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Listeria monocytogenes Serovar 4a is a Possible Evolutionary Intermediate Between *L. monocytogenes* Serovars 1/2a and 4b and *L. innocua*

Chen, Jianshun[1], Lingli Jiang[1], Xueyan Chen[1], Xiaokai Luo[1], Yang Chen[1], Ying Yu[1], Guoming Tian[1], Dongyou Liu[2], and Weihuan Fang[1]*

[1]*Zhejiang University Institute of Preventive Veterinary Medicine, and Zhejiang Provincial Key Laboratory of Prevent Veterinary Medicine, Hangzhou, China*
[2]*College of Veterinary Medicine, Mississippi State University, Mississippi, MS 39762 U.S.A.*

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The genus *Listeria* consists of six closely related species and forms three phylogenetic groups: *L. monocytogenes*-*L. innocua*, *L. ivanovii*-*L. seeligeri*-*L. welshimeri*, and *L. grayi*. In this report, we attempted to examine the evolutionary relationship in the *L. monocytogenes*-*L. innocua* group by probing the nucleotide sequences of 23S rRNA and 16S rRNA, and the gene clusters *lmo0029*-*lmo0042*, *ascB*-*dapE*, *rplS*-*infC*, and *prs*-*ldh* in *L. monocytogenes* serovars 1/2a, 4a, and 4b, and *L. innocua*. Additionally, we assessed the status of *L. monocytogenes*-specific *inlA* and *inlB* genes and 10 *L. innocua*-specific genes in these species/serovars, together with phenotypic characterization by using *in vivo* and *in vitro* procedures. The results indicate that *L. monocytogenes* serovar 4a strains are genetically similar to *L. innocua* in the *lmo0035*-*lmo0042*, *ascB*-*dapE*, and *rplS*-*infC* regions and also possess *L. innocua*-specific genes *lin0372* and *lin1073*. Furthermore, both *L. monocytogenes* serovar 4a and *L. innocua* exhibit impaired intercellular spread ability and negligible pathogenicity in mouse model. On the other hand, despite resembling *L. monocytogenes* serovars 1/2a and 4b in having a nearly identical virulence gene cluster, and *inlA* and *inlB* genes, these serovar 4a strains differ from serovars 1/2a and 4b by harboring notably altered *actA* and *plcB* genes, displaying strong phospholipase activity and subdued *in vivo* and *in vitro* virulence. Thus, by possessing many genes common to *L. monocytogenes* serovars 1/2a and 4b, and sharing many similar gene deletions with *L. innocua*, *L. monocytogenes* serovar 4a represents a possible evolutionary intermediate between *L. monocytogenes* serovars 1/2a and 4b and *L. innocua*.

Keywords: *Listeria*, serovar, phylogeny, evolution, pathogenicity

*Corresponding author
Phone: +86-571-8697-1242; Fax: +86-571-8697-1242;
E-mail: whfang@zju.edu.cn

The genus *Listeria* encompasses six closely related species: *L. monocytogenes. L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, and *L. grayi* [3]. Based upon different genotypic and phenotypic analyses, listeriae can be divided into three major phylogenetic clusters: *L. monocytogenes*-*L. innocua*, *L. ivanovii*-*L. seeligeri*-*L. welshimeri*, and *L. grayi* [14, 15, 32]; 16 serovars (with *L. monocytogenes* consisting of serovars 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7; *L. ivanovii* of serovar 5; and other *Listeria* species of serovars 1/2a, 6a, 6b, and 4c); and three genetic lineages (with lineage I covering serovars 1/2b, 3b, 4b, 4d, and 4e; lineage II containing serovars 1/2a, 1/2c, 3a, and 3c; and lineage III including serovars 4a, 4c, and possibly 7) [1, 20].

Although species within the *Listeria* genus resemble each other morphologically, ecologically, biochemically, and genetically, they show varied pathogenic potential. *L. monocytogenes* is a well-recognized intracellular pathogen of man and animals, *L. ivanovii* infects mainly ruminants, and four other species (*L. seeligeri*, *L. innocua*, *L. welshimeri*, and *L. grayi*) have no pathogenic inclination [4, 37]. Of the 13 *L. monocytogenes* serovars, four (4b, 1/2a, 1/2b, and 1/2c) are responsible for over 98% of human listeriosis cases whereas other serovars (*e.g.*, 4c and 4a) are seldom implicated in listeriosis [12]. Heterogeneity in *L. monocytogenes* pathogenicity has also been observed in mouse models, with serovars 1/2a and 4b being highly virulent and serovar 4a being naturally of low pathogenicity [1, 22].

Interestingly, naturally low virulent *L. monocytogenes* serovar 4a appears to be similar to avirulent *L. innocua* in terms of shared antigenic and genetic structures as well as low virulence [16, 29]. For instance, both *L. monocytogenes* serovar 4a and *L. innocua* have the same flagellar antigen structures [7]. *L. innocua* lacks the *inlGHE* gene cluster, which is also absent in *L. monocytogenes* serovar 4a [5]. However, *L. innocua* differs from *L. monocytogenes* serovar 4a in that it does not harbor the virulence gene cluster [or

Listeria pathogenicity island I (LIPI-1)] and several internalin genes (*e.g.*, *inlA* and *inlB*). This suggests that apart from horizontal gene transfer, deletion of key virulence-associated genes in the genome of a common *Listeria* ancestor may lead to subsequent changes in the corresponding phenotypes including pathogenicity [2, 5, 13].

Nonetheless, the phylogenetic relationship among various *L. monocytogenes* serovars and *L. innocua* are far from clear on the basis of studies on the virulence-associated genes such as *prfA*, *hly*, *plcB*, and internalin genes. To gain new phylogenetic insights into the *L. monocytogenes*-*L. innocua* group, we analyzed the nucleotide sequences of 23S rRNA and 16S rRNA and the gene clusters *lmo0029*-*lmo0042*, *ascB*-*dapE*, *rplS*-*infC*, and *prs*-*ldh* in *L. monocytogenes* serovars 1/2a, 4a, and 4b and *L. innocua*. The rationales to focus on these gene regions were based on the previous observations that the *lmo0029*-*lmo0042* gene region is lost stepwise between *L. monocytogenes* and *L. innocua* [13], and that the PrfA-regulated virulence gene cluster is present in *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri*, but absent in *L. innocua*, *L. welshimeri*, and *L. grayi* [11]. In addition, we assessed the status of *L. monocytogenes*-specific *inlA* and *inlB*, and 10 *L. innocua*-specific genes in these species/serovars, along with phenotypic characterization by using *in vivo* and *in vitro* procedures. These polyphasic approaches have proven fruitful in recent delineation of *Listeria* species and characterization of atypical *L. innocua* and *L. seeligeri* strains [18, 32, 38–40].

Materials and Methods

Bacterial Strains

A total of 32 *Listeria* strains were examined in this study (Table 1). These included 25 *L. monocytogenes* strains/isolates, four of which came from reference collections, and 21 were isolated from food products and processing plants and vessels [41]. In addition, two *L. innocua* (ATCC 33090 and AB2497), one *L. ivanovii* (Li01), one *L. welshimeri* (C15), one *L. seeligeri* (ATCC 35967), and two *L. grayi* (Li07 and Li08) strains were acquired from reference collections (Table 1). *Listeria* strains were refreshed from glycerol stocks maintained at −80°C and cultured on tryptic soy agar plates with 7% sheep blood, followed by growth in brain heart infusion broth (BHI; Oxoid, Hampshire, England) at 37°C.

Mouse Virulence Assay

The virulence potential of 25 *L. monocytogenes* and one *L. innocua* (ATCC 33090) strains was assessed in accordance with a previously reported protocol [17]. Briefly, female ICR mice at 20–22 g (Zhejiang College of Traditional Chinese Medicine, Hangzhou, China) were allowed to acclimatize for 3 days. Five groups of mice (six per group) were inoculated intraperitoneally with 0.2-ml aliquots of appropriately diluted *Listeria* strain resuspended in phosphate-buffered saline (PBS, 0.01 M, pH 7.2). Mice in the control group were injected with 0.2 ml of PBS. The LD_{50} values were calculated by using the trimmed Spearman-Karber method on the basis of mouse mortality data recorded during a 10-day post-injection period, and the relative virulence (%) of these strains was determined as described previously [19].

Plaque-Forming Assay

The ability of *L. monocytogenes* strains to form plaques on mouse fibroblasts L929 cells was assessed as described previously [16]. Cell monolayers were grown to 80% confluence in 2 ml of DMEM containing 10% fetal bovine serum in 6-well plates (Corning, U.S.A.). The overnight *Listeria* cultures were centrifuged and resuspended in PBS. For each strain tested, one well was infected with 5×10^5 CFU and the other was infected with 1.5×10^5 CFU. Upon 1-h incubation at 37°C, the cell monolayers were washed three times with PBS and overlaid with 3 ml of DMEM containing 20 μg/ml gentamicin and 1.4% agarose (Oxoid Ltd., Hampshire, England). Following a 3-day incubation at 37°C, a second 2-ml overlay of DMEM containing 0.02% neutral red solution and 1.4% agarose was added. After a final day of incubation, plaques were photographed by a digital camera. The diameters of 25 plaques were measured using Adobe Photoshop software for each strain. The plaque size of reference strain 10403S was set at 100%.

Assays for Hemolytic and Phospholipase Activities

Hemolytic activity of *Listeria* strains was assayed in sheep blood agar plates as previously described [8]. To titrate the hemolytic activity, supernatant from *Listeria* BHI broth cultures was serially diluted by 2-fold in a 96-well V-bottom microplate with saline (8.5 g/l NaCl). An equal volume of sheep red blood cells in saline was added to each well and the microplates were incubated at 37°C for 1 h. The hemolytic titer of each *Listeria* strain is expressed as the reciprocal of the corresponding dilution of the supernatant required to lyse 50% of the erythrocytes in triplicate wells [16]. Phospholipase activity of *Listeria* strains was examined with the egg yolk assay of Ermolaeva *et al*. [6] without charcoal activation. The BHI agar plates were supplemented with 5% fresh egg yolk suspension in saline. *Listeria* cultures were streaked onto the plates and incubated at 37°C for 48 h, with *L. ivanovii* Li01 being applied as the positive control displaying an opacity zone surrounding the streak [9].

PCR

One ml of each *Listeria* broth culture was transferred to an Eppendorf tube and centrifuged at 12,000 ×*g* for 3 min. The cell pellet was washed twice with milli-Q water (Millipore China Ltd, Beijing, China) and then resuspended in TZ buffer (2% Triton X-100, 2.5 mg/ml NaN_3, and Tris-HCl, pH 8.0). After boiling for 10 min, the bacterial suspension was cooled on ice for 5 min and subsequently centrifuged at 12,000 ×*g* at 4°C for 1 min. The resulting supernatant was used as template DNA. The PCR mixture (in a volume of 30 μl) was made up of 3 μl of 10×PCR 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, custom synthesized by Invitrogen Biotechnology Co. Ltd., Shanghai, China), 0.8 μl of *Taq* DNA polymerase (2 U/μl; TaKaRa Biotech Co. Ltd., Dalian, China), and milli-Q water to a final volume of 28 μl, and 2 μl template DNA. To amplify products larger than 4 kb, *LA Taq* DNA polymerase (TaKaRa) was utilized. The reaction mixtures were subjected to a hot start at 95°C for 3 min prior to 25 cycles of amplification, with a final extension at 72°C for 5 min in a thermal cycler (MJ Research Inc., Boston, MA, U.S.A.). The annealing temperatures varied with specific primer pairs (Supplementary Table 1), and the duration of extension depended on the length of amplicons (1 min per kb, at 72°C). The PCR-

Table 1. Characteristics of *Listeria* strains used in this study.

Strain	Serovar	Source	Hemolytic titer	Relative size of plaque (%)[a]	Mouse mortality (dead/tested)[b]	Relative virulence[c]	$logLD_{50}$[d]
L. monocytogenes EGD	1/2a	Reference strain	2^2	ND	11/30	36.6%	6.64
10403S	1/2a	Reference strain	2^2	100±0	18/30	60%	5.49
NICPBP54006	4a	Reference strain	2^2	0	1/30	3.3%	8.35
NICPBP54007	4b	Reference strain	2^2	ND	11/30	36.6%	6.79
mLm3	4b	Raw milk	2^3	108.3±5.8	28/30	93.3%	3.86
mLm4	4a	Pasteurized milk	2^3	0	2/30	6.6%	8.14
mLm10	1/2a	Pasteurized milk	2^2	95.7±13.1	18/30	60%	5.55
fLm1	1/2a	Beef	2^2	96.3±1.2	14/30	46.6%	6.26
fLm2	1/2b	Pork chops	2^2	88.8±1.3	13/30	43.3%	6.45
fLm3	1/2a	Raw pork	2^2	98.3±3.4	15/30	50%	6.07
fLm4	1/2c	Vegetable	2^2	85.0±1.3	15/30	50%	6.11
fLm5	1/2b	Chicken	2^1	92.0±1.5	16/30	53.3%	5.83
eLm1	1/2a	Seafood plant sewage	2^3	103.7±7.8	18/30	60%	5.53
eLm2	1/2b	Milk plant vessel	2^2	102.8±8.2	12/30	40%	6.46
eLm3	1/2b	Milk plant sewage	2^2	83.7±0.4	12/30	40%	6.43
eLm4	1/2b	Milk plant sewage	2^2	97.0±0.7	13/30	43.3%	6.32
eLm5	1/2a	Milk plant vessel	2^2	89.3±2.3	18/30	60%	5.45
sLm1	4b	American red drum	2^2	84.5±3.9	11/30	36.6%	6.74
sLm2	1/2c	American red drum	2^1	92.0±0.6	14/30	46.6%	6.19
sLm3	4b	American red drum	2^2	85.6±4.5	11/30	36.6%	6.72
sLm4	1/2b	Shelled shrimps	2^2	102.3±3.5	16/30	53.3%	5.94
sLm5	4b	Shelled shrimps	2^2	90.1±0.7	25/30	83.3%	4.40
sLm6	1/2b	Shelled shrimps	2^2	91.9±3.1	17/30	56.6%	5.79
sLm7	1/2b	Shelled shrimps	2^2	100.4±2.2	21/30	70%	5.08
sLm8	1/2a	Shelled shrimps	2^2	98.8±1.4	13/30	43.3%	6.31
L. innocua ATCC 33090	6a	Reference strain	$<2^0$	0	0/30	0%	ND
AB2497	6a	Reference strain	$<2^0$	ND	ND	ND	ND
L. ivanovii Li01	5	Reference strain	2^4	ND	ND	ND	ND
L. welshimeri C15		Reference strain	$<2^0$	ND	ND	ND	ND
L. seeligeri ATCC 35967		Reference strain	2^1	ND	ND	ND	ND
L. grayi Li07		Reference strain	$<2^0$	ND	ND	ND	ND
Li08		Reference strain	$<2^0$	ND	ND	ND	ND

[a] The plaque size of reference strain 10403S was set at 100%.
[b] *Listeria* strain was inoculated i.p. into five groups of six female ICR mice at concentrations of 10^8, 10^7, 10^6, 10^5, and 10^4 colony forming units, and mouse mortality data were collected during a 10-day period post-injection. ND, not done.
[c] Relative virulence (%) was calculated by dividing the number of dead mice with the number of mice tested followed by multiplying with 100 [19].
[d] The trimmed Spearman-Karber method was used to calculate the LD_{50} from the mouse mortality data.

amplified products were electrophoresed on 1.0% agarose gel in the presence of ethidium bromide (0.5 μg/ml) and visualized under UV transillumination. The *L. monocytogenes lmo0029-lmo0042* cluster (and its equivalent in other *Listeria* strains) and three *L. monocytogenes*-specific internalin gene clusters (*inlAB*, *inlC*, and *inlGHE*) were amplified with primers targeting their flanking genes (*i.e.*, *lmo0029*/*lmo0042*, *F0470*/*F0473*, *rplS*/*infC*, and *ascB*/*dapE*). The full-length sequences of LIPI-1 between *prs* and *ldh* were covered by five fragments in separate PCRs. In addition, primers were derived from *L. innocua*-specific genes *lin1073*, *lin1074*, *lin1068*, *lin0198*, *lin0372*, *lin0419*, *lin2454*, *lin2693*, *lin0464*, and *lin0558* [10] for sequence comparison among *Listeria* species (Supplementary Table 1).

Cloning and Sequencing of PCR Products

PCR fragments were purified by using the AxyPrep DNA Gel Extraction Kit (Axygen Inc., U.S.A.) and inserted by T-A cloning strategy into the pMD18-T vector (TaKaRa). The recombinant plasmids were introduced into *Escherichia coli* DH5α and confirmed by PCR and restriction digestion with EcoRI and HindIII. The positive clones were selected and sequenced by the dideoxy method on an ABI-PRISM 377 DNA sequencer.

Genome Walking

Additional primers for genome walking were designed from the gene regions whose sequences became available in the study. Nested PCR was performed by using the TaRaKa Genome Walking Kit in accordance with the procedures recommended by the manufacturer.

Phylogenetic Analysis

Deduced amino acid sequences of the ORFs under investigation were aligned by ClustalX software (version 1.8). The corresponding nucleotide sequences were then trimmed and aligned [32]. Phylogenetic and molecular analyses were undertaken by using the Molecular Evolutionary Genetics Analysis software (MEGA version 3.0) (http:/

/www.megasoftware.net). Phylogenetic trees were constructed and compared by using neighbor-joining (NJ), maximum parsimony (MP), minimum evolution (ME), and UPGMA methods [17, 36]. The robustness of the branching pattern was tested by bootstrap analyses through 1,000 replications.

GenBank Accession Numbers

Forty-five nucleotide sequences covering the genes of *Listeria* strains examined in this study have been deposited in GenBank (Accession Nos. EF392667 to EF392669, EF690661 to EF690672, EU073135 to EU073161, and EU444834 to EU444836) (Supplementary Table 2).

Results

Virulence to Mice

Whereas 23 *L. monocytogenes* strains belonging to serovars 1/2a, 1/2b, 1/2c, and 4b displayed intermediate to high pathogenicity in ICR mice *via* intraperitoneal inoculation (with mortality ranging from 11–28 out of 30 mice tested, relative virulence from 36.3% to 93.3%, and log LD_{50} from 3.86 to 6.79), the other two *L. monocytogenes* strains belonging to serovar 4a (NICPBP54006 and mLm4) exhibited minimal pathogenicity (with mortality between 1 and 2 out of 30 mice tested, relative virulence between 3.3 and 6.6%, and log LD_{50} >8) (Table 1). *L. innocua* ATCC 33090 caused no mortality in the murine intraperitoneal model (Table 1).

Plaque-Forming Ability

In the plaque-forming assay based on L929 cells, 23 *L. monocytogenes* strains belonging to serovars 1/2a, 1/2b, 1/2c, and 4b formed clear plaques with the relative size varying from 83.7% to 108.3%, whereas *L. monocytogenes* serovar 4a strains NICPBP54006 and mLm4 and *L. innocua* ATCC 33090 showed impaired intercellular spread ability (Table 1), which was consistent with the virulence assessment in the mouse model.

Hemolytic and Phospholipase Activities

L. monocytogenes serovar 4a strains (NICPBP54006 and mLm4) displayed potent hemolytic activity on sheep blood

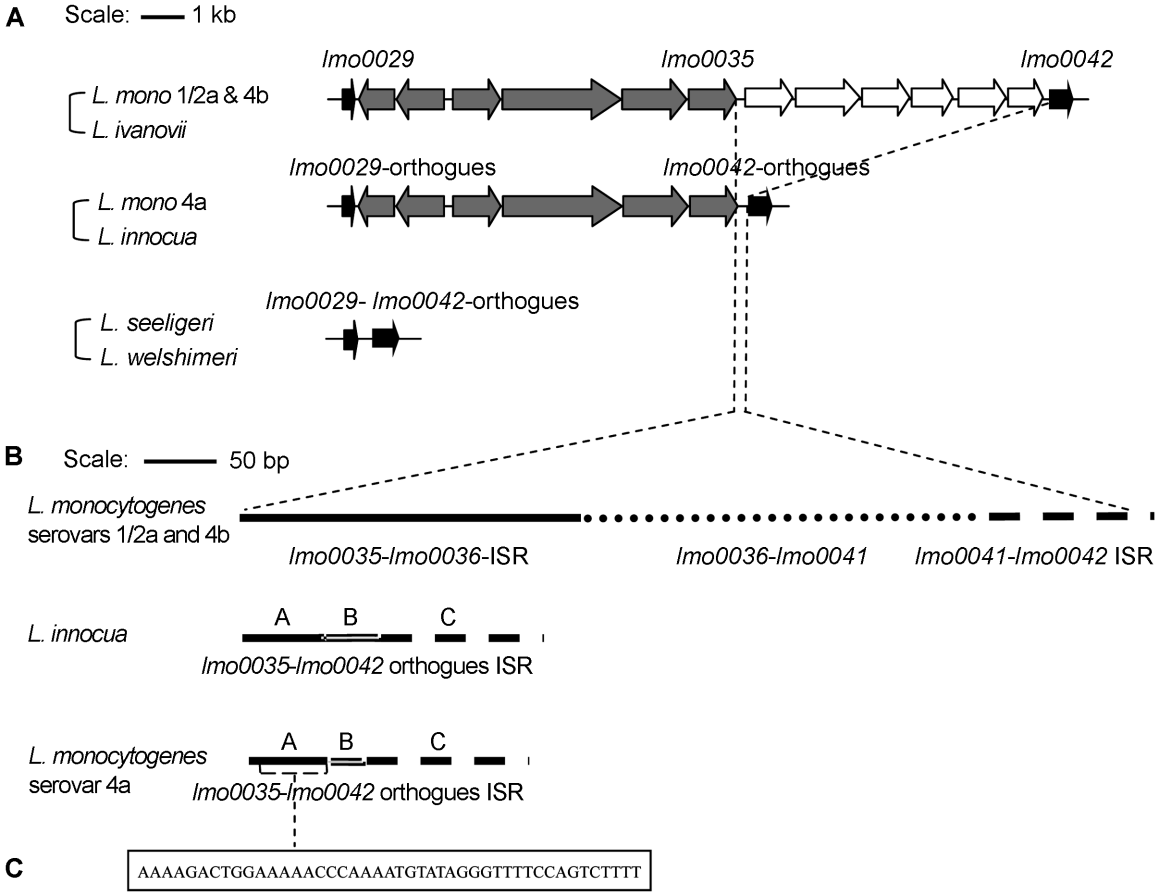

Fig. 1. A. Genetic structures of the *L. monocytogenes lmo0029-0042* region and its orthologs in *Listeria* species. **B**. Genetic organization of the *lmo0035-lmo0042* intergenic spacer region (ISR) of *L. monocytogenes* serovars 1/2a and 4b in relation to those of *L. monocytogenes* serovar 4a and *L. innocua*. The *lmo0035-lmo0042* ISR of *L. monocytogenes* serovar 4a contains three segments from different origins (see text for details). **C**. Alignment of segment A of the *lmo0035-lmo0042* ISR with putative insertion junctions.

Table 2. Comparison of nucleotide sequences in the *lmo0029-0042* locus among *Listeria* species.

Strain	Length (bp)	Nucleotide identity (%)			
		L. monocytogenes		*L. innocua* CLIP11262	*L. welshimeri* SLCC5334
		EGD (1/2a)	F2365 (4b)		
L. monocytogenes 54007 (4b)	15,391	95.3	98.4	85.3	82.6
L. monocytogenes 54006 (4a)	8,735	87.8	88.5	89.2	78.0
L. monocytogenes mLm4 (4a)	8,735	87.6	88.4	89.1	78.1
L. innecua ATCC33090	8,735	85.9	85.8	99.6	77.5
L. welshimeri C15	1,189	82.6	82.2	76.8	98.6
L. seeligeri ATCC35967	1,189	80.8	80.1	72.4	89.1

agar plates and hemolytic titers (from 2^2 to 2^3) in the 96-well plates, similar to *L. monocytogenes* serovars 1/2a, 1/2b, 1/2c, and 4b strains (from 2^1 to 2^3) (Table 1). Among the non-*monocytogenes Listeria* species tested, *L. ivanovii* had a hemolytic titer of 2^4, *L. seeligeri* a hemolytic titer of 2^1, and *L. innocua*, *L. welshimeri*, and *L. grayi* a hemolytic titer of 2^0 (Table 1). On the other hand, *L. monocytogenes* serovar 4a strains (NICPBP54006 and mLm4) demonstrated strong phospholipase activity with a definite zone of opacity surrounding the streak, whereas *L. monocytogenes* serovars 1/2a, 1/2b, 1/2c, and 4b lacked phospholipase activity in the artificial medium without charcoal activation (data not shown).

Genetic Organization of the *lmo0029-lmo0042* Locus

The *lmo0029-lmo0042* locus of *L. monocytogenes* serovars 1/2a and 4b as well as *L. ivanovii* amounted to 15,391 bp in length; *L. monocytogenes* serovar 4a strains (NICPBP54006 and mLm4) along with *L. innocua* possessed a much shortened *lmo0029-lmo0042* locus (measuring only 8,735 bp), with the *lmo0036-lmo0041* region missing; *L. seeligeri* and *L. welshimeri* exhibited an extended reduction in the *lmo0030-lmo0035* region (measuring only 1,189 bp) (Fig. 1A and Table 2). *L. monocytogenes* strain NICPBP54007 (serovar 4b) demonstrated 95.3% and 98.4% nucleotide identities to EGD (serovar 1/2a) and F2365 (serovar 4b), respectively, in the *lmo0029-lmo0042* locus (Table 2). On the other hand, *L. monocytogenes* serovar 4a strains NICPBP54006 and mLm4 showed a higher nucleotide similarity to *L. innocua* CLIP11262 (89.2% and 89.1%) than to *L. monocytogenes* EGD-e (serovar 1/2a) and F2365 (serovar 4b) (87.6–88.5%) in the *lmo0029–lmo0042* locus (Table 2). The *lmo0035–lmo0042* intergenic spacer region (ISR) in *L. monocytogenes* serovar 4a was composed of three segments from different origins (Fig. 1B). Segment A showed 68.8–75% nucleotide identities to the 5' end of the *lmo0035–lmo0036* ISR in *L. monocytogenes* 1/2a and 4b, and 80.7% identity to the corresponding region in *L. innocua* CLIP11262 (Table 3). The segment A also contained repeat sequences (AAAAG-ACTGGAAAAACCCWAWA) (Fig. 1C), which were putative transposon insertion junctions possibly involved in the loss of the *lmo0036–lmo0041* region. This putative transposon-related structure was also present in *L. monocytogenes* serovars 1/2a and 4b and *L. ivanovii*. Segment C exhibits 83.7–83.8% identities to the 3' end of the *lmo0041–lmo0042* ISR in *L. monocytogenes* serovars 1/2a and 4b, and 81.4% identity to that in *L. innocua* CLIP11262 (Table 3). However, it appeared that segment B is unique to *L. monocytogenes* serovar 4a and *L. innocua* (Fig. 1B). Similarly, transposon-related structures were observed within the *lmo0029-lmo0030* ISR in *L. monocytogenes*, *L. innocua*, and *L. ivanovii* (data not shown).

Diversity in *inlC* and *inlG(C2)H(D)E* Gene Clusters

The internalin genes, including *inlA*, *inlB*, *inlC*, *inlG*, *inlH(C2)*, *inlD*, and *inlE*, are scattered in *L. monocytogenes* genomes and contribute to its virulence [10, 28]. The *inlC*

Table 3. Comparison of *L. monocytogenes* serovar 4a segments in the *lmo0035-0042* intergenic spacer regions (ISR) to corresponding fragments (see Fig. 2B for details) in *L. monocytogenes* EGD (1/2a), F2365 (4b), and NICPBP54007 (4b), and *L. innocua* CLIP11262 (6a).

L. monocytogenes 4a segment[a]	Length (bp)	Nucleotide identity (%)			
		CLIP11262 (6a)	EGD (1/2a)	F2365 (4b)	54007 (4b)
A	54	80.7	75.0	72.9	68.8
B	22[b]	32.5	–[c]	–[c]	–[c]
C	112	81.4	83.7	83.8	83.8

[a] *L. monocytogenes* serovar 4a strains (NICPBP54006 and mLm4) share 100% identity in the *lmo0035-0042* ISR.
[b] Segment B of *L. innocua* is 37 bp long.
[c] Segment B of *L. monocytogenes* serovar 4a and *L. innocua* has no ortholog in *L. monocytogenes* 1/2a and 4b.

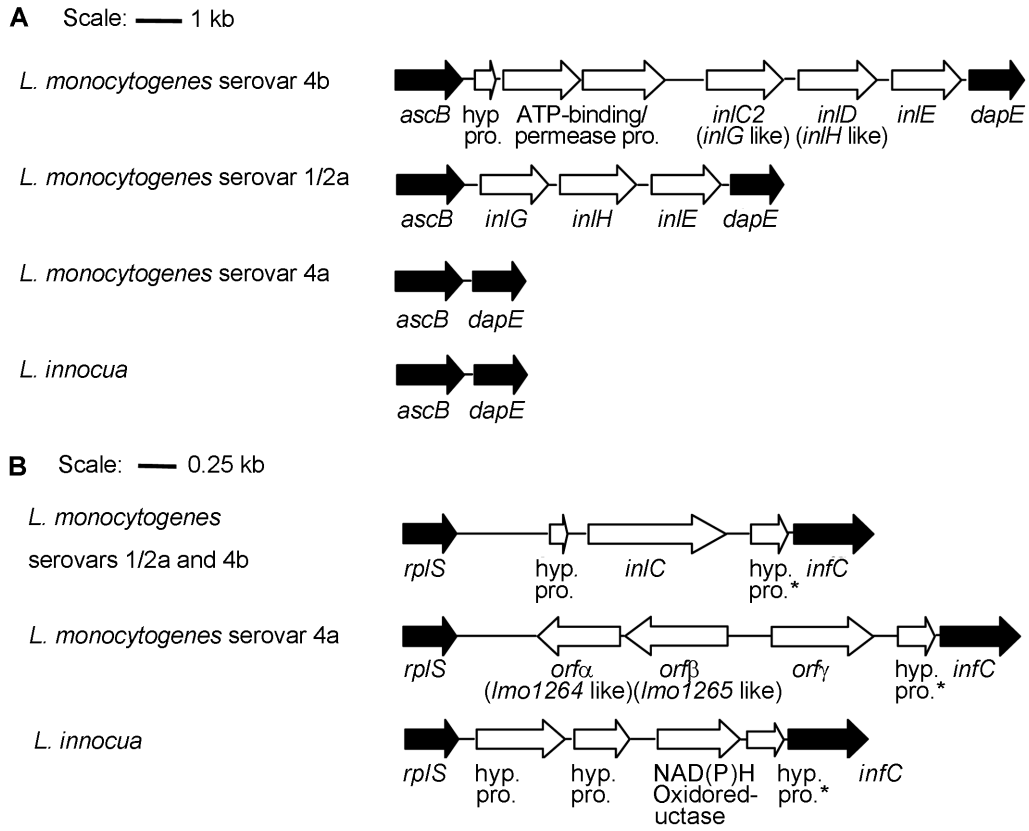

Fig. 2. A schematic diagram of chromosomal regions carrying *L. monocytogenes* internalin genes and the corresponding loci in other *Listeria* species.
The diversity of genetic contents (in white arrows) is delimited by conserved housekeeping genes (in black arrows). **A**. Region between *ascB* and *dapE*. **B**. Region between *infC* and *rplS*. The gene adjacent to *infC* encoding a putative protein is designated with an asterisk, which exhibits conservation among *L. monocytogenes* serovars 1/2a, 4b, and 4a and *L. innocua*.

and *inlG(C2)H(D)E* gene clusters were present in the respective chromosomal regions of *rplS-infC* and *ascB-dapE* in *L. monocytogenes* serovars 1/2a and 4b, but absent in *L. monocytogenes* serovar 4a and *L. innocua* (Fig. 2A). In the locus between *rplS* and *infC*, *inlC* and two unknown genes encoding hypothetical proteins were present in *L. monocytogenes* serovars 1/2a and 4b (Fig. 2B). In *L. innocua*, there existed four genes between *rplS* and *infC*, one resembling an NAD(P)H oxidoreductase and three encoding hypothetical proteins, two of which were specific to this species and the other is similar to that in *L. monocytogenes* serovars 1/2a and 4b (Fig. 2B). *L. monocytogenes* serovar 4a also harbored four ORFs in this region, with one (*hyp.pro*, for hypothetical protein) being similar to *L. monocytogenes* serovars 1/2a and 4b (97.9% nucleotide identity) and *L. innocua* (98.8% nucleotide identity) (Fig. 3B and Table 4), and three others (*orfα*, *orfβ*, and *orfγ*) being different from those in *L. monocytogenes* serovars 1/2a and 4b and *L. innocua* (Fig. 2B). Interestingly, *orfα* and *orfβ*, though in reverse transcription orientation (Fig. 2B), were similar (81.5–83.9%) to the *lmo1264* and *lmo1265* genes in *L. monocytogenes* EGD, the *F1281* and *F1282* genes in *L. monocytogenes* F2365, and the *lin1303* and *lin1304* genes in *L. innocua* CLIP11262 (Table 4), which were nearly 570 kb away from the *rplS-infC* locus. In addition, *orfγ* and the junction region between *rplS* and *orfα* appeared to be unique to *L. monocytogenes* serovar 4a with no homolog being recognized in *L. monocytogenes* serovars 1/2a and 4b or other bacterial species *via* BLAST search. Furthermore, the *inlA* and *inlB* genes existed between *F0470* and *F0473* in *L. monocytogenes* serovar 4a, which exhibited 91.5–97% nucleotide identities to those of EGD, 10403S, and F2365; and they were absent in other *Listeria* species (data not shown).

Comparison of rRNA Gene Sequences

Based on the full-length 16S and 23S rRNA gene sequences (GenBank Accession Nos. X92948-X92954) (Supplementary Table 2) [31], the genus *Listeria* can be divided into two major clusters: one covers *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, and *L. welshimeri*, showing nucleotide similarities between 99.5% and 99.9%; and the other consists of *L. grayi*. Among the five signature regions in the 23S rRNA gene sequences that demonstrated sufficient variation

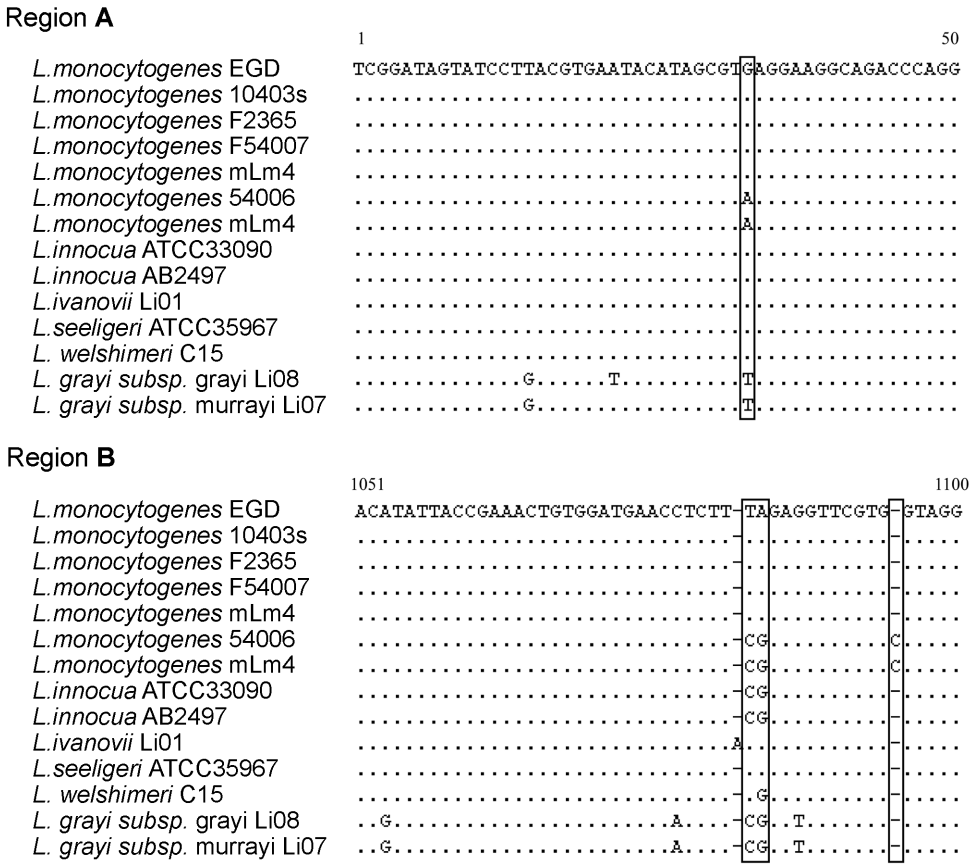

Fig. 3. The signature regions **A** and **B** of *Listeria* 23S rRNA gene sequences.
The sequence of *L. monocytogenes* EGD is used as the reference. Identical nucleotides are indicated by dots, whereas deletions are indicated by dashes. The boxes reveal the critical nucleotide divergence within the genus *Listeria.*

for inter- and intraspecies differentiation of the genus *Listeria*, two (regions A and B) were applicable to the *L. monocytogenes*-*L. innocua* group (Fig. 3). In region A, *L. monocytogenes* serovar 4a differed from *L. monocytogenes* serovars 1/2a and 4b as well as other *Listeria* species by having an A at position 33 instead of G or T. In region B, apart from having a unique insertion of C at position 1,095, *L. monocytogenes* serovar 4a was similar to *L. innocua* by possessing CG at positions 1,083–1,084 instead of TA in *L. monocytogenes* serovars 1/2a and 4b, *L. ivanovii*, and *L. seeligeri*, and TG in *L. welshimeri* (Fig. 3).

Sequence Analysis of the Virulence Gene Cluster LIPI-1

Demarcated by *prs* and *ldh*, LIPI-1 harbors six virulence-associated genes. In the *plcB* and *ldh* intragenic spacer region, there are four additional small ORFs (*orfX*, *orfZ*, *orfB*, and *orfA*; in gray arrows). The *prs* and *orfZ* genes delineated the putative deletion points of LIPI-1 in *L. innocua* (Fig. 4). Apart from some sequence divergence being noted in the *actA* and *plcB* genes, other genes (*i.e.*, *prfA*, *plcA*, *hly*, and *mpl* in the virulence gene cluster and four small ORFs) located between *prs* and *ldh* were similar (94–99% and 82–96%, respectively) among *L. monocytogenes*

Table 4. Comparison of the *orfα*, *orfβ*, and *hyp.pro* (hypothetical protein) genes in *L. monocytogenes* serovar 4a to those in *L. monocytogenes* serovars 1/2a and 4b and *L. innocua.*

L. monocytogenes serovar 4a gene	*L. monocytogenes* EGD (1/2a)			*L. monocytogenes* F2365 (4b)			*L. innocua* CLIP11262		
	Ortholog	Identity (%)		Ortholog	Identity (%)		Ortholog	Identity (%)	
		54006	mLm4		54006	mLm4		54006	mLm4
orfα	*lmo1264*	81.6	81.6	*F1281*	83.9	83.9	*lin1303*	81.8	81.8
orfβ	*lmo1265*	81.9	82.1	*F1282*	81.9	82.1	*lin1304*	81.7	81.5
hyp.pro.	*hyp.pro.*	97.9	97.9	*hyp.pro.*	97.9	97.9	*hyp.pro.*	98.8	98.8

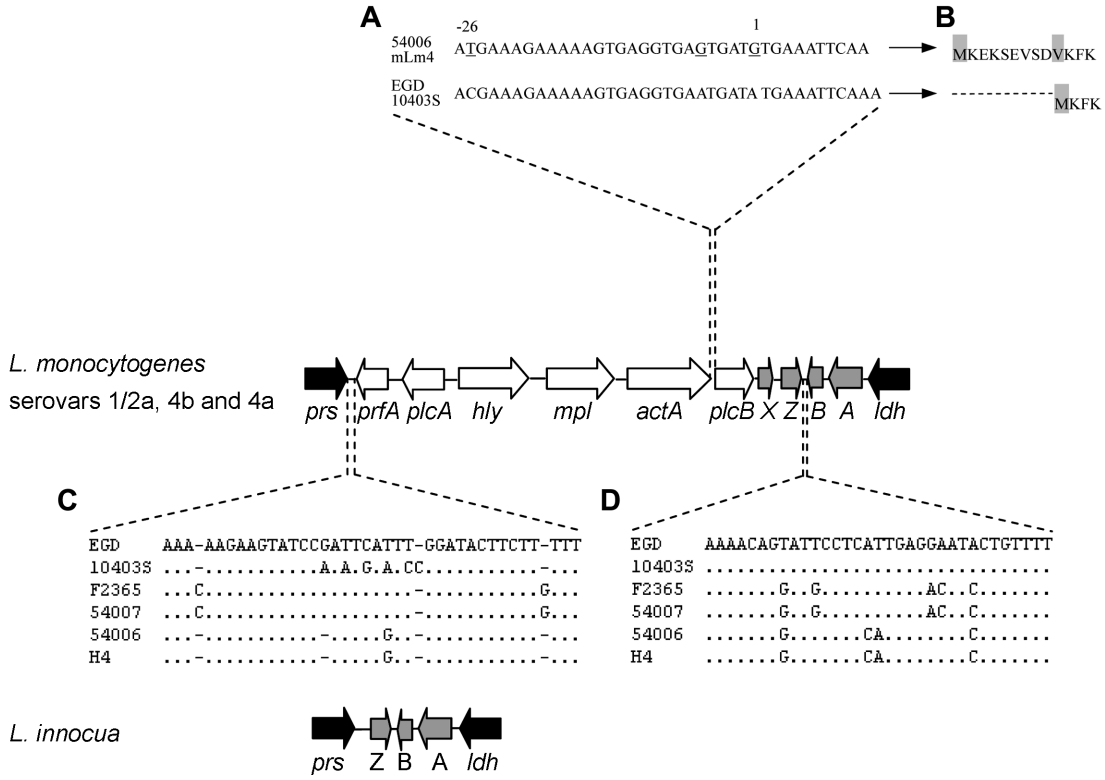

Fig. 4. Analysis of DNA sequences of the virulence gene cluster (LIPI-1).
Specific nucleotide substitution in the *L. monocytogenes* serovar 4a *plcB* gene (**A**) introduces the start codon shift of the ORF (**B**). Alignments of the *prs-prfA* intergenic spacer region (ISR) (**C**) and *orfZ-orfB* ISR (**D**) of *L. monocytogenes* strains reveal putative transposon insertion junctions. In (**C**) and (**D**), identical nucleotides are indicated by dots, whereas deletions are indicated by dashes using *L. monocytogenes* EGD-e sequence as reference.

serovars 4a, 1/2a, and 4b (Supplementary Table 3). It was noteworthy that the *actA* gene of *L. monocytogenes* serovars 4a (NICPBP54006 and mLm4) and 4b (F2365) examined in this study harbored a 105-nucleotide deletion as compared with *L. monocytogenes* serovar 1/2a (EGD and 10403S), leading to reduction of 35 amino acids effectively removing two of the four proline-rich repeats (DFPPPPTDEEL), which were required for binding of the focal contact proteins VASP and Mena to stimulate actin-based motility. Significant mutations of the *plcB* gene in *L. monocytogenes* serovars 4a (NICPBP54006 and mLm4) were found at position 1 (A to G) and at position −26 (C to T) (Fig. 4A), which might introduce the start codon shift in the ORF (Fig. 4B). These changes might have resulted in more efficient operation of the *plcB* gene in *L. monocytogenes* serovars 4a in contrast to *L. monocytogenes* 1/2a and 4b strains, as assessed by *in vitro* phospholipase assay. Sequence alignments of the *prs-prfA* ISR and *orfZ-orfB* ISR in *L. monocytogenes* serovars 4a, 1/2a, and 4b revealed repeat sequences (Figs. 4C and 4D), which were similar to the structures within the *lmo0035-lmo0042* ISR and *lmo0029-lmo0030* ISR referred to above. Existence of such putative transposon insertion junctions implied the possibility of horizontal transfer of the virulence gene cluster [18].

Presence of *L. innocua*-Specific Genes in *L. monocytogenes* Serovar 4a

Among the 10 *L. innocua*-specific genes analyzed (Supplementary Table 1), *lin0372* and *lin1073* were detected in *L. monocytogenes* serovar 4a strains (NICPBP54006 and mLm4), but not in *L. monocytogenes* serovars 1/2a and 4b strains (data not shown). The *lin0372* gene sequence in *L. monocytogenes* serovar 4a strains NICPBP54006 (EU073154) and mLm4 (EU073155) shared a 92.1% nucleotide identity to that in *L. innocua* CLIP11262, whereas the *lin1073* gene sequence in these two strains (EU073152 and EU073153) exhibited 79.3% and 76.2% similarity, respectively, to that in *L. innocua* CLIP11262.

Phylogenetic Analysis of the *L. monocytogenes-L. innocua* Group

The housekeeping gene pairs, *lmo0029*/*lmo0042* (and their orthologs in other *Listeria* species), *ascB*/*dapE*, *rplS*/*infC*, and *prs*/*ldh*, flanking their respective variable regions were conserved at the nucleotide level (Supplementary Table 4). Whereas the *dapE* sequence in *L. monocytogenes* serovar 4a demonstrated a higher similarity to that in *L. innocua* (99%) than to that in *L. monocytogenes* serovars 1/2a and 4b (84.7–87.7%), *lmo0029*, *lmo0042*, *ascB*,

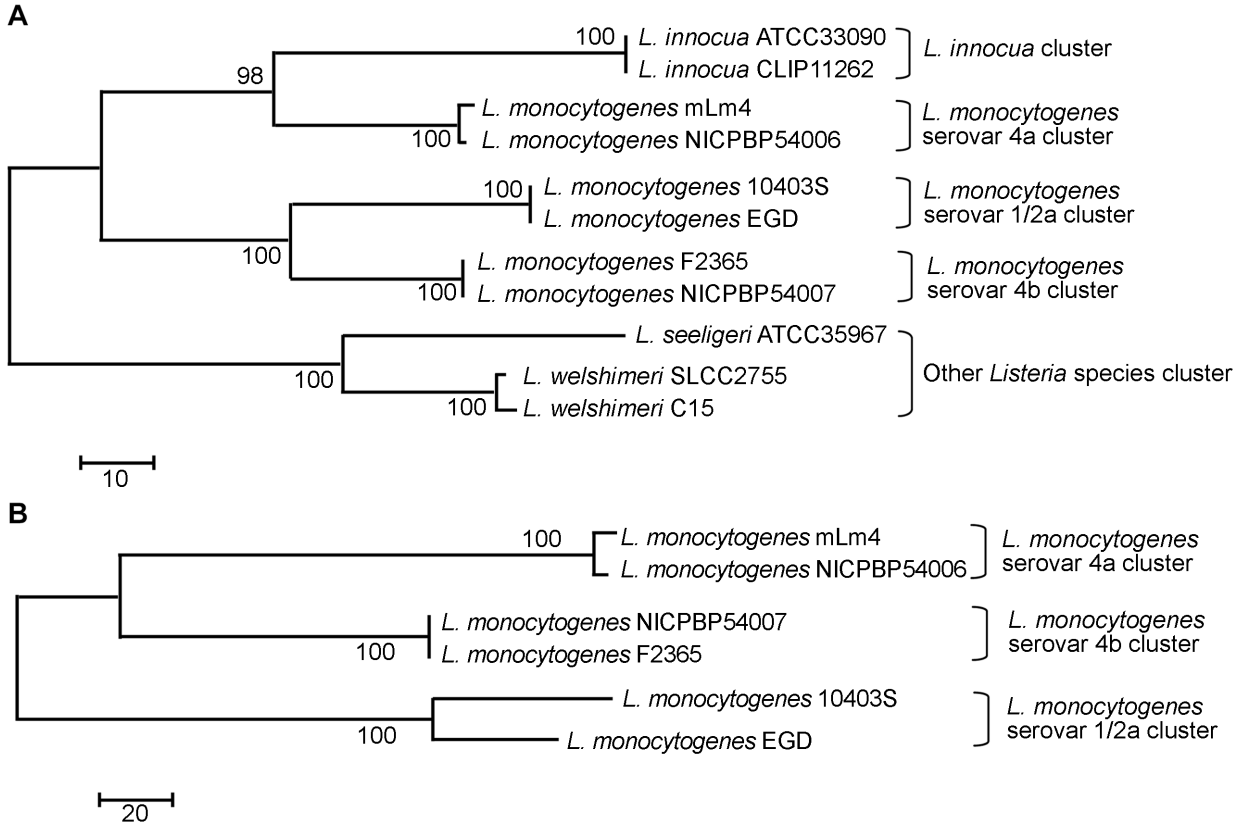

Fig. 5. A. Phylogenetic tree of *L. monocytogenes* serovars 4a, 1/2a, and 4b and other *Listeria* species based on the concatenated data set 23S-rRNA-16S-rRNA-*lmo0029-lmo0042-ascB-dapE-infC-rplS-prs-ldh*. **B**. Phylogenetic tree of selected *L. monocytogenes* serovars based on the concatenated data 23S-rRNA-16S-rRNA-*lmo0029-lmo0042-ascB-dapE-infC-rplS-prs-ldh-prfA-plcA-hly-mpl-actA-plcB-orfX-orfZ-orfB-orfA* including the virulence gene cluster. The values above and below the horizontal lines (expressed as percentages) indicate the robustness of the corresponding branches (which is rooted with *L. monocytogenes* serovar 1/2a strain EGD), as determined by a bootstrap analysis evaluated from 1,000 replications.

rplS, *infC*, *prs*, and *ldh* in *L. monocytogenes* serovar 4a exhibited comparable identities to those in *L. monocytogenes* serovars 1/2a and 4b as well as *L. innocua* (Supplementary Table 4). A composite phylogenetic tree was constructed on the basis of the nucleotide sequences of the concatenated 23S-rRNA-16S-rRNA-*lmo0029-lmo0042-ascB-dapE-infC-rplS-prs-ldh* gene cluster (Fig. 5A). Irrespective of the methods employed (including neighbor-joining, maximun-parsimony, minimum evolution, and UPGMA), *L. monocytogenes* serovar 4a and *L. innocua* were consistently placed as a sister branch, *L. monocytogenes* serovars 1/2a and 4b formed another, and other *Listeria* species occupied the most distinct branch (Fig. 5A). To further illuminate the evolutionary history among *L. monocytogenes* strains covering serovars 1/2a, 4b, and 4a, phylogenetic information in the above gene set as well as the 10 genes (*prfA-plcB-hly-mpl-actA-plcB-orfX-orfZ-orfB-orfA*) covering the whole LIPI-1 was combined. In this scheme, serovars 4a and 4b strains consistently fell into two closely related branches, whereas serovar 1/2a strains had a separate branch (Fig. 5B).

DISCUSSION

To gain further phylogenetic insights on the *L. monocytogenes-L. innocua* group, we comparatively examined the nucleotide sequences of the 23S rRNA and 16S rRNA and the gene clusters *lmo0029-lmo0042*, *ascB-dapE*, *rplS-infC*, and *prs-ldh* as well as *L. monocytogenes*-specific *inlA* and *inlB* and 10 *L. innocua*-specific genes in *L. monocytogenes* serovars 1/2a, 4a, and 4b and *L. innocua*. This was followed by assessment of *in vitro* hemolytic and lecithinase activities and *in vivo* and *in vitro* virulence of these strains. The results suggest that *L. monocytogenes* serovar 4a not only possesses many genetic sequences common to serovars 1/2a and 4b, but also shares some gene deletions and subdued *in vivo* and *in vitro* virulence with *L. innocua*, in addition to harboring a few *L. innocua* genes (*e.g.*, *lin0372* and *lin1073*).

Molecular Characteristics of *lmo0029-lmo0042, ascB-dapE*, and *rplS-infC* Clusters in *L. monocytogenes* Serovar 4a

One of the major findings in this study is the genetic divergences between pathogenic and low virulent *L. monocytogenes* serovars

in the *lmo0029-lmo0042* cluster and internalin clusters, including the *inlGHE* cluster between *ascB* and *dapE*, and *inlC* between *rplS* and *infC*. There is notable loss of the *lmo0036-lmo0041* locus in *L. monocytogenes* serovar 4a and *L. innocua* in relation to *L. monocytogenes* serovars 1/2a and 4b. *L. seeligeri* and *L. welshimeri* harbor a further reduced *lmo0030-lmo0035* locus (Fig. 1A and Table 2). This suggests that the *lmo0029-lmo0042* locus has been successively deleted from *L. monocytogenes* serovars 1/2a and 4b to form *L. monocytogenes* serovar 4a and *L. innocua*, and then *L. seeligeri* and *L. welshimeri*. Occurrence of repeat sequences (AAA-AGACTGGAAAAACCCWAWA) in the *lmo0035-lmo0042* ISR (Fig. 1C), which are also observed in the *prs-prfA* ISR and *orfZ-orfB* ISR flanking LIPI-1 (Figs. 4C and 4D), provides a reminder on the past events involving possible gene transfer and loss [25, 26].

Comparison of *L. monocytogenes* serovars 1/2a and 4b with *L. monocytogenes* serovar 4a and *L. innocua* in the *ascB-dapE* gene region also suggests a single deletion event involving *inlG*(C_2)*H*(*D*)*E* (Fig. 2A). The genetic contents between *rplS* and *infC* reveal significant heterogeneity in the *L. monocytogenes-L. innocua* group (Fig. 2B). The *rplS-infC* region of *L. monocytogenes* serovar 4a seems to have undergone a purge of genetic elements from *L. monocytogenes* serovars 1/2a and 4b: the conserved gene encoding hypothetical proteins adjacent to *infC* is maintained as vestige in this region, and other genes existing in ancestral *L. monocytogenes* strains, such as virulence-associated gene *inlC*, are lost in some descendant *L. monocytogenes* strains (which have evolved later into serovar 4a) and replaced by *orfα* and *orfβ*, the orthologs of *lmo1264* and *lmo1265*, as well as *orfγ*. The *orfγ* gene is unique to *L. monocytogenes* serovar 4a, with no significant homolog in *L. monocytogenes* serovars 1/2a and 4b or other bacterial species, providing clues on the role of DNA uptake in the evolution in the *L. monocytogenes-L. innocua* group. Unexpectedly, no long-repeat sequences or obvious remnants of transposable elements are observed in the close vicinity, and thus we consider that such foreign genes might have been integrated therein by illegitimate recombination, the mechanism of which is described in examples of the restriction-modification (RM) genes and *sly* regions of *Streptococcus suis* [33, 35].

Genetic Features of the Virulence Gene Cluster LIPI-1

The LIPI-1 gene cluster (encompassing *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB*) is found in *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri*, but nonexistent in *L. innocua*, *L. welshimeri*, and *L. grayi* [11]. The low virulent *L. monocytogenes* serovar 4a does contain all the genes within this particular cluster as is the case with the other serovars. The occurence of putative transposon insertion sequences at *prs-prfA* and *orfZ-orfB* flanking the virulence gene cluster suggests that this group of virulence genes might have been transferred horizontally [18]. Within this cluster, the *actA* gene appears to be susceptible for nucleotide alteration [22, 27, 34]. Whereas *L. monocytogenes* serovar 1/2a maintains a complete copy of the *actA* gene, serovars 4b and 4a sometimes harbor a deletion of 105 nucleotides in this gene (Supplementary Table 3). Removal of 105 nucleotides in *actA* in the serovar 4b clinical strain F2365, which was originated from the Jalisco cheese outbreak of 1985 in California [28], implies that a full-length *actA* gene may not be absolutely essential for listerial cell-to-cell spread and virulence as previously thought. Alternatively, *L. monocytogenes* serovar 4b strains with deletions in its *actA* gene may employ other uncharacterized mechanisms for its efficient spread to neighboring cells.

Low Virulence, Apparent Hemolytic Activity, and Phospholipase Activity of *L. monocytogenes* Serovar 4a

Listeria demonstrates both inter- and intraspecies variations in pathogenic potential. Among the four major *L. monocytogenes* serovars, 1/2a, 1/2b, 1/2c, and 4b, causing over 98% of clinical cases of human listeriosis [12], virulence heterogeneity also exists [1]. Virtually all *L. monocytogenes* serovars are capable of forming plaques on mouse fibroblast cells and inducing mouse mortality, with the exception of serovar 4a, which is naturally of low virulence [22, 23]. The fact that 23 *L. monocytogenes* strains belonging to serovars 1/2a, 1/2b, 1/2c, and 4b show remarkable plaque-forming ability on L929 cells and intermediate to high pathogenicity in ICR mice *via* the intraperitoneal route, whereas two strains belonging to serovar 4a lack intercellular spread ability and have minimal pathogenicity in this study, highlights the variable nature of the *L. monocytogenes* pathogenic potential, and confirms the negligible *in vivo* and *in vitro* virulence of serovar 4a, a feature that is shared by *L. innocua* (Table 1).

Listerial hemolytic activity comes mainly from listeriolysin O (LLO). A key role of LLO in *L. monocytogenes* pathogenesis is to lyse the primary vacuole for bacterial release and subsequent replication in the cytosol [37]. Both virulent (1/2a, 1/2b, 1/2c, and 4b) and low virulent (4a) *L. monocytogenes* serovars showed similar levels of *in vitro* hemolytic activity in this study (Table 1), suggesting that the underlying *hly* gene and its regulator PrfA are largely conserved across the species, and fully operational regardless of the pathogenic potential of the serovars. Listerial phospholipase activity derives essentially from a protein, PlcB, which is presumed to assist LLO in the disruption of primary as well as secondary vacuoles [37]. The fact that low virulent *L. monocytogenes* serovar 4a strains (NICPBP54006 and mLm4), but not the virulent serovars 1/2a, 1/2b, 1/2c, and 4b, demonstrated a strong *in vitro* phospholipase activity in the artificial medium indicates the possibility of the latter requiring some stimulation factors for optimal expression of PlcB, such as charcoal as shown in *in vitro* studies [6, 9]. It is possible that specific nucleotide substitutions at positions 1 (A to G) and −26 (C to T) in the *L. monocytogenes* serovar 4a *plcB* gene may result in start codon shift, leading to more efficient production of

PlcB in this serovar (Figs. 4A and 4B). An implication of these results is that using *in vitro* phospholipase activity as a means to ascertain listerial virulence can be unrewarding [22].

Phylogenetic Relationship Within the *L. monocytogenes-L. innocua* Group

On the basis of the composite phylogenetic tree constructed with the nucleotide sequences from the two rRNA operons (23S rRNA and 16S rRNA) and six housekeeping genes (*lmo0029*, *lmo0042*, *ascB*, *dapE*, *infC*, *rplS*, *prs*, and *ldh*) flanking variable gene clusters, it is clear that *L. monocytogenes* is more closely related to *L. innocua* in comparison with *L. seeligeri* and *L. welshimeri* (Fig. 5A), proving additional support for the division of the *Listeria* genus into the *L. monocytogenes-L. innocua*, *L. ivanovii-L. seeligeri-L. welshimeri*, and *L. grayi* phylogenetic clusters [14, 15, 32]. The fact that *L. monocytogenes* serovar 4a show a higher percentage of genetic resemblance to *L. innocua* highlights their close relationship. Through analysis of the concatenated data 23S-rRNA-16S-rRNA-*lmo0029-lmo0042-ascB-dapE-infC-rplS-prs-ldh-prfA-plcA-hly-mpl-actA-plcB-orfX-orfZ-orfB-orfA* including the virulence gene cluster LIPI-1, it is apparent that *L. monocytogenes* serovar 4a appears to be closer genetically to serovar 4b than serovar 1/2a (Fig. 5B).

Although there is ample evidence that suggests the evolution of *L. monocytogenes* serovars 1/2a and 4b to *L. monocytogenes* serovar 4a and *L. innocua via* gene deletion and/or horizontal gene transfer [5], this is by no means a one-step process. For example, available data indicate that transition from serovar 4b to serovar 4a might have to go past serovar 4c [5]. *L. monocytogenes* serovars 4c and 4a, both being classified in lineage III, are seldom involved in human listeriosis. However, they demonstrate different virulence in the mouse intraperitoneal model, with serovar 4c behaving more like serovars 1/2a and 4b [21, 22]. In addition, both serovars 4c and 4a do not have the virulence-specific transcriptional regulator genes *lmo1134* and *lmo2672*, and contain only an altered species-specific transcriptional regulator gene, *lmo0733* [22]. Serovar 4c is similar to those of 1/2a and 4b in having the gene *inlJ*, which was somewhat altered [5, 30]. However, it remains uncertain if *L. monocytogenes* serovar 4c serves as an evolutionary link between serovars 4b and 4a, until full genomic sequence data become available for phylogenomic analysis.

Taken together, horizontal gene transfer might have occurred earlier to form a more ancestral *L. monocytogenes* strain (probably of serovar 1/2a) [5]. *L. monocytogenes* serovar 1/2a then turned into serovar 4b through gene deletion, and the latter gave rise to serovar 4a (possibly *via* serovar 4c), which became *L. innocua via* additional gene deletion events. Theoretically, the loss of some gene clusters related to virulence such as *inlGHE* and *inlC* as well as *lmo0035–0042* could be regarded as adaptive gene loss, because *Listeria* is capable of surviving in the environments without these gene elements [38]. The removal of redundant gene clusters in the evolutionary process might have favored *L. innocua* to adapt to its environmental niches. Nonetheless, available data suggest that *L. innocua* may also contribute to the genetic makeup of *L. monocytogenes* lineage III (covering serovars 4a and 4c). Apart from the identification of *L. innocua*-specific *lin0372* and *lin1073* genes in *L. monocytogenes* serovar 4a in this study, a recent report indicates the presence of a *L. innocua* putative transcriptional regulator gene, *lin0464*, in *L. monocytogenes* serovar 4c [24]. Therefore, by possessing many gene elements common to *L. monocytogenes* serovars 1/2a and 4b, and sharing many similar gene deletions with *L. innocua, L. monocytogenes* serovar 4a represents a possible evolutionary intermediate between *L. monocytogenes* serovars 1/2a and 4b and *L. innocua*.

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