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prfA-Like Transcription Factor Gene *lmo0753* Contributes to L-Rhamnose Utilization in *Listeria monocytogenes* Strains Associated with Human Food-Borne Infections

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***Listeria monocytogenes* is a food-borne bacterial pathogen and the causative agent of human and animal listeriosis. Among the three major genetic lineages of *L. monocytogenes* (i.e., LI, LII, and LIII), LI and LII are predominantly associated with food-borne listeriosis outbreaks, whereas LIII is rarely implicated in human infections. In a previous study, we identified a Crp/Fnr family transcription factor gene, *lmo0753*, that was highly specific to outbreak-associated LI and LII but absent from LIII. Lmo0753 shares two conserved functional domains, including a DNA binding domain, with the well-characterized master virulence regulator PrfA in *L. monocytogenes*. In this study, we constructed *lmo0753* deletion and complementation mutants in two fully sequenced *L. monocytogenes* LII strains, 10403S and EGDe, and compared the flagellar motility, phospholipase C production, hemolysis, and intracellular growth of the mutants and their respective wild types. Our results suggested that *lmo0753* plays a role in hemolytic activity in both EGDe and 10403S. More interestingly, we found that deletion of *lmo0753* led to the loss of L-rhamnose utilization in EGDe, but not in 10403S. RNA-seq analysis of EGDe Δ*0753* incubated in phenol red medium containing L-rhamnose as the sole carbon source revealed that 126 (4.5%) and 546 (19.5%) out of 2,798 genes in the EGDe genome were up- and downregulated more than 2-fold, respectively, compared to the wild-type strain. Genes related to biotin biosynthesis, general stress response, and rhamnose metabolism were shown to be differentially regulated. Findings from this study collectively suggested varied functional roles of *lmo0753* in different LII *L. monocytogenes* strain backgrounds associated with human listeriosis outbreaks.**

Listeria monocytogenes is a Gram-positive, non-spore-forming, facultative anaerobic bacterium and the causative agent of human and animal listeriosis. Listeriosis is often caused by consumption of contaminated food products, such as raw milk, cheese, and ready-to-eat (RTE) meat products (1, 2). Listeriosis in healthy individuals may cause self-limiting gastroenteritis, whereas the disease in immunocompromised individuals, such as the elderly and pregnant women, may cause more severe complications, such as meningitis and encephalitis (1, 3, 4). Due to the high rates of hospitalizations (90%) and deaths (20%) (4, 5), a zero-tolerance policy has been implemented for the RTE meat and poultry industry in the United States (6). *L. monocytogenes* is ubiquitous in nature and is capable of adapting to and proliferating in various environmental niches, such as soil, rotting vegetation, and sewage (1, 3), which makes complete eradication of the persistent pathogen from industrial settings particularly challenging.

The species *L. monocytogenes* can be phylogenetically grouped into at least three major genetic lineages by various genotyping techniques (7). Lineage I (LI) mainly comprises serotypes 1/2b, 3b, 4b, 4d, and 4e; lineage II (LII) contains serotypes 1/2a, 1/2c, 3a, and 3c; and lineage III (LIII) consists of serotypes 4a, some 4b, and 4c (7). Previously reported human listeriosis outbreaks were predominantly caused by LI and LII strains, but rarely by strains in the LIII group (1). In our previous study, we conducted pangenomic analysis of 26 *L. monocytogenes* strains, including eight from LIII (8). We identified 86 disparately distributed genes (DDGs) and eight noncoding small RNAs that were highly conserved in LI and LII genomes but absent in LIII genomes. The majority of these DDGs were associated with cell wall structure, carbohydrate metabolism, and transcriptional regulation. We speculated that some of these DDGs contribute to the environmental persistence and full virulence of LI and LII strains during infection, which led to major food-borne listeriosis outbreaks.

One interesting DDG identified from our previous study was *lmo0753*, which encodes a putative Crp/Fnr family transcription factor. Lmo0753 has two functional domains that are specific to Crp/Fnr transcription factors, similar to the well-characterized positive regulatory factor A, or PrfA. The two domains are an N-terminally located cofactor binding domain and a C-terminally located helix-turn-helix Crp-type DNA binding domain (9). Crp/Fnr family transcription factors function as positive regulators in bacteria and are capable of responding to various environmental signals, such as anoxia, temperature, and oxidative stress (9). Due to its conservation in human outbreak-associated lineages, we hypothesized that *lmo0753* plays a role in regulating environmental survival or virulence-related mechanisms in *L. monocytogenes*.

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TABLE 1 Bacterial strains and plasmids used in this study

L. monocytogenes strain or plasmid	Designation	Reference(s)
Strains		
EGDe	Wild type	
EGDe Δ*0753*		This study
EGDe Δ*0753* (JS-c0753)		This study
10403S	Wild type	
10403S Δ*0753*		This study
10403S Δ*0753* (JS-c0753)		This study
10403S Δ*prfA*		10
10403S *prfA** (G145S)		11, 12
Plasmids		
pKSV7		13
pIMK2		14

TABLE 2 Primers used for construction of deletion mutants and complements

Primer	Sequence (5′ to 3′) (restriction site)[a]
lmo0753 deletion	
0753 A1	GGCGGATCCCGACGTGCCTGTTTATATTTT (BamHI)
0753 A2	ACTAGTTACTCAATAAAAGTAGAATGTTCATTTTGCTTCTCC
0753 B1	GGAGAAGCAAAATGAACATTCTACTTTTATTGAGTAACTAGT
0753 B2	GGCAAGCTTAGCTATCCTTTTTAAATTATT (HindIII)
lmo0753 cloned into pIMK2	
0753 compF	GGCGGAATCTCATGAGCCACCAATCAATA (BamHI)
0753 compR	GCGCCCGGGCCCAGTTCCTTGAAACCAG (SmaI)

[a] Restriction sites are underlined.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are described in Table 1. EGDe (ATCC BAA-679) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). 10403S was provided by Nancy Freitag (University of Illinois). Stocks of bacterial cultures were maintained at −80°C. Unless otherwise stated, all strains were grown overnight (18 h) at 37°C in brain heart infusion (BHI) broth (Becton, Dickinson and Co., Franklin Lakes, NJ) supplemented with chloramphenicol (10 μg ml^{-1}), kanamycin (30 μg ml^{-1}), or streptomycin (200 μg ml^{-1}) when necessary.

Construction of *lmo0753* deletion mutants and complements. *L. monocytogenes* gene deletions in 10403S and EGDe were constructed using methods described by Alonzo et al. (15). All primers used are listed in Table 2. Briefly, upstream and downstream regions of the wild-type gene *lmo0753* were fused via splice overlap extension (SOE) PCR to create a 1,373-bp fragment. The SOE PCR fragment was cloned into the temperature-sensitive shuttle vector pKSV7 using the BamHI and HindIII restriction sites to create plasmid JS-0753. The plasmid was then transformed into 10403S and EGDe as previously described (16), and the Δ*0753* mutation was introduced into the chromosome via allelic exchange (17). The mutation was verified by colony PCR and DNA sequencing.

Complementation strains were constructed by amplifying the entire open reading frame of *lmo0753* by PCR and cloning the fragment into pIMK2 via the BamHI and SmaI restriction sites to generate JS-c0753. The *lmo0753* coding sequence in pIMK2 was under the control of the Phelp promoter, resulting in constitutive gene expression. JS-c0753 was transformed into *L. monocytogenes* Δ*0753* strains (16), and the presence of the *lmo0753* gene was verified by PCR and DNA sequencing.

Broad-range phospholipase C activity. Phospholipase C activity was measured using the method of Alonzo et al. (18). Briefly, single colonies of each strain were streaked onto LB agar containing 2.5% chicken egg yolk. The agar plates were incubated for 24 and 48 h at 37°C, and the diameter of the zone of white precipitate surrounding the streaks was measured. Data were normalized against the negative-control strain 10403S Δ*prfA* to account for bacterial growth. Experiments were repeated at least three times with independent cultures.

Bacterial motility. The flagellar-swimming motility of each strain was assessed using the soft-agar assay previously described by Shetron-Rama et al. (19) with slight modifications. Briefly, 1 μl of an overnight culture of each strain was dropped onto the surfaces of two BHI soft-agar plates containing 0.3% agar. The agar plates were incubated for 24 h at either 37°C or 25°C, and the diameters of the zones of growth for each strain were measured. Experiments were repeated at least three times with independent cultures.

LLO-associated hemolytic activity. Hemolytic activity was measured as described previously by Alonzo et al. (18) with slight modifications. Briefly, overnight BHI cultures of each strain were diluted 1:10 in fresh BHI broth and incubated for 5 h at 37°C with shaking. After 5 h, the optical density at 600 nm (OD_{600}) was measured and normalized for each strain. Serial dilutions of the supernatants were made using phosphate-buffered saline (PBS) containing 1 mM dithiothreitol (pH 5.0), followed by the addition of PBS-washed sheep red blood cells (Innovative Research, Inc., Novi, MI). After 30 min of incubation at 37°C, the mixtures were centrifuged, and the OD_{450} of the supernatant was measured. Hemolytic activity was represented by the OD_{600} divided by the OD_{450}. Experiments were repeated at least three times with independent cultures.

Intracellular growth. Caco-2 human intestinal epithelial cells (ATCC HTB-37) and J774 murine macrophage-like cells were maintained as previously described (20), and intracellular assays were performed using the method of Alonzo et al. (18). Monolayers of cells were grown to confluence on sterile glass coverslips. The cells were infected with *L. monocytogenes* strains at a multiplicity of infection of 100:1 for Caco-2 cells and 0.1:1 for J774 cells. After 1 h, the coverslips were washed three times with 37°C PBS, followed by the addition of fresh 37°C tissue culture medium containing gentamicin (15 μg/ml) to inactivate extracellular bacteria. Thereafter, every 2 h for up to 9 h, the coverslips were removed and eukaryotic cells were lysed by vortexing in 5 ml sterile water. Cell lysates were dilution plated onto LB agar plates to enumerate bacteria. All experiments were repeated at least three times with independent cultures.

Biolog phenotypic microarray. Phenotypic microarray analysis was performed at Biolog Inc. (Hayward, CA). All mutants and wild-type strains were assayed on 20 96-well microtiter plates specific for bacterial metabolic and chemical sensitivity properties according to the manufacturer's specifications (21). Select gained or lost phenotypes of mutants were verified by individual broth assays using HTM minimal medium (22).

L-Rhamnose utilization. L-Rhamnose utilization growth curves were constructed in phenol red minimal medium (10 g pancreatic digest of casein, 5 g sodium chloride, 0.018 g phenol red, and 5 g glucose or rhamnose per liter, pH 7.0) using a Bioscreen C automatic growth curve system (Growth Curves, Piscataway, NJ). Bacterial growth was monitored by recording the cell turbidity every 5 min over a period of 48 h. Experiments were performed at least three times with quadruplicate samples and verified by plate counting. pH curves were drawn using the same medium, and pH values were measured at 8, 24, 32, and 48 h. Experiments were performed three times with independent cultures.

Transcriptomic profiling of L-rhamnose utilization. The *L. monocytogenes* EGDe wild type and its Δ*0753* mutant were grown overnight in BHI broth at 37°C, washed three times with PBS, and subcultured at 1:100 into 100-ml phenol red broth containing either glucose or rhamnose as the sole carbon source. Bacteria were grown at 37°C for 4 h to mid-log phase, and total RNA was extracted using an Ambion RiboPure Bacterial Kit (Life Technologies Corporation, Carlsbad, CA). Total RNA from two biological replicates was prepared from independent cultures on different days to ensure the reproducibility of RNA-seq data. mRNA was purified using the NuGen Prokaryotic RNA-Seq

TABLE 3 Quality control summary statistics for each RNA-seq sample generated via FastQC

Sample	Sample yield (Mb)	% PF[a] clusters	Mismatch rate (%)	% bases ≥Q30[b] (PF)	Mean quality score (PF)	Total no. of reads	No. of reads with at least one reported alignment	% R1 reads with at least one reported alignment
EGDe WT[c] rhamnose-1	2,672	93.86	0	94.66	37.27	56,932,882	43,535,535	76.5
EGDe WT rhamnose-2	2,297	94.15	0	94.87	37.36	48,790,452	34,139,379	70.0
EGDe 0753 rhamnose-1	1,998	94.30	0	94.76	37.31	42,363,954	27,339,106	64.5
EGDe 0753 rhamnose-2	2,614	93.98	0	94.70	37.32	55,633,416	41,570,106	74.7

[a] PF, purity filtered.
[b] Q30, 99.9% accurate.
[c] WT, wild type.

system (NuGen, San Carlos, CA). Deep RNA sequencing was conducted on an Illumina HiSeq 2000 instrument (12.5 million filter-passed 50- plus 50-nucleotide [nt] paired-end reads; Illumina Inc., San Diego, CA). The reads were mapped to the *L. monocytogenes* 10403S sequence (available at http://www.broadinstitute.org/annotation/genome/listeria_group/GenomesIndex.html using GSNAP3) (23). Gene and transcript expression levels were computed using Cufflinks (24–26). Quality control of transcripts was performed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (Table 3).

qRT-PCR. Quantative real-time transcription-PCR (qRT-PCR) was performed as previously described (27). Briefly, six genes showing significant ($P < 0.05$) up- or downregulation identified by RNA-seq were selected for verification by qRT-PCR. Primers were designed with Primer3 software to produce amplicons of approximately 150 bp (28) (Table 4). qRT-PCR was performed using a LightCycler 480 (Roche Applied Science, Indianapolis, IN) with cDNA reverse transcribed from 1 μg of purified total RNA using the Transcriptor first-strand cDNA synthesis kit (Roche Applied Science). The relative expression changes were calculated using the method of Pfaffl (29); the 16S rRNA gene was used as an internal reference for data normalization. Average $\log_2$ values and standard deviations from quadruplicate samples are reported for each experiment. The experiments were conducted three times for statistical analysis.

Statistical analysis. Student's *t* test and analysis of variance (ANOVA) were performed using the GraphPad Prism software package (version 5; GraphPad Software) and Excel software (2010 version; Microsoft).

RNA-seq data accession numbers. RNA-seq data were deposited in the NCBI database with GEO accession numbers GSM1155399 through GSM1155402.

RESULTS

Virulence-associated mechanisms. Using LAlign (30), we found the overall percentage of amino acid identity between Lmo0753 and PrfA was 18%. The C-terminal domains shared 27% identity (65% similarity), and the N-terminal domains shared 25% identity (53% similarity). Figure S1 in the supplemental material depicts a sequence alignment highlighting the helix-turn-helix DNA binding domain in each protein. Using BLAST, we determined that the DNA sequences of *lmo0753* in 10403S and EGDe were 100% identical. To determine if *lmo0753* contributes to virulence-associated mechanisms in *L. monocytogenes* 10403S and EGDe, we performed *in vitro* assays to evaluate the phospholipase C production, flagellar motility, hemolytic activity, and intracellular growth of the mutants. For phospholipase C activity (Fig. 1), assays were conducted for both 24 and 48 h. After 24 h, 10403S Δ*0753* and EGDe Δ*0753* mutants showed no significant difference from their parent strains in phospholipase C activity. After 48 h, 10403S Δ*0753* showed a significant ($P < 0.05$) decrease in phospholipase C activity compared to the parent strain. 10403S *prfA**, a constitutively active form of the protein, showed greater phospholipase C activity at both 24 and 48 h ($P < 0.0001$) than the parent strain. Complementation of 10403S Δ*0753* was able to restore the phenotype of the parent strain. This indicated that *lmo0753* played a role in phospholipase C activity in 10403S after 48 h, but not in EGDe.

Motility assays were conducted for 48 h at both 37°C and 25°C to determine if *lmo0753* is involved in flagellar motility (Fig. 2). Significant motility reduction was observed at 37°C in both strains 10403S Δ*0753* ($P < 0.05$) and 10403S Δ*prfA* ($P < 0.001$). At 25°C, slight but insignificant motility reduction was observed for both 10403S Δ*0753* and EGDe Δ*0753* mutants ($P > 0.05$). The 10403S complementation mutant was able to restore the motility pheno-

TABLE 4 Primers used for qRT-PCR analysis

Gene	Annotation	Primer sequence 5′ to 3′[a]
LMRG_00281	Biotin biosynthesis protein BioY	F: CTATACCACTCGGCCCTATT
		R: TGTCATTCCTTGGAAAACAG
LMRG_01174	Quinolinate synthetase complex A subunit	F: TTTTTGGCGATACGATTTTA
		R: GAAAATCCGCTCTTTTTGTT
LMRG_01948	General stress protein 26	F: TCCGTTTTAATTGGTTACGA
		R: ATAACGACAAAGGATGGTGA
LMRG_00287	Hypothetical protein	F: ATTTGTCGCTGGTATTGTTG
		R: TCCTCGAATCAAGTTGAAAA
LMRG_02489	YukD protein	F: CACTAATTGGGGAGCAAGTA
		R: TCGCTTTATTCGTTGTTTTT
LMRG_00054	Sec-independent protein translocase TatAy	F: GGACCAGGAAGTATTGCTTT
		R: TGGTTTCTTCTTTGGAATCA
LMRG_05501	16S rRNA	F: CAGCTAACGCATTAAGCACT
		R: GTGGTCAAAGGATGTCAAGA

[a] F, forward primer; R, reverse primer.

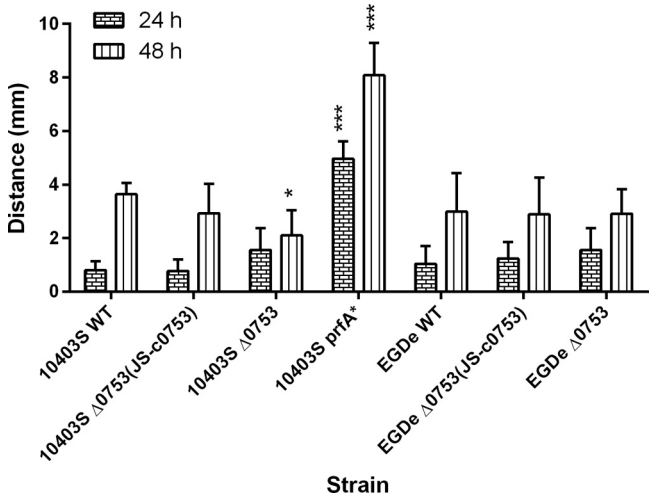

FIG 1 Phospholipase C activity of *L. monocytogenes* strains at 37°C after 24 and 48 h. The error bars represent standard deviations of three independent experiments. Significant differences in comparison to the parent strains under the same experimental condition are shown as follows: *, $P < 0.5$; ***, $P < 0.0001$.

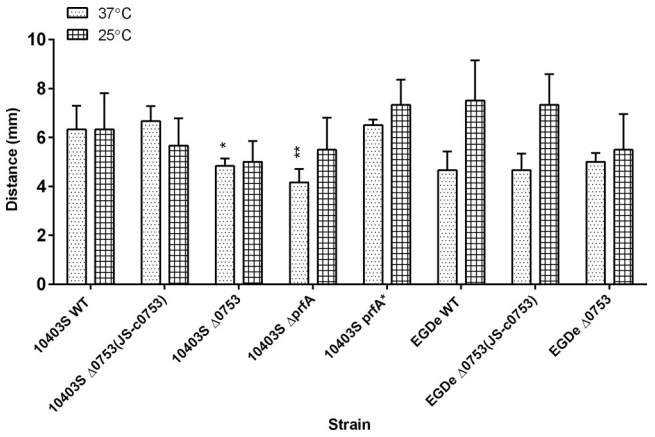

FIG 2 Flagellar-swimming motility of *L. monocytogenes* strains at 37°C and 25°C after 24 h. The error bars represent standard deviations of three independent experiments. Significant differences in comparison to the parent strains under the same experimental condition are shown as follows: *, $P < 0.5$; **, $P < 0.001$.

type to that of the wild-type strain at 37°C. The results indicated that *lmo0753* played a minor role in the motility of *L. monocytogenes* 10403S at 37°C.

Hemolytic-activity assays were performed to determine if *lmo0753* contributed to the hemolytic activity of *L. monocytogenes* 10403S and EGDe (Fig. 3A and B, respectively). Various dilutions of bacteria ranging from 1:10 to 1:320 were used to test the hemolytic activity of each strain at each dilution. At dilutions of 1:10, 1:20, 1:40, and 1:80, significant differences ($P < 0.0001$) were observed for all strains compared to their parent strains. Strains 10403S Δ*0753*, 10403S Δ*prfA*, and EGDe Δ*0753* showed significantly less hemolytic activity than their respective wild-type strains, whereas 10403S *prfA** showed significantly higher hemolytic activity than its wild type (data not shown). Complementation mutants were able to restore the phenotype observed in the wild-type strains. These results suggested that deletion of *lmo0753* had a significant impact on the hemolytic activity of both 10403S and EGDe.

To determine if *lmo0753* is involved in intracellular growth, we compared intracellular growth for all *L. monocytogenes* mutants in both Caco-2 and J774 eukaryotic host cells. 10403S Δ*prfA* was used as a negative control in these assays. No statistical difference was detected when strains were grown in Caco-2 cells (data not shown), and only slight differences were observed when they were grown in J774 cells (Fig. 4). Intracellular growth of both Δ*0753* mutant strains resembled wild-type growth.

L-Rhamnose utilization. *L. monocytogenes* EGDe and 10403S wild types and the respective Δ*0753* mutants were subjected to Biolog phenotypic microarray screening to determine if the deletion had an impact on the metabolic utilization or chemical sensitivity of the bacteria. Twenty standard bacterial plates, which tested for metabolic utilization, such as carbon, nitrogen, phosphorus, and sulfur sources; pH and osmolytes; and antibiotic sensitivity, were used. Compared to its parent strain, the metabolic profile of 10403S Δ*0753* showed increased metabolism in reactions with phenylmethylsufonylfluoride (PMSF) (a protease inhibitor) and crystal violet and decreased metabolism in reactions with 5,7-dichloro-8-hydroxyquinaldine (a lipophilic chelator). EGDe Δ*0753* showed increased metabolism in reactions with domiphen bromide (a cationic detergent) and cesium chloride (a toxic cation) and decreased metabolism in reactions with L-rhamnose, poly-L-lysine (a cationic detergent), and patulin (a microtubulin polymerization inhibitor), in addition to decreased metabolism with nitrogen sources. Select gained/lost phenotypes of mutants were verified by individual broth assays to confirm the Biolog results.

One interesting phenotypic change was the loss of L-rhamnose utilization in EGDe Δ*0753*. L-Rhamnose is often found as a constituent of pectin in dicotyledonous plant cell walls and is an important carbon source for energy metabolism for many soil bacterial species. To verify this phenotypic change, we constructed Bioscreen C growth curves with L-rhamnose as the sole carbon source in phenol red medium. When grown in phenol red medium with glucose as the sole carbon source (Fig. 5A), 10403S and EGDe showed growth patterns similar to those of their respective Δ*0753* mutants. However, when grown in phenol red medium with L-rhamnose as the sole carbon source (Fig. 5B), EGDe Δ*0753* did not grow, whereas other strains showed growth patterns similar to those of the wild-type strains. To ensure that the lack of growth was due to the loss of L-rhamnose utilization, pH determinations were performed. When grown in phenol red medium with glucose as the sole carbon source (Fig. 5C), all strains showed similar decreases in pH to 5.0 after 48 h of incubation, indicating the carbon source was metabolized. When grown in phenol red medium with L-rhamnose as the sole carbon source (Fig. 5D), the pH of EGDe Δ*0753* remained neutral (pH 6.8) after 48 h (statistically significant; $P < 0.0001$), whereas the pH for all other strains dropped to approximately 5.5, suggesting that L-rhamnose was not utilized by EGDe Δ*0753* for metabolism.

Transcriptomic profiling. To explore the transcriptomic response of *L. monocytogenes* when grown in phenol red medium with L-rhamnose as the sole carbon source, we performed RNA-seq analysis on the EGDe wild type and its Δ*0753* mutant. Purified mRNA samples were sequenced on an Illumina HiSeq 2000 instrument, and all samples had a minimum of 42,000,000 paired-

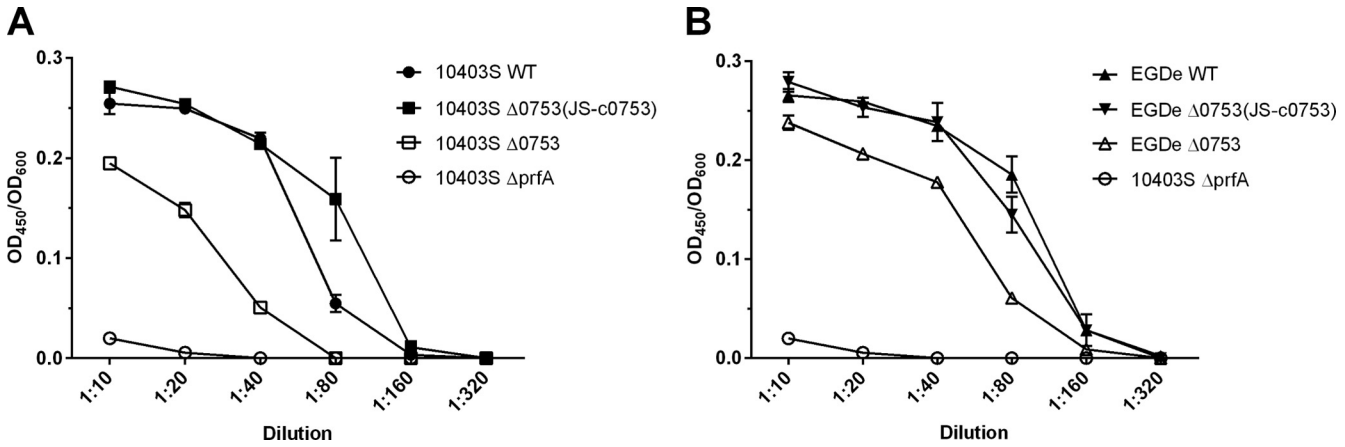

FIG 3 Hemolytic activity of *L. monocytogenes* strains at 37°C represented as the OD_{600} divided by the OD_{450}. The error bars represent standard deviations of three independent experiments.

end reads aligned at >65% efficiency with the reference 10403S genome. 10403S was used the reference genome because detailed genome annotation and analytic tools for the strain were available at the Broad Institute website. qRT-PCR was used to validate the RNA-seq data for select genes. As shown in Fig. 6, the fold change detected by qRT-PCR correlated well with the corresponding RNA-seq results.

Table 3 depicts the quality control summary for each sample sequenced. Out of 2,798 genes in the EGDe genome, 126 (4.5%) genes were found to be upregulated more than 2-fold and 43 (1.5%) genes more than 4-fold under the conditions tested (Table 5). The two most upregulated genes in EGDe Δ*0753* were those encoding the biotin biosynthesis protein BioY (LMRG_00281; upregulated 23.5-fold) and the quinolinate synthetase complex A subunit (LMRG_01174; upregulated 20.3-fold). Other significantly upregulated transcripts included stress-related proteins, such as universal stress protein (LMRG_00196; upregulated 8.2-fold), general stress protein 26 (LMRG_01948; upregulated 8.1-fold), and a starvation-inducible DNA binding protein (LMRG_02041; upregulated 7.8-fold).

A total of 546 (19.5%) genes were found to be downregulated more than 2-fold and 22 (0.8%) genes more than 4-fold under the conditions tested (Table 6). The most significantly downregulated genes in EGDe Δ*0753* included a hypothetical protein (LMRG_00287; downregulated 33.3-fold), YukD protein (LMRG_02489; downregulated 7.7-fold), and Sec-independent protein translocase TatAy (LMRG_00054; downregulated 7.7-fold). Rhamnose operon genes (31) were also downregulated: L-rhamnose 1-epimerase (LMRG_02417) 2.2-fold, rhamnulose-1-phosphate aldolase (LMRG_02418) 1.9-fold, L-rhamnose isomerase (LMRG_02419) 1.9-fold, and rhamnulokinase (LMRG_02420) 2.1-fold.

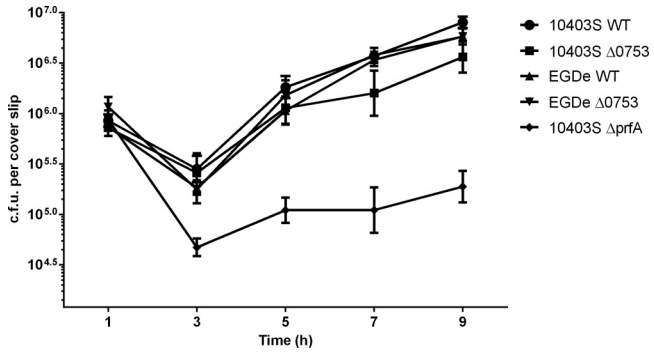

FIG 4 Intracellular growth assays of *L. monocytogenes* strains in J774 host cells. 10403S Δ*prfA* was used as a negative control. The error bars represent standard deviations of three independent experiments.

DISCUSSION

Lmo0753 is a putative Crp/Fnr transcriptional regulator that shares two similar functional domains with PrfA, the master virulence regulator in *L. monocytogenes* (8). The shared functional domains are an N-terminally located cofactor binding domain and a C-terminally located helix-turn-helix domain consisting of two α-helices that are capable of resting in the major groove in DNA (9). Crp/Fnr family transcription factors are a very large and diverse family of proteins, spanning functions such as regulation of virulence, metabolic pathways, and stress response (9). One distinct function of PrfA is its ability to sense environmental changes and switch on various types of virulence factors. When environmental conditions, such as temperature, pH, and access to nutrients, change, PrfA is capable of positively regulating a number of virulence factors and thus aids in pathogenicity (3, 32–34). Because *lmo0753* is highly conserved only in the human outbreak-associated lineages of *L. monocytogenes* (8), we hypothesized that this putative transcription factor likely played a role in environmental-signal sensing and pathogenicity.

It is well known that PrfA regulates *L. monocytogenes* virulence by switching on genes in the PrfA regulon (35). These genes are essential for bacterial invasion of host cells, cell-to-cell spread of the pathogen, and intracellular growth. Broad-range phospholipase C activity, encoded by *plcB*, is important during intracellular growth, mainly for phagosomal-vacuole escape (36). Deletion of *lmo0753* modestly reduced phospholipase C activity in 10403S, but not in EGDe. This indicated that Lmo0753 had different impacts on *plcB*-associated phospholipase C activity in different strain backgrounds. It should be noted that deletion of *prfA* from 10403S also led to less production of phospholipase C and reduced invasive capability of the pathogen; conversely, constitutive activation of *prfA* led to greater phospholipase C activity and thus greater virulence (37). 10403S and EGDe remain motile at body

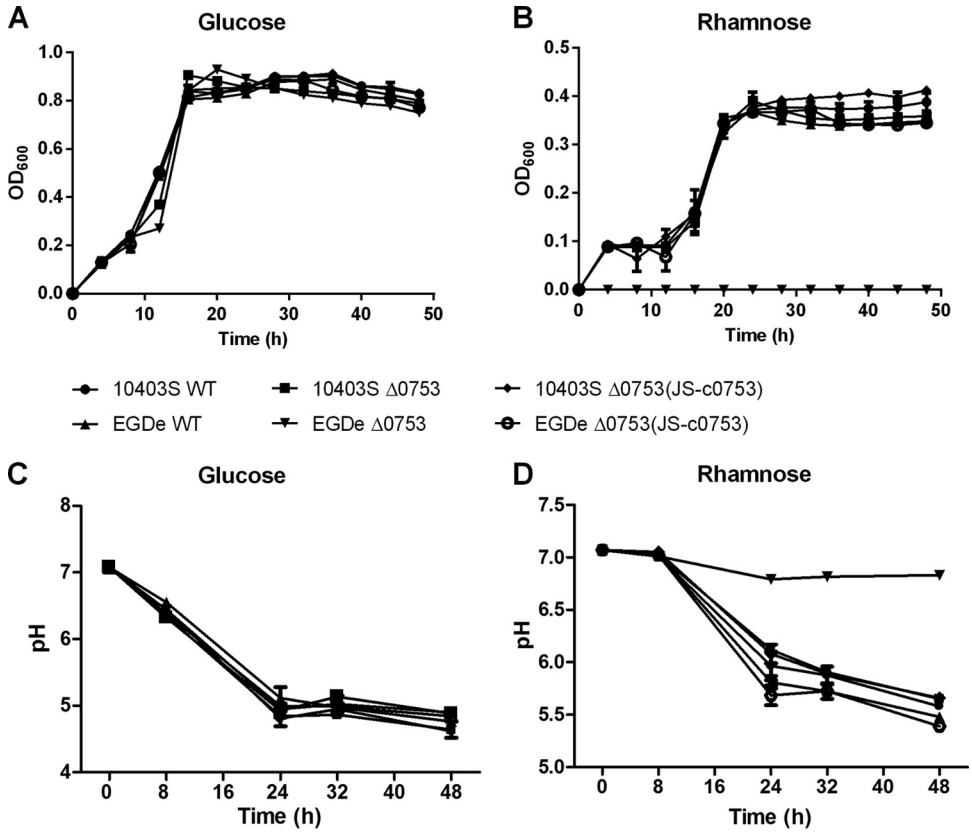

FIG 5 Phenol red minimal medium growth curves and pH carbohydrate assays of *L. monocytogenes* strains. The growth curves were constructed using either glucose (A) or L-rhamnose (B) as the sole carbon source. pH carbohydrate assays were conducted with either glucose (C) or L-rhamnose (D) as the sole carbon source. The error bars represent standard deviations of three independent experiments conducted with quadruplicate samples.

temperature (37°C) (38). Deletion of *lmo0753* resulted in less flagellar-associated motility in 10403S at 37°C, but not in EGDe. Decreased flagellar motility is generally associated with decreased virulence, although this is not always the case (38–40). Our results showed that 10403S Δ*prfA* had decreased flagellar motility compared to the parent strain, which was contradictory to other reports (41). This discrepancy could be explained by the different methods used in different studies for evaluating bacterial motility, such as stab inoculation versus drop inoculation on agar plates. Our study also showed that 10403S *prfA** led to a slight decrease in motility compared with its parent strain. This was consistent with previous reports, although not to the same extent (41).

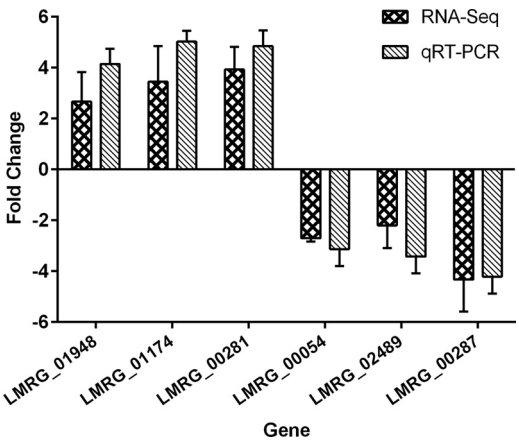

FIG 6 Comparison of gene expression fold changes identified by RNA-seq and qRT-PCR analyses. Six genes (3 upregulated and 3 downregulated) were compared. Fold changes were converted to $\log_2$ values for comparison. 16S rRNA (LMRG_05501) was used as an internal reference. The error bars represent standard deviations of three independent experiments conducted with quadruplicate samples.

Virulent strains of *L. monocytogenes* can effectively escape from the phagosome and enter the cytosol during host cell infection because of the PrfA-dependent gene *hly* (3, 37, 42). Strains lacking *hly* are essentially avirulent (43). We demonstrated that both 10403S Δ*0753* and EGDe Δ*0753* showed reduced hemolytic activity; however, we did not detect a deficiency of either mutant in intracellular growth in Caco-2 cells and only insignificant differences in intracellular growth in J774 cells.

One criterion to differentiate *L. monocytogenes* from other, nonpathogenic *Listeria* species is that *L. monocytogenes* is able to utilize L-rhamnose as a carbon source. Certain atypical LIII strains that are deficient in rhamnose fermentation (44) display attenuated virulence (45), as well as reduced resistance to temperature shifts (46). Rhamnose pathway genes have been shown to be highly upregulated during intracellular growth (47). In this study, EGDe Δ*0753* displayed a marked phenotypic loss of L-rhamnose utilization in the Biolog phenotypic assays. To verify the L-rhamnose deficiency in EGDe Δ*0753*, we constructed growth curves and performed pH assays using L-rhamnose as the sole carbon

TABLE 5 Transcripts with more than a 4-fold increase in EGDe Δ*0753* compared to EGDe WT

Gene	Annotation	Fold change
LMRG_00281	Biotin biosynthesis protein BioY	23.5
LMRG_01174	Quinolinate synthetase complex A subunit	20.3
LMRG_00199	Membrane protein	16.2
LMRG_01972	DNA binding 3-demethylubiquinone-9 3-methyltransferase	15.0
LMRG_01060	Hypothetical protein	13.4
LMRG_01479	Glutamate decarboxylase	12.6
LMRG_01173	Nicotinate-nucleotide diphosphorylase	10.6
LMRG_01172	L-Aspartate oxidase	10.5
LMRG_00557	Cellobiose-specific PTS IIB component	10.3
LMRG_02808	Hypothetical protein	9.6
LMRG_02382	Hypothetical protein	9.0
LMRG_02094	Hypothetical protein	8.8
LMRG_02304	Peptidoglycan-bound protein	8.4
LMRG_00196	Universal stress protein	8.2
LMRG_01948	General stress protein 26	8.1
LMRG_02041	Starvation-inducible DNA binding protein	7.8
LMRG_00236	Hypothetical protein	7.3
LMRG_02013	Succinate-semialdehyde dehydrogenase	6.8
LMRG_00280	Transcription regulator CRP/FNR family protein	6.5
LMRG_02414	Sugar uptake protein	6.2
LMRG_01480	Glutamate/gamma-aminobutyrate antiporter	5.9
LMRG_00710	Hypothetical protein	5.8
LMRG_00885	Glutathione reductase	5.5
LMRG_01794	Hypothetical protein	5.5
LMRG_00335	Magnesium and cobalt transporter CorA	5.4
LMRG_01912	Catalase	5.1
LMRG_02001	Dihydroxyacetone kinase L subunit	5.1
LMRG_00293	Internalin	5.1
LMRG_00221	Tagatose 1,6-diphosphate aldolase	4.9
LMRG_00365	Flagellar biosynthetic protein FliQ	4.9
LMRG_00010	Phosphomethylpyrimidine kinase	4.9
LMRG_00745	Host factor I protein	4.6
LMRG_02602	Hypothetical protein	4.6
LMRG_01030	Chitinase	4.6
LMRG_01444	Hypothetical protein	4.5
LMRG_01763	PspC domain-containing protein	4.4
LMRG_01236	Peptidoglycan binding protein	4.4
LMRG_00278	Hypothetical protein	4.4
LMRG_01602	Hypothetical protein	4.2
LMRG_00472	Mannose-specific PTS IIA component	4.1
LMRG_01574	Hypothetical protein	4.1
LMRG_02611	Succinyl-diaminopimelate desuccinylase	4.1
LMRG_01103	Fur family transcriptional regulator	4.0
LMRG_01849	Hypothetical protein	4.0

TABLE 6 Transcripts with more than a 4-fold decrease in EGDe Δ*0753* compared to EGDe WT

Gene	Annotation	Fold change
LMRG_00287	Hypothetical protein	33.3
LMRG_02489	YukD protein	7.7
LMRG_00054	Sec-independent protein translocase TatAy	7.7
LMRG_02700	Hypothetical protein	6.7
LMRG_01268	Hypothetical protein	6.7
LMRG_00793	Competence protein ComGE	6.3
LMRG_00748	HTH[a]-type transcriptional regulator GlnR	6.3
LMRG_01676	Hypothetical protein	5.9
LMRG_01269	Hypothetical protein	5.9
LMRG_01809	Hypothetical protein	5.9
LMRG_01908	Hypothetical protein	5.3
LMRG_02074	Hypothetical protein	5.3
LMRG_01761	Hypothetical protein	5.3
LMRG_00606	Ethanolamine and carbon dioxide metabolism	5.0
LMRG_01655	Hypothetical protein	4.8
LMRG_01915	Cellobiose-specific PTS IIB component	4.8
LMRG_02072	Membrane protein	4.4
LMRG_00868	Hypothetical protein	4.4
LMRG_02225	K^+-transporting ATPase C subunit	4.2
LMRG_02073	D-Alanine-poly(phosphoribitol) ligase subunit 1	4.0
LMRG_01886	Hypothetical protein	4.0
LMRG_00177	Hypothetical protein	4.0

[a] HTH, helix-turn-helix.

source. EGDe Δ*0753* was found to be deficient in both growth and utilization of this carbon source for metabolism. Interestingly, unlike EGDe Δ*0753*, 10403S Δ*0753* did not display deficiency in L-rhamnose utilization, suggesting that Lmo0753 functions differently in different strain backgrounds.

Transcriptomic profiling via RNA-seq was performed to detect differentially regulated genes when EGDe Δ*0753* was incubated in medium containing L-rhamnose as the sole carbon source. A vast upregulation (23.5-fold) was seen in the biotin biosynthesis protein BioY. Biotin is an essential vitamin that functions as a cofactor in carboxylation and decarboxylation reactions. A recent study suggested roles for the vitamin in cell signaling and gene expression (48). Bacteria typically acquire biotin from exogenous sources (49) and transport it using an energy-coupling-factor transporter that consists of three main components: an S component that provides substrate specificity, a membrane component, and an ATPase A component (50–52). Such an increase in BioY may be due to an increase in stress resulting from starvation of the cells during incubation with L-rhamnose, as seen by the upregulation of three stress-specific genes, including a universal stress protein, general stress protein 26, and a starvation-inducible DNA binding protein. The upregulation of these transcripts was likely due to starvation-associated stress induction and not necessarily L-rhamnose itself.

Two other significantly upregulated genes in EGDe Δ*0753* were those encoding the two components of the quinolinate synthetase complex: quinolinate synthetase complex A subunit (upregulated 20.3-fold) and L-aspartate oxidase (B subunit) (upregulated 10.5-fold). Quinolinate synthetase is an oxygen-sensitive complex required for NADP ($NADP^+$) synthesis (53, 54). Quinolinate synthetase complexes are found in all sequenced genomes of bacilli and other pathogenic microorganisms, including *Streptococcus* and *Enterococcus*, and are necessary for the survival of *Bacillus subtilis* (55). Upregulation of these transcripts may be attributed to the cellular need to increase the *de novo* biosynthetic pathway for metabolic purposes.

YukD protein, a ubiquitin-like transporter (56), was significantly downregulated (7.7-fold) in EGDe Δ*0753* during incubation in medium with L-rhamnose. YukD is an essential part of the type VII WXG100 protein secretory pathway found in *Listeria* and other Gram-positive bacteria (57, 58). WXG100 proteins are relatively small, approximately 100 amino acids in length; have a conserved WXG amino acid motif in the center of the protein; and lack canonical signal peptides (59). The proteins are thought to be

secretory effectors that perform important functions once pathogens are inside host cells (58). This secretion system is deemed critical for cell-to-cell spread in host cells for *Mycobacterium marinum* and is also involved in mechanisms of dissemination and colonization in *Staphylococcus aureus* (60). However, it was reported that this type VII secretion system is not required for *L. monocytogenes* virulence (60). Two additional downregulated transporters were TatAy, a Sec-independent protein translocase (61) (downregulated 7.7-fold) and cellobiose-specific phosphotransferase system (PTS) IIB component, a major carbohydrate active-transport system (62) (downregulated 4.8-fold). Downregulation of protein and carbohydrate secretion systems may be attributed to a stress response mechanism.

In summary, the results from this study clearly demonstrated that *lmo0753* plays a major role in L-rhamnose metabolism in *L. monocytogenes* strain EGDe. The absence of *lmo0753* in *L. monocytogenes* LIII genomes potentially leads to an impaired ability of the bacteria to utilize L-rhamnose as an environmental carbon source, which partially explains the relative infrequency of the lineage in human food-borne disease outbreaks. Although 10403S and EGDe share highly similar genomic contents and belong to the same genetic lineage and serovar of *L. monocytogenes*, we observed marked functional differences of *lmo0753* in the two strains. This observation highlights the need for a thorough examination of gene functions using different strain backgrounds in future studies.

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