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lmo0038 Is Involved in Acid and Heat Stress Responses and Specific for *Listeria monocytogenes* Lineages I and II, and *Listeria ivanovii*

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Abstract

The genus *Listeria* comprises two pathogenic species, *L. monocytogenes* and *L. ivanovii*, as well as four nonpathogenic species, *L. innocua*, *L. weishimeri*, *L. seeligeri*, and *L. grayi*. Within *L. monocytogenes*, lineages I and II are responsible for most listeriosis cases, while lineage III strains are rarely associated with human morbidity but providing important clues for *Listeria* evolution. The gene *lmo0038*, belonging to the peptidylarginine deiminase family, was involved in the optimal growth under stress conditions, including low pH and heat shock (52°C), and virulence potential. Further, this gene was specific to *L. monocytogenes* lineages I and II and *L. ivanovii* with significant similarities at nucleotide and amino acid levels. A novel multiplex PCR, based on *lmo0038* in combination with optimized *iap* migration profiles, was developed for simultaneous identification of *Listeria* species and discrimination of *L. monocytogenes* lineage III, with a detection limit down to 1.0–9.0×10^2 CFU/mL. This assay was evaluated by 119 suspected *Listeria* food-related isolates and corrected 4 and 5 misidentifications by *Listeria* selective agar plate screening and API system, respectively. Therefore, this one-step molecular assay provides a rapid, reliable, and inexpensive screening test to detect *Listeria* species—particularly, the pathogenic species in surveillance programs concerning food safety and foodborne disease cases.

Introduction

Listeriae are members of a group of Gram-positive bacteria with low GC content (G^+_{low}) that include species of the genera *Bacillus*, *Clostridium*, *Enterococcus*, *Streptococcus*, *Staphylococcus*, and *Lactobacillus* (Kalin *et al.*, 2004). Among the six species within the genus *Listeria*, *L. monocytogenes* and *L. ivanovii* are pathogenic (Schmid *et al.*, 2005). *L. ivanovii* is capable of causing abortions and stillbirths in ruminants, while *L. monocytogenes* is a significant human and animal pathogen with clinical manifestations ranging from mild flu-like symptoms and gastroenteritis to septicemia, central nervous infections, and feto-maternal infection (Cossart, 2007; Swaminathan and Gerner-Smidt, 2007). However, *L. monocytogenes* encompasses a diversity of strains with varying virulence and pathogenicity. Serovars 1/2a, 1/2b, 1/2c, and 4b (of lineages I and II) are responsible for over 98% of epidemic and sporadic cases of human listeriosis, whereas lineage III (including serovars 4a and 4c) strains are rarely associated with listeriosis (Buchrieser *et al.*, 2007; Liu *et al.*, 2007). Lineage III is considered a possible novel subspecies from previous studies, and it is tempting to speculate that the

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population structure and biodiversity in this lineage may serve as sources of clues for genome diversification and evolutionary history in *Listeria* (Doumith *et al.*, 2004; Liu *et al.*, 2006a; Chen *et al.*, 2009a).

Because of their widespread occurrence in nature, listeriae have many opportunities to enter the food production and processing environments. They are actually able to multiply in food as a result of their tolerance to extreme pH, temperature, and salt conditions (Gandhi and Chikindas, 2006). However, threat to public health posed by each *Listeria* species or lineage is different due to their host specificity and virulence differences (Wiedmann *et al.*, 1997; Vazquez-Boland *et al.*, 2001; Chen *et al.*, 2009b). Therefore, it is necessary to select suitable targets to develop a one-step molecular assay for rapid and reliable identification of *Listeria* species and discrimination of *L. monocytogenes* lineages.

Differentiation of these closely related *Listeria* species has long been hampered by lack of studies on the characteristics of species other than *L. monocytogenes*. The carbohydrate fermentation tests are time consuming (Liu, 2006). Occurrence of atypical *Listeria* isolates, such as rhamnose-negative *L. innocua* and *L. monocytogenes* strains (Johnson *et al.*, 2004; Liu *et al.*, 2006a; Volokhov *et al.*, 2007), further confused researchers by simple biochemical tests in practical terms. PCR assays targeting 16S and 23S rRNA and some virulence-associated genes have been developed, but further molecular procedures, such as enzyme restriction, sequence-based typing, or other supplementary PCRs, are often required (Vaneechoutte *et al.*, 1998; Cocolin *et al.*, 2002; Liu, 2006; Chen and Knabel, 2007). The *iap* gene is a suitable PCR target for its conserved regions at 3′ end and a species-specific internal region. However, *L. seeligeri*, *L. welshimeri*, and *L. ivanovii* as well as three *L. monocytogenes* lineages could not be distinguished using PCR probing the *iap* gene (Bubert *et al.*, 1999).

Previously, upon examination of 264 genes from 113 *L. monocytogenes* strains by microarray, Doumith *et al.* (2004) have shown that the *lmo0036-0041* locus is especially absent from *L. monocytogenes* lineage III strains. We hypothesized that this locus may be a possible marker to distinguish strains of this particular lineage from other lineages. Sequence analysis indicated that *lmo0038* in this locus had domains similar to those of a member of the peptidylarginine deiminase family involved in bacterial resistance to acid stress (Degnan *et al.*, 2000; Gruening *et al.*, 2006). Therefore, we also examined the roles of *lmo0038* gene in *L. monocytogenes* resistance to acid and thermal stresses.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

Listeria strains mostly from reference sources used in this study were listed in Table 1. Closely related G^{+}_{low} bacterial species *Bacillus cereus*, *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus suis*, and *Streptococcus equi* were used to verify the specificity of the PCR method. *Escherichia coli* DH5α was employed as the host strain for plasmids pUC18 and pKSV7. All strains were cultured in brain heart infusion broth (BHI; Oxoid, Hampshire, England) or BHI agar plates with 5% sheep blood and incubated at 37°C. Stock solutions of ampicillin (100 mg/mL) and chloramphenicol (50 mg/mL) were added to media, where appropriate, at the required levels. Acid stress BHI (pH 4.0 and 5.0) was prepared by adjustment with 2 M hydrochloric acid.

DNA manipulations

DNA extraction was performed as described previously (Jiang *et al.*, 2008). Oligonucleotide primers for PCR were synthesized by Invitrogen Biotechnology (Shanghai, China) (Table 2), and *Taq* DNA polymerase (TaKaRa Biotech, Dalian, China) was used for regular reactions. For products larger than 4 kb, *LA Taq* DNA polymerase (TaKaRa) was employed. PCR reaction was conducted using the PT-200 thermal cycler (MJ Research, Boston, MA). Annealing temperatures varied with primer pairs (Table 2), and the duration of extension depended on the predicted length of amplicons (1 min/kb, at 72°C). PCR fragments were purified using the AxyPrep DNA Gel Extraction Kit (Axygen, Union City, CA) and digested with defined restriction enzymes (TaKaRa) to facilitate the

Table 1. List of *Listeria* Reference Strains and 22 *Listeria monocytogenes* Food-Related Isolates, and Their Relevant Typing Profiles and Virulence to Mice (LD_{50})

Species	*Strains*	*Source*	*Lineage*	*Serovar*	*Log LD_{50}*	lmo0038	iap *Amplicon size (bp)*
L. monocytogenes	SLCC2755	Reference	I	1/2b	5.79	+	720
	ScottA	Reference	I	4b	6.79	+	720
	NICPBP54007	Reference	I	4b	5.25	+	720
	F2-024	Reference	I	4b	ND	+	720
	EGD-e	Reference	II	1/2a	6.80	+	720
	10403S	Reference	II	1/2a	5.49	+	720
	NICPBP54003	Reference	II	1/2a	ND	+	720
	NCTC7973	Reference	II	1/2a	ND	+	720
	AB2483	Reference	II	1/2a	ND	+	720
	NCTC5438	Reference	II	1/2c	6.03	+	720
	NICPBP54002	Reference	II	1/2c	ND	+	720
	NBSC001	Reference	II	1/2c	ND	+	720
	NICPBP54006	Reference	III	4a	>8	−	720
	F2-695	Reference	III	4a	>8	−	720
	J1-168	Reference	III	4a	ND	−	720
	F2-086	Reference	III	4a	ND	−	720
	F2-208	Reference	III	4a	ND	−	720
	J2-071	Reference	III	4c	ND	−	720
	W1-111	Reference	III	4c	ND	−	720
	F2-525	Reference	III	4b	ND	−	720
	P1	Pork	II	1/2a	6.26	+	720
	P2	Pork chops	I	1/2b	6.45	+	720
	P3	Raw pork	II	1/2a	6.07	+	720
	V1	Vegetable	II	1/2c	6.11	+	720
	C17	Chicken	I	1/2b	5.83	+	620
	M1	Milk	I	1/2b	6.46	+	720
	M2	Milk	I	1/2b	6.43	+	720
	M3	Milk	I	1/2b	6.32	+	720
	M4	Milk	II	1/2a	5.45	+	720
	M5	Raw milk	I	4b	3.86	+	720
	M6	Pasteurized milk	II	1/2a	5.55	+	720
	M7	Pasteurized milk	III	4a	>8	−	720
	S1	Seafood	II	1/2a	5.53	+	720
	S2	American red drum	I	4b	6.74	+	720
	S3	American red drum	II	1/2c	6.19	+	720
	S4	American red drum	I	4b	6.72	+	720
	S5	Shelled shrimps	I	1/2b	5.94	+	720
	S6	Shelled shrimps	I	4ab	4.40	+	720
	S7	Shelled shrimps	I	1/2b	5.79	+	720
	S8	Shelled shrimps	I	1/2b	5.08	+	720
	S9	Shelled shrimps	II	1/2a	6.31	+	720
	S19	Seafood	III	4b	7.20	−	720
Listeria innocua	ATCC33090	Reference	ND	6a	>8	−	986
	AB2497	Reference	ND	6a	>8	−	986
	90001	Reference	ND	ND	ND	−	986
	1603	Reference	ND	ND	ND	−	986
Listeria ivanovii	Li01	Reference	ND	5	ND	+	1357
	AB2496	Reference	ND	5	ND	+	1357
Listeria welshimeri	C15	Reference	ND	ND	ND	−	1363
	AB1626	Reference	ND	ND	ND	−	1363
	AB2500	Reference	ND	ND	ND	−	1363
Listeria seeligeri	ATCC35967	Reference	ND	ND	ND	−	1108
Listeria grayi subsp. *grayi*	Li08	Reference	ND	ND	ND	−	479
L. grayi subsp. *murrayi*	Li07	Reference	ND	ND	ND	−	479

LD_{50}, 50% lethal dose; ND, not determined.

Table 2. Details of Primers Used in the Study

(A) Primers used in the sections DNA manipulations, Excision mutagenesis by homologous recombination, and Food-related sample analysis of this article

Target region	*Forward primer 5′–3′*	*Reverse primer 5′–3′*	*Amplicon size (bp)*	*Optimal annealing temperature (°C)*
lmo0038	TTGAAAAACACGCTGGTTGCT	CCAACCACTTCACAGTTTGGA	967(or absent)	62
lmo0036-0041 (lineage I)	GGGTGATTCCGTCTGTTC	TAATAAAATCCACCAGGCAC	6500(or absent)	56
lmo0036-0041 (lineage II)	GGGTGATTCGGTCTGTTC	GGTAATAAAATCCACCAGGC	6500(or absent)	56
lmo0035-0042	TGATTTAGAAGCGTTTATGCA	TTTCCAAACCAGCTATCTTCT	V[a]	55
lmo0029-0042	TTATTCCTGATATTTCACAGG	TTTCCAAACCAGCTATCTTCT	V[a]	55
lmo0038-ab	ATAAGCTTCAAATTTAGTGTCTTGGGCA	ATGATGTTCACTCCTTACAATCGTTCTC	554	62
lmo0038-cd	AAGGAGTGAACATCATGAATCAAAAAAT	TAGAATTCAACGCCACTATCAACCAATT	574	62
lmo0038-ef	GGGGGCAGCAATTCCGACTTC	ACCCCACCTCCACCAGCAGA	1241	62
16S rRNA	TAGCGGTGAAATGCGTAGAT	TTACAAACTCTCGTGGTGTGAC	739	60
23S rRNA	ATAGCGTGCCTTTTGTAGAATG	AGCGCTCCACCAGTCCTT	689	60
hly	GTTGCAAGCGCTTGGAGTGAA	ACGTATCCTCCAGAGTGATGG	420	58
mpl	CAAGGACAGCTTAGGATTAC	TTCTTATTCGCCCATCTCGC	886	56
inlB	ATCACTTTCTTTGGAGCATAATGGT	GCCATCATCACTTATTATTTCTGGA	394	58
lmo0733	TTAAAGCAATCAGAAAATCAAAAG	TCCGCGTTAGAAAAATTCCA	483	57

[a]Species variable size (for details see text).

(B) Primers used for multiplex PCR in the section Assessment of multiplex PCR specific for *Listeria monocytogenes* lineages I and II of this article

Primers	*Target region*	*Sequence 5′–3′*	*Amplicon size (bp)*
LM (F)	*L. monocytogenes-iap*	CAAACTGCTAACACAGCTACT	720
LN (F)	*Listeria innocua-iap*	ACTAGCACTCCAGTTGTTAAAC	986
LS (F)	*Listeria seeligeri-iap*	TACACAAGCGGCTCCTGCTCAAC	1108
LGU (F)	*Listeria grayi-iap*	CCTGCGAAACCAGCAGTTTCT	479
LVSW (F)	*Listeria ivanovii, Listeria welshimeri, L. seeligeri-iap*	TAACTGAGGTAGCGAGCGAA	1357–1366
Ld (R)	Genus *Listeria-iap*	TTATACGCGACCGAAGCCAAC	403
lmo0038-1	*L. monocytogenes-lmo0038, L. ivanovii-i-lmo0038*	TTTCAATTATGTACGCCCAGGTGT	
lmo0038-2		AATATTTCCGCCGCCAAGTAA	

F, forward primer; R, reverse primer.

insertion into vectors. The recombinant plasmids were confirmed by PCR amplification and restriction digestion. Positive clones were then sequenced by dideoxy method on ABI-PRISM 377 DNA sequencer.

Excision mutagenesis by homologous recombination

Homologous recombination strategy was adopted to construct the Δ*lmo0038* deletion mutant using the reference strain EGD-e as the parent strain (Wiedmann *et al.*, 1998). Primer pairs lmo0038-a/lmo0038-b and lmo0038-c/lmo0038-d were used to generate fragments of 554 and 574 bp from flanking regions of *lmo0038*. The two products were gel purified for splicing by overlap extension PCR using external primer pair lmo0038-a/lmo0038-d. The final 1113-bp product containing *Eco*RI and *Hin*dIII was digested and ligated to pUC18, yielding the recombinant plasmid pUC18-Δlmo0038. After sequencing confirmation, the inserted fragment was subcloned into the thermosensitive shuttle vector pKSV7, which was then electrotransformed into the competent *L. monocytogenes* cells (Camilli *et al*., 1993). Electrotransformants were grown at nonpermissive temperature (41°C) in BHI containing chloramphenicol (10 μg/mL) to screen for chromosomal integration, and the cointegrates were passaged successively in BHI without antibiotic selection at a permissive temperature (30°C) to facilitate isolation of derivatives in which integrated plasmids had resolved by a second recombination event. The recombinants were identified as chloramphenicol-sensitive colonies and confirmed by PCR with primers lmo0038-e/lmo0038-f (external to lmo0038-a/lmo0038-d).

Stress response phenotype assays

Stationary-phase bacterial cultures were inoculated into 5 mL BHI and incubated with constant shaking (150 rpm) at 37°C. For thermal stress, cultures of the wild and mutant strains at the mid-log phase were shifted to water bath at 52°C. Acid stress was tested at 37°C by resuspending the mid-log phase cultures in BHI at pH 4.0 or 5.0. Changes of the cell population were determined by plate counting on 20-min basis for 80 min (pH 4.0 and 52°C water bath) and hourly basis for 4 h (pH 5.0). These tests were run in triplicate for three times. Survival rates were calculated and analyzed using two-tailed Student's *t*-test.

Virulence assays

The 50% lethal dose (LD_{50}) of the isolates was estimated in the mouse model as described previously (Jiang *et al.*, 2007).

Assessment of multiplex PCR specific for L. monocytogenes *lineages I and II*

To test the specificity of primers for the multiplex PCR (Table 2B), amplification from the template DNA of strains belonging to the six *Listeria* species as well as other bacterial species was performed in the optimized reaction conditions. The PCR reaction mixture contained 3 μL of buffer [200 mM Tris-HCl (pH 9.0), 100 mM KCl, 20 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, and 1% Triton X-100], 0.6 μL of dNTPs (10 mM), 0.6 μL of each primer (5 μM) on the primer list (Table 2B), 0.8 μL of *Taq* DNA polymerase (2 U/μL), and milli-Q water to a final volume of 28 μL, and 2 μL template DNA of a particular strain. The reaction mixtures were subjected to a hot start (95°C for 3 min) before 25 cycles of amplification (94°C for 45 s, 62°C for 30 s, and 72°C for 1 min) with a final extension (72°C for 5 min).

A 10-fold dilution series of fresh overnight cultures were examined by the multiplex PCR. The bacterial numbers in the dilution series were enumerated by plate counting on BHI agar plates. PCR detection limit was determined according to the lowest bacterial numbers that were sufficient to visualize specific band(s) after amplification.

Food-related sample analysis

The food-related samples collected from Zhejiang and Fujian provinces in eastern China were inoculated on *Listeria* selective agar plates (CHROMAgar, Listeria, Paris, France) to select colonies suspected as *Listeria*. Approximately 50 individual colonies from plates were subjected to phenotyping using the API system (Biomerieux, Marcy l'Etoile, France). Single representative colonies were pure- cultured in BHI, and 1 mL of enriched cultures was taken for DNA extraction

and tested by multiplex PCR. Confirmation tests include, when necessary, sequencing of 16S rRNA, 23S rRNA, and *iap* as well as serotyping, virulence assay, and independent PCRs targeting *L. monocytogenes*-specific genes, including *hly*, *mpl*, *inlB*, and *lmo0733* (Liu, 2006; Zeng *et al.*, 2006; Chen *et al.*, 2009c).

Nucleotide sequence accession numbers

Sequences of *lmo0038* and its adjacent regions of *L. monocytogenes* and *L. ivanovii* strains have been deposited in GenBank with accession numbers EF392667 through EF392669, EU082216 through EU082221, and EU099294 and EU099295.

Results and Discussion

The lmo0038 *gene is specific for pathogenic* Listeria *species*

Thirty-two strains covering six *Listeria* species and six major *L. monocytogenes* serovars (1/2a, 1/2b, 1/2c, 4a, 4b, and 4c) were screened for existence of *lmo0038* by specific PCR using primer set lmo0038 or lmo0036-0041 (Table 2A). The two pathogenic species, *L. monocytogenes* and *L. ivanovii*, contained this gene with 84.2–84.7% and 96.2–96.7% identities at nucleotide and amino acid levels, respectively, and all lineage I and II *L. monocytogenes* serovars tested (serovars 1/2b and 4b of lineage I, and 1/2a and 1/2c of lineage II) harbored highly conserved *lmo0038* with similarities of 96.8–100% at nucleotide level and 98.6–100% at amino acid level (Tables 1 and 3). They displayed higher sequence identities at the intralineage level than at the interlineage level (Table 3). However, lineage III strains (serovars 4a, 4c, and some 4b) and nonpathogenic species *L. innocua*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* lacked this gene (Table 1). BLAST search identified functional domains of *lmo0038* similar to those of a member of the peptidylarginine deiminase family that are involved in stress responses and exist in other Gram-positive or Gram-negative bacteria (e.g., *Pseudomonas aeruginosa*, *E. faecalis*, *Pediococcus pentosaceus*, *Streptococcus*, *Lactococcus*, and *Shewanella*) (Degnan *et al.*, 2000; Gruening *et al.*, 2006).

The long-distance PCR using primer pairs lmo0035(P1)/lmo0042(P3) (Fig. 1A) led to 7538-bp products from *L. monocytogens* strains of lineages I and II, and *L. ivanovii*, and 876-bp products from *L. innocua*, but no products from other species (Fig. 1B). Interestingly, lineage III strains gave the same product size (876 bp) as *L. innocua* (Fig. 1B), among which *L. monocytogenes* NICPBP54006 exhibited 80.1% nucleotide identity to *L. innocua* ATCC33090. In another PCR with primer pairs lmo0029(P2)/lmo0042(P3), 15391-bp amplicons were generated as profile I for *L. monocytogenes* lineages I and II, and *L. ivanovii*, and 8735-bp amplicons as profile II for *L. innocua* and *L. monocytogenes* lineage III strains lacking the *lmo0036–lmo0041* region. Doumith *et al.* (2004) and Volokhov *et al.* (2007) speculated that *L. monocytogenes* and *L. innocua* shared a common ancestry, and *L. innocua* probably evolved from its close relative *L. monocytogenes*. Moreover, *L. monocytogenes* lineage III might constitute the identifiable link

Table 3. Nucleotide (Upper Right Half) and Amino Acid (Bottom Left Half) Identities for *lmo0038* of *Listeria monocytogenes* Strains (Serovars 1/2a, 1/2b, 1/2c, and 4b) and *Listeria ivanovii* Strains

	Strain	*%*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
L. monocytogenes	EGD-e	1		99.3	98.8	97.6	98.8	98.8	97.5	97.5	97.4	84.3	84.5
serovar 1/2a	10403S	2	99.7		99.5	97.1	99.5	99.5	97.0	97.0	96.9	84.4	84.6
	NICPBP54003	3	99.7	99.7		96.8	99.8	99.8	96.7	96.7	96.6	84.2	84.4
serovar 1/2b	SLCC2755	4	98.6	98.6	98.9		96.8	96.8	99.9	99.9	99.8	84.5	84.7
serovar 1/2c	NCTC5438	5	99.7	99.7	100	98.9		100	96.7	96.7	96.6	84.2	84.4
	NICPBP54002	6	99.7	99.7	100	98.9	100		96.7	96.7	96.6	84.2	84.4
serovar 4b	F2365	7	98.6	98.6	98.9	100	98.9	98.9		99.8	99.9	84.6	84.8
	F2024	8	98.6	98.6	98.9	100	98.9	98.9	100		99.9	84.4	84.6
	NICPBP54007	9	98.6	98.6	98.9	100	98.9	98.9	100	100		84.5	84.7
L. ivanovii	Li01	10	96.2	96.2	96.4	96.7	96.4	96.4	96.7	96.7	96.7		99.8
serovar 5	AB2496	11	96.2	96.2	96.4	96.7	96.4	96.4	96.7	96.7	96.7	100	

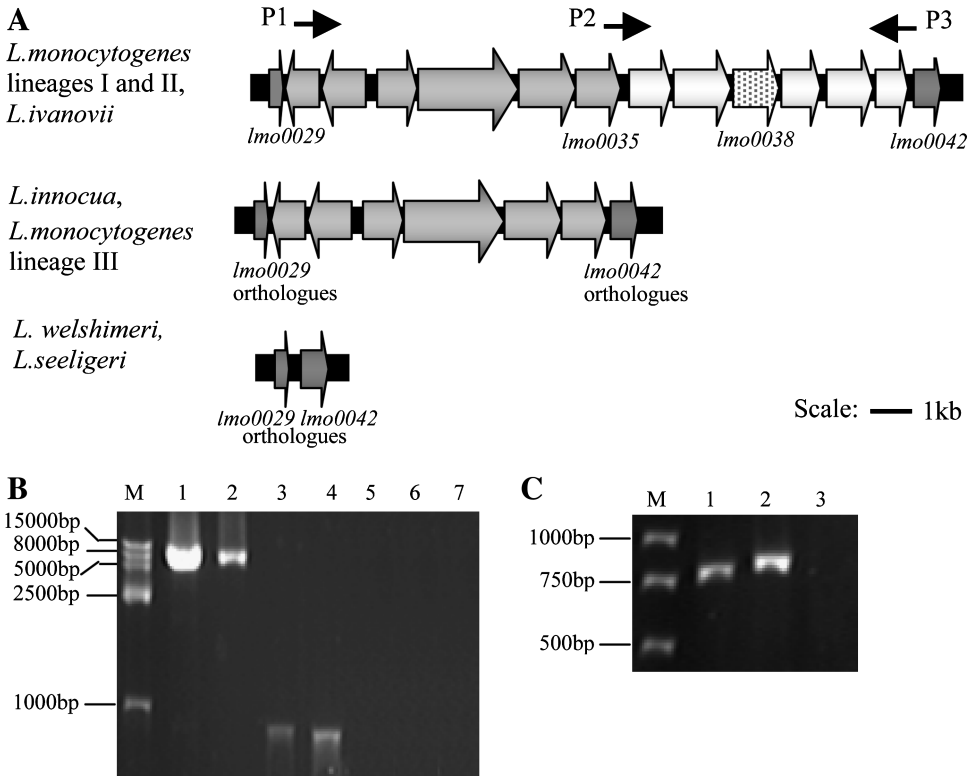

FIG. 1. Genomic organization of the *lmo0038* gene region. (**A**) Comparative analysis of *lmo0029–0042* region and its orthologs in *Listeria* species. *L. monocytogenes* lineages I and II, and *L. ivanovii* harbor the intact set of genes in this region, while *L. monocytogenes* lineage III and *L. innocua* lack *lmo0036–lmo0041*. Such deletion extends to the neighboring genes of the same locus in *L. seeligeri* and *L. welshimeri* genomes. Arrows P1, P2, and P3 indicate primers lmo0029, lmo0035, and lmo0042. (**B**) Amplification of *lmo0035–0042* and its orthologs by long-distance PCR methodology. Lanes: M, molecular weight standard; 1, *L. monocytogenes* EGD-e; 2, *L. ivanovii* Li01; 3, *L. innocua* ATCC33090; 4, *L. monocytogenes* NICPBP54006; 5, *L. seeligeri* ATCC35967; 6, *L. welshimeri* C15; 7, *L. grayi* Li08. (**C**) Amplification of *lmo0029–0042* and its orthologs by long-distance PCR. Lanes: M, molecular weight standard; 1, *L. welshimeri* C15; 2, *L. seeligeri* ATCC35967; 3, *L. grayi* Li08.

between *L. monocytogenes* lineages I and II and *L. innocua* in the evolutionary chain (Doumith *et al.*, 2004; Volokhov *et al.*, 2007; Chen *et al.*, 2009a). Not surprisingly, *L. monocytogenes* lineage III and *L. innocua* displayed some similar genetic arrangements. *L. seeligeri* and *L. welshimeri* fell into profile III of approximately 750 bp with extended reduction of the *lmo0030–lmo0035* region, while *L. grayi* did not harbor any detectable genomic relic in this locus (Fig. 1C). These results indicate that while *lmo0038* was conserved in pathogenic *Listeria* genomes (including *L. monocytogenes* serovars 1/2a, 1/2b, 1/2c, and 4b, and *L. ivanovii*), this gene was absent in nonpathogenic *Listeria* species, and was specifically absent from *L. monocytogenes* lineage III, which was seldom associated with human morbidity (Fig. 1A).

Lmo0038 *is involved in stress responses and virulence potential*

The *lmo0038* mutant, designated EGD-Δlmo0038, was created by deleting the full *lmo0038* sequence of EGD-e. Because *L. monocytogens* is well known for its tolerance to a wide spectrum of temperatures (0–45°C) and extremes of pH conditions (Gandhi and Chikindas, 2006), EGD-Δlmo0038 and its parent strain EGD-e were tested for growth and survival at low pH and 52°C to determine the possible functions that *lmo0038* might have in allowing *L. monocytogenes* to adapt to such stress conditions. When grown at 37°C in BHI at pH 5.0, the mutant had significantly slower growth than the parent strain from hour 2, and much slower at hour 4 (Fig. 2A; $p < 0.05$).

EGD-Δlmo0038 was more sensitive to the acid condition at pH 4.0 (as shown by 65–77% survival) than its parent strain having the survival rate remained unchanged during the 80-min incubation ($p < 0.01$; Fig. 2B). Heat shock stress (52°C) significantly reduced the survival rate of EGD-Δlmo0038 (2.9 log unit decrease in CFU/mL at 60 min) in comparison with its parent strain (1.1 log unit decrease at 60 min; Fig. 3; $p < 0.01$). In addition, both the mutant and parent strains exhibited similar patterns of slow growth at refrigeration temperature (4°C) as examined by turbidimetric method for 1 week (data not shown).

Lmo0038 has domains similar to the peptidylarginine deiminase family involved in the arginine deiminase (ADI) pathway, which has been implicated in bacterial resistance to acidic environments (Degnan *et al.*, 2000; Gruening *et al.*, 2006) and characterized in a number of bacteria (e.g., *P. aeruginosa*, *Strep. suis*, *Strep. pyogenes*, *Streptococcus gordonii*, *E. faecalis*, and *Lactococcus lactis*) (Bourdineaud *et al.*, 1993; Barcelona-Andres *et al.*, 2002; Dong *et al.*, 2002; Budin-Verneuil *et al.*, 2006). The ADI system could catabolize 1 mol of arginine into 2 mol of ammonia, 1 mol of ATP as well as ornithine and carbon dioxide. Ammonia could combine with intracellular cytoplasmic protons to produce ammonium ions ($NH4^+$), thereby increasing intracellular pH and maintaining pH homeostasis. ATP could be used for microbial growth under a variety of environmental conditions or, alternatively, to extrude protons via the F_0F_1ATPase, which had been shown to play a role in pH homeostasis in *Listeria* (Higuchi *et al.*, 1997; Cotter *et al.*, 2000). However, the listerial ADI system did not seem to be as effective in combating the deleterious effects of low pH conditions as the glutamate decarboxylase system (Cotter *et al.*, 2001). Interestingly, Lmo0038 in *L. monocytogenes* was also responsible for heat shock responses, and the mechanism remained unknown.

The ADI system had been implicated in the pathogenesis of *Strep. pyogenes* and *Strep. suis* (Degnan *et al.*, 1998, 2000; Benga *et al.*, 2004). Our study revealed the role for Lmo0038 in listerial pathogenesis using a murine model. Deletion of *lmo0038* resulted in approximately 10-fold increase in LD_{50} compared to the wild-type strain

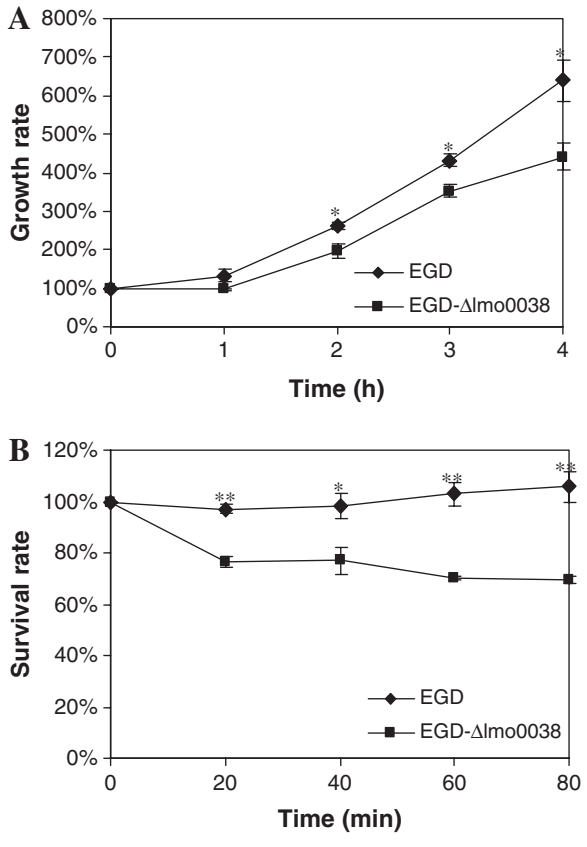

FIG. 2. Survival of *Listeria monocytogenes* strains EGD-e and EGD-Δlmo0038 in brain heart infusion under acid conditions at pH 5.0 (**A**) and pH 4.0 (**B**). These tests were done in triplicate in each run and repeated for three times. Error bars indicate standard deviations. *$p < 0.05$; **$p < 0.01$.

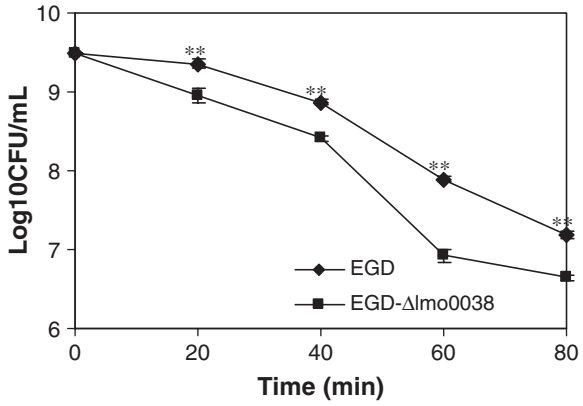

FIG. 3. Effect of heat shock (52°C) on survival of *Listeria monocytogenes* strains EGD-e and EGD-Δlmo0038 for 80 min on 20-min basis. The tests were done in triplicate in each run and repeated for three times. Error bars indicate standard deviations. *$p < 0.05$; **$p < 0.01$.

(log LD_{50} 7.67 for EGD-Δlmo0038 and 6.64 for EGD-e). Arginine is a precursor of nitric oxide, a reactive nitrogen species that plays an important role in host defense against intracellular pathogens, including *L. monocytogenes*. Therefore, reduced arginine in the host cells led to a reduced generation of nitric oxide, and hence a diminished ability to mount an effective immune response to *L. monocytogenes* infection (MacMicking *et al.*, 1997; Bogdan *et al.*, 2000). This experiment represented the first documentation of the contribution of Lmo0038 to bacterial virulence *in vivo*.

Specific PCR identification of Listeria *species and* L. monocytogenes *lineages*

Based on the conserved region of *lmo0038*, primer pair lmo0038-1/lmo0038-2 was designed to amplify a specific 403-bp fragment from chromosomal DNA of *L. monocytogenes* lineages I and II and *L. ivanovii* (lanes 1 and 3, Fig. 4). To further differentiate the species within pathogenic and nonpathogenic groups, *iap* migration profile was optimized by a set of forward primers (LM, LN, LVSW, LGU, and LS) and one common reverse primer (Ld) in relation to the hypervariable internal fragment and conserved portion at the 3′ end of *iap* (Table 2B). Though two fragments of *L. seeligeri-iap* were yielded at 1366 and 1108 bp, respectively, by primer pairs LVSW/Ld and LS/Ld, only the 1108-bp fragment was obtained from *L. seeligeri* when primers LVSW, LS, and Ld were used in combination, allowing differentiation between *L. seeligeri* and *L. welshimeri*. While *L. monocytogenes* lineages I and II led to two bands (403 and 720 bp) regardless of serovars, only *L. monocytogenes* lineage III yielded a 720-bp *iap* fragment. Another pathogenic species, *L. ivanovii*, was identified by the concurrence of the 403-bp and 1357-bp PCR products, and *L. innocua*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* (including *L. grayi* subsp. *grayi* and subsp. *murrayi*) were identified by the 986-bp, 1108-bp, 1363-bp, and 479-bp products, respectively (Fig. 4). Hence, this multiplex PCR procedure based on *lmo0038* and competitive *iap* amplification could identify *Listeria* species and distinguish *L. monocytogenes* lineage III from those of lineages I and II simultaneously on putative colonies from selective agar plates (Tables 1 and 4). Sequencing of these amplicons confirmed the accuracy of this PCR assay (data not shown). Some other G^{+}_{lowGC} bacterial species phylogenetically related to *Listeria*, including *B. cereus*, *B. subtilis*, *E. faecalis*, *Staph. aureus*, *Strep. pyogenes*, *Strep. suis*, and *Strep. equi* (Kalin *et al.*, 2004), did not show any amplicons by this PCR. Further, this procedure was able to detect as low as 1.0–9.0×10^2 CFU/mL of listerial cells of different species (detection limits of *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, *L. grayi* subsp. *Grayi*, and *L. grayi* subsp. *murrayi* were 1.0×10^2 CFU/mL, 1.7×10^2 CFU/mL, 1.0×10^2 CFU/mL, 1.1×10^2 CFU/mL, 9.0×10^2 CFU/mL, 4.8×10^2 CFU/mL, and 4.0×10^2 CFU/mL, respectively).

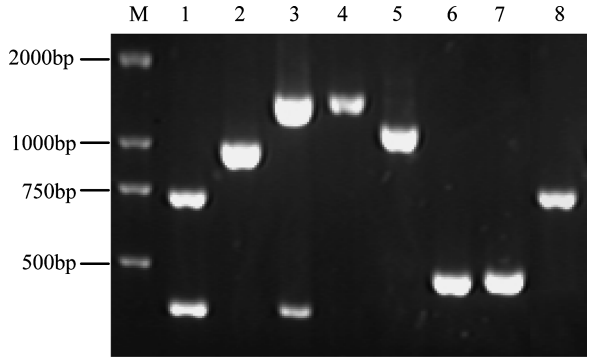

FIG. 4. Differentiation of *Listeria* species and *L. monocytogenes* pathogenicity by multiplex PCR. Lanes: M, molecular weight standard; 1, *L. monocytogenes* EGD-e; 2, *L. innocua* ATCC33090; 3, *L. ivanovii* Li01; 4, *L. welshimeri* C15; 5, *L. seeligeri* ATCC35967; 6, *L. grayi* subsp. *grayi* Li08; 7, *L. grayi* subsp. *murrayi* Li07; 8, *L. monocytogenes* NICPBP54006.

TABLE 4. SEROVAR COMPOSITIONS AND PCR PROFILES OF 86 FOOD-RELATED *LISTERIA MONOCYTOGENES* ISOLATES

Lineage	*Serovar*	*No. (%)*	lmo0038	iap *Amplicon size (bp)*
I	1/2b (or 3b)	22 (25.6%)	+	720[a]
	4b (or 4d, 4e)	6 (7.0%)	+	720
II	1/2a (or 3a)	42 (48.8%)	+	720
	1/2c (or 3c)	14 (16.2%)	+	720
III	4a	1 (1.2%)	−	720
	4b (or 4c)[b]	1 (1.2%)	−	720

[a]One *L. monocytogenes* serovar 1/2b isolate C17 harbored truncated *iap* gene (620 bp).

[b]The *L. monocytogenes* serovar 4b isolate S19 belonging to lineage III might be misclassified by conventional serotyping methods (Liu *et al.*, 2006b).

Identification of Listeria *food-related isolates by multiplex PCR*

Out of all food-related samples from Zhejiang and Fujian provinces in eastern China, 119 isolates were preliminarily suspected as *Listeria* spp., based on colony morphology on *Listeria* selective agar plates. One hundred fifteen isolates showed migration profiles matching those of *Listeria* reference strains as assessed by multiplex PCR developed in this study, allowing easy identification at the species level. *L. monocytogenes* isolates appeared predominant (74.8%, 86/115), which is in agreement with previous reports (Nakamura *et al.*, 2004; Jallewar *et al.*, 2007). *L. innocua* came second (23.5%, 27/115), followed by one *L. seeligeri* isolate and one *L. welshimeri* isolate, but no *L. ivanovii* and *L. grayi*. In 86 *L. monocytogenes* isolates, serovar 1/2a (or 3a) predominated (48.8%, 42/86), followed by serovars 1/2b (or 3b) (25.6%, 22/86), 1/2c (or 3c) (16.2%, 14/86), and 4b (or 4d, 4e) (7.0%, 6/86), while lineage III only accounted for 2.4% (2/86) (Table 4). The serovar compositions of these Chinese food isolates were similar to those of French food isolates (Hong *et al.*, 2007), and the low prevalence of lineage III strains in food-related samples was also consistent with previous reports (Norton *et al.*, 2001; Jiang *et al.*, 2008). In addition, sequencing of 16S rRNA and 23S rRNA revealed that three non-*Listeria* isolates were *E. faecalis*, and the other was *Aerococcus viridians* (data not shown).

API system was also applied for these 115 *Listeria* isolates and revealed 95.7% agreement with PCR results. Discrepancy occurred on the *L. monocytogenes*–*L. innocua* clade and *L. seeligeri*–*L. welshimeri* clade: four isolates supposed to be *L. monocytogenes* by API showed *L. innocua* PCR profile, and one isolate supposed to be *L. seeligeri* by API exhibited *L. welshimeri* PCR profile. Hemolysis test and sequencing of *iap*, 16S rRNA and 23S rRNA supported the results obtained by multiplex PCR (data not shown).

Among these *L. monocytogenes* isolates, amplicons of three isolates were remarkable. Isolate C17 from chicken produced shorter *iap* fragment (approximately 620 bp) than normal one (720 bp), and isolates M7 from pasteurized milk and S19 from seafood lacked *lmo0038* fragment (Table 1). *L. monocytogenes*–specific genes *hly*, *mpl*, *inlB*, and *lmo0733* were present in all these isolates (data not shown). Isolate S19 was determined as serovar 1/2b and exhibited intermediate pathogenicity (log LD_{50} 5.83; Table 1), though deletion in the repeat domain of *iap* including 15 TN units and PSK motif was observed in comparison with that of EGD-e (Bubert *et al.*, 1992), while isolate M7 was determined as serovar 4a and S19 as 4b (lineage III) with lower pathogenicity than other 20 randomly selected *L. monocytogenes* isolates and reference strains in the mouse model (log LD_{50} ranging from 3.86–6.80, Table 1). Long-distance PCRs targeting *lmo0035–0042* and *lmo0029–0042* confirmed the absence of *lmo0038* in this locus. Notably, the *L. monocytogenes* serovar 4b strains belonging to lineage III (e.g., F2-525 and S19) may be misclassified by conventional serotyping methods, and is likely of serovar 4c in the lineage subgroup IIIA (Liu *et al.*, 2006b; Chen *et al.*, 2009c).

In conclusion, the previously uncharacterized *lmo0038* is involved in survival under stress conditions that may be encountered in foods or during infection in the host. Moreover, this gene is specific to *L. monocytogenes* lineages I and II and *L. ivanovii*, and could be explored as the attractive target to separate pathogenic from nonpathogenic *Listeria* species and distinguish *L. monocytogenes* lineage III strains from other lineages. The novel one-step molecular assay targeting *lmo0038* and *iap* could be used for rapid and reliable identification of *Listeria* species and *L. monocytogenes* lineages from food samples, processing environments, or even clinical samples.

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Disclosure Statement

No competing financial interests exist.

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