated for	
	5 min	15 min	5 min	15 min	5 min	15 min	5 min	15 min
PURINES, PYRIMIDINES, NUCLEOSIDES, AND NUCLEOTIDES: PYRIMIDINE RIBONUCLEOTIDE BIOSYNTHESIS	(+)	-	-	(+)*	-	-	-	-
AMINO ACID BIOSYNTHESIS: GLUTAMATE FAMILY	-	-	(-)***	(-)***	-	-	-	-
ENERGY METABOLISM FERMENTATION	-	-	(-)***	(-)**	-	-	-	-
PROTEIN FATE: PROTEIN FOLDING AND STABILIZATION	-	-	(+)**	-	-	-	-	-
AMINO ACID BIOSYNTHESIS AROMATIC AMINO ACID FAMILY	-	-	-	-	-	(+)**	(+)*	-
ENERGY METABOLISM PENTOSE PYRUVATE DEHYDROGENASE	-	-	-	-	-	(+)***	(+)*	(+)*
PROTEIN SYNTHESIS: RIBOSOMAL PROTEINS: SYNTHESIS AND MODIFICATION	-	-	-	-	-	(+)***	(+)***	(+)***
TRANSPORT AND BINDING PROTEINS: CARBOHYDRATES, ORGANIC ALCOHOLS, AND ACIDS	-	-	-	-	-	(+)**	(+)*	-
VIRAL FUNCTIONS: GENERAL	-	-	-	-	(+)***	(+)***	-	-

Table 3.4 (Continued)

[a]categories based on biological function categories (The Institute for Genomic Research Comprehensive Microbial Resource: http://cmr.tigr.org)

[b]Reported changes are based on Gene Set Enrichment analysis. (+) indicates that genes comprising functional category were significantly enriched for genes with higher transcript levels after acid stress relative to non-stressed control in the same growth

Table 3.5. *Listeria monocytogenes* regulons showing enrichment[a] after acid treatment at 37°C

Regulon	Bacteria grown to log phase at:				Bacteria grown to stationary phase at:			
	7°C		37°C		7°C		37°C	
	Acid treated for		Acid treated for		Acid treated for		Acid treated for	
	5 min	15 min	5 min	15 min	5 min	15 min	5 min	15 min
PrfA	(+)***	-	-	-	-	-	-	-
CtsR	-	-	(+)***	(+)***	-	-	-	-
HrcA	-	(+)**	(-)**	(-)*	-	-	-	-
σ^B	(+)***	(+)***	(+)***	(+)***	-	-	(-)***	(-)***
σ^H	-	-	(+)***	(+)***	-	-	(-)***	(-)***
σ^L	-	-	-	-	-	-	-	-

[a]Reported changes are based on Gene Set Enrichment analysis. (+) indicates that genes comprising regulon were significantly enriched for genes with higher transcript levels after acid stress relative to non-stressed control in the same growth phase while

showed significantly higher transcript levels after acid stress in 37°C-grown log phase cells (Table 3.2).

Consistent with σ^B activation after acid stress, genes involved in chemotaxis (i.e., *cheR*) and flagellar biosynthesis genes (lmo0676, lmo0680, lmo0681), which have been shown to be indirectly negatively regulated by σ^B (Raengpradub *et al.*, 2008; Toledo-Arana *et al.*, 2009), were down-regulated after acid exposure in 37°C-grown log phase cells (Table 3.2).

Genes differentially expressed after acid treatment in *L. monocytogenes* grown to stationary phase at 37°C. For *L. monocytogenes* grown to stationary phase at 37°C, 62 genes showed differential transcription after 5 or 15 min after acid challenge (See Tables 3.2 and 3.3 for a list of select genes and Table S3.6 for a full list of genes differentially expressed after acid stress for 37°C-grown stationary phase cells)

(i) genes with higher transcript levels after acid treatment. For *L. monocytogenes* grown to stationary phase at 37°C, 13 genes showed higher transcript levels after 5 or 15 min after acid challenge (Table S3.4). There were two operons with at least two genes showing higher transcript levels after acid treatment (Table 3.2). GSEA of fold changes after 5 min acid treatment showed positive enrichments in gene categories "protein synthesis: ribosomal proteins: synthesis and modification," "amino acid biosynthesis: aromatic amino acid family," "transport and binding proteins: carbohydrates, organic alcohols, and acids," and "energy metabolism: pentose pyruvate dehydrogenase" (Table 3.4). Both operons that showed significantly higher transcript levels after acid treatment encode ribosomal proteins (Table 3.2).

(ii) genes with lower transcript levels after acid treatment. In *L. monocytogenes* grown at 37°C to stationary phase, there were three operons with at

least two genes showing lower transcript levels after acid treatment (Table 3.2), and GSEA showed negative enrichments for σ^B and σ^H regulons after 5 or 15 min of acid treatment (Table 3.5), suggesting that for 37°C-grown stationary phase cells the activities of these regulators are likely reduced after acid treatment.

Examples of genes showing lower transcript levels after acid treatment of stationary phase 37°C-grown cells are Na^+/H^+ antiporter lmo2378 and lmo1605 (*murC*), which is involved in cell wall biosynthesis (Table 3.3).

Genes differentially expressed after acid treatment in *L. monocytogenes* grown to log phase at 7°C. For *L. monocytogenes* grown to log phase at 7°C, 29 genes showed differential transcription after acid stress (i.e. after 5 or 15 min. acid exposure) (See Tables 3.6 and 3.7 for lists of select genes and Table S3.7 for a full list of genes differentially expressed after acid stress for 7°C-grown log phase cells).

(i) genes with higher transcript levels after acid treatment. For *L. monocytogenes* grown to log phase at 7°C, there were two operons with at least two genes showing higher transcript levels after acid treatment (Table 3.6). After 5 min acid treatment, GSEA showed significant positive enrichments in functional categories "purines, pyrimidines, nucleosides, and nucleotides: pyrimidine ribonucleotide biosynthesis" (Table 3.4). The PrfA and HrcA regulons were positively enriched after 5 min acid treatment (Table 3.5).

(ii) genes with lower transcript levels after acid treatment. For *L. monocytogenes* grown at 7°C to log phase, there were no gene categories or regulons that were negatively enriched after acid stress (Tables 3.4 and 3.5), and only 13 genes had lower transcript levels after acid stress (Table S3.7). There was one operon with at least two genes showing lower transcript levels after acid treatment (Table 3.6). Genes showing lower transcript levels after acid stress included lmo0133, which

encodes a protein similar to YjdI in *E. coli,* a ribulose-5-phosphate 3-epimerase, a gene encdoding a protein similar to YbtB from *Bacillus subtilis*, and lmo1639, which encodes a dna-3-methyladenine glycosidase (Table 3.7), a class of protein involved in DNA repair (Hosfield *et al.*, 2001).

Genes differentially expressed after acid treatment in *L. monocytogenes* grown to stationary phase at 7°C. For *L. monocytogenes* grown to stationary phase at 7°C, 46 genes showed differential expression levels after acid treatment. See Tables 3.6 and 3.7 for lists of select genes and Table S3.8 for a full list of genes differentially expressed after acid stress for 7°C-grown stationary phase cells.

(i) genes with higher transcript levels after acid treatment. There were five operons with at least two genes showing higher transcript levels after acid treatment (Table 3.6). GSEA showed significant positive enrichments in gene categories "protein synthesis: ribosomal proteins: synthesis and modification," "amino acid biosynthesis aromatic amino acid family," "transport and binding proteins: carbohydrates, organic alcohols, and acids," and "energy metabolism: pentose pyruvate dehydrogenase" after 15 min of acid treatment and "viral functions: general" after 5 min and 15 min of acid treatment. (Table 3.4). Two large groups of bacteriophage genes showed significantly higher transcript levels after acid treatment (Table 3.6). lmo0115-lmo0129 is one large operon containing *lmaA-lmaD*. *lmaA* has been studied for its association with pathogenic *Listeria* spp. (Gohmann *et al.*, 1990; Schaferkordt and Chakraborty, 1997). The second group consists of three operons comprised of genes encoding proteins from a temperate bacteriophage similar to listeriophage A118. The increased transcript levels of genes encoding phage-related proteins suggests that the response to acid for cells grown to stationary phase at 7°C may involve prophage induction.

Table 3.6. Operons differentially expressed[a] after 5 or 15 min acid treatment[b] in *Listeria monocytogenes* grown at 7°C to log or stationary phase

Gene Name	Gene Function[c]	Fold change[d] after acid treatment				Putative Binding Sites[e]
		Log phase		Stationary phase		
		5 min	15 min	5 min	15 min	
lmo0115 (*lmaD*)	similar to Antigen D	-	-	1.99 ***	2.13 ***	-
lmo0117 (*lmaB*)	antigen B	-	-	5.12 ***	5.32 ***	-
lmo0118 (*lmaA*)	antigen A	-	-	5.02 ***	5.05 ***	-
lmo0119	unknown	-	-	3.75 ***	4.04 ***	L
lmo0120	unknown	-	-	3.80 ***	4.09 ***	-
lmo0121	similar to bacteriophage minor tail proteins	-	-	4.56 **	3.88 **	-
lmo0122	similar to phage proteins	-	-	3.84 ***	3.73 ***	-
lmo0123	similar to protein gp18 from Bacteriophage A118	-	-	4.86 ***	4.51 ***	-
lmo0124	unknown	-	-	4.02 ***	4.12 ***	-
lmo0125	unknown	-	-	4.20 ***	4.12 ***	-
lmo0126	unknown	-	-	4.99 ***	5.10 ***	-
lmo0127	weakly similar to protein gp20 from Bacteriophage A118	-	-	5.21 ***	5.09 ***	-
lmo0128	similar to a protein from Bacteriophage phi-105 (ORF 45)	-	-	4.20 ***	4.89 ***	-
lmo0129	similar to autolysin: N-acetylmuramoyl-L-alanine amidase	-	-	3.47 ***	3.94 ***	-
lmo2278 (*lysA*)	L-alanoyl-D-glutamate peptidase	-	-	-	1.72 **	-
lmo2279	holin [Bacteriophage A118]	-	-	-	1.74 *	-
lmo2282	protein gp21 [Bacteriophage A118]	-	-	-	1.75 *	-
lmo2283	protein gp20 [Bacteriophage A118]	-	-	-	2.07 **	-

Table 3.3 (Continued)

Gene Name	Gene Function[c]	Fold change[d] after acid treatment				Putative Binding Sites[e]
		Log phase		Stationary phase		
		5 min	15 min	5 min	15 min	
lmo2286	Protein gp17 [Bacteriophage A118]	-	-	1.68 **	1.64 *	-
lmo2288	Protein gp15 [Bacteriophage A118]	-	-	2.83 ***	2.61 **	-
lmo2290	Portein gp13 [Bacteriophage A118]	1.53 ***	-	3.22 **	3.14 **	-
lmo2291	major tail shaft protein [Bacteriophage A118]	1.70 ***	-	4.05 ***	5.26 ***	-
lmo2292	Portein gp11 [Bacteriophage A118]	1.65 ***	1.54 ***	4.04 ***	3.42 ***	-
lmo2293	Protein gp10 [Bacteriophage A118]	-	-	2.89 ***	2.70 ***	-
lmo2295	Protein gp8 [Bacteriophage A118]	-	-	2.41 ***	2.35 ***	-
lmo2297	putative scaffolding protein [Bacteriophage A118]	-	-	3.23 ***	2.98 ***	-
lmo2299	putative portal protein [Bacteriophage A118]	-	-	-	1.69 *	-
lmo2303	Protein gp66 [Bacteriophage A118]	-	-	2.08 **	2.02 ***	-
lmo2317	similar to protein gp49 [Bacteriophage A118]	-	-	-	2.01 **	-
lmo2322	gp44 [Bacteriophage A118]	-	-	1.98 *	-	-
lmo2326	similar to protein gp41 [Bacteriophage A118]	-	-	2.18 *	2.27 *	-
lmo2408	similar to repressor protein	-1.55 ***	-1.64 ***	-	-	-
lmo2409	unknown	-2.22 ***	-2.26 ***	-	-	1

Table 3.3 (Continued)

[a]Operons with at least two genes showing a fold change with an absolute value ≥ 1.5 and an adjusted adj. p value ≤ 0.05 after acid treatment

[b]Acid treatment was done at 37°C in BHI adjusted to pH 3.5 with HCl

[b]gene functions were based on annotation provided by ListiList (http://genolist.pasteur.fr/ListiList).

[d]Superscripts are adjusted p values: "***" (< 0.001), "**" (< 0.01), "*" (≤ 0.05)

[e]Putative binding sites for Sigma B (B,b), Sigma H (H,h), Sigma L (L, l), CtsR (III,iii), HrcA (I, i) are based on a recombing of the HMM results reported in Hu 2007a, Hu 2007b, Raengpradub 2008, and (Chatanurongakul 2010 submitted). Hits were considered signifcant if they had an E value ≤ 0.02 and ≤ 300 bp upstream of the respective coding sequence. Uppercase denotes genes for which regulation has been confirmed by other studies (i.e. differential transcription in an isogenic deletion mutant vs. wild-type).

Table 3.7. Selected genes differentially transcribed[a] after 5 or 15 min. acid treatment[b] in *L. monocytogenes* grown at 7 °C

Name	Gene Function[c]	Fold-change after acid stress[d]				Putative Binding Sites[e]
		Log phase		Stationary phase		
		5 min.	15 min.	5 min	15 min	
lmo0066	similar to toxin components	1.51 ***	-	-	-	h
lmo0133	similar to *E. coli* YjdI protein	-	1.50 *	-	-	-
lmo0217	similar to *B. subtilis* DivIC protein	-	-	-	-1.65 *	-
lmo0299	similar to PTS beta-glucoside-specific enzyme IIB	-	-2.24 *	-	-	-
lmo0320	similar to surface protein (peptidoglycan bound, LPXTG motif)	-	3.64 *	-	-	l
lmo0326	similar to transcriptional regulators	-	2.87 **	-	-	h
lmo0669	similar to oxidoreductase	1.35 ***	1.55 ***	-	-	-
lmo0728	similar to riboflavin kinase / FAD synthase	-	-	-	-1.81 ***	-
lmo0735	similar to Ribulose-5-Phosphate 3-Epimerase	-	-3.57 **	-	-	-
lmo0883	similar to *B. subtilis* YbtB protein	-1.41 **	-1.72 ***	-1.83 *	-	b
lmo0875	similar to PTS system, beta-glucoside enzyme IIB component	1.61 ***	-	-	-	-
lmo1049	similar to molybdopterin biosynthesis protein MoeB	-	-	-	-1.59 ***	-
lmo1114	highly similar to TN916 ORF23	-	-2.02 *	-	-	-
lmo1151	similar to *Salmonella* Typhimurium PduA protein	1.57 **	-	-	-	-
lmo1631 (*trpD*)	highly similar to anthranilate phosphoribosyltransferase	-	-	-	3.09 **	-
lmo1639	similar to dna-3-methyladenine glycosidase	-	-1.55 ***	-	-	-
lmo1749	similar to shikimate kinase	-1.67 ***	-1.93 ***	-	-	-

Table 3.7 (Continued)

Name	Gene Function[c]	Fold-change after acid stress[d]				Putative Binding Sites[e]
		Log phase		Stationary phase		
		5 min.	15 min.	5 min	15 min	
lmo1856 (*deoD*)	purine nucleoside phosphorylase	-	-	-	-1.63 *	-
lmo1883	similar to chitinases	1.52 *	-	-	-	-
lmo1926	similar to chorismate mutase	-	-	-	-1.61 *	-
lmo1972	similar to pentitol PTS system enzyme II B component	-	1.62 *	-	-	-
lmo2009	similar to putative transport system integral membrane protein	-	1.63 *	-	-	-
lmo2191	similar to unknown proteins	-	-	-	-1.55 ***	-
lmo2300	putative terminase large subunit from Bacteriophage A118	-	4.32 *	-	-	-
lmo2445	similar to internalin	-	-	-	-2.75 *	-
lmo2536 (*atpI*)	highly similar to ATP synthase subunit i	-	-	-	-1.66 ***	-

[a]genes showing a fold change with an absolute value ≥ 1.5 after acid treatment and an adj. p value ≤ 0.05 after acid treatment

[b]Acid treatment was BHI-MOPS adjusted to pH 3.5 with HCl followed by incubation at 37°C

[c]Gene functions were based on annotation provided by TIGR (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi).

[d]Superscripts are adjusted p values: "***" (< 0.001), "**" (< 0.01), "*" (≤ 0.05)

[e]Putative binding sites for Sigma B (B,b), Sigma H (H,h), Sigma L (L, l), CtsR (III,iii), HrcA (I, i) are based on a recombing of the HMM results reported in Hu 2007a, Hu 2007b, Raengpradub 2008, and (Chatanurongakul 2010 submitted). Hits were considered signifcant if they had an E value ≤ 0.02 and located ≤ 300 bp upstream of the respective coding sequence. Uppercase denotes genes for which regulation has been confirmed in another study (i.e. differential transcription in an isogenic deletion mutant vs. wild-type).

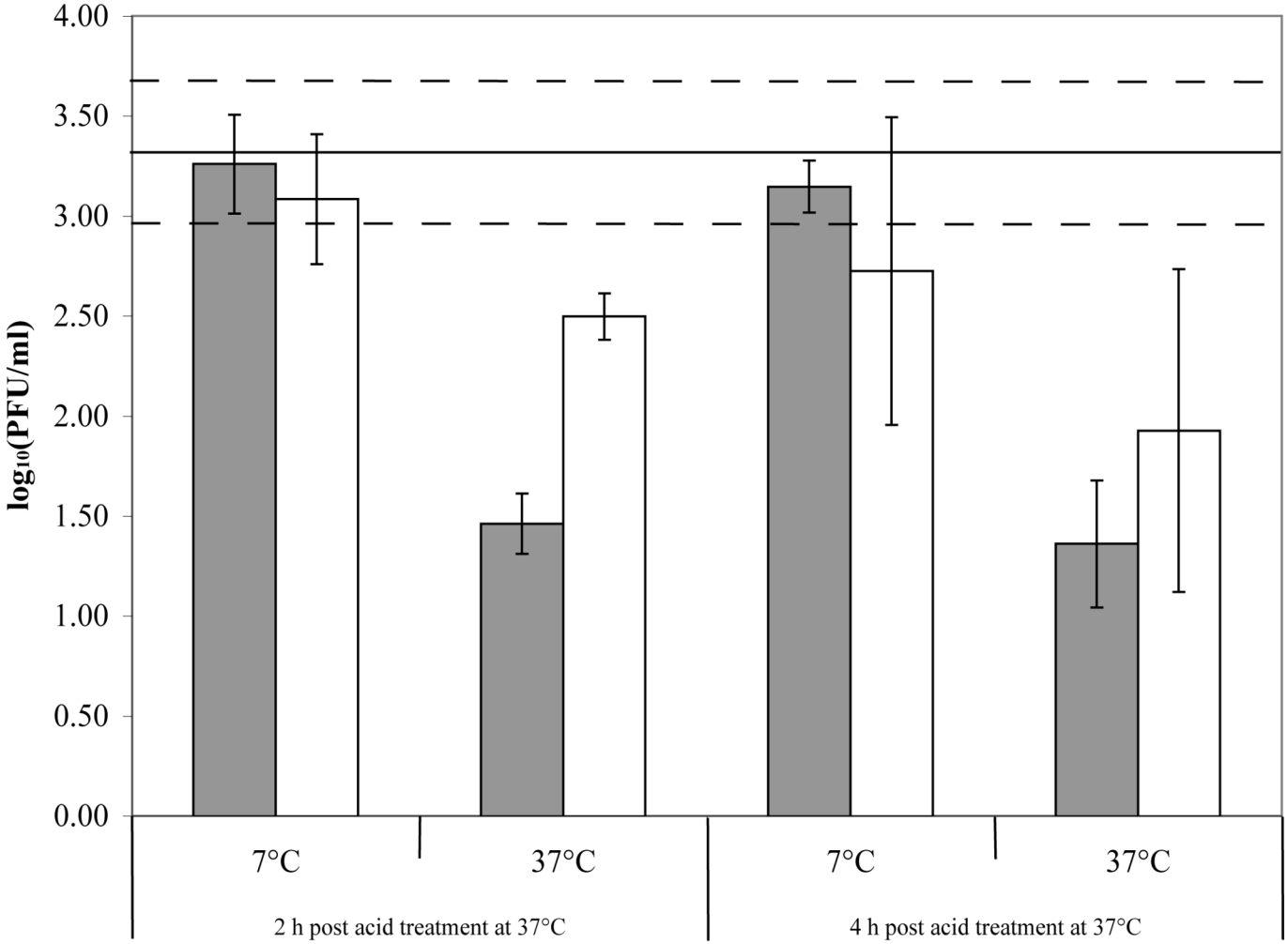
(ii) genes with lower transcript levels after acid treatment. For *L. monoctygoenes* grown to 7°C to stationary phase, no significant gene categories or regulon negative enrichments were found (Tables 3.4 and 3.5) after acid treatment. There were no operons with at least two genes showing lower transcript levels after acid treatment (Table 3.6). Among the 13 genes that showed lower transcript levels after acid stress were those encoding proteins similar to *B. subtilis* DivIC and YbtB (lmo0217 and lmo0883, respectively) and a number of metabolism genes: lmo0278 (similar to riboflavin kinase/FAD synthase), lmo1049 (molybdopterin biosynthesis protein MoeB), lmo1631 (*trpD*; anthranilate phosphoribosyltransferase), lmo1856 (*deoD*; purine nucleoside phosphorylase), and lmo1929 (similar to chorismate mutase). Also showing significantly lower expression were lmo2445, which encodes an internalin-like protein, and *atpI* (lmo2536), which encodes ATP synthase subunit i (Tables 3.6 and 3.7).

Prophage induction occurs in stationary phase *L. monocytogenes* grown at 7°C. When *L. monocytogenes* grown at 7°C to stationary phase were exposed to a sudden downshift in extracellular pH (from approx 7.0 to 3.5) at 37°C, GSEA showed a significant enrichment of genes categorized as "viral functions: general," and transcript levels of two large groups of genes encoding bacteriophage-like proteins showed higher transcript levels after 5 min acid treatment, suggesting a possible acid shock-triggered prophage induction. To determine whether virulent bacteriophage were present in supernatants of 7°C-grown stationary phase *L. monocytogenes* and to determine how exposure to pH 3.5 for 15 min may affect the supernatent phage titer, cells were grown at 7°C to stationary then acid treated (pH 3.5) for 15 min at 37°C, neutralized with the addition of NaOH, then monitored for plaque forming substances over time at 7°C or 37°C. At time 0 (i.e., before acid treatment), 10403S had an

average titer of 3.29 log PFU/ml (Figure 3.3). After acid treatment for 15 min at 37°C and incubation at 7°C for 2 hr, the average titer was 3.20 log PFU/ml (Figure 3.3). After 4 h at 7°C, acid-treated cultures had an average phage titre of 3.15 log PFU/ml (Figure 3.3). Cultures grown to 7°C and not exposed to acid and incubated at 7°C for 2 and 4 h had average phage titers of 3.08 log PFU/ml and 2.73 log PFU/ml, respectively (Figure 3.3). The effect of acid treatment on change in log PFU/ml (from initial titer) was not significant at either 2 h or 4 h incubation at 7°C ($p = 0.2221$ for 2 h, $p = 0.3298$ for 4 h; matched pairs analysis). These results indicate that for *L. monocytogenes* grown to stationary phase at 7°C there was no measurable induction of plaque forming substances after acid treatment. We were unable to isolate plaque-forming substances from 37°C stationary phase cultures before acid treatment, suggesting that stationary phase prophage expression in *L. monocytogenes* 10403S is dependent on growth at lower temperatures.

For cultures incubated at 37°C for 2 h post acid treatment, the average titer was 1.46 log PFU/ml compared to non-acid-treated cells incubated at 37°C, which had an average titre of 2.5 log PFU/ml (Figure 3.3) ($p = 0.0139$; matched pairs analysis), indicating that for cells incubated at 37°C after acid treatment, the acid treatment had a negative effect on phage titre. Acid-treated cultures incubated for 4 h at 37°C, had an average titer of 1.36 log PFU/ml, compared to non-acid-treated cultures, which had an average titer of 1.93 log PFU/ml (Figure 3.3). There was no significant effect of acid treatment on change in phage titer after 4 h at 37°C ($p = 0.2586$; matched pairs analysis). With one exception (i.e. 4 h with no acid treatment; $p = 0.0647$; matched pairs analysis), *L. monocytogenes* incubated at 7°C had significantly higher titers than cells incubated at 37°C ($p < 0.05$; matched pairs analysis), regardless of acid treatment, consistent with our microarray results showing that transcription levels of multiple bacteriophage genes were higher at 7°C compared to 37°C (Table S3.1).

Figure 3.3. Plaque forming substances present in the supernatants of *Listeria monocytogenes* grown to stationary phase at 7°C then exposed to acid shock. *L. monocytogenes* 10403S was to grown to stationary phase at 7°C, then either exposed to pH 3.5 for 15 min (followed by neutralization of medium pH; gray bars) or not (white bars) and incubated at 7°C or 37°C for 2 or 4 hr. The solid line at 3.29 $\log_{10}$(PFU/ml) represents the mean count at time zero (i.e. stationary phase culture with no acid stress or subsequent incubation). Hashed lines represent the standard deviation of the counts at time zero. After 2 h, cultures incubated at 7°C showed higher $\log_{10}$ (PFU/ml) than cultures incubated at 37°C ($p < 0.05$; matched pairs analysis). At none of the time points were counts significantly higher in acid-treated cultures than in non-acid-treated cultures. Bars represent the average of four biological replicates. Error bars are standard deviation.


4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
log10(PFU/ml)
7°C
37°C
7°C
37°C
2 h post acid treatment at 37°C
4 h post acid treatment at 37°C

A sample of a purified phage stock prepared from a single plaque obtained from *L. monocytogenes* culture grown at 7°C to stationary phase was subjected to transmission electron microscopy. The phage had similar morphology to *Listeria* bacteriophage A118 [i.e. an isomeric capsid with a long, non-contractile tail; (Zink and Loessner, 1992)].

DISCUSSION

The results of this study show that growth temperature and growth phase of *L. monocytogenes* affect its survival and transcriptional response to a sudden downshift in pH. Specifically, we found that (i) *L. monocytogenes* grown at ≥ 30°C are more resistant to acid treatment than cells grown at 7°C; (ii) *L. monocytogenes* grown to log phase show an acid response involving induction of positive and negative regulators, and (iii) the acid response of stationary phase *L. monocytogenes* is distinct from that of log phase and involves growth temperature-dependent induction of bacteriophage genes.

***L. monocytogenes* grown at ≥ 30°C are more resistant to acid treatment than cells grown at 7°C.** *L. monocytogenes* cells grown at 7°C were more susceptible to sudden acid treatment than cells grown at 37°C. Other studies have shown that foodborne pathogens grown at low temperatures mount less effective ATR and are less resistant to acid challenge than cells grown at higher temperatures. For example, growth of *L. monocytogenes*, *Salmonella* Typhimurium and *E. coli* 0157:H7 at 10°C reduced both the effectiveness of the ATR and their survival after challenge (pH 3.5; 25°C) with lactic or acetic acids (Samelis *et al.*, 2003). Also, *L. monocytogenes* grown at 10°C had a significantly shorter D-value when exposed to pH 2.5 (at 30°C and at 10°C) than cells grown at 30°C then acid stressed under the same conditions (Patchett *et al.*,

1996). Therefore, our results and the results of others suggest that *L. monocytogenes* grown at refrigeration temperatures may be more susceptible to gastric killing. Conversely, *L. monocytogenes* grown at temperatures greater than 7°C (e.g., in temperature-abused food product) may be more resistant to gastric killing.

We detected higher transcript levels at 37°C compared to 7°C for genes encoding proteins that have been implicated in acid stress response. For example, GSEA revealed positive enrichments of σ^H-dependent genes at 37°C in stationary phase and σ^B-dependent genes in log phase. σ^B-dependent lmo2362 and lmo2363, which encode a GABA transporter and associated glutamate decarboxylase, respectively, have been shown to contribute to the survival of *L. monocytogenes* LO28 in low pH environments. (Cotter *et al.*, 2001; Cotter *et al.*, 2005). lmo2362 and lmo2363 showed higher transcript levels at 37°C compared to 7°C, as did *groEL*, the product of which has been shown to be up-regulated after acid exposure (Phan-Thanh and Mahouin, 1999). Increased expression of these stress response genes at 37°C may explain the increased resistance of 37°C-grown cells to acid stress compared to 7°C-grown cells.

Studies have shown that stationary phase *L. monocytogenes* cells are more resistant to acid stress than log phase cells (Davis *et al.*, 1996; O'Driscoll *et al.*, 1996; Samelis *et al.*, 2003), and Patchett *et al*. (1996) showed that *L. monocytogenes* growing at slower rates were more resistant to acid treatment than cells growing at faster rates. Consistent with these findings, we found stationary phase cells grown at 7°C showed nearly 10-fold higher survival than log phase cells after 15 min acid treatment. However, for cells grown at 37°C, survival between stationary and log phase cells was similar.

***L. monocytogenes* grown to log phase show an acid response involving the induction of positive and negative regulators.** *L. monocytogenes* grown at 37°C to log phase and acid treated for 5 or 15 min showed a transcriptional response involving negative regulators HrcA and CtsR and positive regulators σ^B and σ^H.

For 37°C-grown log phase cells, GSEA showed enrichment for genes encoding proteins in the category protein fate: folding and stabilization, suggesting that exposure to acid induced protein repair mechanisms. Supporting this finding, GSEA showed a positive enrichment of the CtsR regulon, which is comprised of type III stress genes, indicating that CtsR regulon is likely de-repressed after acid treatment. Genes encoding Class III stress proteins have been shown to be transcribed at higher levels after acid shock in other gram-positive bacteria, including *Streptococcus pneumoniae* (Martin-Galiano *et al.*, 2005), *Streptococcus mutans* (Gong *et al.*, 2009), and *Lactobacillus reuteri* (Wall *et al.*, 2007) indicating that this category of proteins is likely necessary for adaptation to acid environments in *L. monocytogenes* and other gram-positive bacteria.

L. monocytogenes is a facultative intracellular pathogen and has the capability to induce phagocytosis by non-phagocytic intestinal epithelial cells and phagocytic macrophages (Vazquez-Boland *et al.*, 2001). CtsR-regulated Class III genes *clpB* and *clpP*, which were significantly up-regulated in log-phase 37°C-grown cells after acid treatment, have been shown to contribute to intracellular survival and escape from the host phagosome. Specifically, ClpP is necessary for functional LLO secretion (Gaillot *et al.*, 2000), which is vital to escape from the primary host cell vacuole (Scortti *et al.*, 2007), and a Δ*clpC* strain was shown to be impaired in escape from the macrophage phagosome (Rouquette *et al.*, 1998). Therefore, CtsR is likely an important regulator of the initial response to acid shock in *L. monocytogenes* and its activation may be advantageous to subsequent intracellular survival.

For 37°C-grown log phase cells, GSEA showed negative enrichment of HrcA-repressed genes after acid treatment, suggesting that HrcA is activated after acid treatment in *L. monocytogenes*. However, for cells grown at 37°C to log phase, six genes that were up-regulated after acid treatment were found to have putative upstream HrcA binding sites, and certain HrcA-regulated genes (e.g. *grpE* and *hrcA*), were up-regulated after acid stress. HrcA-regulated Class I heat shock genes (Narberhaus, 1999; Hu *et al.*, 2007a), are involved in acid stress response in *L. monocytogenes* and other organisms (Hanawa *et al.*, 1999; Frees *et al.*, 2007; Kress *et al.*, 2009). For example, in *L. monocytogenes*, a Δ*dnaK* mutant was more sensitive to acid shock at pH 3.5 than its parent strain (Hanawa *et al.*, 1999), and a 2-D gel analysis revealed that DnaK expression was induced in *Lactobacillus delbrueckii* subsp. *bulgaricus* after acidification of the growth medium to pH 4.5 with lactic acid (Lim *et al.*, 2000). DnaK has also been shown to be critical for phagocytosis (Hanawa *et al.*, 1999), and increased transcript levels of *dnaK* have been observed in the intracellular environment (Chatterjee *et al.*, 2006).

For log phase cells grown at 7°C or 37°C, GSEA showed positive enrichment of σ^B- and σ^H-regulated genes at both 5 and 15 min post acid exposure. σ^B has been shown to be important for acid stress survival of *L. monocytogenes* (Ferreira *et al.*, 2003) and has been shown to regulate transcription of genes that are involved in acid survival, including glutamate decarboxylase (gad) genes. There are two complete gad systems in *L. monocytogenes*, GadD1T1 (*lmo0443*-*lmo0444*) and GadT2D2 (*lmo2362*-*lmo2363*) as well as a lone decarboxylase GadD3 (*lmo2434*) (Cotter *et al.*, 2005). Though lmo2434 (Kazmierczak *et al.*, 2003)and lmo2362 have been shown to be σ^B-dependent (Wemekamp-Kamphuis *et al.*, 2004), neither of these genes were transcribed at significantly higher levels after acid treatment. Surprisingly, we observed lower transcript levels of lmo2363 and its associated transporter lmo2362

after acid treatment of 37°C-grown log phase cells. However, functional overlap among the gad systems may compensate during adaptation to low pH and Gad activity has been shown to vary among different strains of *L. monocytogenes* (Dykes and Moorhead, 2000). σ^H levels have been shown to increase after acid stress in *L. monocytogenes* (Phan-Thanh and Mahouin, 1999), and our results showed significant enrichment of the σ^H regulon after acid stress. Therefore, our results, with the results of others, indicate that σ^H is an important contributor to the acid stress response of *L. monocytogenes*. Recently our group showed that the regulons of σ^B and σ^H contain significant overlap, indicating some functional redundancy between these sigma factors (Chaturongakul et al., submitted for publication). However, there were several genes that were differentially expressed in 37°C-grown log phase cells after acid stress had predicted σ^H-dependent promoters, but not σ^B-dependent promoter (e.g., lmo0181, *hemC*, *pyrD, minD*), indicating that there are both combined and individual contributions of σ^B and σ^H to the early acid response of *L. monocytogenes*.

For *L. monocytogenes* grown at 37°C to log phase, approximately two thirds of the significantly induced or repressed operons had genes with putative binding sites for one or more of the regulators that were induced after acid stress suggesting that, for cells grown to log phase at 37°C, the *L. monocytogenes* acid shock response likely involves a network of transcriptional regulators. For example, five of the six HrcA-dependent genes that showed higher transcript levels after acid treatment of 37°C log phase cells have binding sites for at least one other regulator tested in this study. For example, there are putative binding sites for HrcA, σ^B and CtsR upstream of the *dnaK-grpE-hrcA* operon, which was significantly up-regulated after acid treatment. Also, transcript levels of lmo2362 and lmo2363, both of which were shown here to be down-regulated after acid stress in 37°C-grown log phase cells, have been shown to be down-regulated by σ^B (Raengpradub *et al.*, 2008) and HrcA (Hu *et al.*, 2007a).

Our results showed activation of regulators and enrichment of functional categories that have been shown to contribute to intracellular survival. After phagocytosis, *L. monocytogenes* can grow by scavenging intracellular nutrient sources. For 37°C grown log-phase cells GSEA showed enrichment in pyrimidine ribonucleotide biosynthesis genes after 15 min acid treatment. Examples of pyrimidine metabolism genes showing higher transcript levels after acid treatment were *pyrD* and *pyrAB*. Klarsfeld et al (Klarsfeld *et al.*, 1994) found that a *pyrE*, which is cotranscribed with *pyrD* and *pyrAB*, to be translated at higher levels in the intracellular environment compared to growth in BHI. Therefore, pyrimidine biosynthesis genes, several of which we found to be transcribed after acid treatment, may be important for utilizing nucleic acids as a carbon source in the cytosol of host cells. Acid adaptation has been shown to increase the survival of *L. monocytogenes* in activated macrophages (Conte *et al.*, 2002). Therefore, up-regulation of pyrimidine biosynthesis genes and activation of other stress gene regulators during gastric passage may prime cells for rapid phagosomal escape and intracellular growth and thus contribute to intracellular survival of the organism.

Though, for cells grown at 7°C to log phase, GSEA showed significant enrichment of the σ^B and PrfA regulons after 5 min acid treatment and enrichment of σ^B and HrcA regulons after 15 min, none of the functional gene categories tested were significantly enriched, and none of the genes encoding heat shock proteins or other common acid response genes that were induced in 37°C-grown cells were significantly differentially expressed by 15 min after acid addition. Furthermore, by 15 min we observed a 90% reduction in survival for 7°C-grown log phase cells. Therefore, cells grown at 7°C to log phase may be unable to mount a response to sudden acid stress and thus may be more susceptible to inactivation in low pH environments such as the gastric environment.

The acid response of stationary phase *L. monocytogenes* is distinct from that of log phase and involves growth temperature dependent induction of bacteriophage genes. Unlike in log phase cells, GSEA showed no significant positive enrichments of CtsR, σ^B, or σ^H regulons after acid stress of either 7°C- or 37°C-grown stationary phase cells. Interestingly, GSEA showed a significant negative enrichment of the σ^B and σ^H regulons after acid stress, which suggests reduced roles in gene transcription for these alternative sigma factors after acid stress. However, stationary phase cells were at least as resistant as log-phase cells to acid exposure, suggesting that any negative effect of acid stress on σ^B and σ^H activities does not affect the ability of *L. monocytogenes* to survive acid stress. Stress regulators may be activated upon entry into stationary phase, providing protection against subsequent acid stress and leading to higher baseline transcription of acid-response genes. For example, σ^B and σ^H have been shown to be involved in stationary phase stress survival in *Bacillus subtilis* (Gaidenko and Price, 1998). Furthermore, σ^B-dependent genes have been shown to be expressed at higher levels in stationary phase compared to log phase (Kazmierczak *et al.*, 2006) and σ^B has been shown to play a role in stationary phase acid survival of *L monocytogenes* (Wiedmann *et al.*, 1998). Finally, *L. monocytogenes* cells have been shown to develop an acid resistance with entry stationary phase (Davis *et al.*, 1996). Therefore, the activation of acid response genes upon entry into stationary phase may explain the similar or greater acid resistance in stationary phase cells compared to log phase. Four functional categories were positively enriched after acid stress for both 37°C-grown stationary phase cells and 7°C stationary phase cells, suggesting that the genes involved in acid stress response of stationary phase cells are functionally similar between growth temperatures. However, our survival study showed that after 30 min (AGF) or 60 min (ABHI) cells

grown to stationary phase at 7°C are significantly more susceptible to acid stress than cells grown at 37°C to stationary phase.

Only the category "viral functions:general," was significantly enriched after acid stress for 7°C-grown stationary phase cells but for not for 37°C-grown stationary phase cells, and two large operons of genes encoding bacteriophage proteins were induced in 7°C-grown stationary phase cells after acid stress. These two large groups of genes that were expressed at higher levels after acid stress were from a prophage similar to Listeriaphage A118 and from an operon that includes *lmaDCBA* through lmo0129, which has been identified as a cryptic prophage (Nelson *et al.*, 2004). LmaA was shown to be associated with pathogenic *Listeriae* (Schaferkordt and Chakraborty, 1997) and to elicit a delayed-type hypersensitivity reaction in *L. monocytogenes* immune mice (Gohmann *et al.*, 1990). *lmaA* has, therefore, been labeled a potential virulence gene (Schaferkordt and Chakraborty, 1997). LmaA has also been shown to be localized to the bacterial surface and secreted into the medium at 20°C, but not at 37°C (Schaferkordt and Chakraborty, 1997), which is consistent with the temperature-dependent transcription of this operon observed in our study. Bacteriophage A118 is classified in the Siphoviridae family based on its morphologically characteristics [i.e. having a long, flexible non-contractile tail and isomeric capsid (Zink *et al.*, 1995)]. Its genome consists of double stranded DNA, encodes 72 ORFS, and its sequence has been determined (Loessner *et al.*, 2000). It is integrated into the *comK* gene which is similar to a competence development gene in *Bacillus subtilis* (van Sinderen *et al.*, 1995). *L. monocytogenes* H7858 and F6854, harbor a prophage integrated into the *comK* site (Nelson *et al.*, 2004), though the nucleotide identity to A118 is varied (59.2% for F6854 and 16.6% for H7858). We were able to isolate a bacteriophage with a morphology similar to A118 from the supernatant of 10403S cultured at 7°C, indicating constitutive induction of the A118-

like prophage at 7°C in stationary phase, though we did not observe a reproducible increase in phage titre after acid stress. Increased levels of bacteriophage gene transcripts after short-term acid stress (i.e. 5 and 15 min.) have been reported in at least one other organism [i.e. *Lactobacillus reuterii* (Wall *et al.*, 2007)] indicating that this response is not specific to *L. monocytogenes*. A 17 ORF cryptic prophage has been identified in similar locations in genomes of *L. monocytogenes* strains EGD-e, F2365, H7858, and F6858 (Nelson *et al.*, 2004). Therefore, *L. monocytogenes* strains harboring these bacteriophage genes may have selective advantages in mixed populations for stress survival and niche adaptation. Prophage induction can be linked to the bacterial SOS response (Waldor and Friedman, 2005) and there is evidence that the SOS response is induced after acid stress in *L. monocytogenes* (van der Veen *et al.*, 2010). Therefore induction of bacteriophage gene expression in *L. monocytogenes* grown to 7°C may be evidence of SOS response induction. The induction of bacteriophage genes upon acid stress may also lead to exchange of genetic material among strains in a mixed population and may represent an important mechanism for the acquisition of stress resistant mechanisms. The products of the putative incomplete (cryptic) bacteriophage operon that was induced here after acid treatment has been shown to specifically inhibit the growth other strains of *L. monocytogenes* (Zink *et al.*, 1995). Therefore a strain harboring these genes, which produce proteins or bacteriophage with bacteriocin-like function, may have an advantage in a mixed culture, thus contributing to its environmental persistence. Further study is needed to determine whether bacteriophage gene expression is linked to the SOS response in *L. monocytogenes* and what role, if any, this induction plays in environmental stress survival.

CONCLUSION

L. monocytogenes grown at 7°C showed a drastically different response to acid stress than cells grown at 37°C, in terms of survival and changes in gene transcription. The contact of *L. monocytogenes* with intestinal epithelial cells, its main route of infection, is contingent upon the organism surviving gastric stress. The results of this study show that adaptation to food-relevant temperatures, specifically refrigeration, can impact *L. monocytogenes* acid stress response, which is vital to its transmission from food to humans. Specifically, cells grown at refrigeration temperatures are more susceptible to inactivation by acid treatment. Therefore, *L. monocytogenes* present in refrigerated ready-to-eat food may be more susceptible to gastric stress.

The transcriptional response to acid treatment differed significantly between *L. monocytogenes* grown at 7°C compared to 37°C. In the *L. monocytogenes* ATR, changes in gene expression induced during growth in sub-lethal acidic conditions confer a resistance to subsequent acid challenge or other stress (Ryan *et al.*, 2008). Acid treatment of cells grown to log phase at 37°C induced transcriptional regulators involved in pH homeostasis or macromolecule integrity similarly to cells adapting to the intracellular environment. Many of the genes found be differentially expressed after acid treatment have putative upstream binding sites for multiple different transcriptional regulators, indicating that regulatory networks may be at the core of the response to sudden acid stress in *L. monocytogenes*.

The acid response of 7°C-grown cells involved the induction of a cryptic and fully virulent bacteriophage potentially capable of inactivating other strains of *L. monocytogenes*. Bacteriocin-like phage protein induction may represent a mechanism by which some strains or serotypes are able to persist in harsh food environments better than others. Further study is needed to determine the relationship between prophage harborage and niche adaptation.

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CHAPTER FOUR

Conclusions

The development of more effective strategies to reduce the incidence of listeriosis is dependent upon an improved understanding of the transient environmental response of *L. monocytogenes* throughout the various stages of its transmission, including through food systems. The results of this study show that adaptation to different environmental temperatures affects the stress survival and virulence of *L. monocytogenes*. Specifically, growth at environmental temperatures outside the host can influence the expression of genes and associated phenotypes, which include interactions between transcriptional regulators and functional synergisms between proteins during acid stress and invasion of intestinal epithelial cell invasion.

An accurate estimation inactivation efficacy for a treatment against *L. monocytogenes* is dependent upon the selection of growth parameters for the test strain. The United States Food and Drug Administration, in its report entitled, "Safe Practices for Food Processes," regarding growth of an organism for a challenge study, states,

"The challenge cultures should be grown in media and under conditions suitable for optimal growth of the specific challenge culture. In some studies, specific challenge organisms may be adapted to certain conditions. Such adaptation will be tailored to the specific food."

Our study has shown that growth temperature impacts how *L. monocytogenes* reacts to subsequent environmental stimulus. Therefore, growth temperature of the test organism and temperature during treatment should be considered as important parameters and these parameters should be considered during challenge studies.

A recent study suggested that *E. coli* may have evolved mechanisms to adapt to a series of environmental stresses associated with transmission from the environment into the host (Bonneau *et al.*, 2007). Considerable overlap was found between genes differentially regulated after a temperature up-shift and those differentially regulated under oxygen-restricted conditions, indicating that an up-shift in environmental temperature similar to that which the organism encounters during ingestion, triggers genes involved in response to oxygen restriction, a stress condition the organism encounters during passage through the gastrointestinal system. Stress cross-protection has been observed in *L. monocytogenes* (Lou and Yousef, 1997; Skandamis *et al.*, 2008) (Koutsoumanis *et al.*, 2003), and we have shown in this study that contributions of certain host cell invasion factors at human body temperature are dependent on growth of the organism at temperatures lower than body temperature. Furthermore, we've shown that exposure to low pH environments similar to gastric passage trigger the expression of transcriptional regulators that are vital to survival inside the host cell. *L. monocytogenes* may, therefore, have evolved predictive mechanisms that, when triggered during growth in food environments or during ingestion and gastric passage, give the organism an advantage against host defenses. Therefore, in addition to further study of how environmental transitions affect gene expression and pathogenesis of foodborne pathogens and further characterization of infection-related stimulomes is vital to the development of improved models of how these organisms survive stress during transmission and to the development of more efficient strategies to reduce the global burden of foodborne illness.

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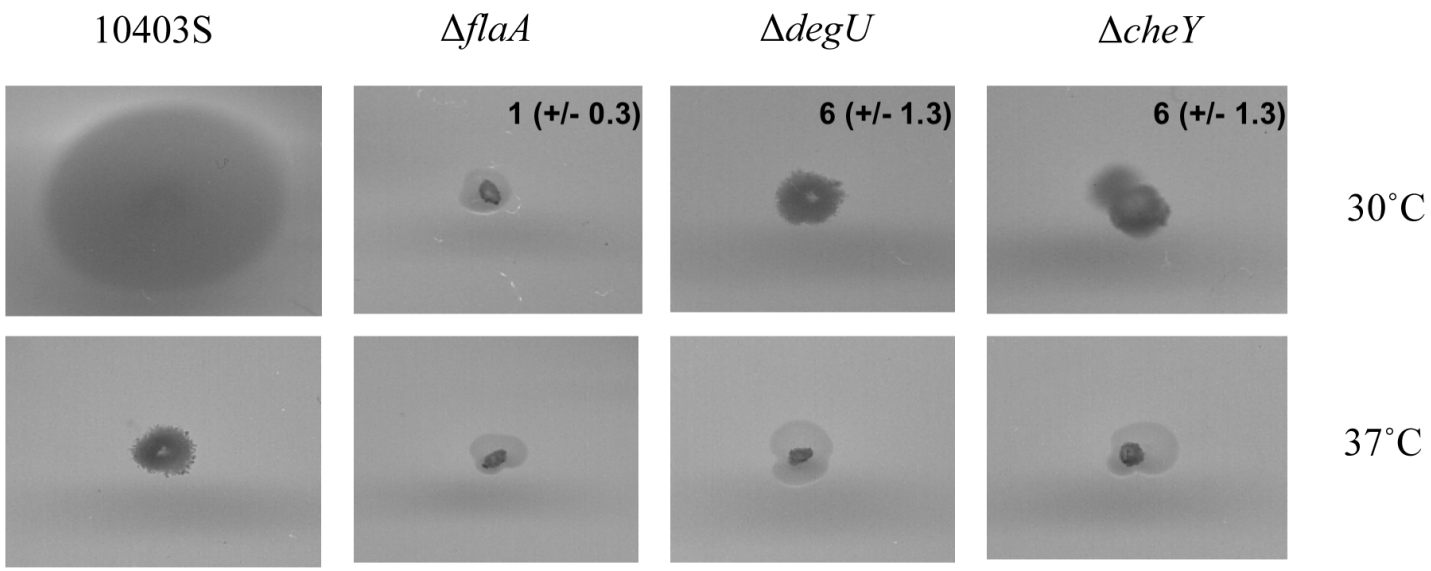
APPENDIX ONE

Supplemental Figures

Figure A1[S2.1]. Swarming behavior of *Listeria monocytogenes* 10403S, Δ*degU*, Δ*cheY*, and Δ*flaA* strains grown at 30°C or 37°C. Three independent trials of the swarming assays were performed and each strain was tested in triplicate in a given trial. Images show representative results. Pixel counts of the swarming areas were determined to quantitate swarming areas, and relative swarming of the mutant strain was expressed relative to parent strain swarming (which was set at 100%). Relative swarming is shown for mutant strains grown at 30°C (values shown represent average +/- standard deviation). No detectable swarming was observed for the mutants at 37°C and hence no relative swarming values are shown.


10403S
ΔflaA
ΔdegU
ΔcheY
1 (+/- 0.3)
6 (+/- 1.3)
6 (+/- 1.3)
30˚C
37˚C

APPENDIX TWO

Supplemental Tables

Table A2[S2.1]. Primers used for the generation of response regulator null mutants

Strain Designation	Genotype	Primer Name	Primer Sequences 5' to 3'[a]
B4-007	Δ*lisRK*[b]	KDBlisRKSoeA	**CGG** *GGTACC* TCTTAAAAACTTACTACTTGATCC
		KDBlisRKSoeB	TATTCTATTCATTTGGCCTAACCC
		KDBlisRKSoeC	GGGTTAGGCCAAATGAATAGAATA
			ATGCGTACATGACGACTAGCC
		KDBlisRKSoeD	**CGC** *GGATCC* ATTATCATCTGCTACAATCGGCAT
B2-078	Δ*agrA*	CRL01agrAsoeA	**G** *GAATT* CGCTCAAGAGTTGAAAATTGATGC
		CRL02agrAsoeB	CGGTAGCATAAATTCATCCCC
		CRL03agrAsoeC	GGGGATGAATTTATGCTACCG
			AGCTTGAGTTTATAAAAGTGGCC
		CRL04agrAsoeD	**CG** *GGATCC* CACTACGCTTAATCCGATACGT
B2-080	Δ*resD*	SRMresDSoeA	**CGC** *GGATCC* AAACGAAAGATAAAGCCATTG
		SRMresDSoeB	CACTCTAACTTGTTCACTC
		SRMresDSoeC	GAGTGAACAAGTTAGAGTG
			GGATATAAATTTGAAGTTCCAG
		SRMresDSoeD	**CGG** *GGTACC* TGTCTTGAATTAATTTATTGGC
B2-086	Δ*lmo1022*	BB7 lmo1022SoeA	**GG** *GGTACCT* GCCAATCGAAGTAGAATGGC
		BB8 lmo1022SoeB	TATCATTCGCTTTCCACGTCC
			GGACGTGGAAAGCGAATGATA
		BB9 lmo1022SoeC	GACCTAGTAGAAAAAAAGTAGCA
		BB10	
		lmo1022SoeD	**GG** *AAGCTT* GGCATGCTTATCATTTGTCGC

B2-096	Δ*lmo1060*	BB11 lmo1060SoeA	**GG** *GGTACC* AACACCAGAAAAAACACGTG
		BB12 lmo1060SoeB	GAGTATTTTTTCCATATTTGCC
		BB13 lmo1060SoeC	GGCAAATATGGAAAAAATACTC AAAATTCAAACGATTAGAGGT
		BB14 lmo1060SoeD	**G** *TCTAGA* ATTTTTTGTTTCTGTTCCACC
C5-017	Δ*lmo2010*	YC10 lmo2010A	**A** *GGATCC* CCAAGGTTTGCTCTGCAG
		YC11 lmo2010B	ACCTTTGAGAATAAGGGGCTC
		YC12 lmo2010C	GAGCCCCTTATTCTCAAAGGT TCTGGTTATACCGATATGGCTTAT
		YC13 lmo2010D	**C** *GAATTC* CACCCAGTCACTGCTCAT
C5-019	Δ*lmo2583*	YC6 lmo2583A	**G** *GGATCC* CGGCACTATGTAGTTCAGC
		YC7 lmo2583B	CACAAGTTTAAGTATATGCCGATC
		YC8 lmo2583C	GATCGGCATATACTTAAACTTGTG CCCGTGAAAGAATTTGAGCT
		YC9 lmo2583D	**T** *GAATTC* AAAGAAATCGTAGCATTTGACTCG
B2-100	Δ*phoP*	WS5 phoPA	**GA** *GGTACC* CACGGATTGAAATACCAACG
		WS6 phoPB	TTCGGTTATAAAATGGAGAACG
		WS7 phoPC	GTTCTCCATTTTATAACCGAA AAGAATTTTTACCAACGTACTTCC
		WS8 phoPD	**CT** *AAGCTT* GCTTGTAGTTTTGGGTGC

C5-041	Δ*virR*	YC41 lmo1745A	**G** *GGATCC* ACTTCAAATTAGTTACAGATGCTG
		YC42 lmo1745B	TACTACACCAATCTCAAATCCC
		YC43 lmo1745C	GGGATTTGAGATTGGTGTAGTA
			GCAGAAATTGGTTTGAGCG
		YC44 lmo1745D	**T** *GAATTC* CTCAACTAAAGCTCGACCT
C5-036	Δ*lmo1507*	YC35 lmo1507A	**A** *GGATCC* GCCGATTGAATGGTAAAACCTGA
		YC36 lmo1507B	CGCTTCATAACCCATTTTGCC
		YC37 lmo1507C	GGCAAAATGGGTTATGAAGCG
			CGTTTACGCCAAAAAATCGC
		YC38 lmo1507D	**GTA** *GTCGAC* GAGAAATCCTTAGGTAAAGGCTGA
C5-033	Δ*degU*	YC29 lmo2515A	**G** *GGATCC* GTGTTTTCCATCGCTATGGATG
		YC30 lmo2515B	AATTCGCTTGATACCTTCGC
		YC31 lmo2515C	GCGAAGGTATCAAGCGAATT
			GTAACGGCAATCAAGCACG
		YC32 lmo2515D	**CAT** *GTCGAC* CAATGGCTCGTTTGCCAA
B2-104	Δ*kdpE*	WS1 lmo2678A	**CT** *GGTACC* CCATACGATTCTCCAGCGAG
		WS2 lmo2678B	GTTGGGGTTGGATACCGG
		WS3 lmo2678C	CCGGTATCCAACCCCAAC
			TAGCACAAGCCGCTTGCTG
		WS4 lmo2678D	**GT** *AAGCTT* GACACTGATTGAGCAAGTGC
B2-105	Δ*cheY*	BB27 cheYSoeA	**G** *TCTAGA* CAACTAAAGCATCTGCTTC
		BB28 cheYSoeB	ATTCTTAATCATCGTACGCAT
		BB29 cheYSoeC	ATGCGTACGATGATTAAGAAT
			GACCGAGTTTTAGAGGCG

		BB30 cheYSoeD	**GG** *GGTACC* AATTTGAATGGCAATACGGTA
B2-102	Δ*cesR*	BB3 cesRSoeA	**GG** *GGTACC* CTGTCATCGCAATTCTAACGG
		BB4 cesRSoeB	AGAAGTTGTCATACTCATTGTCC
			GGACAATGAGTATGACAACTTCT
		BB5 cesRSoeC	TACAAAATTGAAATCTAAACTGG
		BB6 cesRSoeD	**G** *TCTAGA* GTTCCCGCATATTTTCGATG

[a]Clamp sequences for SOE-A and SOE-D primers are bolded; restriction sites for SOE-A and SOE-D primers are italicized; overhangs complementary to SOE-B primers are underlined.

[b]This mutant includes an internal deletion of the *lisR* gene, which also removed a portion of the ribosome binding site of *lisK* sensory kinase gene.

Table A2 [S2.2]. Summary of gene deletion effects on transcription levels of various *Listeria monocytogenes* stress and virulence genes

Gene	p-values for the effects of various gene deletion effects on *L. monocytogenes* gene transcription measured in stationary phase at:					
	30°C			37°C		
	sigB[a]	prfA	sigB*prfA	sigB	prfA	sigB*prfA
inlA	<0.0001***	0.2961	0.3139	<0.0001***	0.1282	0.0799
flaA	0.0003***	0.7317	0.7263	0.4051	0.9589	0.4016
plcA	0.0475	0.6952	0.5122	0.2593	<0.0001***	0.1808
gadA	<0.0001***	0.9887	0.8895	<0.0001***	0.6512	0.8698

[a] The variables listed in this column represent either single-gene deletions (e.g., "sigB") or interactions between two gene deletions (e.g. "sigB*prfA"). The p-values for the single-gene deletions measure the individual effect of deleting each respective gene. The "gene*gene" variable measures synergistic deletion effects by comparing the effect of deleting both genes to the effect of deleting either one gene or the other; significant values are marked with (***) p-value ≤ 0.001, (**) p-value ≤ 0.01, or (*) p-value ≤ 0.05. The actual data used for these analyses are presented in Figure 2.4.

Table A2 [S3.1]. *Listeria monocytogenes* genes transcribed at higher levels[a] at 7°C compared to 37°C

Name[b]	Gene Function[c]	Fold change[d] (7°C/37°C)	
		Log phase	Stationary phase
lmo0005 (*recF*)	RecF protein	2.14 ***	-
lmo0013 (*qoxA*)	AA3-600 quinol oxidase subunit II	2.05 **	-
lmo0019	unknown	-	5.10 ***
lmo0042	similar to *E. coli* DedA protein	4.48 ***	-
lmo0047	unknown	2.99 ***	2.82 *
lmo0066	similar to toxin components	-	2.66 ***
lmo0076	similar to *E. coli* Ada protein (O6-methylguanine-DNA methyltransferase)	-	2.68 ***
lmo0114	similar to putative repressor C1 from lactococcal bacteriophage Tuc2009	-	2.02 ***
lmo0117 (*lmaB*)	antigen B	7.36 ***	-
lmo0118 (*lmaA*)	antigen A	5.08 ***	-
lmo0119	unknown	5.98 ***	-
lmo0120	unknown	6.12 ***	-
lmo0121	similar to bacteriophage minor tail proteins	3.47 ***	-
lmo0122	similar to phage proteins	-	2.03 ***
lmo0123	similar to protein gp18 from Bacteriophage A118	4.35 ***	-
lmo0124	unknown	3.13 ***	-
lmo0125	unknown	3.40 ***	-
lmo0126	unknown	4.81 ***	-
lmo0127	weakly similar to protein gp20 from Bacteriophage A118	3.50 ***	-
lmo0129	similar to autolysin: N-acetylmuramoyl-L-alanine amidase	4.73 ***	-
lmo0131	conserved hypothetical protein	-	2.89 ***
lmo0133	similar to *E. coli* YjdI protein	-	2.60 ***
lmo0134	similar to *E. coli* YjdJ protein	-	5.02 ***

lmo0137	similar to oligopeptide ABC transporter, permease protein	-	2.11 ***
lmo0193	unknown	4.22 ***	-
lmo0194	ABC transporter, ATP-binding protein	4.47 ***	-
lmo0195	similar to membrane protein (putative ABC transporter component)	4.20 ***	-
lmo0200 (*prfA*)	listeriolysin positive regulatory protein	-	4.60 ***
lmo0249 (*rplA*)	ribosomal protein L1	-	2.10 ***
lmo0250 (*rplJ*)	ribosomal protein L10	-	2.34 ***
lmo0251 (*rplL*)	ribosomal protein L12	-	2.54 ***
lmo0256	conserved hypothetical protein	-	2.06 ***
lmo0266	similar to transcriptional regulators	-	3.70 ***
lmo0267	similar to other proteins	-	4.63 ***
lmo0268	similar to phosphoglycerate mutase	-	3.59 ***
lmo0269	similar to transporter	2.07 ***	-
lmo0273	unknown	-	2.47 ***
lmo0283	similar to ABC transporter permease protein	2.81 ***	-
lmo0284	similar to ABC transporter (ATP-binding	2.24 ***	-
lmo0285	putative lipoprotein	2.43 ***	-
lmo0312	similar to unknown proteins	2.11 *	-
lmo0321	similar to unknown proteins	2.49 ***	4.82 ***
lmo0322	similar to unknown proteins	2.06 **	-
lmo0352	highly similar to regulatory proteins	2.12 ***	-
lmo0361	similar to conserved hypothetical integral membrane protein	4.67 ***	-
lmo0362	similar to conserved hypothetical protein	6.11 ***	-
lmo0365	similar to conserved hypothetical protein	6.74 ***	-
lmo0366	conserved hypothetical protein, putative	7.20 ***	-
lmo0367	conserved hypothetical protein similar to *B. subtilis* YwbN protein	6.08 ***	-

lmo0377	unknown	2.05 ***	-
lmo0391	unknown	-	2.19 ***
lmo0433 (*inlA*)	Internalin A	-	2.05 ***
lmo0484	unknown	4.37 ***	-
lmo0485	unknown	2.48 ***	-
lmo0515	conserved hypothetical protein	-	4.01 ***
lmo0518	similar to unknown protein	2.76 ***	-
lmo0519	similar to multidrug resistance protein	4.47 ***	-
lmo0525	unknown	3.08 ***	-
lmo0540	similar to penicillin-binding protein	-	3.48 ***
lmo0541	similar to ABC transporter (binding protein)	13.44 ***	-
lmo0553	unknown	-	2.28 ***
lmo0561	similar to phosphorybosil-AMP-cyclohydrolase (HisI2 protein)	-	2.94 ***
lmo0562	similar to phosphoribosyl-AMP cyclohydrolase (HisI1 protein)	-	4.71 ***
lmo0563	highly similar to cyclase HisF	-	3.47 ***
lmo0564	highly similar to phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	-	3.47 ***
lmo0565	similar to amidotransferases	-	2.20 ***
lmo0570	similar histidinol phosphate phosphatase	-	2.14 ***
lmo0579	similar to unknown protein	-	2.32 ***
lmo0581	conserved hypothetical protein	2.27 ***	-
lmo0590	similar to a fusion of two types of conserved hypothetical proteinconserved hypothetical	2.00 ***	-
lmo0592	unknown	2.15 **	-
lmo0593	similar to transport proteins (formate)	2.21 **	-
lmo0595	similar to O-acetylhomoserine sulfhydrylase	2.31 ***	-

lmo0596	similar to unknown proteins	2.26 **	-
lmo0597	similar to transcription regulator	5.18 ***	-
lmo0599	conserved hypothetical protein	2.86 ***	-
lmo0600	unknown	2.24 ***	-
lmo0604	similar to *B. subtilis* YvlA protein	5.22 ***	-
lmo0609	similar to *E. coli* phage shock protein E	-	2.14 ***
lmo0610	similar to internalin proteins, putative peptidoglycan bound protein (LPXTG	-	4.41 ***
lmo0617	unknown	-	2.14 ***
lmo0623	unknown	-	2.92 ***
lmo0624	similar to unknown proteins	2.16 ***	3.42 ***
lmo0625	unknown	-	7.19 ***
lmo0626	similar to unknown protein	-	2.85 ***
lmo0656	conserved hypothetical protein	2.30 ***	-
lmo0661	similar to unknown proteins	-	2.28 ***
lmo0665	unknown	2.02 ***	-
lmo0666	similar to unknown protein	2.98 ***	-
lmo0669	similar to oxidoreductase	-	2.04 ***
lmo0672	similar to unknown protein	2.64 ***	-
lmo0680	similar to flagella-associated protein flhA	2.22 ***	-
lmo0681	similar to flagellar biosynthesis protein FlhF	2.20 **	-
lmo0683	similar to chemotactic methyltransferase CheR	2.37 **	-
lmo0684	unknown	2.24 **	-
lmo0685	similar to motility protein (flagellar motor rotation) MotA	2.51 **	-
lmo0686 (*motB*)	similar to motility protein (flagellar motor rotation) MotB	2.17 ***	-
lmo0687	unknown	2.69 ***	-
lmo0688	similar to unknown protein	2.43 ***	-
lmo0689	similar to CheA activity-modulating chemotaxis protein CheV	2.49 ***	-

lmo0734	similar to transcriptional regulator (LacI family)	2.34 ***	-
lmo0753	similar to transcription regulator Crp/Fnr family	-	3.05 ***
lmo0754	weakly similar to a bile acid 7-alpha dehydratase	-	4.37 ***
lmo0757	similar to hypothetical proteins	-	2.37 ***
lmo0759	unknown	-	2.97 ***
lmo0760	unknown	-	2.53 ***
lmo0778	unknown	2.92 ***	-
lmo0780	unknown	2.68 ***	-
lmo0781	similar to mannose-specific phosphotransferase system (PTS) component IID	-	4.26 ***
lmo0782	similar to mannose-specific phosphotransferase system (PTS) component IIC	-	2.79 ***
lmo0783	similar to mannose-specific phosphotransferase system (PTS) component IIB	-	5.29 ***
lmo0784	similar to mannose-specific phosphotransferase system (PTS) component IIA	-	4.63 ***
lmo0795	conserved hypothetical protein	2.22 ***	-
lmo0799	unknown	2.41 ***	-
lmo0800	similar to *B. subtilis* YqkB protein	-	5.35 ***
lmo0806	similar to transcription regulator	2.89 **	-
lmo0808	similar to spermidine/putrescine ABC transporter, permease protein	2.42 **	-
lmo0809	similar to spermidine/putrescine ABC transporter, permease protein	2.25 ***	-
lmo0810	similar to spermidine/putrescine-binding protein	2.17 ***	-
lmo0811	similar to carbonic anhydrase	2.32 ***	-
lmo0818	similar to cation transporting ATPase	2.17 ***	-
lmo0823	similar to oxydoreductases	-	3.82 ***

lmo0830 (*fbp*)	highly similar to fructose-1,6-bisphosphatase	2.30 **	-
lmo0835	putative peptidoglycan bound protein (LPXTG motif)	2.37 *	-
lmo0836	similar to *B. subtilis* YrkR protein	2.56 ***	-
lmo0843	similar to *B. subtilis* protein YsdA	-	2.46 ***
lmo0844	conserved hypothetical protein	3.61 ***	-
lmo0850	hypothetical protein	2.57 ***	3.68 ***
lmo0851	unknown	-	2.10 ***
lmo0867	unknown	3.08 ***	-
lmo0869	unknown	2.00 **	-
lmo0873	Similar to transcriptional regulator	-	2.03 ***
lmo0875	similar to PTS system, beta-glucoside enzyme IIB component	-	2.03 ***
lmo0894 (*rsbW*)	sigma-B activity negative regulator RsbW	-	2.07 ***
lmo0903	conserved hypothetical protein	4.50 ***	-
lmo0905	unknown	2.10 ***	3.08 ***
lmo0906	similar to glutathione Reductase	-	3.75 ***
lmo0907	similar to phosphoglycerate mutase	-	2.06 ***
lmo0910	unknown	-	2.22 ***
lmo0911	unknown	-	2.25 ***
lmo0912	similar to transporters (formate)	-	3.76 ***
lmo0913	similar to succinate semialdehyde dehydrogenase	-	2.70 ***
lmo0915	similar to phosphotransferase system enzyme IIC	-	2.06 ***
lmo0937	unknown	-	2.21 ***
lmo0939	unknown	-	2.47 ***
lmo0944	similar to *B. subtilis* YneR protein	2.04 ***	-
lmo0945	similar to C-terminal part of B. subtilis ComEC protein and to ComEA	-	2.51 ***
lmo0954	unknown	-	3.66 ***
lmo0955	unknown	-	2.13 ***
lmo0994	unknown	-	2.48 ***

lmo1000	similar to phytoene dehydrogenase	2.30 ***	-
lmo1001	similar to *B. subtilis* protein YkvS	4.41 ***	-
lmo1007	unknown	18.96 ***	-
lmo1015 (*gbuB*)	highly similar to glycine betaine ABC transporters (permease)	4.27 ***	-
lmo1016 (*gbuC*)	highly similar to glycine betaine ABC transporters (glycine betaine-binding protein)	3.85 ***	-
lmo1023	similar to a bacterial K(+)-uptake system	-	2.31 ***
lmo1056	unknown	2.02 ***	-
lmo1064	similar to membrane and transport	2.35 ***	-
lmo1069	similar to *B. subtilis* YlaI protein	-	2.93 ***
lmo1091	similar to glysosyltransferases	-	2.39 ***
lmo1112	highly similar to TN916 ORF21	4.34 *	-
lmo1131	similar to ABC transporters, ATP-binding	2.20 *	-
lmo1132	similar to ABC transporters, ATP-binding	4.37 ***	-
lmo1137	unknown	5.23 **	-
lmo1145	similar to *Salmonella enterica* PduV protein	2.23 **	-
lmo1151	similar to *Salmonella* Typhimurium PduA	-	2.38 ***
lmo1172	similar to similar to two-component response regulator	2.22 ***	3.84 ***
lmo1173	similar to two-component sensor histidine kinase	2.13 **	3.75 ***
lmo1175 (*eutB*)	similar to ethanolamine ammonia-lyase, heavy chain	-	2.18 ***
lmo1176 (*eutC*)	similar to ethanolamine ammonia-lyase, light chain	-	2.39 ***
lmo1177	similar to putative carboxysome structural protein (eutL)	3.23 ***	-
lmo1179	similar to acetaldehyde dehydrogenase / alcohol dehydrogenase	-	4.19 ***
lmo1180	similar to putative carboxysome structural protein	-	3.90 ***
lmo1181	similar to cobalamin adenosyl transferase	5.08 **	2.10 **

lmo1182	similar to *Salmonella enterica* PduL protein	5.82 **	2.38 **
lmo1183	unknown	9.23 ***	2.10 **
lmo1184	similar to carbon dioxide concentrating mechanism protein	6.31 **	3.17 **
lmo1185	similar to *Salmonella enterica* PduT protein	5.68 ***	-
lmo1186	similar to ethanolamine utilization protein EutH - *E. coli*	2.70 **	2.02 **
lmo1231	similar to DNA polymerase beta, to B. subtilis YshC protein	-	2.38 ***
lmo1232	similar to MutS protein (MutS2)	-	2.17 ***
lmo1245	unknown	7.80 ***	-
lmo1246	similar to ATP-dependent RNA helicase (DEAD motif)	2.27 ***	-
lmo1249	unknown	2.65 *	-
lmo1252	similar to *B. subtilis* YxkD protein	3.15 ***	-
lmo1257	unknown	-	2.11 ***
lmo1340	similar to *B. subtilis* YqgU protein	-	7.34 ***
lmo1428 (*opuCA*)	similar to glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)	2.16 ***	-
lmo1431	similar to ABC transporter (ATP-binding	2.15 ***	-
lmo1450	similar to ATP-dependent RNA helicase, DEAD-box family (deaD)	2.03 ***	-
lmo1479 (*lepA*)	highly similar to GTP-binding protein LepA	2.39 ***	-
lmo1485	similar to unknown proteins	2.05 ***	-
lmo1486	unknown	2.02 **	-
lmo1498	similar to O-methyltransferase	-	2.28 *
lmo1499	similar to unknown proteins	-	3.89 **
lmo1512	similar to putative tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase	-	2.28 ***
lmo1513	similar to iron-sulfur cofactor synthesis protein	-	2.54 ***
lmo1518	unknown	-	2.37 ***

lmo1519 (*aspS*)	aspartyl-tRNA synthetase	-	3.78 ***
lmo1526	similar to unknown proteins	2.62 ***	-
lmo1537	conserved GTP binding protein	2.01 ***	-
lmo1558	similar to hypothetical GTP binding protein	2.02 **	-
lmo1567 (*citZ*)	highly similar to citrate synthase subunit II	2.15 ***	-
lmo1568	similar to unknown proteins	2.08 ***	-
lmo1583	similar to thiol peroxidases	2.14 ***	-
lmo1617	similar to multidrug-efflux transporter	2.98 ***	-
lmo1618	similar to transcription regulator MarR family	2.32 **	-
lmo1649	unknown	-	2.44 ***
lmo1650	similar to hypothetical proteins	-	2.50 ***
lmo1690	similar to hypothetical proteins	-	2.19 ***
lmo1692	unknown	-	2.66 ***
lmo1713	similar to cell-shape determining proteins	2.31 ***	-
lmo1722	similar to ATP-dependent RNA helicases	2.24 ***	2.46 ***
lmo1762	unknown	2.71 ***	-
lmo1791	unknown	-	2.79 ***
lmo1826	unknown	2.07 ***	-
lmo1828	similar to conserved hypothetical protein	2.06 ***	-
lmo1829	similar to fibronectin binding proteins	2.23 ***	-
lmo1832 (*pyrF*)	highly similar to orotidine 5-phosphate decarboxylases	-	2.55 **
lmo1835 (*pyrAB*)	highly similar to carbamoyl-phosphate synthetase (catalytic subunit)	2.17 **	-
lmo1836 (*pyrAa*)	highly similar to carbamoyl-phosphate synthetase (glutaminase subunit)	2.39 **	-
lmo1838 (*pyrB*)	highly similar to aspartate carbamoyltransferase	2.36 **	-
lmo1840 (*pyrR*)	highly similar to pyrimidine operon regulatory protein	2.28 **	-
lmo1843	similar to conserved hypothetical proteins	2.66 ***	-
lmo1844 (*lsp*)	highly similar to signal peptidase II	2.30 ***	-

lmo1845	similar to conserved hypothetical proteins	-	4.95 ***
lmo1864	similar to hemolysinIII proteins, putative integral membrane protein	2.76 ***	3.29 ***
lmo1865	similar to conserved hypothetical proteins	2.41 ***	-
lmo1868	similar to conserved hypothetical proteins	-	2.74 ***
lmo1870	similar to alkaline phosphatase	2.80 ***	-
lmo1887	similar to conserved hypothetical proteins	2.09 ***	-
lmo1891 (*recU*)	similar to DNA repair and homologous recombination protein	2.31 ***	-
lmo1936 (*gpsA*)	similar to NAD(P)H-dependent glycerol-3-phosphate dehydrogenase	2.23 **	2.64 ***
lmo1937	similar to unknown protein	2.24 **	-
lmo1951	similar to unknown proteins	-	2.22 ***
lmo1957 (*fhuG*)	similar to ferrichrome ABC transporter	2.29 ***	-
lmo1958 (*fhuB*)	similar to ferrichrome ABC transporter	3.11 ***	-
lmo1960 (*fhuC*)	similar to ferrichrome ABC transporter (ATP-binding protein)	3.84 ***	-
lmo1961	similar to oxidoreductases	2.25 ***	-
lmo1962	similar to transcription regulators (TetR family)	-	2.49 ***
lmo1963	similar to unknown proteins	-	2.78 ***
lmo1964	similar to ABC transporter, ATP-binding protein	-	2.68 ***
lmo1972	similar to pentitol PTS system enzyme II B component	-	2.50 ***
lmo1978	similar to glucose-6-phosphate 1-dehydrogenase	-	4.19 ***
lmo1984 (*ilvB*)	similar to acetolactate synthase (acetohydroxy-acid synthase) (large subunit)	2.02 **	-
lmo1985 (*ilvN*)	similar to acetolactate synthase (acetohydroxy-acid synthase) (small subunit)	3.13 ***	-

lmo1986 (*ilvC*)	similar to ketol-acid reductoisomerase (acetohydroxy-acid isomeroreductase)	2.63 **	-
lmo1988 (*leuB*)	similar to 3-isopropylmalate dehydrogenase	2.69 *	-
lmo1990 (*leuD*)	similar to 3-isopropylmalate dehydratase (small subunit)	2.31 *	-
lmo1991 (*ilvA*)	similar to threonine dehydratase	2.31 **	-
lmo1992	similar to alpha-acetolactate decarboxylase	4.22 ***	5.73 ***
lmo1997	similar to PTS mannose-specific enzyme IIA component	-	3.42 ***
lmo1998	similar to opine catabolism protein	-	2.20 ***
lmo2006 (*alsS*)	similar to alpha-acetolactate synthase	3.20 ***	-
lmo2057 (*ctaB*)	highly similar to heme A farnesyltransferase	2.17 ***	-
lmo2058 (*ctaA*)	similar to heme O oxygenase	2.23 ***	-
lmo2065	unknown	2.31 ***	-
lmo2071	unknown	2.22 ***	-
lmo2087	similar to unknown proteins	2.26 ***	-
lmo2114	similar to ABC transporter (ATP-binding	2.06 ***	-
lmo2181	similar to unknown protein	2.73 **	-
lmo2185	unknown	4.63 ***	-
lmo2186	unknown	8.36 ***	-
lmo2191	similar to unknown proteins	2.46 ***	-
lmo2197	unknown	2.16 ***	-
lmo2199	similar to unknown protein	5.95 ***	-
lmo2200	similar to transcription regulator	7.03 ***	-
lmo2210	unknown	5.07 ***	-
lmo2220	similar to S. aureus Cbf1 protein	-	2.08 ***
lmo2232	similar to unknown proteins	-	2.69 ***
lmo2256	similar to unknown proteins	-	5.75 ***
lmo2258	unknown	2.05 **	-
lmo2263	similar to unkown proteins	-	2.03 ***
lmo2269	unknown	-	8.56 ***
lmo2289	Protein gp14 [Bacteriophage A118]	-	2.19 ***

lmo2291	major tail shaft protein [Bacteriophage A118]	2.62 **	-
lmo2292	Portein gp11 [Bacteriophage A118]	2.10 *	-
lmo2301	similar to putative terminase small subunit from Bacteriophage A118	-	2.21 ***
lmo2303	Protein gp66 [Bacteriophage A118]	2.33 **	-
lmo2304	Bacteriophage A118 gp65 protein	2.34 **	-
lmo2323	gp43 [Bacteriophage A118]	6.68 **	-
lmo2324	similar to anti-repressor [Bacteriophage A118]	2.69 *	-
lmo2335 (*fruA*)	highly similar to phosphotransferase system (PTS) fructose-specific enzyme IIABC component	12.35 **	-
lmo2336 (*fruB*)	fructose-1-phosphate kinase	21.42 ***	-
lmo2337	similar to regulatory protein DeoR family	20.71 ***	-
lmo2338 (*pepC*)	aminopeptidase C	-	3.20 ***
lmo2345	conserved hypothetical protein	2.36 **	-
lmo2347	similar to amino acid ABC transporter	2.64 **	-
lmo2348	similar to amino acid ABC-transporter	2.32 ***	-
lmo2349	similar to amino acid ABC transporter (binding protein)	3.58 ***	-
lmo2350	similar to *B. subtilis* YtmI protein	2.09 **	-
lmo2352	similar to LysR family transcription regulator	4.04 ***	-
lmo2357	similar to unknown protein	-	2.14 ***
lmo2358	similar to N-acetylglucosamine-6-phosphate isomerase	-	2.62 ***
lmo2360	transmembrane protein	3.47 ***	-
lmo2361	conserved hypothetical protein	3.01 ***	-
lmo2372	similar to ABC-transporter ATP binding proteins	2.36 ***	-
lmo2375	unknown	3.70 ***	-
lmo2378	similar to proteins involved in resistance to cholate and to NA(+) and in pH homeostasis	2.40 ***	-

lmo2379	similar to proteins involved in resistance to cholate and to NA(+) and in pH homeostasis	2.17 ***	-
lmo2380	similar to proteins involved in resistance to cholate and to NA(+) and in pH homeostasis	2.14 ***	-
lmo2409	unknown	2.05 ***	-
lmo2432	unknown	-	2.25 ***
lmo2454	unknown	-	3.56 ***
lmo2462	similar to dipeptidases	-	2.68 ***
lmo2463	similar to transport protein	-	2.18 ***
lmo2468 (*clpP*)	ATP-dependent Clp protease proteolytic subunit	2.87 ***	-
lmo2480	similar to acetyltransferase	2.00 ***	-
lmo2486	unknown	3.06 *	-
lmo2487	similar to B. subtilis YvlB protein	3.03 ***	4.71 ***
lmo2494	similar to negative regulator of phosphate regulon	3.05 ***	-
lmo2495	similar to phosphate ABC transporter (ATP-binding protein)	4.10 ***	-
lmo2498	similar to phosphate ABC transporter (permease protein)	2.63 *	-
lmo2522	similar to hypothetical cell wall binding protein from B. subtilis	-	2.31 ***
lmo2535 (*atpB*)	highly similar to H+-transporting ATP synthase chain a	-	2.11 ***
lmo2557	conserved hypothetical protein	2.54 ***	-
lmo2567	unknown	4.57 ***	-
lmo2568	unknown	3.02 **	-
lmo2569	similar to dipeptide ABC transporter (dipeptide-binding protein)	2.60 ***	-
lmo2588	similar to drug-export proteins	2.23 **	-
lmo2602	conserved hypothetical protein	-	2.87 ***
lmo2603	unknown	-	2.28 ***
lmo2613 (*rplO*)	ribosomal protein L15	-	2.41 ***
lmo2614 (*rpmD*)	ribosomal protein L30	-	2.92 ***

lmo2615 (*rpsE*)	ribosomal protein S5	-	3.08 ***
lmo2616 (*rplR*)	ribosomal protein L18	-	3.09 ***
lmo2617 (*rplF*)	ribosomal protein L6	-	2.88 ***
lmo2618 (*rpsH*)	ribosomal protein S8	-	3.09 ***
lmo2619 (*rpsN*)	ribosomal protein S14	-	3.10 ***
lmo2620 (*rplE*)	ribosomal protein L5	-	3.14 ***
lmo2621 (*rplX*)	ribosomal protein L24	-	2.73 ***
lmo2622 (*rplN*)	ribosomal protein L14	-	2.81 ***
lmo2623 (*rpsQ*)	ribosomal protein S17	-	2.27 ***
lmo2624 (*rpmC*)	ribosomal protein L29	-	2.42 ***
lmo2625 (*rplP*)	ribosomal protein L16	-	2.06 ***
lmo2626 (*rpsC*)	ribosomal protein S3	-	2.03 ***
lmo2628 (r*plV*)	ribosomal protein S19	-	2.01 ***
lmo2648	similar to Phosphotriesterase	-	2.34 ***
lmo2649	similar to hypothetical PTS enzyme IIC	-	2.33 ***
lmo2650	similar to hypothetical PTS enzyme IIB	-	2.52 ***
lmo2669	unknown	-	4.25 ***
lmo2673	conserved hypothetical protein	-	2.72 ***
lmo2674	similar to ribose 5-phosphate epimerase	-	4.30 ***
lmo2683	similar to cellobiose phosphotransferase enzyme IIB component	2.22 **	-
lmo2689	highly similar to Mg2+ transport ATPase	2.15 ***	-
lmo2694	similar to lysine decarboxylase	-	2.35 ***
lmo2745	similar to ABC transporter (ATP-binding protein)	-	2.10 ***
lmo2761	similar to beta-glucosidase	2.18 **	-
lmo2766	similar to hypothetical transcriptional regulator	3.01 ***	-
lmo2784	similar to lichenan operon transcription antiterminator licR	-	2.45 ***
lmo2786 (*bvrC*)	bvrC	-	5.27 ***
lmo2787 (*bvrB*)	beta-glucoside-specific phosphotransferase enzyme II ABC component	-	2.74 **

lmo2811	similar to GTPase	2.28 ***	-
lmo2830	similar to thioredoxin	-	5.56 ***
lmo2852	unknown	4.15 ***	-

[a]Genes were considered to be up-regulated at 7°C if the fold change compared to 37°C was ≥ 2.0 with an adjusted p-value ≤ 0.05

[b]Gene names are from ListiList (http://genolist.pasteur.fr/ListiList). Predicted operons are boxed. Operon predictions are from ListiList and Toledo-Arana *et. al.*, 2009.

[c]gene functions were based on annotation provided by ListiList

[d]Superscripts are adjusted p values: "***" (< 0.001), "**" (< 0.01), "*" (≤ 0.05), "NS" (> 0.05)

Table A2 [S3.2]. *Listeria monocytogenes* genes transcribed at lower levels[a] at 7°C compared to 37°C

Name[b]	Gene Function[c]	Fold change[d] (7°C/37°C)	
		Log phase	Stationary phase
lmo0013 (*qoxA*)	AA3-600 quinol oxidase subunit II	-	-2.04 ***
lmo0014 (*qoxB*)	AA3-600 quinol oxidase subunit I	-	-2.05 ***
lmo0018	beta-glucosidase	-2.84 **	-
lmo0021	similar to PTS system, fructose-specific IIA component	-2.14 **	-
lmo0022	similar to PTS system, fructose-specific IIB component	-4.39 *	-
lmo0024	similar to PTS system, mannose-specific IID component	-4.76 **	-
lmo0027	similar to PTS system, beta-glucosides specific enzyme IIABC	-6.84 *	-
lmo0044 (*rpsF*)	ribosomal protein S6	-	-3.07 ***
lmo0045 (*ssb*)	highly similar to single-strand binding protein (SSB)	-	-3.99 ***
lmo0046 (*rpsR*)	ribosomal protein S18	-	-4.16 ***
lmo0053 (*rplI*)	50S ribosomal protein L9	-	-2.44 ***
lmo0101	similar to transcription regulator	-	-2.02 ***
lmo0104	unknown	-2.12 ***	-
lmo0109	similar to transcriptional regulatory proteins, AraC family	-4.35 **	-2.65 ***
lmo0110	similar to lipase	-5.17 **	-
lmo0180	similar to sugar ABC transporter, permease protein	-2.39 **	-
lmo0181	similar to sugar ABC transporter, sugar-binding protein	-3.50 **	-
lmo0183	similar to alpha-glucosidase	-3.64 **	-
lmo0184	similar to oligo-1,6-glucosidase	-3.11 **	-
lmo0196	similar to *B. subtilis* SpoVG protein	-	-3.29 ***
lmo0197	similar to *B. subtilis* SpoVG protein	-	-2.24 ***
lmo0201 (*plcA*)	phosphatidylinositol-specific phospholipase c	-2.72 ***	-
lmo0202 (*hly*)	listeriolysin O precursor	-14.43 ***	-3.80 ***
lmo0204 (*actA*)	actin-assembly inducing protein precursor	-2.38 **	-
lmo0205 (*plcB*)	phospholipase C	-3.12 ***	-
lmo0208	conserved hypothetical protein	-2.45 ***	-
lmo0217	similar to *B. subtilis* DivIC protein	-	-2.03 ***

lmo0238 (*cysE*)	similar to serine O-acetyltransferase	-2.65 ***	-2.23 ***
lmo0239 (*cysS*)	cysteinyl-tRNA synthetase	-3.24 ***	-3.23 ***
lmo0240	highly similar to *B. subtilis* YazC protein	-3.21 ***	-3.20 ***
lmo0241	similar to conserved hypothetical proteins like to *B. subtilis* YacO protein	-3.35 ***	-
lmo0242	similar to *B. subtilis* Yacp protein	-3.61 ***	-2.06 ***
lmo0243 (*sigH*)	RNA polymerase sigma-30 factor (sigma-H)	-2.78 ***	-
lmo0244	similar to ribosomal protein L33 type II	-	-2.03 ***
lmo0245 (*secE*)	highly similar to preprotein translocase subunit	-	-2.15 ***
lmo0258 (*rpoB*)	RNA polymerase (beta subunit)	-	-3.57 ***
lmo0259 (*rpoC*)	RNA polymerase (beta subunit)	-	-2.66 ***
lmo0291	conserved hypothetical protein similar to *B. subtilis* YycJ protein	-	-2.38 ***
lmo0295	similar to FMN-containing NADPH-linked nitro/flavin reductase	-2.93 *	-
lmo0298	similar to PTS beta-glucoside-specific enzyme IIC component	-4.38 *	-
lmo0299	similar to PTS beta-glucoside-specific enzyme IIB component	-9.09 *	-
lmo0300	similar to phospho-beta-glucosidase and phospho-beta-galactosidase	-6.81 *	-
lmo0301	similar to PTS beta-glucoside-specific enzyme IIA component	-2.06 **	-
lmo0307	unknown	-2.76 ***	-
lmo0319	similar to phospho-beta-glucosidase	-4.41 **	-
lmo0323	similar to unknown proteins	-2.05 *	-
lmo0342	similar to transketolase	-2.16 *	-
lmo0343	similar to transaldolase	-3.20 *	-
lmo0345	similar to sugar-phosphate isomerase	-2.90 *	-2.17 **
lmo0346	similar to triosephosphate isomerase	-3.69 **	-
lmo0347	similar to dihydroxyacetone kinase	-2.10 *	-2.68 **
lmo0348	similar to dihydroxyacetone kinase	-	-2.37 ***

lmo0349	unknown	-	-4.05 **
lmo0351	similar to unknown proteins	-	-4.17 **
lmo0354	similar to fatty-acid--CoA ligase	-3.09 ***	-
lmo0355	similar to Flavocytochrome C Fumarate Reductase chain A	-10.10 ***	-
lmo0386	similar to *B. subtilis* IolD protein, to acetolactate synthase	-2.73 *	-
lmo0387	similar to *B. subtilis* YhdG protein	-	-2.20 ***
lmo0388	unknown	-	-2.06 ***
lmo0398	similar to phosphotransferase system enzyme IIA	-9.25 *	-
lmo0399	similar to fructose-specific phosphotransferase enzyme IIB	-3.22 *	-
lmo0400	similar to fructose-specific phosphotransferase enzyme IIC	-15.17 *	-
lmo0401	highly similar to *E. coli* YbgG protein, a putative sugar hydrolase	-4.82 *	-
lmo0402	similar to transcriptional antiterminator (BglG family)	-3.69 *	-
lmo0408	unknown	-	-2.58 ***
lmo0424	similar to *Staphylococcus xylosus* glucose uptake protein	-4.27 *	-
lmo0425	similar to transcription antiterminator BglG family	-15.82 **	-
lmo0426	similar to PTS fructose-specific enzyme IIA component	-25.39 **	-
lmo0427	similar to PTS fructose-specific enzyme IIB component	-19.37 **	-
lmo0428	similar to PTS fructose-specific enzyme IIC component	-12.30 **	-
lmo0429	similar to sugar hydrolase	-9.76 **	-
lmo0431	similar to acetyltransferase	-2.08 **	-
lmo0443	similar to *B. subtilis* transcription	-	-5.31 ***
lmo0471	unknown	-5.30 *	-
lmo0472	unknown	-13.43 *	-
lmo0493	similar to acylase	-2.03 **	-
lmo0517	similar to phosphoglycerate mutase	-4.54 *	-
lmo0536	similar to 6-phospho-beta-glucosidase	-2.67 **	-

lmo0539	similar to tagatose-1,6-diphosphate aldolase	-	-4.57 ***
lmo0593	similar to transport proteins	-	-3.05 ***
lmo0636	similar to unknown proteins	-2.21 ***	-
lmo0641	similar to heavy metal-transporting ATPase	-2.95 **	-
lmo0647	unknown	-	-2.80 ***
lmo0707	similar to flagellar hook-associated protein 2 FliD	-	-2.12 ***
lmo0711	similar to flagellar basal-body rod protein flgC	-	-2.04 ***
lmo0721	putative fibronectin-binding protein	-2.73 ***	-
lmo0726	Hypothetical CDS	-	-3.26 ***
lmo0727	similar to L-glutamine-D-fructose-6-phosphate amidotransferase	-	-3.09 ***
lmo0735	similar to Ribulose-5-Phosphate 3-Epimerase	-	-2.31 ***
lmo0736	similar to ribose 5-phosphate isomerase	-	-2.71 ***
lmo0737	unknown	-	-2.54 ***
lmo0738	similar to phosphotransferase system (PTS) beta-glucoside-specific enzyme IIABC component	-	-3.38 ***
lmo0739	similar to 6-phospho-beta-glucosidase	-	-7.74 ***
lmo0781	similar to mannose-specific phosphotransferase system (PTS) component IID	-2.04 *	-
lmo0782	similar to mannose-specific phosphotransferase system (PTS) component IIC	-2.47 **	-
lmo0788	unknown	-3.67 **	-
lmo0792	similar to conserved hypothetical protein	-	-2.04 ***
lmo0794	similar to *B. subtilis* YwnB protein	-	-2.60 ***
lmo0796	conserved hypothetical protein	-	-3.71 ***
lmo0813	similar to fructokinases	-3.31 ***	-
lmo0814	similar to oxidoreductases	-2.36 ***	-
lmo0822	similar to transcriptional regulators	-	-2.09 ***
lmo0829 (*nifJ*)	highly similar to pyruvate-flavodoxin oxidoreductase	-3.11 ***	-
lmo0864	unknown	-2.86 *	-
lmo0865	similar to phosphomannomutase	-2.18 *	-

lmo0912	similar to transporters (formate)	-4.23 ***	-
lmo0914	similar to PTS system, IIB component	-2.99 *	-
lmo0916	similar to phosphotransferase system enzyme IIA	-12.87 **	-
lmo0917	similar to beta-glucosidase	-6.00 *	-
lmo0931	similar to lipoate protein ligase A	-	-2.55 ***
lmo0940	unknown	-2.68 ***	-
lmo0941	unknown	-2.47 ***	-
lmo0942	similar to heat shock protein HtpG	-2.27 ***	-
lmo0943 (*fri*)	non-heme iron-binding ferritin	-	-2.08 ***
lmo0944	similar to *B. subtilis* YneR protein	-	-4.57 ***
lmo0953	unknown	-	-2.28 ***
lmo0956	similar to N-acetylglucosamine-6P-phosphate deacetylase (EC 3.5.1.25)	-2.73 **	-
lmo0957	similar to glucosamine-6-Phoasphate isomerase (EC 5.3.1.10)	-	-2.91 ***
lmo0960	similar to proteases	-2.28 *	-
lmo0961	similar to proteases	-2.20 *	-
lmo0971 (*dltD*)	DltD protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid	-2.20 ***	-
lmo0972 (*dltC*)	D-alanyl carrier protein	-2.02 **	-
lmo0973 (*dltB*)	DltB protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid	-2.13 ***	-
lmo0974 (*dltA*)	D-alanine-activating enzyme (dae), D-alanine-D-alanyl carrier protein ligase (dcl)	-2.13 **	-
lmo1002 (*ptsH*)	PTS phosphocarrier protein Hpr (histidine containing protein)	-	-3.44 ***
lmo1013	similar to conserved hypothetical proteins like to *B. subtilis* YkuT protein	-3.00 ***	-
lmo1043	similar to molybdopterin-guanine dinucleotide biosynthesis MobB	-2.31 *	-
lmo1046	similar to molybdenum cofactor biosynthesis protein C	-2.37 *	-
lmo1047	similar to molybdenum cofactor biosynthesis protein A	-2.04 **	-
lmo1051	similar to formylmethionine deformylase and to B. subtilis YkrB protein	-	-2.32 ***
lmo1057	similar to L-lactate dehydrogenase	-	-3.93 ***
lmo1058	similar to *B. subtilis* YktA protein	-	-4.23 ***

lmo1059	unknown	-	-4.42 ***
lmo1086	similar to CDP-ribitol pyrophosphorylase	-	-2.44 ***
lmo1087	similar to glucitol dehydrogenase	-	-2.20 *
lmo1120	unknown	-2.11 *	-
lmo1121	unknown	-2.56 **	-
lmo1123	unknown	-2.96 **	-
lmo1124	unknown	-2.40 *	-
lmo1140	unknown	-2.66 **	-2.62 ***
lmo1253	similar to transcription regulator GntR family	-	-2.66 ***
lmo1254	similar to alpha,alpha-	-9.60 ***	-
lmo1255	similar to PTS system trehalose specific enzyme IIBC	-5.19 **	-
lmo1257	unknown	-36.15 ***	-
lmo1261	unknown	-	-2.98 ***
lmo1293 (*glpD*)	similar to glycerol 3 phosphate dehydrogenase	-5.25 **	-
lmo1303	similar to B. subtilis YneA protein	-2.12 ***	-
lmo1348	similar to aminomethyltransferase	-3.32 *	-2.37 ***
lmo1349	similar to glycine dehydrogenase (decarboxylating) subunit 1	-4.62 **	-3.93 ***
lmo1350	similar to glycine dehydrogenase (decarboxylating) subunit 2	-5.26 **	-4.74 ***
lmo1369	similar to phosphotransbutyrylase	-3.36 **	-
lmo1371	similar to branched-chain alpha-keto acid dehydrogenase E3 subunit	-2.05 ***	-2.09 ***
lmo1372	similar to branched-chain alpha-keto acid dehydrogenase E1 subunit (2-oxoisovalerate dehydrogenase alpha subunit)	-2.33 ***	-
lmo1373	similar to branched-chain alpha-keto acid dehydrogenase E1 subunit (2-oxoisovalerate dehydrogenase beta subunit)	-2.29 ***	-
lmo1380	unknown	-	-2.44 ***
lmo1388 (*tcsA*)	CD4+ T cell-stimulating antigen, lipoprotein	-	-2.70 **
lmo1389	similar to sugar ABC transporter, ATP-binding protein	-2.02 **	-
lmo1391	similar to sugar ABC transporter, permease protein	-2.07 **	-
lmo1399	similar to unknown protein	-	-2.13 ***
lmo1406 (*pflB*)	pyruvate formate-lyase	-2.48 ***	-

lmo1413	putative peptidoglycan bound protein (LPXTG motif)	-2.02 **	-
lmo1423	unknown	-	-2.35 ***
lmo1424	similar to manganese transport proteins NRAMP	-2.26 ***	-5.39 ***
lmo1460	similar to *B. subtilis* RecO protein involved in DNA repair and homologous recombination	-2.55 ***	-3.41 ***
lmo1463	similar to cytidine deaminase	-	-2.57 ***
lmo1464	similar to diacylglycerol kinase	-	-2.35 ***
lmo1465	similar to unknown proteins	-	-2.19 ***
lmo1466	similar to unknown proteins	-	-2.33 ***
lmo1522	similar to unknown proteins	-	-2.82 ***
lmo1535	similar to unknown proteins	-	-2.28 **
lmo1538	similar to glycerol kinase	-3.62 **	-
lmo1539	similar to glycerol uptake facilitator	-4.65 **	-
lmo1570 (*pykA*)	highly similar to pyruvate kinases	-	-3.26 ***
lmo1589 (*argB*)	highly similar to N-acetylglutamate 5-phosphotransferase	-2.72 *	-
lmo1590 (*argJ*)	highly similar to ornithine acetyltransferase and amino-acid acetyltransferases	-3.31 *	-
lmo1591 (*argC*)	similar to N-acetylglutamate gamma-semialdehyde dehydrogenases	-9.92 **	-
lmo1604	similar to 2-cys peroxiredoxin	-	-2.31 ***
lmo1605 (*murC*)	similar to UDP-N-acetyl muramate-	-2.22 ***	-
lmo1606	similar to DNA translocase	-2.47 ***	-
lmo1608	similar to unknown proteins	-	-2.18 ***
lmo1614	similar to unknown proteins	-2.13 ***	-
lmo1626	unknown	-2.29 ***	-
lmo1627 (*trpA*)	highly similar to tryptophan synthase (alpha subunit)	-3.699 ***	-
lmo1628 (*trpB*)	highly similar to tryptophan synthase (beta subunit)	-3.283 ***	-
lmo1630 (*trpC*)	highly similar to indol-3-glycerol phosphate synthases	-2.665 **	-
lmo1631 (*trpD*)	highly similar to anthranilate phosphoribosyltransferase	-3.16 ***	-

lmo1632 (*trpG*)	highly similar to anthranilate synthase beta subunit	-4.314 ***	-
lmo1634	similar to Alcohol-acetaldehyde dehydrogenase	-24.49 ***	-
lmo1657 (*tsf*)	translation elongation factor	-	-2.07 ***
lmo1658 (*rpsB*)	30S ribosomal protein S2	-	-2.07 ***
lmo1683	similar to transcription regulators (Fur family), PerR in B. subtilis	-	-2.65 ***
lmo1687	similar to hypothetical proteins	-	-6.32 ***
lmo1703	similar to similar to RNA methyltransferases	-	-3.14 ***
lmo1727	similar to transcription regulators (LacI family)	-2.94 **	-
lmo1728	some similarities to cellobiose-phosphorylase	-2.41 *	-
lmo1730	similar to sugar ABC transporter binding protein	-3.37 *	-2.16 **
lmo1731	similar to sugar ABC transporter,	-2.21 *	-
lmo1768 (*purF*)	glutamine phosphoribosylpyrophosphate amidotransferase	-2.07 *	-
lmo1793	similar to putative 16S rRNA processing protein RimM	-	-2.35 ***
lmo1825	similar to pantothenate metabolism flavoprotein homolog	-	-2.09 ***
lmo1848	similar metal cations ABC transporter (permease protein)	-	-2.48 ***
lmo1849	similar to metal cations ABC transporter, ATP-binding proteins	-	-2.42 ***
lmo1862	similar to hypothetical proteins	-	-2.47 ***
lmo1863	similar to hypothetical proteins	-	-2.88 ***
lmo1865	similar to conserved hypothetical proteins	-	-8.51 ***
lmo1866	similar to conserved hypothetical proteins	-	-3.82 ***
lmo1867	similar to pyruvate phosphate dikinase	-	-10.04 ***
lmo1879 (*cspD*)	similar to cold shock protein	-8.15 **	-
lmo1883	similar to chitinases	-9.03 **	-5.16 ***
lmo1893	unknown	-	-2.21 ***
lmo1917 (*pflA*)	similar to pyruvate formate-lyase	-2.78 *	-
lmo1941	similar to unknown proteins	-	-2.48 ***

lmo1956 (*fur*)	similar to transcriptional regulator (Fur family)	-	-3.01 ***
lmo1975	similar to E. coli DNA-damage-inducible protein dinP	-2.88 ***	-
lmo1994	similar to transcription regulators (LacI family)	-2.39 ***	-
lmo1995 (*dra*)	similar to deoxyribose-phosphate aldolase	-2.68 ***	-
lmo1997	similar to PTS mannose-specific enzyme IIA component	-12.64 **	-
lmo1998	similar to opine catabolism protein	-12.57 **	-
lmo1999	weakly similar to glucosamine-	-7.81 **	-
lmo2000	similar to PTS mannose-specific enzyme IID component	-36.08 **	-
lmo2001	similar to PTS mannose-specific enzyme IIC component	-16.00 **	-
lmo2002	similar to PTS mannose-specific enzyme IIB component	-3.76 **	-
lmo2003	similar to transcription regulator GntR family	-12.52 **	-
lmo2004	similar to transcription regulator GntR family	-7.72 **	-
lmo2016 (*cspB*)	similar to major cold-shock protein	-2.28 **	-15.39 ***
lmo2039 (*pbpB*)	similar to penicillin-binding protein	-2.03 ***	-
lmo2046	weakly similar to ketopantoate reductase involved in thiamin biosynthesis	-	-2.06 ***
lmo2063	unknown	-2.57 ***	-
lmo2068 (*groEL*)	class I heat-shock protein (chaperonin) GroEL	-	-2.02 ***
lmo2083	unknown	-2.08 **	-
lmo2090 (*argG*)	similar to argininosuccinate synthase	-4.80 *	-
lmo2091 (*argH*)	similar to argininosuccinate lyase	-2.04 **	-
lmo2099	similar to transcription antiterminator	-2.51 *	-
lmo2105	similar to ferrous iron transport protein B	-2.36 *	-
lmo2119	similar to unknown proteins	-	-2.72 ***
lmo2125	similar to maltose/maltodextrin ABC-transporter (binding protein)	-2.23 *	-
lmo2132	unknown	-	-2.26 ***
lmo2159	similar to oxidoreductase	-9.05 **	-

lmo2160	similar to unknown proteins	-8.43 **	-
lmo2161	unknown	-7.33 *	-
lmo2162	similar to unknown proteins	-5.39 **	-
lmo2163	similar to oxidoreductase	-6.22 *	-
lmo2173	similar to sigma-54-dependent transcriptional activator	-3.93 ***	-
lmo2175	similar to dehydrogenase	-6.32 **	-
lmo2213	similar to unknown protein	-2.03 *	-
lmo2225 (*citG*)	similar to fumarate hydratase	-	-2.09 ***
lmo2238	similar to transport system permease protein	-3.16 **	-
lmo2251	similar to amino acid ABC transporter (ATP-binding protein)	-2.76 *	-
lmo2332 (*int*)	putative integrase [Bacteriophage A118]	-2.08 ***	-
lmo2335 (*fruA*)	highly similar to phosphotransferase system (PTS) fructose-specific enzyme IIABC component	-	-2.78 ***
lmo2340	similar to *Erwinia chrysanthemi* IndA protein	-2.35 **	-2.94 ***
lmo2362	similar to amino acid antiporter (acid resistance)	-7.78 **	-8.17 ***
lmo2363	similar to glutamate decarboxylase	-5.95 ***	-7.59 ***
lmo2385	similar to B. subtilis YuxO protein	-	-2.00 **
lmo2391	conserved hypothetical protein similar to B. subtilis YhfK protein	-	-2.12 ***
lmo2406	similar to *B. subtilis* YunF protein	-	-2.72 ***
lmo2410	unknown	-2.42 ***	-
lmo2451	similar to preprotein translocase subunit SecG	-	-2.78 ***
lmo2452	similar to carboxylesterase	-	-2.90 ***
lmo2453	similar to lipolytic enzyme	-	-4.28 ***
lmo2484	similar to *B. subtilis* YvlD protein	-	-2.26 **
lmo2539 (*glyA*)	highly similar to glycine hydroxymethyltransferase	-	-2.11 ***
lmo2540	similar to phosphatases	-	-2.17 ***
lmo2579	conserved hypothetical protein	-2.46 ***	-

lmo2584	similar to formate dehydrogenase associated protein	-2.83 *	-
lmo2585	similar to *B. subtilis* YrhD protein	-4.45 *	-
lmo2586	similar to formate dehydrogenase alpha chain	-2.05 **	-
lmo2596 (*rpsI*)	ribosomal protein S9	-	-2.11 ***
lmo2646	unknown	-71.90 ***	-
lmo2647	similar to creatinine amidohydrolase	-14.48 **	-
lmo2648	similar to Phosphotriesterase	-21.38 **	-
lmo2649	similar to hypothetical PTS enzyme	-23.67 **	-
lmo2650	similar to hypothetical PTS enzyme	-51.77 *	-
lmo2651	similar to mannitol-specific PTS enzyme IIA component	-28.46 **	-
lmo2652	similar to transcriptional	-3.39 ***	-
lmo2666	similar to PTS system galactitol-specific enzyme IIB component	-2.03 *	-
lmo2667	similar to PTS system galactitol-specific enzyme IIA component	-2.02 *	-
lmo2675	unknown	-4.51 ***	-2.18 ***
lmo2676	similar to UV-damage repair protein	-3.79 ***	-2.45 ***
lmo2695	similar to dihydroxyacetone kinase	-3.20 *	-
lmo2696	similar to hypothetical	-3.85 *	-
lmo2697	unknown	-2.83 *	-
lmo2730	similar to phosphatase	-2.02 ***	-
lmo2731	similar to transcription regulator (RpiR family)	-2.39 ***	-
lmo2771	similar to beta-glucosidase	-2.42 **	-
lmo2772	similar to beta-glucoside-specific	-2.14 *	-
lmo2773	similar to transcription antiterminator	-2.77 ***	-
lmo2785 (*kat*)	catalase	-	-3.72 ***
lmo2788 (*bvrA*)	transcription antiterminator	-2.10 ***	-
lmo2795	similar to *E. coli* RpiR transcription regulator	-3.38 **	-
lmo2796	similar to transcription regulator	-2.46 *	-

lmo2797	similar to phosphotransferase system mannitol-specific enzyme IIA	-2.06 *	-
lmo2799	similar to phosphotransferase system mannitol-specific enzyme IIBC	-10.46 *	-
lmo2800	similar to dehydrogenase	-7.41 *	-
lmo2809	hypothetical secreted protein	-2.71 *	-
lmo2820	amino-terminal domain similar to transcription regulators	-2.04 ***	-
lmo2828	unknown	-2.24 ***	-2.87 ***
lmo2851	similar to AraC-type regulatory	-	-2.63 ***

[a]Genes were considered to be up-regulated at 7°C if the fold change compared to 37°C was ≥ 2.0 with an adjusted p-value ≤ 0.05

[b]Gene names are from ListiList (http://genolist.pasteur.fr/ListiList). Predicted operons are boxed. Operon predictions are from ListiList and Toledo-Arana et. al. 2009.

[c]Gene functions are based on annotation provided by ListiList.

[c]Superscripts are adjusted p values: "***" (< 0.001), "**" (< 0.01), "*" (≤ 0.05), "NS" (> 0.05)

Table A2 [S3.3]. *Listeria monocytogenes* gene biological function categories[a] showing positive or negative enrichment[b] at 7°C compared to 37°C

Functional Category	Gene Set Enrichment Analysis q-values	
	Log phase	**Stationary phase**
AMINO ACID BIOSYNTHESIS AROMATIC AMINO ACID FAMILY	(-)***	-
AMINO ACID BIOSYNTHESIS: GLUTAMATE FAMILY	(-)*	-
CELLULAR PROCESSES CHEMOTAXIS AND MOTILITY	(+)**	-
ENERGY METABOLISM AMINO ACIDS AND AMINES	(-)*	-
ENERGY METABOLISM ATP-PROTON MOTIVE FORCE INTERCONVERSION	-	(+)**
ENERGY METABOLISM BIOSYNTHESIS AND DEGRADATION OF POLYSACCHARIDES	(-)*	-
ENERGY METABOLISM FERMENTATION	(-)*	-
ENERGY METABOLISM PENTOSE PYRUVATE DEHYDROGENASE	(-)***	-
ENERGY METABOLISM SUGARS	(-)**	-
PROTEIN SYNTHESIS: TRNA AMINOACYLATION	(-)*	-
TRANSPORT AND BINDING PROTEINS: CARBOHYDRATES, ORGANIC ALCOHOLS, AND ACIDS	(-)***	-

[a]categories based on biological function categories (The Institute for Genomic Research Comprehensive Microbial Resource: http://cmr.tigr.org)

[b]Reported changes are based on Gene Set Enrichment analysis. (+) indicates that genes comprising regulon were significantly enriched for genes with higher transcript levels at 7°C compared to 37°C in the same growth phase while (-) indicates significant enrichment for lower transcript levels at 7°C. ***$q < 0.001$, **$q < 0.01$, *$q < 0.05$

Table A2 [S3.4]. *Listeria monocytogenes* regulons showing positive or negative enrichment at 7°C compared to 37°C

Regulon	Growth Phase	
	Log	Stationary
CodY	(-)***	(+)**
CtsR	-	(-)*
HrcA	(-)**	-
σ^B	(-)**	-
σ^H	(+)*	(-)**
σ^L	(-)***	(-)*

[a]Reported changes are based on Gene Set Enrichment analysis. (+) indicates that genes comprising regulon were significantly enriched for genes with higher transcript levels at 7°C compared to 37°C in the same growth phase while (-) indicates significant enrichment for lower transcript levels at 7°C. ***$q < 0.001$, **$q < 0.01$, *$q < 0.05$

Table A2 [S3.5] Genes differentially transcribed after 5 or 15 min. acid treatment[a] in *L. monocytogenes* grown to log phase at 37 °C.

Name[b]	Description of encoded protein[c]	Fold-change[d] 5 min	15 min
lmo0047	unknown	1.51 *	1.63 **
lmo0111	unknown	-1.93 ***	-2.32 ***
lmo0130	similar to 5-nucleotidase, putative peptidoglycan bound protein (LPXTG motif)	1.63 **	1.65 **
lmo0135	similar to oligopeptide ABC transport system substrate-binding proteins	2.36 **	2.27 **
lmo0136	similar to oligopeptide ABC transporter, permease protein	2.27 **	2.10 **
lmo0137	similar to oligopeptide ABC transporter, permease protein	1.64 **	1.54 *
lmo0152	similar to oligopeptide ABC transporter-binding protein	2.15 **	2.36 **
lmo0180	similar to sugar ABC transporter, permease protein	1.71 *	1.80 *
lmo0181	similar to sugar ABC transporter, sugar-binding protein	1.75 *	1.87 **
lmo0200 (*prfA*)	listeriolysin positive regulatory protein	1.72 *	1.80 **
lmo0217	similar to *B. subtilis* DivIC protein	-1.56 **	-1.96 ***
lmo0230	similar to *B. subtilis* YacH protein	1.53 *	1.56 *
lmo0231	similar to arginine kinase	1.43 NS	1.60 **
lmo0232 (*clpC*)	endopeptidase Clp ATP-binding chain C	1.59 **	1.67 **
lmo0238 (*cysE*)	similar to serine O-acetyltransferase	-1.52 *	-1.65 **
lmo0269	similar to transporter	-1.70 NS	-2.13 **
lmo0278	similar to sugar ABC transporter, ATP-binding protein	1.73 NS	1.79 *
lmo0279	highly similar to anaerobic ribonucleoside-triphosphate reductase	-1.63 *	-1.64 *
lmo0304	unknown	-1.53 *	-1.61 **
lmo0307	unknown	-2.36 **	-2.04 *
lmo0354	similar to fatty-acid--CoA ligase	-2.15 **	-2.19 **
lmo0372	similar to beta-glucosidase	1.08 NS	1.59 *

lmo0412	unknown	-1.98 ^{NS}	-2.51 *
lmo0449	unknown	-1.85 ***	-2.01 ***
lmo0455	similar to unknown proteins	-1.54 *	-1.64 **
lmo0490	similar to shikimate 5-dehydrogenase	-2.67 **	-2.96 **
lmo0496	similar to *B. subtilis* YnzC protein	1.49 ^{NS}	1.52 **
lmo0533	similar to unknown proteins	-1.57 ***	-1.71 ***
lmo0573	conserved hypothetical protein	1.73 *	1.97 **
lmo0604	similar to *B. subtilis* YvlA protein	1.54 *	1.61 *
lmo0618	similar to protein kinase	-1.51 **	-1.56 **
lmo0676	similar to flagellar biosynthesic protein FliP	-1.92 ***	-2.49 ***
lmo0680	similar to flagella-associated protein flhA	-1.59 **	-1.87 ***
lmo0681	similar to flagellar biosynthesis protein FlhF	-1.54 *	-1.84 **
lmo0683	similar to chemotactic methyltransferase CheR	-2.16 **	-2.68 **
lmo0721	putative fibronectin-binding protein	-1.56 **	-1.54 **
lmo0773	similar to alcohol dehydrogenase	-1.79 ***	-1.81 ***
lmo0800	similar to *B. subtilis* YqkB protein	1.41 ^{NS}	1.52 *
lmo0806	similar to transcription regulator	1.84 ^{NS}	2.03 *
lmo0808	similar to spermidine/putrescine ABC transporter, permease protein	1.87 *	2.08 *
lmo0809	similar to spermidine/putrescine ABC transporter, permease protein	1.90 **	2.07 **
lmo0810	similar to spermidine/putrescine-	2.27 **	2.25 ***
lmo0811	similar to carbonic anhydrase	2.55 **	2.78 **
lmo0814	similar to oxidoreductases	-1.97 **	-1.78 *
lmo0847	similar to Glutamine ABC transporter (binding and transport protein)	2.27 **	2.29 **
lmo0848	similar to amino acid ABC transporter, ATP-binding protein	1.82 **	1.91 **
lmo0883	similar to B. subtilis YbtB protein	-2.22 ***	-2.81 ***
lmo0903	conserved hypothetical protein	2.61 **	2.65 **
lmo0912	similar to transporters (formate)	-3.98 **	-4.14 ***

lmo0952	unknown	-1.56 ***	-1.75 ***
lmo0960	similar to proteases	-2.37 ***	-2.10 **
lmo0997 (*clpE*)	ATP-dependent protease	2.01 **	1.83 *
lmo0998	similar to hypothetical protein	-1.48 **	-1.89 ***
lmo1066	similar to extragenic suppressor protein SuhB and to myo-inositol-1(or 4)-monophosphatase	-1.64 **	-1.69 **
lmo1089 (*tagD*)	highly similar to glycerol-3-phosphate cytidylyltransferase (gct), CDP-glycerol pyrophosphorylase (teichoic acid biosynthesis protein D)	-1.86 ***	-2.03 ***
lmo1090	similar to glycosyltransferases	-1.51 **	-1.64 ***
lmo1120	unknown	-1.61 NS	-1.90 *
lmo1138	similar to ATP-dependent Clp protease proteolytic component	1.63 **	1.61 **
lmo1257	unknown	-5.50 *	-4.50 *
lmo1266	unknown	-2.04 *	-2.17 *
lmo1294 (*miaA*)	similar to tRNA isopentenylpyrophosphate transferase	-1.32 NS	-1.67 ***
lmo1360 (*folD*)	highly similar to methylenetetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase	-1.81 ***	-2.02 ***
lmo1369	similar to phosphotransbutyrylase	-1.80 **	-1.67 **
lmo1406 (*pflB*)	pyruvate formate-lyase	-4.03 *	-4.30 *
lmo1407 (*pflC*)	pyruvate-formate lyase activating enzyme	-2.86 *	-2.72 *
lmo1441	similar to putative peptidoglycan acetylation protein	-1.50 NS	-1.86 **
lmo1474 (*grpE*)	heat shock protein GrpE	1.84 **	1.71 **
lmo1475 (*hrcA*)	transcription repressor of class I heat-shock gene HrcA	1.83 **	1.76 **
lmo1500	similar to unknown proteins	-1.37 NS	-1.70 ***
lmo1544 (*minD*)	highly similar to cell division inhibitor (septum placement) protein MinD	-1.41 NS	-1.52 ***

lmo1556 (*hemC*)	highly similar to porphobilinogen deaminases (hydroxymethylbilane synthase)	-1.44 NS	-1.59 *
lmo1557 (*hemA*)	highly similar to glutamyl-tRNA reductase	-1.72 ***	-1.87 ***
lmo1589 (*argB*)	highly similar to N-acetylglutamate 5-phosphotransferase	-3.41 NS	-4.37 *
lmo1590 (*argJ*)	highly similar to ornithine acetyltransferase and amino-acid acetyltransferases	-6.76 *	-4.84 *
lmo1591 (*argC*)	similar to N-acetylglutamate gamma-semialdehyde dehydrogenases	-25.44 *	-11.04 *
lmo1597	unknown	-2.69 ***	-3.04 ***
lmo1625	similar to putative transporters	-1.45 NS	-1.53 ***
lmo1634	similar to Alcohol-acetaldehyde dehydrogenase	-6.66 *	-6.24 *
lmo1639	similar to dna-3-methyladenine glycosidase	-1.54 **	-1.71 **
lmo1696	similar to unknown proteins	-1.44 NS	-1.50 **
lmo1710	similar to putative flavodoxin	-1.42 NS	-1.60 ***
lmo1744	similar to unknown proteins	-1.81 **	-2.18 ***
lmo1749	similar to shikimate kinase	-2.46 ***	-2.88 ***
lmo1750	similar to unknown protein	-1.66 *	-1.61 *
lmo1752	unknown	1.42 NS	1.55 **
lmo1803	similar to FtsY of E. coli and SRP receptor alpha-subunit	1.67 **	1.70 **
lmo1828	similar to conserved hypothetical protein	-1.50 **	-1.91 ***
lmo1833 (*pyrD*)	highly similar to dihydroorotase	1.42 NS	1.57 **
lmo1835 (*pyrAB*)	highly similar to carbamoyl-phosphate synthetase (catalytic subunit)	1.83 *	1.91 **
lmo1840 (*pyrR*)	highly similar to pyrimidine operon regulatory protein	1.46 NS	1.57 *
lmo1856 (*deoD*)	purine nucleoside phosphorylase	-1.35 NS	-1.59 ***
lmo1857	similar to hypoyhetical protein	-1.43 NS	-1.70 ***
lmo1867	similar to pyruvate phosphate dikinase	1.45 NS	1.53 **

lmo1884	similar to xanthine permeases	1.54 *	1.53 *
lmo1917 (*pflA*)	similar to pyruvate formate-lyase	-4.54 *	-3.47 NS
lmo1926	similar to chorismate mutase	-1.93 **	-2.52 ***
lmo1943	similar to unknown proteins	-1.52 **	-1.57 **
lmo1965	similar to unknown proteins	-1.89 NS	-2.52 *
lmo1977	similar to unknown proteins	-1.45 NS	-1.78 ***
lmo1992	similar to alpha-acetolactate decarboxylase	1.68 ***	1.65 ***
lmo2006 (*alsS*)	similar to alpha-acetolactate synthase protein, AlsS	1.46 NS	1.52 **
lmo2040 (*ftsL*)	similar to cell-division protein FtsL	-1.40 NS	-1.51 ***
lmo2041	similar to unknown proteins	-1.49 NS	-1.68 ***
lmo2057 (*ctaB*)	highly similar to heme A farnesyltransferase	1.60 **	1.61 ***
lmo2063	unknown	-1.78 ***	-1.83 ***
lmo2090 (*argG*)	similar to argininosuccinate synthase	-8.74 *	-10.59 *
lmo2100	similar to transcriptional regulator (GntR family) and to aminotransferase (MocR-like)	1.61 *	-2.79 **
lmo2114	similar to ABC transporter (ATP-binding protein)	2.28 **	2.25 **
lmo2115	similar to ABC transporter (permease)	2.23 **	2.16 **
lmo2152	similar to thioredoxin	1.52 **	1.49 **
lmo2156	unknown	1.94 ***	1.89 ***
lmo2173	similar to sigma-54-dependent transcriptional activator	-3.11 *	-3.23 *
lmo2184	similar to ferrichrome ABC transporter (binding protein)	1.46 NS	1.50 **
lmo2190 (*mecA*)	competence negative regulator mecA	1.39 NS	1.52 **
lmo2200	similar to transcription regulator	1.51 *	1.49 *
lmo2238	similar to transport system permease protein	-3.46 *	-3.75 **
lmo2241	similar to transcriptional regulators (GntR family)	-1.42 NS	-1.60 **
lmo2250 (*arpJ*)	similar to amino acid ABC transporter, permease protein	-3.89 *	-3.53 *
lmo2258	unknown	1.56 *	1.69 **
lmo2260	similar to unknown proteins	1.87 *	1.78 *

lmo2261	similar to unknown proteins	1.63 *	1.42 NS
lmo2293	Protein gp10 [Bacteriophage A118]	-2.34 *	-1.80 NS
lmo2295	Protein gp8 [Bacteriophage A118]	-2.88 *	-2.39 *
lmo2296	similar to coat protein [Bacteriophage SPP1]	-2.32 *	-1.23 NS
lmo2352	similar to LysR family transcription regulator	1.50 NS	1.51 *
lmo2362	similar to amino acid antiporter (acid	-2.97 **	-2.80 **
lmo2363	similar to glutamate decarboxylase	-2.59 *	-2.28 *
lmo2380	similar to proteins involved in resistance to cholate and to NA(+) and in pH homeostasis	1.45 NS	1.51 *
lmo2393	similar to B. subtilis YuzD protein	1.71 **	1.68 **
lmo2408	similar to repressor protein	-1.61 ***	-1.94 ***
lmo2409	unknown	-3.28 **	-4.21 ***
lmo2416	unknown	-1.78 **	-1.68 **
lmo2420	unknown	-1.73 ***	-2.00 ***
lmo2433	similar to acetylesterase	1.98 *	2.10 **
lmo2439	unknown	1.63 **	1.61 **
lmo2468 (*clpP*)	ATP-dependent Clp protease	1.58 ***	1.64 ***
lmo2484	similar to B. subtilis YvlD protein	1.80 NS	2.11 *
lmo2487	similar to B. subtilis YvlB protein	1.56 *	1.74 **
lmo2569	similar to dipeptide ABC transporter (dipeptide-binding protein)	1.74 **	1.78 **
lmo2587	conserved hypothetical proteins	-1.43 NS	-1.61 **
lmo2591	surface protein (GW repeat) similar to N-acetylmuramidase	1.58 **	1.53 **
lmo2642	unknown	-1.89 *	-2.03 **
lmo2690	similar to transcription regulator, TetR family	1.47 NS	1.54 **
lmo2718 (*cydA*)	highly similar to cytochrome D ubiquinol oxidase subunit I	-1.59 *	-1.47 NS
lmo2720	similar to acetate-CoA ligase	-2.07 **	-1.79 **
lmo2769	similar to ABC transporter, ATP-	-2.55 **	-3.28 ***
lmo2827	similar to transcriptional regulator	1.50 *	1.39 NS

lmo2829	similar to yeast protein Frm2p involved in fatty acid signaling	1.51 ^{NS}	1.60 *
lmo2851	similar to AraC-type regulatory protein	1.73 *	1.43 ^{NS}

[a]Acid treatment was BHI-MOPS adjusted to pH 3.5 with HCl followed by incubation at 37°C

[b]Gene names are from ListiList (http://genolist.pasteur.fr/ListiList). Predicted operons are boxed. Operon predictions are from ListiList and Toledo Arana et. al. 2009.

[c]gene functions were based on annotation provided by ListiList

[d]Superscripts are adjusted p values: "***" (< 0.001), "**" (< 0.01), "*" (≤ 0.05), "NS" (> 0.05)

Table A2 [S3.6]. Genes differentially transcribed after 5 or 15 min. acid treatment[a] in *L. monocytogenes* grown to stationary phase at 37 °C

Name[b]	Gene Function[c]	Fold change[d]	
		5 min	15 min
lmo0104	unknown	-1.46 NS	-1.64 *
lmo0113	similar to protein gp35 from Bacteriophage A118	-1.56 NS	-1.81 *
lmo0114	similar to putative repressor C1 from lactococcal bacteriophage Tuc2009	-1.38 NS	-1.57 *
lmo0189	highly similar to B. subtilis Veg protein	1.45 NS	1.66 **
lmo0217	similar to B. subtilis DivIC protein	-2.37 *	-3.03 ***
lmo0321	similar to unknown proteins	-1.54 NS	-2.13 **
lmo0351	similar to unknown proteins	-1.60 *	-1.85 **
lmo0523	similar to B. subtilis YybC protein	1.02 NS	6.34 *
lmo0524	similar to putative sulfate transporter	-1.76 NS	-1.72 *
lmo0578	putative conserved membrane protein	-1.94 NS	-2.15 **
lmo0648	similar to membrane proteins	-1.43 NS	-1.83 **
lmo0770	similar to transcriptional regulator (LacI family)	-1.62 NS	-1.68 *
lmo0904	unknown	-1.29 NS	-1.67 *
lmo0998	similar to hypothetical protein	-1.63 *	-1.60 *
lmo1041	similar to molybdate ABC transporter binding protein	-1.36 NS	-1.66 *
lmo1046	molybdenum cofactor biosynthesis protein C	-1.41 NS	-1.58 *
lmo1049	similar to molybdopterin biosynthesis protein MoeB	-1.44 NS	-1.75 **
lmo1166	similar to NADPH-dependent butanol dehydrogenase	-1.59 NS	-1.84 **
lmo1219	unknown	-1.53 NS	-1.66 *
lmo1227	similar to uracil-DNA glycosylase	-1.42 NS	-1.55 **
lmo1261	unknown	-2.89 *	-4.46 ***
lmo1348	similar to aminomethyltransferase	-2.15 NS	-2.62 *
lmo1397 (*cinA*)	similar to competence-damage inducible protein CinA	-1.37 NS	-1.54 *
lmo1411	unknown	-1.39 NS	-1.66 *
lmo1453	conserved hypothetical protein	-1.86 NS	-2.77 **
lmo1541	similar to unknown protein	1.60 *	1.57 **
lmo1542 (*rplU*)	ribosomal protein L21	1.51 *	1.69 ***

lmo1597	unknown	-1.51 NS	-1.75 *
lmo1604	2-cys peroxiredoxin	-1.55 NS	-1.51 *
lmo1605 (*murC*)	UDP-N-acetyl muramate-alanine ligases	-1.44 NS	-1.63 *
lmo1614	similar to unknown proteins	1.12 NS	3.99 *
lmo1639	similar to dna-3-methyladenine glycosidase	-2.06 *	-1.83 *
lmo1828	similar to conserved hypothetical protein	-2.42 ***	-2.07 **
lmo1856 (*deoD*)	purine nucleoside phosphorylase	-1.59 *	-1.65 **
lmo1857	similar to hypoyhetical protein	-1.45 NS	-1.57 **
lmo1929 (*ndk*)	similar to nucleoside diphosphate kinase	-1.45 NS	-1.73 ***
lmo1932	heptaprenyl diphosphate synthase component I	-1.36 NS	-1.51 *
lmo1939 (*cmk*)	similar to cytidylate kinase	-1.38 NS	-1.62 *
lmo1965	similar to unknown proteins	-1.66 NS	-1.90 *
lmo2020 (*divIVA*)	similar to cell-division initiation protein (septum placement)	-1.61 NS	-1.93 **
lmo2113	similar to unknown proteins	-1.34 NS	-1.58 **
lmo2129	unknown	-1.57 NS	-1.94 *
lmo2139	similar to ABC transporter (ATP-binding	-1.45 NS	-1.54 **
lmo2176	similar to transcriptional regulator (tetR	-1.68 *	-1.91 **
lmo2207	similar to unknown protein	-1.47 NS	-1.68 **
lmo2210	unknown	-1.07 NS	2.73 *
lmo2248	similar to unknown proteins	-1.30 NS	-1.57 **
lmo2255	unknown	-1.30 NS	-1.63 *
lmo2293	Protein gp10 [Bacteriophage A118]	-1.02 NS	4.28 *
lmo2304	Bacteriophage A118 gp65 protein	1.13 NS	3.03 *
lmo2334	similar to transcriptional regulator	-1.62 NS	-1.84 *
lmo2362	similar to amino acid antiporter (acid resistance)	1.85 NS	1.85 *
lmo2378	similar to proteins involved in resistance to cholate and to NA(+) and in pH homeostasis	-1.35 NS	-1.56 *
lmo2448	conserved hypothetical protein	-1.46 NS	-1.84 ***
lmo2536 (*atpI*)	highly similar to ATP synthase subunit i	-1.16 NS	-1.56 ***
lmo2586	similar to formate dehydrogenase alpha chain	-1.57 NS	-1.79 *
lmo2625 (*rplP*)	ribosomal protein L16	1.46 NS	1.69 *
lmo2630 (*rplW*)	ribosomal protein L23	1.27 NS	1.50 *

lmo2632 (*rplC*)	ribosomal protein L3	1.33 [NS]	1.50 [*]
lmo2633 (*rpsJ*)	ribosomal protein S10	1.51 [NS]	1.64 [*]
lmo2658	similar to spermidine/spermine N1-acetyl transferase	-1.46 [NS]	-1.65 [**]
lmo2741	similar to drug-efflux transporters	-1.79 [NS]	-2.62 [**]
lmo2773	similar to transcription antiterminator	1.53 [*]	1.72 [***]

[a]Acid treatment was BHI-MOPS adjusted to pH 3.5 with HCl followed by incubation at 37°C

[b]Gene names are from ListiList (http://genolist.pasteur.fr/ListiList). Predicted operons are boxed. Operon predictions are from ListiList and Toledo-Arana et. al. 2009.

[c]gene functions were based on annotation provided by ListiList

[d]Superscripts are adjusted p values: "***" (< 0.001), "**" (< 0.01), "*" (< 0.05), "NS" (> 0.05)

Table A2 [S3.7]. Genes differentially transcribed after 5 or 15 min. acid treatment[a] in *L. monocytogenes* grown to log phase at 7 °C

Name	Description of encoded protein[b]	Fold change[c]	
		5 min.	15 min.
lmo0066	similar to toxin components	1.51 ***	-1.35 NS
lmo0071	unknown	-1.14 NS	-7.13 *
lmo0111	unknown	-1.57 ***	-1.62 ***
lmo0133	similar to *E. coli* YjdI protein	1.14 NS	1.50 *
lmo0299	similar to PTS beta-glucoside-specific enzyme IIB component	-1.08 NS	-2.24 *
lmo0306	unknown	-1.60 **	-1.55 *
lmo0320	similar to surface protein (peptidoglycan bound, LPXTG motif)	1.05 NS	3.64 *
lmo0326	similar to transcriptional regulators	1.12 NS	2.87 **
lmo0418	unknown	2.63 ***	2.14 ***
lmo0545	unknown	-1.00 NS	1.66 NS
lmo0669	similar to oxidoreductase	1.35 ***	1.55 ***
lmo0735	similar to Ribulose-5-Phosphate 3-Epimerase	-1.11 NS	-3.57 **
lmo0875	similar to PTS system, beta-glucoside enzyme IIB component	1.61 ***	1.29 NS
lmo0879	unknown	1.09 NS	3.03 NS
lmo0883	similar to *B. subtilis* YbtB protein	-1.41 **	-1.72 ***
lmo0952	unknown	-1.56 ***	-1.61 **
lmo1114	highly similar to TN916 ORF23	1.09 NS	-2.02 *
lmo1151	similar to Salmonella typhimurium PduA protein	1.57 **	-1.46 NS
lmo1266	unknown	-1.66 ***	-1.80 ***
lmo1639	similar to dna-3-methyladenine glycosidase	-1.49 ***	-1.55 ***
lmo1749	similar to shikimate kinase	-1.67 ***	-1.93 ***
lmo1883	similar to chitinases	1.52 *	-1.22 NS
lmo1972	similar to pentitol PTS system enzyme II B component	1.36 NS	1.62 *
lmo2009	similar to putative transport system integral membrane protein	-1.17 NS	1.63 *
lmo2290	Portein gp13 [Bacteriophage A118]	1.53 ***	1.48 NS
lmo2291	major tail shaft protein [Bacteriophage A118]	1.70 ***	-1.46 NS
lmo2292	Portein gp11 [Bacteriophage A118]	1.65 ***	1.54 ***
lmo2300	putative terminase large subunit from Bacteriophage A118	1.01 NS	4.32 *
lmo2408	similar to repressor protein	-1.55 NS	-1.64 ***
lmo2409	unknown	-2.22 NS	-2.26 ***
lmo2836	similar to alcohol dehydrogenase	1.02 NS	2.00 **

[a]Acid treatment was BHI-MOPS adjusted to pH 3.5 with HCl followed by incubation at 37°C

[c]gene functions were based on annotation provided by ListiList (http://genolist.pasteur.fr/ListiList).

[c]Superscripts are adjusted p values: "***" (< 0.001), "**" (< 0.01), "*" (≤ 0.05), "NS" (> 0.05)

Table A2 [S3.8]. Genes differentially transcribed after 5 or 15 min. acid treatment[a] in *L. monocytogenes* grown to stationary phase at 7 °C

Name[b]	Gene Function[c]	Fold-change[d]	
		5 min	15 min
lmo0049	unknown	-1.49 NS	-1.56 *
lmo0115 (*lmaD*)	similar to Antigen D	1.99 ***	2.13 ***
lmo0117 (*lmaB*)	antigen B	5.12 ***	5.32 ***
lmo0118 (*lmaA*)	antigen A	5.02 ***	5.05 ***
lmo0119	unknown	3.75 ***	4.04 ***
lmo0120	unknown	3.80 ***	4.09 ***
lmo0121	similar to bacteriophage minor tail proteins	4.56 **	3.88 **
lmo0122	similar to phage proteins	3.84 ***	3.73 ***
lmo0123	similar to protein gp18 from Bacteriophage A118	4.86 ***	4.51 ***
lmo0124	unknown	4.02 ***	4.12 ***
lmo0125	unknown	4.20 ***	4.12 ***
lmo0126	unknown	4.99 ***	5.10 ***
lmo0127	weakly similar to protein gp20 from Bacteriophage A118	5.21 ***	5.09 ***
lmo0128	similar to a protein from Bacteriophage phi-105 (ORF 45)	4.20 ***	4.89 ***
lmo0129	similar to autolysin: N-acetylmuramoyl-L-alanine amidase	3.47 ***	3.94 ***
lmo0217	similar to *B. subtilis* DivIC protein	-1.32 NS	-1.65 *
lmo0328	unknown	1.06 NS	-2.07 *
lmo0728	similar to riboflavin kinase / FAD synthase	-1.42 NS	-1.81 ***
lmo0883	similar to *B. subtilis* YbtB protein	-1.83 *	-1.49 NS
lmo0903	conserved hypothetical protein	2.35 ***	3.15 ***
lmo1049	similar to molybdopterin biosynthesis protein MoeB	-1.35 NS	-1.59 ***
lmo1631 (*trpD*)	highly similar to anthranilate phosphoribosyltransferase	-1.05 NS	3.09 **
lmo1828	similar to conserved hypothetical protein	-1.21 NS	-1.53 *
lmo1856 (*deoD*)	purine nucleoside phosphorylase	-1.31 NS	-1.63 *
lmo1926	similar to chorismate mutase	-1.45 NS	-1.61 *
lmo2191	similar to unknown proteins	-1.28 NS	-1.55 ***
lmo2210	unknown	-1.09 NS	-1.53 *

lmo2278 (*lysA*)	L-alanoyl-D-glutamate peptidase	1.41 ^{NS}	1.72 **
lmo2279	holin [Bacteriophage A118]	1.54 ^{NS}	1.74 *
lmo2282	protein gp21 [Bacteriophage A118]	1.35 ^{NS}	1.75 *
lmo2283	protein gp20 [Bacteriophage A118]	1.77 ^{NS}	2.07 **
lmo2286	Protein gp17 [Bacteriophage A118]	1.68 **	1.64 *
lmo2288	Protein gp15 [Bacteriophage A118]	2.83 ***	2.61 **
lmo2290	Portein gp13 [Bacteriophage A118]	3.22 **	3.14 **
lmo2291	major tail shaft protein [Bacteriophage A118]	4.05 ***	5.26 ***
lmo2292	Portein gp11 [Bacteriophage A118]	4.04 ***	3.42 ***
lmo2293	Protein gp10 [Bacteriophage A118]	2.89 ***	2.70 ***
lmo2295	Protein gp8 [Bacteriophage A118]	2.41 ***	2.35 ***
lmo2297	putative scaffolding protein [Bacteriophage A118]	3.23 ***	2.98 ***
lmo2299	putative portal protein [Bacteriophage A118]	1.43 ^{NS}	1.69 *
lmo2303	Protein gp66 [Bacteriophage A118]	2.08 **	2.02 ***
lmo2317	similar to protein gp49 [Bacteriophage A118]	1.40 ^{NS}	2.01 **
lmo2322	gp44 [Bacteriophage A118]	1.98 *	1.85 ^{NS}
lmo2326	similar to protein gp41 [Bacteriophage A118]	2.18 *	2.27 *
lmo2445	similar to internalin	1.05 ^{NS}	-2.75 *
lmo2536 (*atpI*)	highly similar to ATP synthase subunit i	-1.40 ^{NS}	-1.66 ***

[a]Acid treatment was BHI-MOPS adjusted to pH 3.5 with HCl followed by incubation at 37°C

[b]Gene names are from ListiList (http://genolist.pasteur.fr/ListiList). Predicted operons are boxed. Operon predictions are from ListiList and Toledo-Arana et. al. 2009.

[c]Gene functions were based on annotation provided by ListiList

[d]Superscripts are adjusted p values: "***" (< 0.001), "**" (< 0.01), "*" (≤ 0.05), "NS" (> 0.05)