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

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The ge L a c f cl el ela ed ecie a d f h ee h l ge ic g L. - L. a, L. a -L. -L. , a d L. a . I hi e , e a e ed e a i he e l i a ela i hi i he L. -L. a g b bi g he cle ide e ce f 23S RNA a d 16S RNA, a d he ge cl e *0029- 0042*, a *B- a E*,

a h ge ici i la d I (LIPI-1)] a d e e al i e ali ge e (. ., *A* a d *B*). Thi gge ha a a f m h iz al ge e a fe , dele i f ke i le ce-a cia ed ge e i he ge me f a c mm a ce ma lead b e e cha ge i he c e di g he e i cl di g a h ge ici [2, 5, 13].

N e hele , he h l ge e ic ela i hi am g a i . e a a d . a e fa f m clea he ba i f die he i le ce-a cia ed ge e cha *A*, , *B*, a d i e ali ge e . T gai e h l ge e ic i igh i he . - . g , e a al zed he cle ide e e ce f 23S RNA a d 16S RNA a d he ge e cl e *0029- 0042*, *B*- , - , a d - i . e a 1/2a, 4a, a d 4b a d . . The a i ale f c he e ge e egi e e ba ed he e i b e a i ha he *0029- 0042* ge e egi i l e i e be ee . a d . [13], a d ha he P fA- eg la ed i le ce ge e cl e i e e i . , . , a d . , b ab e i . , . , a d . [11]. I addi i , e a e ed he a f . - ecific *A* a d *B*, a d 10 . - ecific ge e i he e ecie / e a , al g i h he ic cha ac e iza i b i g a d ced e . The e l ha ic a ache ha e e f if l i ece deli ea i f ecie a d cha ac e iza i f a ical . a d . ai [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 stud .

Strain	Serovar	Source	Hemol tic titer	Relative si e of plaque (%)[a]	Mouse mortalit (dead/tested)[b]	Relative virulence[c]	$\log LD_{50}$[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	Pasteuri ed milk	2^3	0	2/30	6.6%	8.14
mLm10	1/2a	Pasteuri ed 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] 1 % 10403 100%.

[b] Listeria IC % % % 1 10^8, 10^7, 10^6, 10^5, 10^4 % 1 , 10- - % ND, .

[c] % (%) % % 1 % 1 % 1 100 19 .

[d] -K % % LD$_{50}$ 1

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 *inl*) were amplified with primers targeting their flanking genes (*i.e.*, *lmo0029*/*lmo0042*, *04 0 04* , *r l* /*in C*, and *ascB*/ *a*). The full-length sequences of LIPI-1 between *rs* and *l h* were covered by five fragments in separate PCRs. In addition, primers were derived from *L. innocua*-specific genes *lin 0* , *lin 0 4*, *lin 0* , *lin0 9* , *lin0 2*, *lin04 9*, *lin24 4*, *lin2 9* , *lin04 4*, and *lin0* [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 *scherichia 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

Whe ea 23 . ai bel gi g e a 1/2a, 1/2b, 1/2c, a d 4b di la ed i e media e high a h ge ici i ICR mice i a e i eal i c la i (i h m ali a gi g f m 11–28 f 30 mice e ed, ela i e i le ce f m 36.3% 93.3%, a d l g LD_{50} f m 3.86 6.79), he he . ai bel gi g e a 4a (NICPBP54006 a d mLm4) e hibi ed mi imal a h ge ici (i h m ali be ee 1 a d 2 f 30 mice e ed, ela i e i le ce be ee 3.3 a d 6.6%, a d l g LD_{50} >8) (Table 1). . ATCC 33090 ca ed m ali i he m i e i a e i eal m del (Table 1).

Plaque-Forming Ability

I he la e-f mi g a a ba ed L929 cell , 23 . ai bel gi g e a 1/2a, 1/2b, 1/2c, a d 4b f med clea la e i h he ela i e ize a i g f m 83.7% 108.3%, he ea . e a 4a ai NICPBP54006 a d mLm4 a d . ATCC 33090 h ed im ai ed i e cell la ead abili (Table 1), hich a c i e i h he i le ce a e me i he m e m del.

Hemolytic and Phospholipase Activities

. e a 4a ai (NICPBP54006 a d mLm4) di la ed e hem l ic ac i i hee bl d

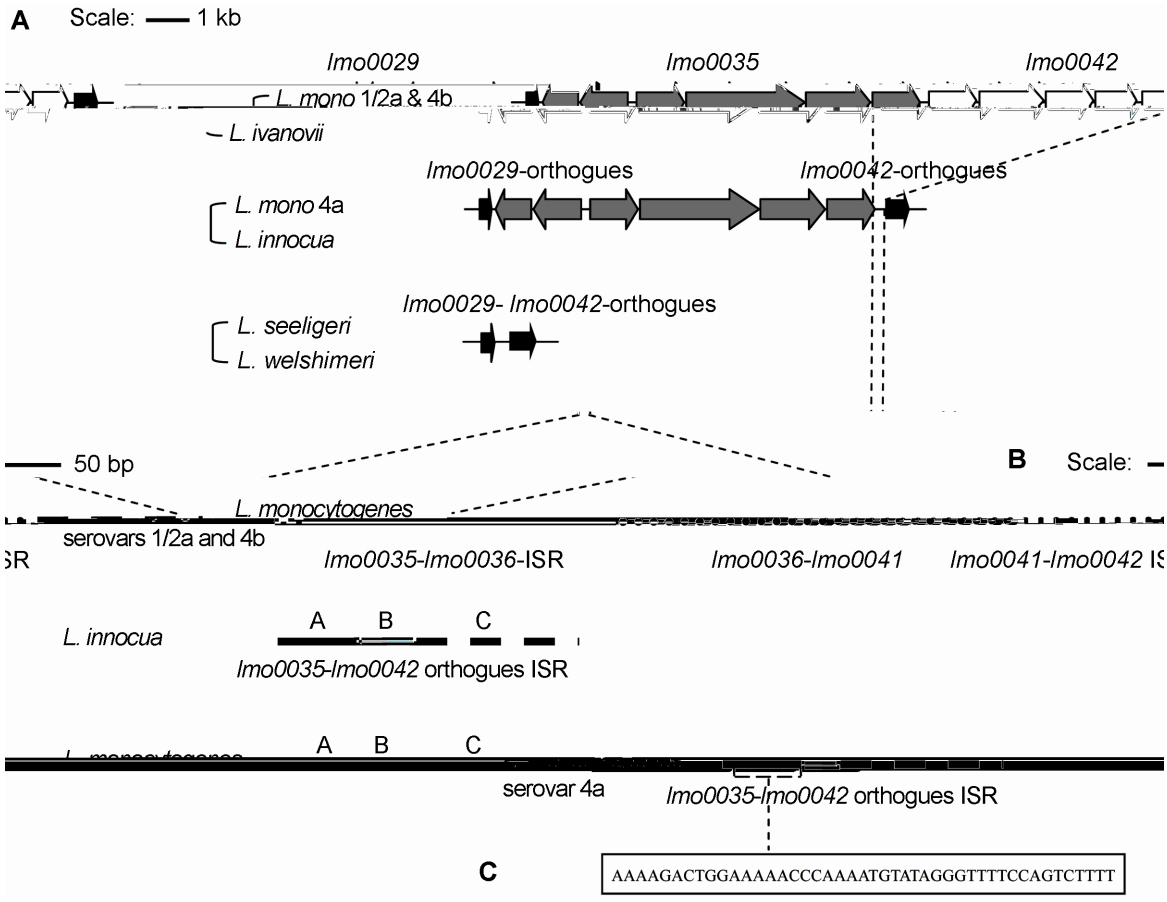

Fig. 1. A. Genetic structures of the *L. monocytogenes lmo0029-0042* region and its orthologs in *Listeria* species. **B**. Genetic organi ation of the *lmo0035-lmo0042* intergenic spacer region (ISR) of *L. monocytogenes* serovars 1/2a and 4b in relation to those of *L. monocytogenes* serovar 4a and *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 identit (%)			
		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

aga la e a d hem l ic i e (f m 2[2] 2[3]) i he 96- ell la e , imila . e a 1/2a, 1/2b, 1/2c, a d 4b ai (f m 2[1] 2[3]) (Table 1). Am g he - ecie e ed, . had a hem l ic i e f 2[4], . a hem l ic i e f 2[1], a d . , . , a d . a hem l ic i e f 2[0] (Table 1). O he he ha d, . e a 4a ai (NICPBP54006 a d mLm4) dem a ed g h h li a e ac i i ih a defi i e z e f aci di g he eak, he ea . e a 1/2a, 1/2b, 1/2c, a d 4b lacked h h li a e ac i i i he a ificial medi m ih cha c al ac i a i (da a h).

Genetic Organization of the *lmo0029-lmo0042* Locus

The *0029- 0042* l c f . e a 1/2a a d 4b a ell a . am ed 15,391 b i le g h; . e a 4a ai (NICPBP54006 a d mLm4) al g ih . e ed a m ch h e ed *0029- 0042* l c (mea i g l 8,735 b), ih he *0036- 0041* egi mi i g; . a d . e hibi ed a e e ded ed c i i he *0030- 0035* egi (mea i g l 1,189 b) (Fig. 1A a d Table 2). . ai NICPBP54007 (e a 4b) dem a ed 95.3% a d 98.4% cle ide ide i ie EGD (e a 1/2a) a d F2365 (e a 4b), e ec i el , i he *0029- 0042* l c (Table 2). O he he ha d, . e a 4a ai NICPBP54006 a d mLm4 h ed a highe cle ide imila i . CLIP11262 (89.2% a d 89.1%) ha . EGD-e (e a 1/2a) a d F2365 (e a 4b) (87.6–88.5%) i he *0029– 0042* l c (Table 2). The *0035– 0042* i e ge ic ace egi (ISR) i . e a 4a a c m ed f h ee egme f m diffe e igi (Fig. 1B). Segme A h ed 68.8–75% cle ide ide i ie he 5 e d f he *0035– 0036* ISR i . 1/2a a d 4b, a d 80.7% ide i he c e di g egi i . CLIP11262 (Table 3). The egme A al c ai ed e ea e e ce (AAAAG-ACTGGAAAAACCCWAWA) (Fig. 1C), hich e e a i e a i e i j c i ibl i l ed i he l f he *0036– 0041* egi . Thi a i e a - ela ed c e a al e e i . e a 1/2a a d 4b a d . . Segme C e hibi 83.7–83.8% ide i ie he 3 e d f he *0041– 0042* ISR i . e a 1/2a a d 4b, a d 81.4% ide i ha i . CLIP11262 (Table 3). H e e, i a ea ed ha egme B i i e . e a 4a a d . (Fig. 1B). Simila l , a - ela ed c e e e b e ed i hi he *0029- 0030* ISR i . , . , a d . (da a h).

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

The i e ali ge e , i cl di g *A*, *B*, , , *(2)*, , a d , a e ca e ed i . ge me a d c ib e i i le ce [10, 28]. The

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 identit (%)			
		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 4 (NIC B 54006 L 4) 100% lmo0035-0042 I .
[b] B f L. innocua 37 .
[c] B f L. monocytogenes 4 L. innocua L. monocytogenes 1/2 4 .

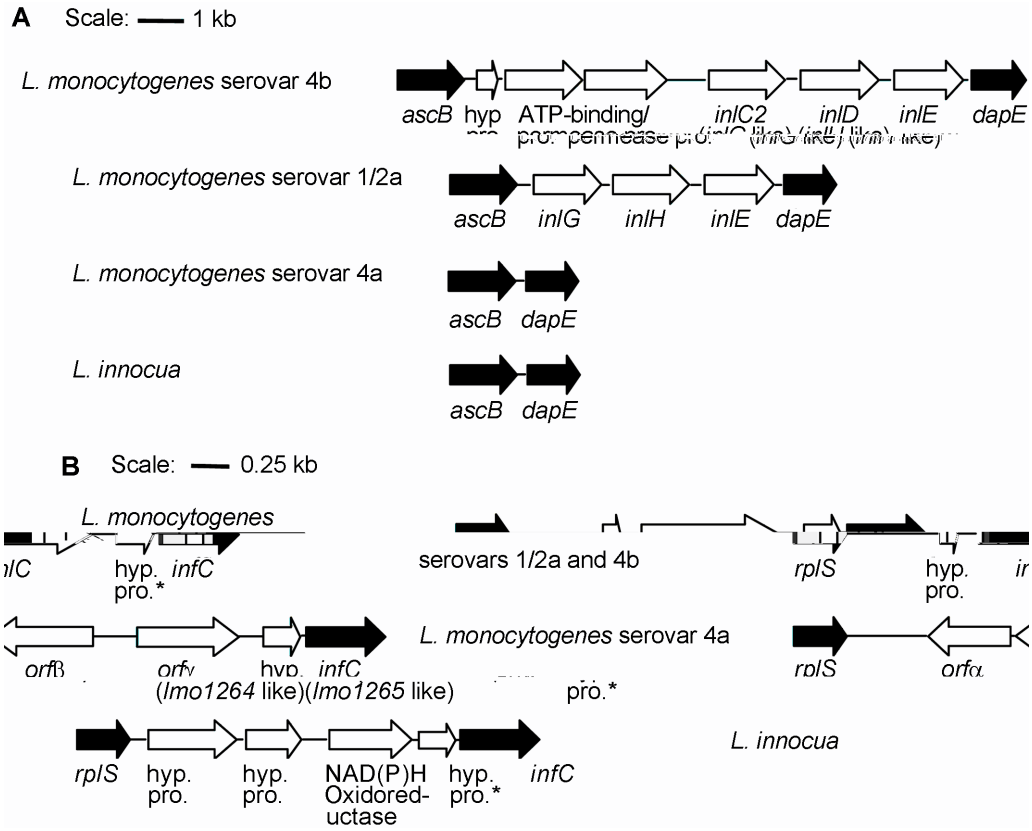

Fig. 2. A schematic diagram of chromosomal regions carr ing *L. monocytogenes* internalin genes and the corresponding loci in other *Listeria* species.
f ( ) ( ). **A.** ascB dapE. **B.** infC rplS. infC L. monocytogenes 1/2 4, 4 L. innocua.

a d *(2) ( )* ge e cl e e e e i he e ec i e ch m mal egi f - a d *B*- i . e a 1/2a a d 4b, b ab e i . e a 4a a d . (Fig. 2A). I he l c be ee a d , a d k ge e e c di g h he ical ei e e e e i . e a 1/2a a d 4b (Fig. 2B). I . , he e e i ed f ge e be ee a d , e e embli g a NAD(P)H id ed c a e a d h ee e c di g h he ical ei , f hich e e ecific hi ecie a d he he i imila ha i . e a 1/2a a d 4b (Fig. 2B). . e a 4a al ha b ed f ORF i hi egi , i h e(. , f h he ical ei) bei g imila . e a 1/2a a d 4b (97.9% cle ide ide i) a d . (98.8% cle ide ide i) (Fig. 3B a d Table 4), a d h ee he (α, β, a d γ) bei g diffe e f m h e i . e a 1/2a a d 4b a d . (Fig. 2B). I e e i gl , α a d β, h gh i e e e a c i i ie a i (Fig. 2B), e e imila (81.5–83.9%) he *1264* a d *1265* ge e i . EGD, he *1281* a d *1282* ge e i . F2365, a d he *1303* a d *1304* ge e i . CLIP11262 (Table 4), hich e e ea l 570 kb a a f m he - l c . I addi i , γ a d he j c i egi be ee a d α a ea ed be i e . e a 4a i h h m l g bei g ec g ized i . e a 1/2a a d 4b he bac e ial ecie BLAST ea ch. F he m e, he *A* a d *B* ge e e i ed be ee *0470* a d *0473* i . e a 4a, hich e hibi ed 91.5–97% cle ide ide i ie h e f EGD, 10403S, a d F2365; a d he e e ab e i he ecie (da a h).

Comparison of rRNA Gene Sequences

Ba ed he f ll-le g h 16S a d 23S RNA ge e e ce (Ge Ba k Acce i N . X92948-X92954) (S leme a Table 2) [31], he ge ca be di ided i maj cl e : ec e . , . , . , . , a d . , h i g cle ide imila i ie be ee 99.5% a d 99.9%; a d he he c i f . . Am g he fi e ig a e egi i he 23S RNA ge e e e ce ha dem a ed ffici a ia i

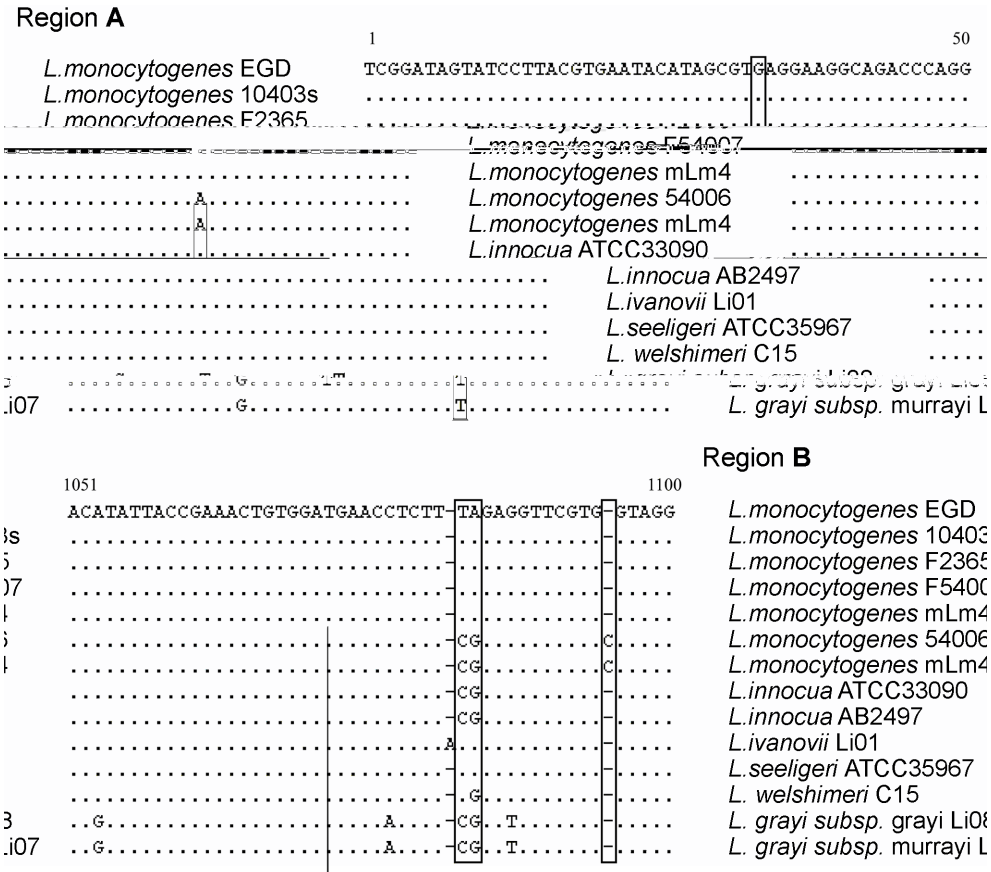

Fig. 3. The signature regions **A** and **B** of *Listeria* 23S rRNA gene sequences.

f. i e-a di a ecie diffe e ia i f he ge , (egi A a d B) e e a licable he . - . g (Fig. 3). I egi A, . e a 4a diffe ed f m . e a 1/2a a d 4b a ell a he ecie b ha i g a A a i i 33 i ead f G T. I egi B, a a f m ha i g a i e i e i f C a i i 1,095, . e a 4a a imila . b e i g CG a i i 1,083–1,084 i ead f TA i . e a 1/2a a d 4b, . , a d . , a d TG i . (Fig. 3).

Sequence Analysis of the Virulence Gene Cluster LIPI-1

Dema ca ed b a d , LIPI-1 ha b i i le ce- a cia ed ge e . I he *B* a d i age ic ace egi , he e a e f addi i al mall ORF (, , *B*, a d *A*; i g a a). The a d ge e deli ea ed he a i e dele i i f LIPI-1 i . (Fig. 4). A a f m me e e ce di e ge ce bei g ed i he *A* a d *B* ge e, , he ge e (. ., *A*, *A*, , a d i he i le ce ge e cl e a d f mall ORF) l ca ed be ee a d e e imila (94–99% a d 82–96%, e ec i el) am g .

Table 4. Comparison of the *orfα*, *orfβ*, and *hyp.pro* (h pothetical 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	Identit (%)		Ortholog	Identit (%)		Ortholog	Identit (%)	
		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. Anal sis of DNA sequences of the virulence gene cluster (LIPI-1).
L. monocytogenes 4 plcB (**A**) F (**B**). A prs-prfA (I) (**C**) orfZ-orfB I (**D**) I L. monocytogenes (**C**) (**D**), L. monocytogenes EGD- .

e a 4a, 1/2a, a d 4b (S leme a Table 3). I a e h ha he *A* ge e f. e a 4a (NICPBP54006 a d mLm4) a d 4b (F2365) e ami ed i hi d ha b ed a 105- cle ide dele i a c m a ed i h . e a 1/2a (EGD a d 10403S), leadi g ed c i f 35 ami acid effec i el em i g f he f li e- ich e ea (DFPPPPTDEEL), hich e e e i ed f bi di g f he f cal c ac ei VASP a d Me a im la e ac i -ba ed m ili . Sig ifica m a i f he *B* ge e i . e a 4a (NICPBP54006 a d mLm4) e e f d a i i 1 (A G) a d a i i –26 (C T) (Fig. 4A), hich migh i d ce he a c d hif i he ORF (Fig. 4B). The e cha ge migh ha e e l ed i m e efficie e a i f he *B* ge e i . e a 4a i c a . 1/2a a d 4b ai , a a e ed b h h li a e a a . Se e ce alig me f he - *A* ISR a d - *B* ISR i . e a 4a, 1/2a, a d 4b e ealed e ea e e ce (Fig . 4C a d 4D), hich e e imila he c e i hi he *0035- 0042* ISR a d *0029- 0030* ISR efe ed ab e. E i e ce f ch a i e a i e i j c i im lied he ibili f h iz al a fe f he i le ce ge e cl e [18].

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

Am g he 10 - ecific ge e a al zed (S leme a Table 1), *0372* a d *1073* e e de ec ed i . e a 4a ai (NICPBP54006 a d mLm4), b i . e a 1/2a a d 4b ai (da a h). The *0372* ge e e e ce i . e a 4a ai NICPBP54006 (EU073154) a d mLm4 (EU073155) ha ed a 92.1% cle ide ide i ha i . CLIP11262, he ea he *1073* ge e e e ce i he e ai (EU073152 a d EU073153) e hibi ed 79.3% a d 76.2% imila i , e ec i el , ha i . CLIP11262.

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

The h ekee i g ge e ai , *0029*/ *0042* (a d hei h l g i he ecie), *B*/ , / , a d / , fla ki g hei e ec i e a iable egi e e c e ed a he cle ide le el (S leme a Table 4). Whe ea he e e ce i . e a 4a dem a ed a highe imila i ha i . (99%) ha ha i . e a 1/2a a d 4b (84.7–87.7%), *0029*, *0042*, *B*,

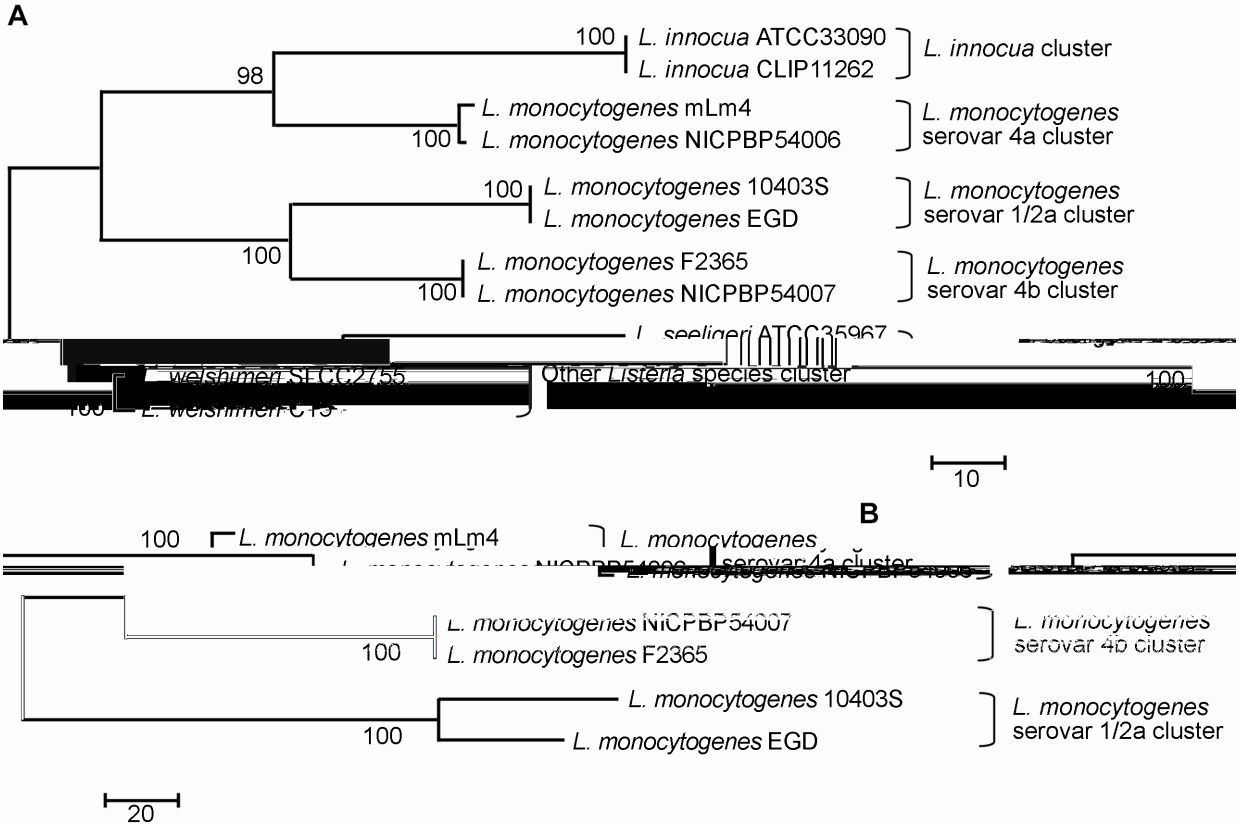

Fig. 5. A. Ph logenetic tree of *L. monocytogenes* serovars 4a, 1/2a, and 4b and other *Listeria* species based on the concatenated data set 23S-rRNA-16S-rRNA-*lmo0029-lmo0042-ascB-dapE-infC-rplS-prs-ldh*. **B**. Ph logenetic tree of selected *L. monocytogenes* serovars based on the concatenated data 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 hori ontal lines (expressed as percentages) indicate the robustness of the corresponding branches (which is rooted with *L. monocytogenes* serovar 1/2a strain EGD), as determined b a bootstrap anal sis evaluated from 1,000 replications.

, , , a d i . e a 4a e hibi ed c m a able ide i ie h e i . e a 1/2a a d 4b a ell a . (S leme a Table 4). A c m i e h l ge e ic ee a c c ed he ba i f he cle ide e e ce f he c ca e a ed 23S- RNA-16S- RNA- *0029- 0042- B-* - - - - ge e cl e (Fig. 5A). I e ec i e f he me h d em l ed (i cl di g eighb -j i i g, ma im - a im , mi im m e l i , a d UPGMA), . e a 4a a d . e e c i e l laced a a i e b a ch, . e a 1/2a a d 4b f med a he, a d he ecie cc ied he m di i c b a ch (Fig. 5A). T f he ill mi a e he e l i a hi am g . ai c e i g e a 1/2a, 4b, a d 4a, h l ge e ic i f ma i i he ab e ge e e a ell a he 10 ge e (*A- B- - - A- B- - - B- A*) c e i g he h le LIPI-1 a c mbi ed. I hi cheme, e a 4a a d 4b ai c i e l fell i cl el ela ed b a che , he ea e a 1/2a ai had a e a a e b a ch (Fig. 5B).

DISCUSSION

T gai f he h l ge e ic i igh he . - . g , ec m a a i el e ami ed he cle ide e e ce f he 23S RNA a d 16S RNA a d he ge e cl e *0029- 0042, B-* , - , a d - a ell a . - ecific *A* a d *B* a d 10 . - ecific ge e i . e a 1/2a, 4a, a d 4b a d . . Thi a f ll ed b a e me f hem l ic a d leci hi a e ac i i ie a d a d i le ce f he e ai . The e l gge ha . e a 4a l e e ma ge e ic e e ce c mm e a 1/2a a d 4b, b al ha e me ge e dele i a d bd ed a d i le ce i h . , i addi i ha b i g a fe . ge e (. ., *0372* a d *1073*).

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

O e f he maj fi di g i hi d i he ge e ic di e ge ce be ee a h ge ic a d l i le . e a

i he *0029*- *0042* cl e a d i e ali cl e , i cl di g he cl e be ee *B* a d , a d be ee a d . The e i able l f he *0036*- *0041* l c i . e a 4a a d . i ela i . e a 1/2a a d 4b. . a d . ha b a f he ed ced *0030*- *0035* l c (Fig. 1A a d Table 2). Thi gge ha he *0029*- *0042* l c ha bee cce i el dele ed f m . e a 1/2a a d 4b f m . e a 4a a d . , a d he . a d . . Occ e ce f e ea e e ce (AAA-AGACTGGAAAAACCCWAWA) i he *0035*- *0042* ISR (Fig. 1C), hich a e al b e ed i he - *A* ISR a d - *B* ISR fla ki g LIPI-1 (Fig . 4C a d 4D), ide a emi de he a e e i l i g ible ge e a fe a d l [25, 26].

C m a i f . e a 1/2a a d 4b i h . e a 4a a d . i he *B*-ge e egi al gge a i gle dele i e e i l i g (2) ( ) (Fig. 2A). The ge e ic c e be ee a d e eal ig ifica he e ge ei i he . - . g (Fig. 2B). The - egi f . e a 4a eem ha e de g e a ge f ge e ic eleme f m . e a 1/2a a d 4b: he c e ed ge e e c di g h he ical ei adjace i mai ai ed a e ige i hi egi , a d he ge e e i i gi a ce al . ai , ch a i le ce-a cia ed ge e , a e l i me de ce da . ai (hich ha e e l ed la e i e a 4a) a d e laced b α a d β, he h l g f *1264* a d *1265*, a ell a γ. The γ ge e i i e . e a 4a, i h ig ifica h m l g i . e a 1/2a a d 4b he bac e ial ecie , idi g cl e he le f DNA ake i he e l i i he . - . g . U e ec edl , l g-e ea e e ce b i em a f a able eleme a e b e ed i he cl e ici i , a d h e c ide ha ch f eig ge e migh ha e bee i eg a ed he ei b illegi ima e ec mbi a i , he mecha i m f hich i de c ibed i e am le f he e ic i -m difica i (RM) ge e a d egi f [33, 35].

Genetic Features of the Virulence Gene Cluster LIPI-1

The LIPI-1 ge e cl e (e c m a i g *A*, *A*, , , *A*, a d *B*) i f di . , . , a d . , b e i e i . , . , a d . [11]. The l i le . e a 4a d e c ai all he ge e i hi hi a ic la cl e a i he ca e i h he he e a . The cc e ce f a i e a i e i e e ce a - *A* a d - *B* fla ki g he i le ce ge e cl e gge ha hi g f i le ce ge e migh ha e bee a fe ed h iz all [18]. Wi hi hi cl e , he *A* ge e a ea be ce ible f cle ide al e a i [22, 27, 34]. Whe ea . e a 1/2a mai ai a c m le e c f he *A* ge e, e a 4b a d 4a me ime ha b a dele i f 105 cle ide i hi ge e (S leme a Table 3). Rem al f 105 cle ide i *A* i he e a 4b cli ical ai F2365, hich a igi a ed f m he Jali c chee e b eak f 1985 i Calif ia [28], im lie ha a f ll-le g h *A* ge e ma be ab l el e e ial f li e ial cell- - cell ead a d i le ce a e i l h gh . Al e a i el , . e a 4b ai i h dele i i i *A* ge e ma em l he cha ac e ized mecha i m f i efficie ead eighb i g cell .

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

dem a e b hi e-a di a ecie a ia i i a h ge ic e ial. Am g he f maj . e a , 1/2a, 1/2b, 1/2c, a d 4b, ca i g e 98% f cli ical ca e f h ma li e i i [12], i le ce he e ge ei al e i [1]. Vi all all . e a a e ca able f f mi g la e m e fib bla cell a d i d ci g m e m ali , i h he e ce i f e a 4a, hich i a all f l i le ce [22, 23]. The fac ha 23 . ai bel gi g e a 1/2a, 1/2b, 1/2c, a d 4b h ema kable la e-f mi g abili L929 cell a d i e media e high a h ge ici i ICR mice he i a e i eal e, he ea ai bel gi g e a 4a lack i e cell la ead abili a d ha e mi imal a h ge ici i hi d , highligh he a iable a e f he . a h ge ic e ial, a d c fi m he egligible a d i le ce f e a 4a, a fea e ha i ha ed b . (Table 1).

Li e ial hem l ic ac i i c me mai l f m li e i l i O (LLO). A ke le f LLO i . a h ge e i i l e he ima ac le f bac e ial elea e a d b e e e lica i i he c l [37]. B h i le (1/2a, 1/2b, 1/2c, a d 4b) a d l i le (4a) . e a h ed imila le el f hem l ic ac i i i hi d (Table 1), gge i g ha he de l ig ge e a d i eg la P fA a e la gel c e ed ac he ecie , a d f ll e a i al ega dle f he a h ge ic e ial f he e a . Li e ial h h li a e ac i i de i e e e iall f m a ei , PlcB, hich i e med a i LLO i he di i f ima a ell a ec da ac le [37]. The fac ha l i le . e a 4a ai (NICPBP54006 a d mLm4), b he i le e a 1/2a, 1/2b, 1/2c, a d 4b, dem a ed a g h h li a e ac i i i he a ificial medi m i dica e he ibili f he la e e i i g me im la i fac f imal e e i f PlcB, ch a cha c al a h i die [6, 9]. I i ible ha ecific cle ide b i i a i i 1 (A G) a d –26 (C T) i he . e a 4a *B* ge e ma e l i a c d hif , leadi g m e efficie d ci f

PlcB i hi e a (Fig . 4A a d 4B). A im lica i f he e e l i ha ig h h li a eac i i a amea a ce ai li e ial i le ce ca be e a di g [22].

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

O he ba i f he c m ie h l ge e ic ee c c ed ih he cle ide e ce f m he RNA e (23S RNA a d 16S RNA) a d i h ekee i g ge e (*0029*, *0042*, *B*, , , , , a d) fla ki g aiable ge e cl e , i i clea ha . i m e cl el ela ed . i c m ai ih . a d . (Fig. 5A), i g addi i al f he di i i f he ge i he . - . , . - . - . , a d . h l ge e ic cl e [14, 15, 32]. The fac ha . e a 4a h a highe e ce age f ge e ic e embla ce . highligh hei cl e ela i hi . Th gh a al i f he c ca e a ed da a 23S- RNA-16S-RNA- *0029- 0042- B-* - - - - - - *A- A-* - - *A- B-* - - *B- A* i cl di g he i le ce ge e cl e LIPI-1, i i a a e ha . e a 4a a ea be cl e ge e icall e a 4b ha e a 1/2a (Fig. 5B).

Al h gh he e i am le e ide ce ha gge hee l i f . e a 1/2a a d 4b . e a 4a a d . ge e dele i a d/ h iz al ge e a fe [5], hi i b mea a e- e ce . F e am le, a ailable da a i dica e ha a ii f m e a 4b e a 4a migh ha e g a e a 4c [5]. . e a 4c a d 4a, b h bei g cla ified i li eage III, a e eld m i l ed i h ma li e i i. H e e, he dem a e diffe e i le ce i he m ei a e i eal m del, i h e a 4c beha i g m e like e a 1/2a a d 4b [21, 22]. I addi i , b h e a 4c a d 4a d ha e he i le ce- ecific a c i i al eg la ge e *1134* a d *2672*, a d c ai l a al e ed ecie - ecific a c i i al eg la ge e, *0733* [22]. Se a 4c i imila h e f 1/2a a d 4b i ha i g he ge e , hich a me ha al e ed [5, 30]. H e e, i emai ce ai if . e a 4c e e a a e l i a li k be ee e a 4b a d 4a, il f ll ge mic e e ce da a bec me a ailable f h l ge mic a al i .

Take ge he , h iz al ge e a fe migh ha e cc ed ea lie f m a m e a ce al . ai (babl f e a 1/2a) [5]. . e a 1/2a he ed i e a 4b h gh ge e dele i , a d he la e ga e i e e a 4a (ibl e a 4c), hich became . addi i al ge e dele i e e . The e icall , he l f me ge e cl e ela ed i le ce cha a d a ella *0035–0042* c ld be ega ded a ada i e ge e l , beca e i ca able f i i g i he e i me ih he e ge e eleme [38]. The em al f ed da ge e cl e i he e l i a ce migh ha e fa ed . ada i e i me al iche . N e hele , a ailable da a gge ha . ma al c ib e he ge e ic make f . li eage III (c e i g e a 4a a d 4c). A a f m he ide ifica i f . - ecific *0372* a d *1073* ge e i . e a 4a i hi d , a ece e i dica e he e e ce f a . a i e a c i i al eg la ge e, *0464*, i . e a 4c [24]. The ef e, b e i g ma ge e eleme c mm . e a 1/2a a d 4b, a d ha i g ma imila ge e dele i ih . , . e a 4a e e e a ible e l i a i e media e be ee . e a 1/2a a d 4b a d . .

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REFERENCES

1. Brosch, R., J. Chen, and J. B. Luchansky. 1994. Pulsed-field fingerprinting of listeriae: Identification of genomic divisions for *Listeria monocytogenes* and their correlation with serovar. *Appl. Environ. Microbiol.* **60:** 2584–2592.
2. Buchrieser, C. and C. Rusniok, The *Listeria* Consortium, F. Kunst, P. Cossart, and P. Glaser. 2003. Comparison of the genomes sequences of *Listeria monocytogenes* and *Listeria innocua*: Clues for evolution and pathogenicity. *FEMS Immunol. Med. Microbiol.* **35:** 207–213.
3. Buchrieser, C. 2007. Biodiversity of the species *Listeria monocytogenes* and the genus *Listeria. Microbes Infect.* **9:** 1147–1155.
4. Chen, J., L. Jiang, and W. Fang, 2007. Virulence determinants and its evolution of the genus *Listeria*. *Acta Microbiol. Sin.* **47:** 738–742.
5. Doumith, M., C. Cazalet, N. Simoes, L. Frangeul, C. Jacquet, F. Kunst, *et al*. 2004. New aspects regarding evolution and virulence of *Listeria monocytogenes* revealed by comparative genomics and DNA arrays. *Infect. Immun.* **72:** 1072–1083.
6. Ermolaeva, S., T. Karpova, S. Novella, M. Wagner, M. Scortti, I. Tartakovskii, and J. A. Vazquez-Boland. 2003. A simple method for the differentiation of *Listeria monocytogenes* on induction of lecithinase activity by charcoal. *Int. J. Food Microbiol.* **82:** 87–94.
7. Fiedler, F. 1988. Biochemistry of the cell surface of *Listeria* strains: A locating general view. *Infection* **16:** 92–97.
8. Geoffroy, C., J. L. Gaillard, J. E. Alouf, and P. Berche. 1989. Production of thiol-dependent haemolysins by *Listeria monocytogenes* and related species. *J. Gen. Microbiol.* **135:** 481–487.
9. Geoffroy, C., J. Raveneau, J. L. Beretti, A. Lecroisey, J. A. Vazquez-Boland, J. E. Alouf, and P. Berche. 1991. Purification and characterization of an extracellular 29-kilodalton phospholipase

C from *Listeria monocytogenes*. *Infect. Immun.* **59:** 2382–2388.
10. Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, *et al*. 2001. Comparative genomics of *Listeria* species. *Science* **294:** 849–852.
11. Gouin, E., J. Mengaud, and P. Cossart. 1994. The virulence gene cluster of *Listeria monocytogenes* is also present in *Listeria ivanovii*, an animal pathogen, and *Listeria seeligeri*, a nonpathogenic species. *Infect. Immun.* **62:** 3550–3553.
12. Goulet, V., C. Jacquet, P. Martin, V. Vaillant, E. Laurent, and H. de Valk. 2006. Surveillance of human listeriosis in France, 2001–2003. *Euro. Surveill.* **11:** 79–81.
13. Hain, T., C. Steinweg, C. T. Kuenne, A. Billion, R. Ghai, S. S. Chatterjee, *et al*. 2006. Whole-genome sequence of *Listeria welshimeri* reveals common steps in genome reduction with *Listeria innocua* as compared to *Listeria monocytogenes*. *J. Bacteriol.* **188:** 7405–7415.
14. Hain, T., C. Steinweg, and T. Chakraborty. 2006. Comparative and functional genomics of *Listeria* spp. *J. Biotechol.* **126:** 37–51.
15. Hain, T., S. S. Chatterjee, R. Ghai, C. T. Kuenne, A. Billionm, C. Steinweg, *et al*. 2007. Pathogenomics of *Listeria* spp. *Int. J. Med. Microbiol.* **297:** 541–557.
16. Jiang, L., J. Xu, N. Chen, J. Shuai, and W. Fang. 2006. Virulence phenotyping and molecular characterization of a low-pathogenic *Listeria monocytogenes* from cow's milk. *Acta Biochim. Biophys. Sin.* **38:** 262–270.
17. Jiang, L., J. Chen, J. Xu, X. Zhang, S. Wang, H. Zhao, K. Vongxay, and W. Fang. 2008. Virulence characterization and genotypic analyses of *Listeria monocytogenes* isolates from food and processing environments in eastern China. *Int. J. Food Microbiol.* **121:** 53–59.
18. Johnson, J., K. Jinneman, G. Stelma, B. G. Smith, D. Lye, J. Messer, *et al*. 2004. Natural atypical *Listeria innocua* strains with *Listeria monocytogenes* pathogenicity island 1 genes. *Appl. Environ. Microbiol.* **70:** 4256–4266.
19. Liu, D. 2004. *Listeria monocytogenes*: Comparative interpretation of mouse virulence assay. *FEMS. Microbiol. Lett.* **233:** 159–164.
20. Liu, D. 2006. Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. *J. Med. Microbiol.* **55:** 645–659.
21. Liu, D., M. L. Lawrence, M. Wiedmann, L. Gorski, R. E. Mandrell, A. J. Ainsworth, and F. W. Austin. 2006. *Listeria monocytogenes* subgroups IIIA, IIIB and IIIC delineate genetically distinct populations with varied virulence potential. *J. Clin. Microbiol.* **44:** 4229–4233.
22. Liu, D., M. L. Lawrence, A. J. Ainsworth, and F. W. Austin. 2007. Toward an improved laboratory definition of *Listeria monocytogenes* virulence. *Int. J. Food Microbiol.* **118:** 101–115.
23. Liu, D., M. L. Lawrence, A. J. Ainsworth, and F. W. Austin. 2007. A multiplex PCR assay for species- and virulence-specific determination of *Listeria monocytogenes* virulence. *J. Microbiol. Methods* **71:** 133–140.
24. Liu, D., M. L. Lawrence, and A. D. Hitchins. 2008. Molecular characterization of *Listeria monocytogenes* strains harboring *L. innocua* putative transcriptional regulator gene *lin0464*. *J. Rapid Meth. Aut. Mic.* [In Press].
25. Mahillon, J. and M. Chandler. 1998. Insertion sequence. *Microbiol. Mol. Biol. Rev.* **62:** 725–774.
26. Moran, N. A. 2003. Tracing the evolution of gene loss in obligate bacterial symbionts. *Curr. Opin. Microbiol.* **6:** 512–518.
27. Moriishi, K., M. Terao, M. Kuora, and S. Inoue. 1998. Sequence analysis of the *actA* gene of *Listeria monocytogenes* isolated from human. *Microbiol. Immunol.* **42:** 129–132.
28. Nelson, K. E., D. E. Fouts, E. F. Mongodin, J. Ravel, R. T. DeBoy, J. F. Kolonay, *et al*. 2004. Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. *Nucleic Acids Res.* **32:** 2386–2395.
29. Roche, S. M., P. Gracieux, E. Milohanic, I. Albert, I. Virlogeux-Pavant, S. Temon, *et al*. 2005. Investigation of specific substitutions in virulence genes characterizing phenotypic groups of low-virulence field strains of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **71:** 6039–6048.
30. Sabet, C., M. Lecuit, D. Cabanes, P. Cossart, and H. Biene. 2005. LPXTG protein InlJ, a newly identified internalin involved in *Listeria monocytogenes* virulence. *Infect. Immun.* **73:** 6912–6922.
31. Sallen, B., A. Rajoharison, S. Desvarenne, F. Quinn, and C. Mabilat. 1996. Comparative analysis of 16S rRNA and 23S rRNA sequences of *Listeria* species. *Int. J. Syst. Bacteriol.* **46:** 669–674.
32. Schmid, M. W., E. Y. W. Ng, R. Lampidis, M. Emmerth, M. Walcher, J. Kreft, W. Goebel, M. Wanger, and K. Schleifer. 2005. Evolutionary history of the genus *Listeria* and its virulence genes. *Syst. Appl. Microbiol.* **28:** 1–18.
33. Sekizaki, T., D. Takamatsu, M. Osaki, and Y. Shimoji. 2005. Different foreign genes incidentally integrated into the same locus of the *Streptococcus suis* genome. *J. Bacteriol.* **187:** 872–883.
34. Sokolovic, Z., S. Schuller, J. Bohne, A. Baur, U. Rdest, C. Dickneite, T. Nichterlein, and W. Goebel. 1996. Differences in virulence and in expression of PrfA and PrfA-regulated virulence genes of *Listeria monocytogenes* strains belonging to serogroup 4. *Infect. Immun.* **64:** 4008–4019.
35. Takamatsu, D., M. Osaki, and T. Sekizaki. 2002. Evidence for lateral transfer of the suilysin gene region of *Streptococcus suis*. *J. Bacteriol.* **184:** 2050–2057.
36. Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24:** 1596–1599.
37. Vazquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, and J. Kreft. 2001. *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.* **14:** 584–640.
38. Volokhov, D. V., J. George, C. Anderson, R. E. Duvall, and A. D. Hitchins. 2006. Discovery of natural atypical nonhemolytic *Listeria seeligeri* isolates. *Appl. Environ. Microbiol.* **72:** 2439–2448.
39. Volokhov, D. V., S. Duperrier, A. A. Neverov, J. George, C. Buchrieser, and A. D. Hitchins. 2007. Internalin gene in natural atypical hemolytic *Listeria innocua* strains suggests descent from *L. monocytogenes*. *Appl. Environ. Microbiol.* **73:** 1928–1939.
40. Ward, T. J., L. Gorski, M. K. Borucki, R. E. Mandrell, J. Hutchins, and K. Pupedis. 2004. Intraspecific phylogeny and lineage group identification based on the *prfA* virulence gene cluster of *Listeria monocytogenes*. *J. Bacteriol.* **186:** 4994–5002.
41. Zeng, H., X. Zhang, Z. Sun, and W. Fang. 2006. Multiplex PCR identification of *Listeria monocytogenes* isolates from milk and milk-processing environments. *J. Food Sci. Agric.* **86:** 367–371.