Construction of *Listeria monocytogenes* Mutants with In-Frame Deletions in Putative ATP-Binding Cassette (ABC) Transporters and Analysis of Their Growth under Stress Conditions

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Abstract

Listeria monocytogenes is a food-borne pathogen that is difficult to eliminate since it can survive under different stress conditions such as low pH and high salt. Understanding its survival under stress conditions is important in controlling this pathogen in food. ABC transporters have been shown to be induced in *L. monocytogenes* subjected to high pressure and nisin treatments; therefore, we hypothesized that genes encoding the ABC transporters may be involved in general stress responses. To study the function of these genes, deletion mutants of ABC transporter genes (LMOF2365_1875, LMOF2365_1877) were created in *L. monocytogenes* F2365, and these deletion mutants were tested under different stress conditions. Compared to the wild type, Δ LMOF2365_1875 and Δ LMOF2365_1877 showed slower growth under nisin (250 μ g/ml) and acid (pH 5) treatments. Under salt treatment (5% NaCl in minimal medium), Δ LMOF2365_1877 showed slower growth whereas Δ LMOF2365_1875 had growth similar to the wild type. Moreover, Δ LMOF2365_1875 had an increased capacity to form biofilms compared to the wild type. Our results indicate that these deletion mutants may be more sensitive to multiple stress conditions compared to the wild type, suggesting that LMOF2365_1875 and LMOF2365_1877 may contribute to the general stress response in *L. monocytogenes*. An understanding of the growth of these mutants under multiple stress conditions and their ability to form biofilms may help in the development of intervention strategies to control *L. monocytogenes* in food and in the environment.

Keywords: ABC transporter; *Listeria monocytogenes*; Stress response

Introduction

Listeria monocytogenes is a food-borne pathogen responsible for human listeriosis, an invasive infection with high mortality rates. Listeriosis is responsible for an estimated 2500 serious illnesses and 500 deaths each year in the United States, accounting for 28% of annual deaths attributable to known food-borne pathogens, second only to deaths due to *Salmonella* infections [1]. The disease affects primarily pregnant women, newborns, the elderly and immune-compromised individuals, due to uncontrolled intracellular replication within host cells [2]. Transmission of *L. monocytogenes* is generally through eating contaminated food, in particular, dairy products made from unpasteurized milk and ready-to-eat meat and fish products [3]. In addition, *L. monocytogenes* has been found in a variety of raw foods such as uncooked meats and vegetables, as well as in processed foods that become contaminated after processing, such as soft cheeses and cold cuts at the deli counter [3,4]. Potential sources of *L. monocytogenes* contamination in foods include the incoming product, food handlers, consumers, and environmental sources, such as utensils and equipment, which may harbor pathogenic microorganisms or serve as vehicles of contamination if cleaning and sanitation procedures are poor [5]. *L. monocytogenes* is widely distributed in the environment, forms biofilms, and is relatively resistant to acid and high salt concentrations [6]. Unlike most food-borne pathogens, *L. monocytogenes* multiplies readily in refrigerated foods [7].

In response to changes in the natural environment, bacteria undergo a complex program of differential gene expression. A number of transcriptional regulators important for stress response gene expression have been identified in *L. monocytogenes* [8-10], and the pathogen has developed efficient strategies for survival under stress conditions, such

as starvation, wide variations in temperature, pH, and osmolarity [11]. The regulation of gene expression in response to environmental stress conditions is essential for bacterial survival [12]. Proteins induced by heat shock and other stress conditions include proteases and ATP-dependent chaperones. Energy dependent proteolysis plays an important role in the turnover of both abnormal proteins and naturally short-lived, specific regulatory proteins [13,14]. In the Gram-positive model organism, *B. subtilis*, expression of heat shock genes involves at least four different regulatory mechanisms [15,16]. Class I heat shock genes encode classical chaperones, such as GroES, GroEL, and DnaK, and they are controlled by the HrcA repressor. Class II genes encode general stress proteins whose transcription is dependent on the alternative σ^B [16]. Class III heat shock genes encode ClpP and two Hsp100 Clp ATPases, ClpC and ClpE, and these form part of the CtsR stress response regulon. These genes are negatively controlled by CtsR, the product of the first gene of the ClpC operon [15,17,18].

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Class IV includes stress response genes such as *ftsH*, *lonA*, *clpX*, and *htpG* whose expression is independent of *HrcA*, σ^B or *CtsR* and whose regulatory mechanisms remain to be identified [15,16].

In 2001, the genome sequence of the prototype *L. monocytogenes* serotype 1/2a strain EGDe [19] was published, which heralded the launch of the post-genomic era for *L. monocytogenes* research. Three years later, the complete sequence of the epidemic serotype 4b clone F2365 was deciphered [20]. More recently, a bank of 16 animal, food, and environmental *L. monocytogenes* isolates was sequenced by the BROAD Institute MIT. The resulting sequences should provide an invaluable source of information for identification of genetic determinants involved in stress responses and the environmental biology of the organism.

A typical ABC transporter consists of several subunits with the following structural domains: a nucleotide-binding domain, a transmembrane domain, or a solute-binding domain [21]. All ABC transporters are either exporters or importers. There are more than 30 copies of different ABC transporters present in the genome of *L. monocytogenes*. Some ABC transporters have been shown to be involved in biofilm formation, and in responses to acid and salt stress [22-25]. ABC transporters were also involved in resistance to antimicrobials such as nisin that acts on the cell envelope [26,27].

LMOF2365_1875 (ABC transporter, manganese-binding protein), LMOF2365_1876 (manganese ABC transporter; permease protein) and LMOF2365_1877 (manganese ABC transporter; ATP-binding protein) were highly induced in milk [28]; however, this operon was inhibited in ready-to-eat meats [29]. These gene complexes are involved in manganese transport. Manganese is involved in a number of cellular functions such as virulence and oxidative stress [30]. For example, deletion of the putative Mn (II) ABC transporter (*MntA*) in *B. anthracis* resulted in increased sensitivity to oxidative stress [31]. Since this ABC transporter operon was induced with a number of treatments such as high pressure and nisin [32], we hypothesized that this operon may be involved in general stress responses. Therefore, the in-frame deletion mutants, LMOF236_1875 and LMOF2365_1877, were constructed and mutant growth was tested under different stress treatments such as exposure to nisin, acid, and salt.

Materials and Methods

Bacteria and growth conditions

L. monocytogenes strain F2365 isolated from Mexican-style soft cheese that was implicated in an outbreak of listeriosis in California in 1985 [33] was used in this study since its genome is fully sequenced and annotated [20]. Glycerol stock cultures of *L. monocytogenes* F2365 and isogenic mutants of this parent strain (Table 1) stored at -80°C were streaked onto Brain Heart Infusion (BHI) (Sigma-Aldrich St. Louis, MO) agar plates and grown at 37°C prior to each experiment.

Construction of in-frame deletion mutants LMOF2365_1875 and LMOF2365_1877 in *L. monocytogenes* F2365

Gene deletion fragments were constructed by splice overlap extension (SOE) PCR [34] to generate two 400-bp fragments: one upstream including the ATG codon (AB product) and one downstream beginning from the stop codon (CD product). The sequences and positions of PCR primers are listed in table 1 and figure 1. The 5' end of the SOEB primers had about 20 bp complementary to the SOEC primers. The initial PCR products were diluted 1:20 in PCR-grade water, and 1 µl of each product was used as a template in a second round of PCR with the SOEA and SOED primers to generate an 800-bp product (PCR product AD). The 2nd round PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) and quantified using a Nanodrop ND100 UV-Vis spectrophotometer (Thermo Scientific, Inc., Wilmington, DE). The 2nd round PCR products and pKSV7 vector (a gift from S. Kathariou, North Carolina State University) were digested with KpnI and XbaI restriction enzymes, cloned into the pKSV7 vector and transformed into *E. coli* competent cells (C4040-03, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Transformants were selected on Luria-Bertani (LB) agar containing 100 µg/ml ampicillin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Calbiochem, Merck, Darmstadt, Germany). White colonies were screened by colony PCR, using primers A and D and then sequenced. The plasmids containing PCR product AD were electroporated into *L. monocytogenes* F2365 as described [35]. The transformants were serially passaged at 40°C in BHI with 10 µg/ml chloramphenicol to select for the cells in

Strains/plasmids/primers	Description	Source or reference
E. coli strains		
TOP10	competent cells	Invitrogen
DH5α	competent cells	Invitrogen
Plasmids		
pKSV7	Temperature-sensitive integration vector; Cm ^r	gift from S. Kathariou
L. monocytogenes strains		
F2365	Wild-type serotype 4b strain, genome sequenced	Nelson et al. [20]
ΔLMOF2365_1875	LMOF2365_1875 deletion	This study
ΔLMOF2365_1877	LMOF2365_1877 deletion	This study
Primers		
F2365_1875SOEA	5' GGGGTACC GCTTTCCCGCTTAGTGTTC3'	This study
F2365_1875SOEB	5' <u>GGGAGGTTATCGAATGAAATAAAAAAGACGCAGAGATTAAC</u> 3'	This study
F2365_1875SOEC	5'TTTCATTTCGATAACCTCCC3'	This study
F2365_1875SOED	5' GCTCTAGAG CGAAAAGTAGTACAGATTG3'	This study
F2365_1877SOEA	5' GGGGTACC ACTCCGCACCGCAAGCAC3'	This study
F2365_1877SOEB	5' <u>GTGGGAAAAGGGTGGTTTATGATGTAATAGATGTTGTTTTAGAAGG</u> 3'	This study
F2365_1877SOEC	5' CATCTAAACCACC CTTTTCCAC3'	This study
F2365_1877SOED	5' GCTCTAGAG AATTAGCTAAAACGCTTG3'	This study

Restriction sites (KpnI and XbaI) are highlighted in bold. Regions overlapping complementary to SOEC primers are underlined

Table 1: Strains, plasmids and primers used in this study.

which the plasmid had integrated into the chromosome by homologous recombination. Colonies obtained during subsequent passages at 30°C in BHI without chloramphenicol were screened for chloramphenicol sensitivity (colonies that did not grow on the BHI/ chloramphenicol plates) that indicates a second homologous recombination event with loss of the plasmids. Chloramphenicol sensitive colonies were screened by colony PCR using SOEA and SOED primers to identify isolates with the mutant alleles. The size of the fragment in the deletion mutant was about 800 bp while the sizes in the wild type were 800 bp plus the size of the deleted genes.

Growth assays for *L. monocytogenes* F2365 wild type and Δ LMOF2365_1875, Δ LMOF2365_1877 under different stress conditions

L. monocytogenes F2365 (wild type), and the Δ LMOF2365_1875 and Δ LMOF2365_1877 mutants were used for growth assays. To make a log-phase culture, one *L. monocytogenes* colony was inoculated into 5 mL BHI and grown at 37°C with agitation at 200 rpm overnight. A 50- μ l aliquot of overnight culture was added into 5 mL BHI and grown at 37°C with agitation at 180 rpm for 3 h until the OD₆₀₀ was 0.4. Growth assays were performed in a 96-well plate format using log-phase bacteria. The lid of the microtiter plate was pre-treated with 0.05% Triton X-100 in 20% ethanol to eliminate liquid condensation. After 15-30 s, the treatment solution was poured off and the cover was leaned against a vertical surface and allowed to air-dry [36]. For the nisin inhibition assay, nisin (containing 2.5% pure nisin, balance sodium chloride and denatured milk solids, activity of 1X10⁶ IU/g, according to the manufacturer) from *Lactococcus lactis* was purchased from Sigma-Aldrich (N5764). Nisin at a concentration of 250 μ g/ml in BHI was used for growth studies, and 0.02N HCl was used as a negative control. For the salt tolerance assays, log-phase cells were harvested by centrifugation at 5000 rpm at 4°C for 10 min and resuspended in 5 mL of Minimal Medium (MM) [37] with 0.5% glucose. MM with 5% NaCl and 0.5% glucose was used for the growth studies. BHI at pH 5 was used for the acid tolerance assays, and BHI at pH 7 was used as a control. The plate was placed into a Safire II spectrophotometer (Tecan) at 37°C, with OD readings at λ 600 nm taken every hour for 18 h.

Biofilm assays

Five milliliters of Mueller-Hinton broth overnight cultures of *L. monocytogenes* F2365 (wild type), Δ LMOF2365_1875, and Δ LMOF2365_1877 were initiated from plate grown isolates. The overnight cultures were incubated at 32°C with agitation (200 rpm). The next day, the overnight cultures were diluted 1:100 into fresh Mueller-Hinton broth. Flat bottom cell culture 96-well microtiter plates (Greiner Bio-one, Monroe, NC) were washed with 100% ethanol and allowed to air dry in a biological hood until all residual ethanol had evaporated. For each strain, 100 μ l of the freshly diluted culture were placed in 8 different wells. Additionally, 100 μ l sterile Mueller-Hinton broths were also placed in 8 additional wells as the negative control. The plates were incubated statically at 32°C for 48 h. The wells were then observed to determine if visible biofilms were present in the *L. monocytogenes* inoculated wells. The medium was then removed from the microtiter plate wells, and the individual wells were washed 5 times with 150 μ l of sterile distilled water. The plates were then allowed to air dry for 45 min, and then 150 μ l of 1% crystal violet solution were added to each of the wells. After 45 min, the stain was removed, and the wells were washed 5 times with 150 μ l sterile distilled water. The wells were then destained of the residual biofilm-bound crystal violet with

200 μ l of 95% ethanol. One hundred microliters of the destain mixture were then transferred to a new microtiter plate, and the OD 590 nm was measured for each well. The resulting data for three separate trials were subjected to an analysis of variance. The individual trials were considered as a block when performing the mean separations using the least significant difference technique at a $p < 0.05$ level [38].

Results and Discussion

Genetic organization of the ABC operon and *in silico* analysis of LMOF2365_1875, 1876, and 1877

The whole genome of *L. monocytogenes* F2365 has been sequenced [20]. DNA sequence analysis indicates that the composition of the ABC transporter operon is comprised of three genes: LMOF2365_1875 (encoding for ABC transporter, manganese-binding protein), LMOF2365_1876 (encoding for manganese ABC transporter, permease protein) and LMOF2365_1877 (encoding for manganese ABC transporter, ATP binding protein). This operon is transcribed from the opposite direction (3' to 5') in the genome. Blast searches from NCBI databases indicated that the protein encoded by LMOF2365_1876 is predicted to contain 8 transmembrane domains. A conserved domain search (<http://www.ncbi.nlm.nih.gov/cdd>) indicated that LMOF2365_1875 encodes the domain for the metal binding protein, PsaA. LMOF2365_1876 encodes for transmembrane subunit (TM) of periplasmic binding protein (PBP)-dependent ATP-Binding Cassette (ABC) transporters, and LMOF2365_1877 contains the domain for the ATP-binding cassette domain of the metal-type transporters.

We were unable to obtain a deletion mutant for LMOF2365_1876. The in-frame deletion mutants for LMOF2365_1875 and LMOF2365_1877 were constructed using SOE primers (Table 1). The mutants were generated by an in-frame deletion of the entire gene with only the first or second amino acid and the stop codon remaining. Δ LMOF2365_1875 had amino acids 3 to 310 removed, and Δ LMOF2365_1877 had amino acids 2 to 236 removed. These in-frame deletions were verified by PCR (Figure 1) and confirmed by sequencing.

Effect of different stress conditions (nisin, acid, and salt) on mutant growth

Growth phenotypes of the LMOF2365 parental strain and its isogenic Δ LMOF2365_1875, and Δ LMOF2365_1877 mutants were tested under types of stress potentially experienced by this pathogen in food-associated environments: nisin, salt, and acid. As shown in figure 2A, with the treatment with 250 μ g/ml nisin, the OD₆₀₀ values of Δ LMOF2365_1875 and Δ LMOF2365_1877 were significantly lower than that of the wild type between 0 and 16 h at 37°C. The fact that mutants grew slower compared to the wild type at the concentration of 250 μ g/ml nisin indicates that mutants were more sensitive to nisin. At pH 5, Δ LMOF2365_1875 and Δ LMOF2365_1877 began to grow more slowly after 6-8 h, and the difference in growth between the mutants and the wild type was maximum at 12 h (Figure 2B). Under salt (5% NaCl in minimal medium) treatment, Δ LMOF2365_1875 grew similarly to the wild type whereas Δ LMOF2365_1877 grew slower than the wild type. The difference in growth was maximum at 10 h (Figure 2C). We also tested growth of the mutants in the presence of verapamil (0-30 mM) (a known ABC transporter inhibitor) and in food (apple juice and milk); however, the mutants grew similarly as compared to the wild type under these conditions (data not shown).

Δ LMOF2365_1877 mutant had an increased capacity for biofilm formation

ABC transporters have been shown to be associated with biofilm formation in *L. monocytogenes* [22,23]. Biofilm assays were performed to determine if biofilm formation of Δ LMOF2365_1875 and Δ LMOF2365_1877 was different from that of the wild type. As shown in figure 3, Δ LMOF2365_1875 formed more biofilm than the wild type whereas biofilm formation by Δ LMOF2365_1877 was similar to that of the wild type (Figure 3).

Discussion

In this paper, in-frame deletions of ABC transporters (LMOF2365_1875 and LMOF2365_1877) were constructed, and the growth of these deletion mutants was tested under food-related stress conditions. We were unable to obtain a deletion mutant of LMOF2365_1876 through multiple attempts because this gene may be essential to cell survival. The effect of nisin was investigated because it has antimicrobial activity and can be used as a food preservative [39]. The effect of salt was studied because salt is often used as a general preservative and food additive to enhance the flavor and shelf life of food [40]. pH 5 was selected for the acid tolerance assay because human gastric pH is between 3.0 and 5.0 during food digestion, and *L. monocytogenes* is generally consumed with contaminated food [41]. Furthermore, the pH of cheese that is often implicated in listeriosis

Figure 1: Schematic diagram of the ABC transporter (LMOF23651875-1877) operon in *L. monocytogenes* F2365 genome (A) and generation of chromosomal deletion mutants (B). A. LMOF2365_1875 encoded a manganese ABC transporter; ATP-binding protein, LMOF2365_1876 encodes for manganese ABC transporter; permease protein. LMOF2365_1877 encodes for ABC transporter; manganese-binding protein. Arrows indicate the position of primers used for mutant generation (not drawn to scale). B. PCR products of the wild-type genes (LMOF2365_1875, LMOF2365_1877) and the genes from the respective deletion mutants (Δ LMOF2365_1875 and Δ LMOF2365_1877) amplified using specific primers (SOEA and SOED) flanking each gene. Lane 1, 1kb Plus DNA ladder, Lane 2, LMOF2365_1875 gene and flanking region of the wild type. Lane 3, flanking region of Δ LMOF2365_1875. Lane 4, LMOF2365_1877 gene and flanking region of the wild type. Lane 5, flanking region of Δ LMOF2365_1877. The predicted sizes are as follows: LMOF2365_1875, 1978bp; Δ LMOF2365_1875, 1054bp; LMOF2365_1877, 1565bp; Δ LMOF2365_1877, 718bp.

Figure 2: Growth curves of *L. monocytogenes* F2365, Δ LMOF2365_1875 and Δ LMOF2365_1877 at 37°C in (A) nisin (250 μ g/ml in BHI), (B) acid (BHI acidified to pH 5 with HCl), and (C) salt (5% NaCl in minimal medium). Cell growth was measured spectrophotometrically by monitoring the OD₆₀₀ at 2-h intervals for 16 h at 37°C. Data shown here are averages of three independent experiments.

outbreaks is usually pH 5 [42], and *L. monocytogenes* mounts an acid tolerance response after exposure to this pH [43]. Biofilm formation was also tested since it is a major reason for the persistence of *L. monocytogenes* on food contact surfaces and imposes important challenges to the production of safe food by the food industry [44].

LMOF2365_1875, 1876, 1877 showed over a ten-fold induction using a DNA microarray assay and over 70-fold induction by real-time quantitative PCR assays [28]. The current work extends our previous studies through the construction of the knock-out mutants to determine a phenotype. We were puzzled by the fact that although LMOF2365_1875 and LMOF2365_1877 were highly induced in milk, the knock-out mutants of LMOF2365_1875 and LMOF2365_1877 grew similarly in milk (data not shown). Our results indicate that the induction of genes by microarray and/or real-time PCR assays does not necessarily result in a strong phenotype at the growth level.

Although the mutants grew more slowly than the wild type under stress conditions such as nisin, salt, and acid treatments in laboratory medium, they may behave differently in food matrices. Studying

Figure 3: Growth curves of *L. monocytogenes* F2365, ΔLMOF2365_1875 and ΔLMOF2365_1877 at 37°C in (A) nisin (250 µg/ml in BHI), (B) acid (BHI acidified to pH 5 with HCl), and (C) salt (5% NaCl in minimal medium). Cell growth was measured spectrophotometrically by monitoring the OD₆₀₀ at 2-h intervals for 16 h at 37°C. Data shown here are averages of three independent experiments.

bacterial growth in food matrices is much more complex than in culture media. For example, LMOF2365_1877 was induced in milk [28] but inhibited in meat [29]. Food matrices may provide different factors to regulate gene expression, and further studies in our laboratory will continue to explore growth of the *L. monocytogenes* mutants on food matrices.

Although the deletion mutants showed some sensitivity to nisin, acid, and salt, the differences compared to the wild type were not large. The growth of mutants was not affected by verapamil (data not shown), which is a well-known ABC transporter inhibitor [45]. The effect of another antimicrobial compound (bacitracin) on mutant growth was also investigated (data not shown) and resulted in no growth difference. Oxidative stress assays using hydrogen peroxide also showed no differences in growth between the mutants and wild type (data not shown). Growth with different carbon sources including mannose, fructose, raffinose, sorbitol, sucrose, xylose and glucose did not affect mutant growth (data not shown). Finally, phenotypic array (Biolog, Inc., Hayward, CA) screening for these two deletion mutants and the wild type was performed and only minor differences were observed (data not shown). The lack of an identifiable phenotype may possibly be due to the fact that activity of the genes can be replaced by other similar genes in the cell since there are other ABC transporters present in the genome. Our notion is supported by the fact that deletion of an ABC transporter in *Lactobacillus plantarum* also resulted in no phenotype change [46]. This may suggest that *L. monocytogenes* is highly adaptive in response to inactivation of these genes or, alternatively, that other ABC transporters that have not yet been identified are also involved in sensitivity to acid and salt in *L. monocytogenes*.

In *L. monocytogenes*, one ABC transporter (Im.G_1771) encoding a putative ABC transporter permease has been reported to be involved in negative regulation of biofilm formation since deletion of this gene resulted in increased capacity to form biofilms [22]. The Δ1771 mutant was further characterized by genomic and proteomic analysis. DNA microarrays and two-dimensional gel electrophoresis revealed 49 and 11 differentially expressed genes and proteins in Δ1771, respectively [23]. In our study, ΔLMOF2365_1875 also showed increased capacity for biofilm formation compared to the wild type, although LMOF2365_1875 showed very little homology to Im.G_1771. In *Streptococcus mutans*, deletion of an ABC transporter resulted

in impaired capacity to form biofilms [25]. ΔLMOF2365_1875 and ΔLMOF2365_1877 were sensitive to nisin, indicating that these genes might be responsible for nisin resistance. Consistent with our data, deletion of another ABC transporter (*anrB*) in *L. monocytogenes* [27] also showed sensitivity to nisin. However, the ABC operon examined in the current study has a different chromosomal location and showed little homology in sequences to the ABC transporters described by Collins et al. [27]. ABC transporters are located on cell membranes that are usually targets for the action of nisin. Our findings also suggest that putative ABC transporters have the potential to be used as targets for the development of new antimicrobials against *L. monocytogenes*.

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