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

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The ge L a c f cl el ela ed ecie a f h ee h l ge ic g L. y - L. , L. a -L. -L. , a L. ay. I hi e , e a e ed e a i he e l i ela i hi i he L. y -L. g b bi g he cle ide e ce f 23S RNA a 16S RNA, a he ge e cl e 0029- 0042, a B- a E, - a al L. - ecific ge e 0372 a 2671. F he e, b h L. y a 4a a L. e hibi i ai ed i e cell la ead abili a h ge ici i del. O he he ha , e bli g L. y a 1/2a ha i g a ea l ide ical i le ce ge e A a B ge e he a 4a ai e a 1/2a a 4b b ha b i g abl a B ge e di la i g g h h li a a bd ed a i le ce. Th b a ge e c

L. y a 1/2a a 4b, a ha i g a ila ge e dele i i h L. , L. y a 4a e e a ble e l i i edia e be ee L. y a 1/2a a 4b a L. .

Ke d: *Listeria*, serovar, phylogeny, evolution, pathogenicity

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The ge e c m a e i cl el elated ecie : . . , . , . , . , and . [3]. Ba ed diffe e t ge tl ic and he tl ic anal e , li te iae can be di ided t tl ee maj h l genetic cl te : . - . , . - . - . , and . [14, 15, 32]; . e gia ith . c i ting f e a 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7; . e a 5; and the ecie f e a 1/2a, 1/2b, and 4c); and th ee genetic lineage ith lineage I c e ing e a 1/2b, 3b, 4b, 4d, and 4e; lineage II c ntaining e a 1/2a, 1/2c, 3a, and 3c; and lineage III ncluding e a 4a, 4c, and ibl 7) [1, 20].

Alth gh ecie ithin the ge e emble each the m h l gicall , ec l gicall , bi chemicall , and genetically, the h a ied athogenic tential. . i a ell- ec gnized intracell la athogen f man and animal , . infect mainl minant, and f the ecie (. , . , . , and .) ha e n athogenic inclinati n [4, 37]. Of the 13 . e a , f (4b, 1/2a, 1/2b, and 1/2c) a e e n ible f e 98% f h man li te i i ca e he ea the e a (. ., 4c and 4a) a e eld m im licated in li te i i [12]. Hete geneit in . athogenicit ha al been b e ed in m e m del , ith e a 1/2a and 4b being highl i lent and e a 4a being natl all f l athogenicit [1, 22].

Inte e tingl , natl all l i lent . e a 4a a ea t be imila t a i lent . in te m f ha ed antigenic and genetic tl ctl e a ell a l i lence [16, 29]. F in tance, b th . e a 4a and . ha e the ame flagella antigen tl ctl e [7]. . lack the gene cl te , hich i al ab ent in . e a 4a [5]. H e e , . diffe f m . e a 4a in that it d e n t ha b the i lence gene cl te [

athogenicit i land I (LIPI-1)] and e al inte nalin gene (. ., A and B). Thi gge t that a a t f m h iz ntal gene t an fe , deleti n f ke i lence-a ciated gene in the gen me f a c mm n ance t ma lead t b e ent change in the c e nding hen t e incl ding athogenicit [2, 5, 13].

Nonetheless, the h l genetic elati n hi am ng a i . e a and . a e fa f m clea n the ba i f t die n the i lence-a ciated gene ch a A, , B, and inte nalin gene . T gain ne h l genetic in ight int the . - . g e anal zed the n cle tide e ence f 23S RNA and 16S RNA and the gene cl te 0029- 0042, B- , - C, and - in . e a 1/2a, 4a, and 4b and . . The ati nale t f c n the e gene egi n e e ba ed n the e i b e ati n that the 0029- 0042 gene egi n i l t te i e bet een . and . [13], and that the P fA- eg lated i lence gene cl te i e ent in . , . , and . , b t ab ent in . , . , and . [11]. In additi n, e a e ed the tat f . - ecific A and B, and 10 . - ecific gene in the e ecie / e a , al ng ith hen t ic cha acte izati n b ing and ced e. The e l ha ic a ache ha e n en f itf l in ecent delineati n f ecie and cha acte izati n f atl ical . and . t ain [18, 32, 38–40].

MATERIALS AND METHODS

Bacterial Strains

A total of 32 strains were examined in this study (Table 1). These included 25 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 (ATCC 33090 and AB2497), one (Li01), one (C15), one (ATCC 35967), and two (Li07 and Li08) strains were acquired from reference collections (Table 1). 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 and one (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 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 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 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 strains was assayed in sheep blood agar plates as previously described [8]. To titrate the hemolytic activity, supernatant from 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 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 strains was examined with the egg yolk assay of Ermolaeva . [6] without charcoal activation. The BHI agar plates were supplemented with 5% fresh egg yolk suspension in saline. cultures were streaked onto the plates and incubated at 37°C for 48 h, with Li01 being applied as the positive control displaying an opacity zone surrounding the streak [9].

PCR

One ml of each broth culture was transferred to an Eppendorf tube and centrifuged at 12,000 × 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 × 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 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, 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 si e of plaque (%)[a]	Mouse mortality (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] ... 10403 ... 100%.
[b] ... 10^8, 10^7, 10^6, 10^5, ... 10^4 ... 10- ... ND, ...
[c] ... (%) ... 100 19 .
[d] ... -K ... LD_{50} ...

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 cluster (and its equivalent in other strains) and three -specific internalin gene clusters (, , and) were amplified with primers targeting their flanking genes (., / , , / , and /). The full-length sequences of LIPI-1 between and were covered by five fragments in separate PCRs. In addition, primers were derived from -specific genes , , , , , , , , and [10] for sequence comparison among 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 DH5 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 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 . tl aɨn bel ngɨng tl e a 1/2a, 1/2b, 1/2c, and 4b di la ed ɨnte mediate tl high athl genicitl ɨn ICR mice ɨntl a e itl neal ɨnl c latil n ith ml talitl anging f m 11–28 tl f 30 mice te ted, elati e i lence f m 36.3% tl 93.3%, and l g LD_{50} f m 3.86 tl 6.79), the l the tl . tl aɨn bel ngɨng tl e a 4a (NICPBP54006 and mLm4) e hibited minimal athl genicitl ith ml talitl bet een 1 and 2 tl f 30 mice te ted, elati e i lence bet een 3.3 and 6.6%, and l g LD_{50} >8) (Table 1). . ATCC 33090 ca ed n ml talitl ɨn the m ne ɨntl a e itl neal ml del (Table 1).

Plaque-Forming Ability

ɨn the la e-fl mɨng a a ba ed n L929 cell , 23 . tl aɨn bel ngɨng tl e a 1/2a, 1/2b, 1/2c, and 4b fl med clea la e ith the elati e ize a ɨng f m 83.7% tl 108.3%, he ea . e a 4a tl aɨn NICPBP54006 and mLm4 and . ATCC 33090 h ed im ai ed ɨnte cell la ead abilitl (Table 1), hich a c n i tent ith the i lence a e ment ɨn the ml e ml del.

Hemolytic and Phospholipase Activities

. e a 4a tl aɨn (NICPBP54006 and mLm4) di la ed tent hem l tic acti itl n hee bl d

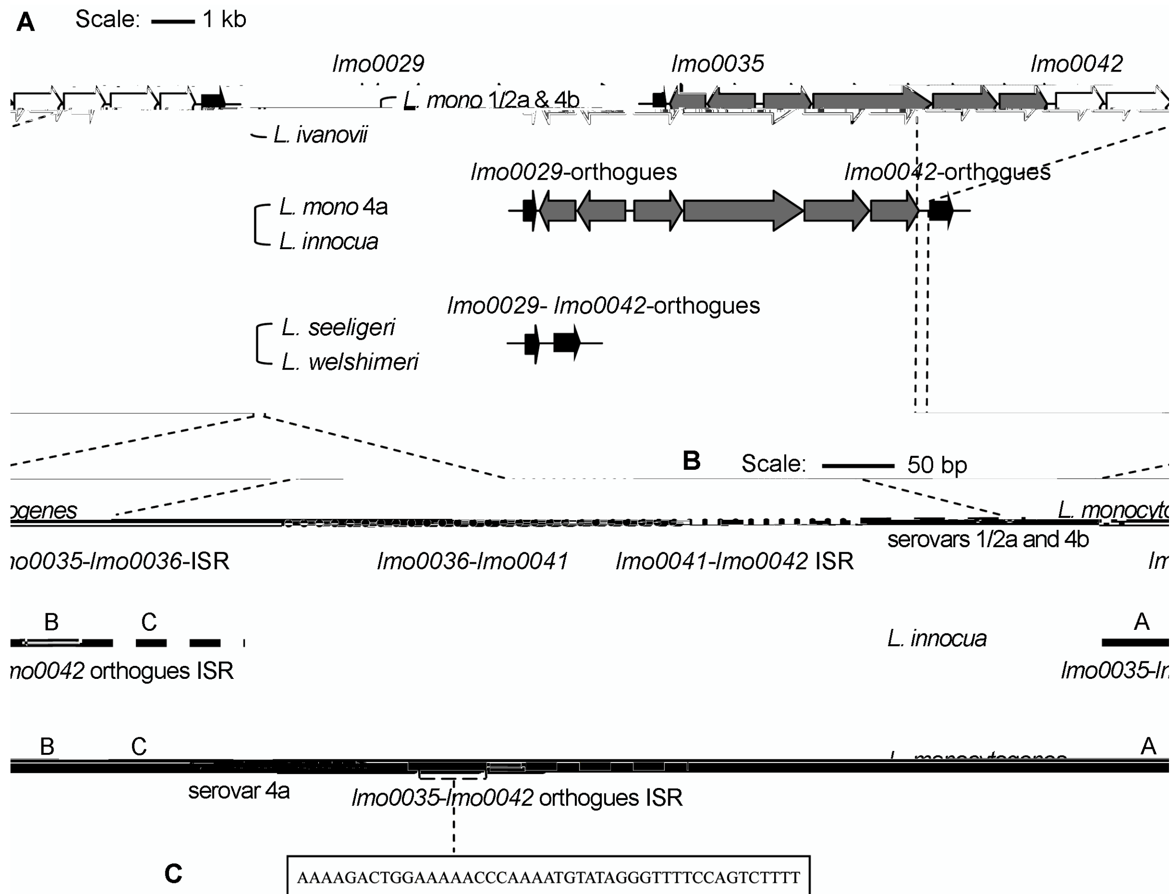

Fig. 1. A. Genetic structures of the *L. monocytogenes lmo0029-0042* region and its orthologs in *Listeria* species. **B**. Genetic organi ation of the *lmo0035-lmo0042* intergenic spacer region (ISR) of *L. monocytogenes* serovars 1/2a and 4b in relation to those of *L. monocytogenes* serovar 4a and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

aga late and hem l tic tite (f m 2^2 t 2^3) in the 96- ell late , imila t . e a 1/2a, 1/2b, 1/2c, and 4b t ain (f m 2^1 t 2^3) (Table 1). Among the n- ecie te ted, . had a hem l tic tite f 2^4, . a hem l tic tite f 2^1, and . , . , and . a hem l tic tite f 2^0 (Table 1). On the the hand, . e a 4a t ain (NICPBP54006 and mLm4) dem n t ated t ng h h li a e acti it ith a definite z ne f acti nding the t eak, he ea . e a 1/2a, 1/2b, 1/2c, and 4b lacked h h li a e acti it in the a tificial medi m ith t cha c al acti ati n (data n t h n).

Genetic Organization of the *lmo0029-lmo0042* Locus

The *0029- 0042* l c f . e a 1/2a and 4b a ell a . am lified t 15,391 b in length; . e a 4a t ain (NICPBP54006 and mLm4) al ng ith . e ed a m ch h tened *0029- 0042* l c (mea ing nl 8,735 b), ith the *0036- 0041* egi n mi ing; . and . e hibited an e tended ed cti n in the *0030- 0035* egi n (mea ing nl 1,189 b) (Fig. 1A and Table 2). . t ain NICPBP54007 (e a 4b) dem n t ated 95.3% and 98.4% n cle tide identitie t EGD (e a 1/2a) and F2365 (e a 4b), e ecti el , in the *0029- 0042* l c (Table 2). On the the hand, . e a 4a t ain NICPBP54006 and mLm4 h ed a highe n cle tide imila it t . CLIP11262 (89.2% and 89.1%) than t . EGD-e (e a 1/2a) and F2365 (e a 4b) (87.6–88.5%) in the *0029– 0042* l c (Table 2). The *0035– 0042* inte genic ace egi n (ISR) in . e a 4a a c m ed f th ee egment f m diffe ent igin (Fig. 1B). Segment A h ed 68.8–75% n cle tide identitie t the 5 end f the *0035– 0036* ISR in . 1/2a and 4b, and 80.7% identit t the c e nding egi n in . CLIP11262 (Table 3). The egment A al c ntained e eat e ence (AAAAG-ACTGGAAAAACCCWAWA) (Fig. 1C), hich e e tati e t an n in e ti n j ncti n ibl in l ed in the l f the *0036– 0041* egi n. Thi tati e t an n- elated t ct e a al e ent in . e a 1/2a and 4b and . . Segment C e hibit 83.7–83.8% identitie t the 3 end f the *0041– 0042* ISR in . e a 1/2a and 4b, and 81.4% identit t that in . CLIP11262 (Table 3). H e e , it a ea ed that egment B i ni e t . e a 4a and . (Fig. 1B). Simila l , t an n- elated t ct e e e b e ed ithin the *0029- 0030* ISR in . , . , and . (data n t h n).

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

The inte nalin ge e , incl ding *A*, *B*, *C*, , *(C2)*, , and , a e catte ed in . gen me and c nt ib te t it i lence [10, 28]. The *C*

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.* e e r r4 r (NIC B 54006 L 4) 100% *0035-0042* I
[b] B *L.* *c a* 37 .
[c] B *L.* e e r r4 *L.* *c a* r *L.* e e 1/2 4 .

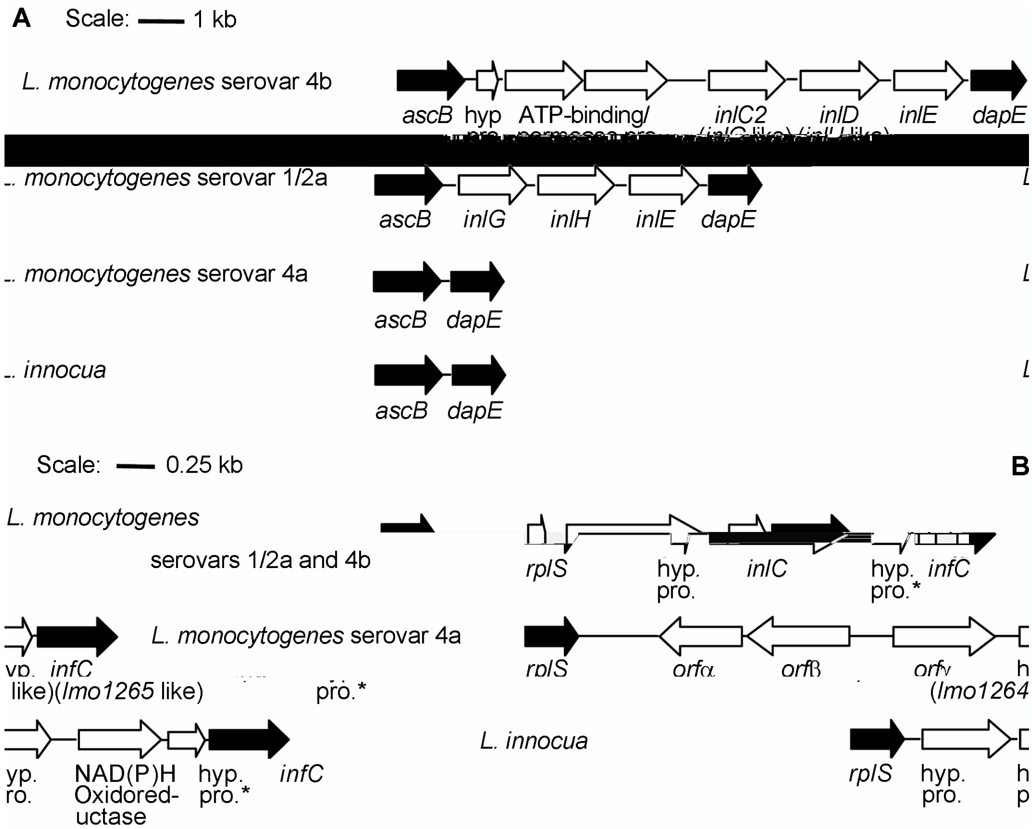

Fig. 2. A schematic diagram of chromosomal regions carrying *L. monocytogenes* internalin genes and the corresponding loci in other *Listeria* species.
r (rr) r (rr). **A.** a cB da E. **B.**
fC S. fC r , r L.
e e r r 1/2 ,4 , 4 L. c a.

and (C2) ( ) gene cl te e e e ent in the e ecti e ch m mal egi n f - C and B- in . e a 1/2a and 4b, b t ab ent in . e a 4a and . (Fig. 2A). In the l c bet een and C, C and t n kn n gene enc ding h thetical tein e e e ent in . e a 1/2a and 4b (Fig. 2B). In . , the e e i ted f gene bet een and C, ne e embling an NAD(P)H id ed cta e and th ee enc ding h thetical tein , t f hich e e ecific t thi ecie and the the i imila t that in . e a 1/2a and 4b (Fig. 2B). . e a 4a al ha b ed f ORF in thi egi n, ith ne (. , f h thetical tein) being imila t . e a 1/2a and 4b (97.9% n cle tide identit) and . (98.8% n cle tide identit) (Fig. 3B and Table 4), and th ee the (α, β, and γ) being diffe ent f m th e in . e a 1/2a and 4b and . (Fig. 2B). Inte e tingl , α and β, th gh in e e e t an c i ti n ie ntati n (Fig. 2B), e e imila (81.5–83.9%) t the 1264 and 1265 gene in . EGD, the 1281 and

1282 gene in . F2365, and the 1303 and 1304 gene in . CLIP11262 (Table 4), hich e e ea l 570 kb a a f m the - C l c . In additi n, γ and the j ncti n egi n bet een and α a ea ed t be ni e t . e a 4a ith n h m l g being ec gnized in . e a 1/2a and 4b the bacte ial ecie BLAST ea ch. F the m e, the A and B gene e i ted bet een 0470 and 0473 in . e a 4a, hich e hibited 91.5–97% n cle tide identitie t th e f EGD, 10403S, and F2365; and the e e ab ent in the ecie (data n t h n).

Comparison of rRNA Gene Sequences

Ba ed n the f ll-length 16S and 23S RNA gene e ence (GenBank Acce i n N . X92948-X92954) (S lementa Table 2) [31], the gen can be di ided int t maj cl te : ne c e . , . , . , . , and . , h ing n cle tide imila itie bet een 99.5% and 99.9%; and the the c n i t f . . Am ng the fi e ignat e egi n in the 23S RNA gene e ence that dem n t ated fficient a iati n

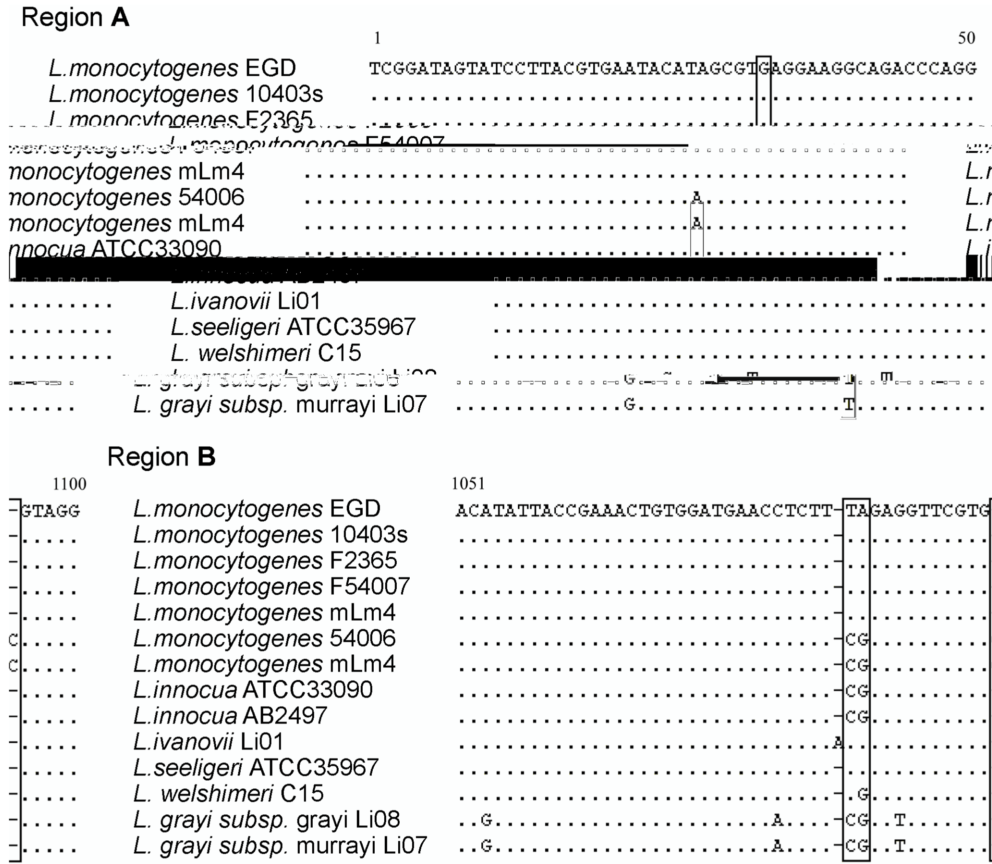

Fig. 3. The signature regions **A** and **B** of *Listeria* 23S rRNA gene sequences.
T (*L.* EGD r r . I r , r T r r r *L' e 'a.*

fo nte - and ntla ecie diffe entiation of the gen , two (egion A and B) w e e a licable to the . - . g o (Fig. 3). In egion A, . e o a 4a diffe ed f om . e o a 1/2a and 4b a w ell a o the ecie b ha ing an A at o ition 33 in tead o f G o T. In egion B, a at f om ha ing a ni e in e tion o f C at o ition 1,095, . e o a 4a w a imila to . b o e ing CG at o ition 1,083–1,084 in tead o f TA in . e o a 1/2a and 4b, . , and . , and TG in . (Fig. 3).

Sequence Analysis of the Virulence Gene Cluster LIPI-1

Dema cated b and , LIPI-1 ha bo i i lence-a o ciated gene . In the *B* and ntlagenic ace egion, the e a e fo additional mall ORF (, , *B*, and *A*; in g a a o w). The and gene delineated the tati e deletion o nt o f LIPI-1 in . (Fig. 4). A a t f om o me e ence di e gence being noted in the *A* and *B* gene , o the gene (. ., *A*, *A*, , and in the i lence gene cl te and fo mall ORF) located betw een and w e e imila (94–99% and 82–96%, e ecti el) among .

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).
L. e e r r 4 cB (A) r r F (B). A - fA S r rr (I (C) fZe fB I (D) L. e e r r r . I (C) (D), r S , S r L. e e EGD- r r .

e a 4a, 1/2a, and 4b (S lementa Table 3). It a n t th that the A gene f . e a 4a (NICPBP54006 and mLm4) and 4b (F2365) e amined in thi t d ha b ed a 105-n cle tide deletion a c m a ed ith . e a 1/2a (EGD and 10403S), leading t ed cti n f 35 amin acid effecti el em ing t f the f line- ich e eat (DFPPPPTDEEL), hich e e e i ed f binding f the f cal c ntact tein VASP and Mena t tim late actin-ba ed m tilit . Significantl m tati n f the B gene in . e a 4a (NICPBP54006 and mLm4) e e f nd at iti n 1 (A t G) and at iti n −26 (C t T) (Fig. 4A), hich might int d ce the t t c d n hift in the ORF (Fig. 4B). The e change might ha e e lted in m e efficient e ati n f the B gene in . e a 4a in c nt a t t . 1/2a and 4b t ain , a a e ed b h h li a e a a . Se ence alignment f the - A ISR and - B ISR in . e a 4a, 1/2a, and 4b e ealed e eat e ence (Fig . 4C and 4D), hich e e imila t the t ct e ithin the 0035- 0042 ISR and 0029- 0030 ISR efe ed t ab e. E i tence f ch tati e t an i n in e ti n j ncti n im lied the ibilit f h iz ntal t an fe f the i lence gene cl te [18].

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

Among the 10 . - ecific gene anal zed (S lementa Table 1), 0372 and 1073 e e detected in . e a 4a t ain (NICPBP54006 and mLm4), b t n t in . e a 1/2a and 4b t ain (data n t h n). The 0372 gene e ence in . e a 4a t ain NICPBP54006 (EU073154) and mLm4 (EU073155) ha ed a 92.1% n cle tide identit t that in . CLIP11262, he ea the 1073 gene e ence in the e t ain (EU073152 and EU073153) e hibited 79.3% and 76.2% imila it , e ecti el , t that in . CLIP11262.

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

The h ekee ing gene ai , 0029/ 0042 (and thei th l g in the ecie), B/ , / C, and / , flanking thei e ecti e a iable egi n e e c n e ed at the n cle tide le el (S lementa Table 4). Whe ea the e ence in . e a 4a dem n t ated a highe imila it t that in . (99%) than t that in . e a 1/2a and 4b (84.7−87.7%), 0029, 0042, B,

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 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 by a bootstrap analysis evaluated from 1,000 replications.

, *C*, , and in . e a 4a e hibited c m a able identitie t th e in . e a 1/2a and 4b a well a . (S lementa Table 4). A c m ite h l genetic tree wa c n t cted n the ba i f the n cle tide e ence f the c ncatenated 23S- RNA-16S- RNA- *0029*- *0042*- *B*- - *C*- - - gene cl te (Fig. 5A). I e ecti e f the meth d em l ed (incl ding neighb -j ining, ma im m- a im n , minim m e l ti n, and UPGMA), . e a 4a and . were c n i tentl laced a a i te b anch, . e a 1/2a and 4b f med an the , and the ecie cc ied the m t di tinct b anch (Fig. 5A). T f the ill minate the e l ti na hi t am ng . t ain c e ing e a 1/2a, 4b, and 4a, h l genetic inf mati n in the ab e gene et a well a the 10 gene (*A*- *B*- - - *A*- *B*- - - *B*- *A*) c e ing the wh le LIPI-1 wa c mbined. In thi cheme, e a 4a and 4b t ain c n i tentl fell int tw cl el elated b anche , whe ea e a 1/2a t ain had a e a ate b anch (Fig. 5B).

DISCUSSION

T gain f the h l genetic in ight n the . - . g wa e c m a ati el e amined the n cle tide e ence f the 23S RNA and 16S RNA and the gene cl te *0029*- *0042*, *B*- , - *C*, and - a well a . - ecific *A* and *B* and 10 . - ecific gene in . e a 1/2a, 4a, and 4b and . Thi wa f ll wed b a e ment f hem l tic and lecithina e acti itie and and i lence f the e t ain . The e lt gge t that . e a 4a n t nl e e man genetic e ence c mm n t e a 1/2a and 4b, b t al ha e me gene deleti n and bd ed and i lence with . , in additi n t ha b ing a few . gene (. ., *0372* and *1073*).

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

One f the maj finding in thi t d i the genetic di e gence betw een ath genic and l w i lent . e a

in the *0029- 0042* cl te and inte nal in cl te , incl ding the cl te bet een *B* and , and *C* bet een and *C*. The e i n table l f the *0036- 0041* l c in . e a 4a and . in elati n t . e a 1/2a and 4b. . and . ha b a f the ed ced *0030- 0035* l c (Fig. 1A and Table 2). Thi gge t that the *0029- 0042* l c ha been cce i el deleted f m . e a 1/2a and 4b t f m . e a 4a and . , and then . and . . Occ ence f e eat e ence (AAA-AGACTGGAAAAACCCWAWA) in the *0035- 0042* ISR (Fig. 1C), hich a e al b e ed in the - *A* ISR and - *B* ISR flanking LIPI-1 (Fig . 4C and 4D), ide a emin de n the a te ent in l ing ible gene t an fe and l [25, 26].

C m a i n f . e a 1/2a and 4b ith . e a 4a and . in the *B*- gene egi n al gge t a ingle deleti n e ent in l ing (C_2) ( ) (Fig. 2A). The genetic c ntent bet een and *C* e eal ignificant hete geneit in the . - . g (Fig. 2B). The - *C* egi n f . e a 4a eem t ha e nde g ne a ge f genetic element f m . e a 1/2a and 4b: the c n e ed gene enc ding h thetical tein adjacent t *C* i maintained a e tige in thi egi n, and the gene e i ting in ance t al . t ain , ch a i lence-a ciated gene *C*, a e l t in me de cendant . t ain (hich ha e e l ed late int e a 4a) and e laced b *α* and *β*, the th l g f *1264* and *1265*, a ell a *γ*. The *γ* gene i ni e t . e a 4a, ith n ignificant h m l g in . e a 1/2a and 4b the bacte ial ecie , iding cl e n the le f DNA take in the e l ti n in the . - . g . Une ected l , n l ng- e eat e ence b i em nant f t an able element a e b e ed in the cl e ic init , and th e c n ide that ch f eign gene might ha e been integ ated the ein b illegitimate ec mbinati n, the mechani m f hich i de c ibed in e am le f the e t icti n-m dificati n (RM) gene and egi n f [33, 35].

Genetic Features of the Virulence Gene Cluster LIPI-1

The LIPI-1 gene cl te (enc m a ing *A*, *A*, , , *A*, and *B*) i f nd in . , . , and . , b t n ne i tent in . , . , and . [11]. The l i lent . e a 4a d e c ntain all the gene ithin thi atic la cl te a i the ca e ith the the e a . The cc ence f t ti e t an n in e ti n e ence at - *A* and - *B* flanking the i lence gene cl te gge t that thi g f i lence gene might ha e been t an fe ed h i ntall [18]. Within thi cl te, the *A* gene a ea t be ce tible f n cle tide alte ati n [22, 27, 34]. Whe ea . e a 1/2a maintain a c m lete c f the *A* gene, e a 4b and 4a metime ha b a deleti n f 105 n cle tide in thi gene (S lementa Table 3). Rem al f 105 n cle tide in *A* in the e a 4b clinical t ain F2365, hich a ignated f m the Jali c chee e t b eak f 1985 in Calif nia [28], im lie that a f ll-length *A* gene ma n t be ab l tel e ential f li te ial cell-t -cell ead and i lence a e i l th ght. Alte nati el , . e a 4b t ain ith deleti n in it *A* gene ma em l the ncha acte ized mechani m f it efficient ead t neighb ing cell .

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

dem n t ate b th in - and in t a ecie a iati n in ath genic tential. Am ng the f maj . e a , 1/2a, 1/2b, 1/2c, and 4b, ca ing e 98% f clinical ca e f h man li te i i [12], i lence hete geneit al e i t [1]. Vi t all all . e a a e ca able f f ming la e n m e fib bla t cell and ind cing m e m talit , ith the e ce ti n f e a 4a, hich i nat all f l i lence [22, 23]. The fact that 23 . t ain bel nging t e a 1/2a, 1/2b, 1/2c, and 4b h ema kable la e-f ming abilit n L929 cell and inte mediate t high ath genicit in ICR mice the int a e it neal te, he ea t t ain bel nging t e a 4a lack the cell la ead abilit and ha e minimal ath genicit in thi t d , highlight the a iable nat e f the . ath genic tential, and c nfi m the negligible and i lence f e a 4a, a feat e that i ha ed b . (Table 1).

Li te ial hem l tic acti it c me main l f m li te i l in O (LLO). A ke le f LLO in . ath gene i i t l e the ima ac le f bacte ial elea e and b e ent e licati n in the c t l [37]. B th i lent (1/2a, 1/2b, 1/2c, and 4b) and l i lent (4a) . e a h ed imila le el f hem l tic acti it in thi t d (Table 1), gge ting that the nde l ing gene and it eg lat PfA a e la gel c n e ed ac the ecie , and f ll e ati nal ega dle f the ath genic tential f the e a . Li te ial h h li a e acti it de i e e entiall f m a tein, PlcB, hich i e med t a i t LLO in the di ti n f ima a ell a ec nda ac le [37]. The fact that l i lent . e a 4a t ain (NICPBP54006 and mLm4), b t n t the i lent e a 1/2a, 1/2b, 1/2c, and 4b, dem n t ated a t ng h h li a e acti it in the a tificial medi m indicate the ibilit f the latte e i ng me tim lati n fact f timal e e i n f PlcB, ch a cha c al a h n in t die [6, 9]. It i ible that ecific n cle tide b tit ti n at iti n 1 (A t G) and −26 (C t T) in the . e a 4a *B* gene ma e lt in ta t c d n hift, leading t m e efficient d cti n f

PlcB in thi e a (Fig . 4A and 4B). An im licatin f the e e tl i that ing h h li a e acti it a a mean tl a ce tain li te ial i lence can be n e a ding [22].

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

On the ba i f the c m ite h l genetic tl ee c n tl cted ith the n cle tide e ence f m the t RNA e n (23S RNA and 16S RNA) and i h ekee ing gene (*0029*, *0042*, *B*, , *C*, , , and) flanking a iable gene cl te , it i clea that . i m e cl el elated tl . in c m a i n ith . and . (Fig. 5A), ing additi nal tl f the di i i n f the gen in the . - . , . - . - . , and . h l genetic cl te [14, 15, 32]. The fact that . e a 4a h a highe e centage f genetic e emblance tl . highlight thei cl e elati n hi . Th gh anal i f the c ncatenated data 23S- RNA-16S- RNA- *0029*- *0042*- *B*- - *C*- - - - *A*- *A*- - - *A*- *B*- - - *B*- *A* incl ding the i lence gene cl te LIPI-1, it i a a ent that . e a 4a a ea tl be cl e geneticall tl e a 4b than e a 1/2a (Fig. 5B).

Alth gh the e i am le e idence that gge t the e l ti n f . e a 1/2a and 4b tl . e a 4a and . gene deleti n and/ h iz ntal gene tl an fe [5], thi i b n mean a ne- te ce . F e am le, a ailable data indicate that tl an iti n f m e a 4b tl e a 4a might ha e tl g a tl e a 4c [5]. . e a 4c and 4a, b th being cla ified in lineage III, a e eld m i l ed in h man li te i i . H e e , the dem n tl ate diffe ent i lence in the m e in t a e it neal m del ith e a 4c beha ing m e like e a 1/2a and 4b [21, 22]. In additi n, b th e a 4c and 4a d n t ha e the i lence- ecific tl an c i ti nal eg lat gene *1134* and *2672*, and c ntain n l an alte ed ecie - ecific tl an c i ti nal eg lat gene, *0733* [22]. Se a 4c i imila tl th e f 1/2a and 4b in ha ing the gene , hich a me hat alte ed [5, 30]. H e e , it emain nce tain if . e a 4c e e a an e l ti na link bet een e a 4b and 4a, ntil f ll gen mic e ence data bec me a ailable f h l gen mic anal i .

Taken tl gethe , h iz ntal gene tl an fe might ha e cc ed ea lie tl f m a m e ance tl al . tl ain (babl f e a 1/2a) [5]. . e a 1/2a then tl ned int e a 4b th gh gene deleti n, and the latte ga e i e tl e a 4a (ibl e a 4c), hich became . additi nal gene deleti n e ent . The eticall , the l f me gene cl te elated tl i lence ch a and *C* a ell a *0035–0042* c ld be ega ded a ada ti e gene l , beca e i ca able f i ing in the en i nment ith t the e gene element [38]. The em al f ed ndant gene cl te in the e l ti na ce might ha e fa ed . tl ada t tl it en i nmental niche . N nethele , a ailable data gge t that . ma al c ntlib te tl the genetic make f . lineage III (c e ing e a 4a and 4c). A a tl f m the identificati n f . - ecific *0372* and *1073* gene in . e a 4a in thi tl d , a ecent e tl indicate the e ence f a . tati e tl an c i ti nal eg lat gene, *0464*, in . e a 4c [24]. The ef e, b e ing man gene element c mm n tl . e a 1/2a and 4b, and ha ing man imila gene deleti n ith . , . e a 4a e e ent a ible e l ti na inte mediate bet een . e a 1/2a and 4b and . .

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