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Deciphering the Biodiversity of *Listeria monocytogenes* Lineage III Strains by Polyphasic Approaches[§]

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***Listeria monocytogenes* is a foodborne pathogen of humans and animals. The majority of human listeriosis cases are caused by strains of lineages I and II, while lineage III strains are rare and seldom implicated in human listeriosis. We revealed by 16S rRNA sequencing the special evolutionary status of *L. monocytogenes* lineage III, which falls between lineages I and II strains of *L. monocytogenes* and the non-pathogenic species *L. innocua* and *L. marthii* in the dendrogram. Thirteen lineage III strains were then characterized by polyphasic approaches. Biochemical reactions demonstrated 8 biotypes, internalin profiling identified 10 internalin types clustered in 4 groups, and multilocus sequence typing differentiated 12 sequence types. These typing schemes show that lineage III strains represent the most diverse population of *L. monocytogenes*, and comprise at least four subpopulations IIIA-1, IIIA-2, IIIB, and IIIC. The *in vitro* and *in vivo* virulence assessments showed that two lineage IIIA-2 strains had reduced pathogenicity, while the other lineage III strains had comparable virulence to lineages I and II. The IIIB strains are phylogenetically distinct from other subpopulations, providing additional evidence that this sublineage represents a novel lineage. The two biochemical reactions L-rhamnose and L-lactate alkalinization, and 10 internalins were identified as potential markers for lineage III subpopulations. This study provides new insights into the biodiversity and population structure of lineage III strains, which are important for understanding the evolution of the *L. monocytogenes-L. innocua* clade.**

***Keywords*:** *L. monocytogenes*, lineage III, biodiversity, virulence, subpopulation

The genus *Listeria* comprises a number of species including *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* (Buchrieser, 2007), as well as two new species *L. marthii* (Graves *et al*., 2010) and *L. rocourtiae* (Leclercq *et al*., 2010). *L. monocytogenes* is the only species capable of causing life-threatening infections in animals and humans. *L. innocua* is closely related to *L. monocytogenes* and has once been postulated as the nonpathogenic variant of *L. monocytogenes* (Glaser *et al*., 2001; Chen *et al*., 2010a; Velge and Roche, 2010). The morphological, ecological, biochemical, and genetic resemblance, and clear difference of virulence between *L. monocytogenes* and *L. innocua* make this bacterial clade attractive as models to examine the evolution of pathogenicity (Johnson *et al*., 2004; Chen *et al*., 2010a).

By using various phenotypic and genotypic methods, *L. monocytogenes* can be divided into at least three phylogenetic lineages, of which lineage I encompasses major serovars 1/2b and 4b, lineage II includes major serovars 1/2a and 1/2c, and lineage III consists of sublineages IIIA, IIIB, and IIIC covering serovars 4a, 4c, and atypical 4b (Wiedmann *et al*., 1997; Roberts *et al*., 2006). While lineages I and II strains are responsible for 99% of listeriosis cases in humans (Swaminathan and Gerner-Smidt, 2007), lineage III strains are extremely rare and seldom implicated in human listeriosis (Roberts *et al*., 2006; Chen *et al*., 2009a; Orsi *et al*., 2010).

One noticeable aspect of lineage III is the strikingly higher level of phenotypic and genetic diversity among strains compared to those of lineages I and II (Doumith *et al*., 2004; Liu *et al*., 2006; Roberts *et al*., 2006). Recently, two low-pathogenic lineage III strains were identified (Chen *et al*., 2009b). By possessing many genes common to *L. monocytogenes* lineages I and II, and sharing many similar gene deletions with *L. innocua*, these two strains were considered as evolutionary intermediates between *L. monocytogenes* and *L. innocua* (Chen *et al*., 2009b). It is tempting to speculate that analysis of the biodiversity and population structure in lineage III may facilitate better understanding of the evolutionary history in the *L. monocytogenes* - *L. innocua* clade. Although lineage III is known to contain three sublineages (Roberts *et al*., 2006), systematic knowledge on strain diversity and further subdivision of this lineage is still limited.

This study used polyphasic approaches, including 21-gene-based multilocus sequence typing, 46-reaction-based biochemical typing, 37-gene-based internalin profiling and *in vitro* and *in vivo* virulence assessments, in an attempt to (i) compare the pathogenic potential of *L. monocytogenes* lineage III strains; (ii) depict the phenotypic and genetic characteristics of lineage III strains; and (iii) determine further partitioning of lineage III.

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Materials and Methods

Bacterial strains

A total of 92 *Listeria* strains from our strain collection were examined in this study (Supplementary data Table 1), 58 *L. monocytogenes* strains covering lineages I (n=32), II (n=13), and III (n=13), and 34 *L. innocua* strains including subgroups A (n=19), B (n=13), C (n=1), and D (n=1). All stock cultures were stored at -80°C in 20% glycerol, and grown in brain heart infusion broth (BHI; Oxoid, England) at 37°C.

DNA manipulations

Genomic DNA was extracted as described previously (Chen *et al*., 2009c). Oligonucleotide primers for PCR were synthesized by Invitrogen Biotechnology (China), and *Taq* DNA polymerase (TaKaRa, China) was used for regular PCR reactions. For products larger than 4 kb, *LA Taq* DNA polymerase (TaKaRa) was employed. PCR reactions were conducted using the PT-200 thermal cycler (MJ, USA). PCR fragments were purified using the AxyPrep DNA Gel Extraction kit (Axygen, USA) and ligated into pMD18-T (TaKaRa). The recombinant plasmids were then sequenced by the dideoxy method on an ABI-PRISM 377 DNA sequencer.

Construction of phylogenetic tree based on 16S rRNA

The complete 16S rRNAs in 92 *L. monocytogenes* and *L. innocua* strains were amplified and sequenced. The sequences of 4 *L. marthii* strains were obtained from the NCBI database (EU545980 to EU545983) (Graves *et al*., 2010). These 96 sequences were used to construct a phylogenetic tree via MEGA 4.0 by using the neighbor-joining method (Tamura *et al*., 2007).

Multilocus sequence typing

Twenty-one genes were selected for the multilocus sequence typing (MLST) of 24 *L. monocytogenes* strains (6 belonging to lineage I, 5 to lineage II and 13 to lineage III), including 14 housekeeping genes (*gyrB*, *prs*, *ldh*, *dapE*, *bglA*, *hisJ*, *ribC*, *lhkA*, *daaA*, *purM*, *gap*, *tuf*, *abcZ*, *kat*), 3 stress-response genes (*arcA*, *betL*, *sigB*) and 4 virulence genes (*prfA*, *actA*, *inlA*, *inlB*) (Table 1). MEGA 4.0 was used to construct a neighbor-joining tree of *L. monocytogenes* strains using the number of nucleotide differences in the concatenated sequences of 21 loci

Table 1. PCR primers used for multilocus sequence typing

Locus	Putative function	Forward primer	Reverse primer	Length (bp)	Reference
gyrB	DNA gyrase subunit B	TGGTGCATCGGTAGTTAATGC	CAACATCTGGGTTTTCCATCAT	657	Chen *et al*. (2010b)
arcA	Arginine deiminase	AATGATTAAAGAATCCAATCAA	CTTGATTTCGATCATATGTAATTA	759	This study
prs	Phosphoribosyl pyrophosphate synthetase	GAAAGTATCCGTGGTTGTCATG	CTTCTGGAAGAGCGATGGAG	630	This study
prfA	Positive regulatory protein	CCCAAGTAGCAGGACATGCTA	ACGCTCAAGCAGAAGAATTCA	630	This study
actA	Actin-assembly inducing protein precursor	GGTACGTGATAAAATCGACGA	TAGTTATGTCACTTATCAGAGC	414	Wiedman *et al.* (1997)
ldh	L-lactate dehydrogenase	GAAAATAGTATCCATTGCACCT	TCTAGTTACGCATTTGCTTGT	522	This study
dapE	Succinyl diaminopimelate desuccinylase	GTAAATATTGATTCGACTAATG	CACTAGCACTTGTTTCACTG	669	Chen *et al*. (2010b)
bglA	Phospho-beta-glucosidase	GTGCTCATGTAATAGCTGAAGGA	TCAAGGTTTTCCGTACTTCCA	627	This study
inlA	Internalin A	TAATATAAGTGATATAAGCCCAG	TTTATCCGTACTGAAATTCC	549	Chen *et al*. (2009d)
inlB	Internalin B	AACCCAACCACTGAAAGAGGT	GTAGTAATTGGTGTTTTGGCTTC	564	This study
hisJ	Histidinol phosphate phosphatase	TCCACATGGTACGCATGAT	GGACATGTCAAAATGAAAGATC	714	Chen *et al*. (2010b)
sigB	Stess responsive alternative sigma factor B	CCAAAAGTATCTCAACCTGAT	CATGCATTTGTGATATATCGA	642	Chen *et al*. (2010b)
ribC	Riboflavin kinase and FAD synthase	AAGACGATATACTTACATCAT	GTCTTTTTCTAACTGAGCA	633	Chen *et al*. (2010b)
lhkA	Two-component sensor histidine kinase	ATTTTTACCAAAGCCAAGTAGA	TTGTCGTTCAATTTCTTCTTG	576	This study
daaA	D-amino acid aminotransferase	GCTTGAACGATTTCTTCTACAA	GCGGATATCAGTTTGGTGAT	711	This study
purM	Phosphoribosyl aminoimidazole synthase	CAAGCTCCACTTTGACAGCTAA	TAAAGCAGGCGTGGACGTA	693	Chen *et al*. (2010b)
betL	Glycine betaine transporter	ACAGAACATTATCCAAATGAGTT	ACGTTGTGATTTTTTCGGTC	534	Chen *et al*. (2010b)
gap	Glyceraldehyde 3-phosphate dehydrogenase	CTGGATCAGAAGCTGCTTCCA	GTCGTATTCAAAATGTGGAAGGA	621	Chen *et al*. (2010b)
tuf	Translation elongation factor	CATTTCTACTCCAGTTACTACT	GCTCTAAACCCCATGTTA	681	Chen *et al*. (2010b)
abcZ	ABC transport	GTAGTTTTTGCGACATACCTTCA	AGAGCTAACCATCCACCGAAG	687	This study
kat	Catalase	TGACCGTCACGTTGGTTGT	TCATTCGTGATGCGATTAAGT	630	This study
Subtotal				13143	

with 1,000 bootstrap tests (Tamura *et al*., 2007). An unrooted tree was constructed because a suitable outgroup that is closely related to *L. monocytogenes* and that contains orthologues to all 21 genes was not available. Nucleotide diversity indices and Tajima's D (for testing if nucleotide sequences have evolved according to the neutral theory) were calculated using DNAsp v4.8 (Rozas *et al*., 2003). Discrimination index (D.I.) values were calculated according to the method previously described (Hunter and Gaston, 1988).

Plaque forming assay

A plaque forming assay was performed to determine the ability of 15 *Listeria* strains (1 each in lineages I and II, and 13 in lineage III) to spread from cell to cell as represented by plaque size (Liu *et al*., 2007). This assay was conducted in mouse fibroblasts L929 cells as previously described (Chen *et al*., 2009d). These experiments were repeated three times, in triplicate wells for each strain. The plaque size of serovar 4b strain M5 was set at 100%.

Mouse virulence assay

The *in vivo* virulence of 15 *Listeria* strains was assessed in conventional ICR mice (Chen *et al*., 2009d) or immunocompromised ICR mice intraperitoneally injected with 200 mg/kg carrageenan 24 h before challenge (Stelma *et al*., 1987). The mice (6/group) were inoculated intraperitoneally with 10-fold serial dilutions of *Listeria* cells suspended in phosphate-buffered saline (PBS, pH 7.2). Mice receiving PBS were included as control. The LD_{50} values were calculated by using the trimmed Spearman-Karber method on the basis of mouse mortality data recorded during a ten-day post-injection period (Finney, 1985). The animal experiments were approved by the Animal Welfare Committee of Zhejiang University.

Biochemical patterns

Forty-six biochemical reactions were performed using Vitek32 (bioMérieux, France) according to the manufacturer's instructions, including reactivities of D-xylose, D-sorbitol, D-galactose, lactose, D-ribose, D-mannitol, D-mannose, D-maltose, D-raffinose, sucrose, D-trehalose, L-rhamnose, N-acetyl-D-glucosamine, pullulan, cyclodextrin, D-amygdalin, methyl-β-D-glucopyranoside, salicin, α-glucosidase, α-galactosidase, β-galactosidase, β-galactopyranosidase, α-mannosidase, β-glucuronidase, β-D-glucuronidase, Ala-Phe-Pro arylamidase, L-proline arylamidase, L-aspartate arylamidase, leucine arylamidase, alanine arylamidase, tyrosine arylamidase, L-pyrrolidonyl-arylamidase, phosphatase, urease, arginine dihydeolase 1, and arginine dihydrolase 2, as well as β-hemolysis, PIPLC, PCPLC, polymixin B resistance, novobiocin resistance, optochin resistance, bacitracin resistance, O/129 resistance, L-lactate alkalinization and growth in 6.5% NaCl. A distinct biochemical type (BT) number was given to each different combination of biochemical activities.

Internalin profiling

Presence or absence of 19 *L. monocytogenes*-specific, 4 *L. innocua*-specific, and 14 *L. monocytogenes-L. innocua*-common internalin

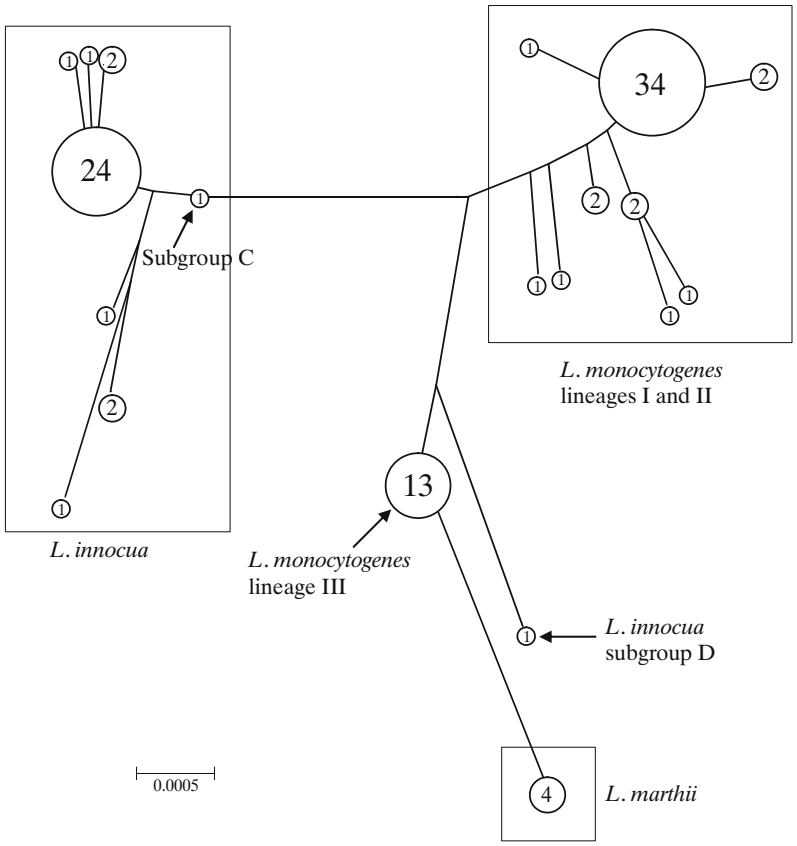

Fig. 1. A Neighbor-joining cladogram of 58 *L. monocytogenes*, 34 *L. innocua*, and 4 *L. marthii* strains based on the complete sequences of 16S rRNA.

Table 2. Descriptive analysis of nucleotide sequences of 21 genes for *L. monocytogenes* lineage III strains

Gene	Size (bp)	No. of strains	No. of alleles	No. (%) of polymorphic sites	π	D. I.	Tajima's D
gyrB	657	13	12	42 (6.39)	0.02073	0.99	-0.1477
arcA	759	13	11	77 (10.14)	0.03265	0.97	-0.4725
prs	630	13	9	24 (3.81)	0.01330	0.95	0.3069
prfA	630	13	10	30 (4.76)	0.01746	0.95	0.2225
actA	414	13	11	43 (10.39)	0.03971	0.97	0.4100
ldh	522	13	12	38 (7.28)	0.02713	0.99	0.4425
dapE	669	13	10	105 (15.70)	0.06909	0.95	1.0062
bglA	627	13	11	62 (9.89)	0.03851	0.97	0.4519
inlA	549	13	9	51 (9.29)	0.03635	0.94	0.6057
inlB	564	13	10	34 (6.03)	0.02007	0.96	-0.0539
hisJ	714	13	9	65 (9.10)	0.03101	0.94	-0.1286
sigB	642	13	6	56 (8.72)	0.04432	0.82	2.2302[b]
ribC	633	13	9	43 (6.79)	0.02592	0.94	0.6975
lhkA	576	13	12	50 (8.68)	0.02836	0.99	-0.1325
daaA	711	13	11	37 (5.20)	0.02244	0.97	1.0675
purM	693	13	11	52 (7.50)	0.03190	0.97	0.7668
betL	534	13	11	85 (15.92)	0.08197	0.97	1.5220
gap	621	13	4	7 (1.13)	0.00548	0.68	1.8980[a]
tuf	681	13	9	13 (1.91)	0.00644	0.92	0.1669
abcZ	687	13	11	43 (6.26)	0.02711	0.97	1.3983
kat	630	13	11	49 (7.78)	0.03074	0.97	0.4710
Concatenated	13143	13	12	1006 (7.65)	0.03012	0.99	0.6283
Concatenated, IIIA	13143	6	5	304 (2.31)	0.01071	0.93	0.2964
Concatenated, IIIB	13143	5	5	338 (2.57)	0.01221	1.00	-0.1699
Concatenated, IIIC	13143	2	2	149 (1.13)	0.01142	1.00	N/A[c]

[a] $0.05<p<0.10$
[b] $p<0.05$
[c] Limited number of strains, resulting in inability to compute Tajima's test.

genes were examined by PCR as previously described (Chen *et al.*, 2010a) in 18 *L. monocytogenes* strains (3 in lineage I, 2 in lineage II, and 13 in lineage III) as well as 4 *L. innocua* strains. Due to conserved repeats present in the internalin multigene family, primers were designed based on the distinguishable regions through sequence comparison. A distinct internalin type (IT) number was given to each different internalin composition.

Statistical analysis

The two-tailed Student's *t*-test was used for comparison of data, where necessary, and P values≤0.01 were considered as statistically significant.

Results and Discussion

L. monocytogenes lineage III was placed between *L. monocytogenes*, *L. innocua*, and *L. marthii* based on 16S rRNA sequences

The 16S rRNA molecule is useful for determining phylogenetic relationships of prokaryotes (Cohan, 2001). In this study, the dendrogram based on the complete sequences of 16S rRNA revealed three major clusters, corresponding to three species *L. monocytogenes*, *L. innocua*, and *L. marthii*. *L. monocytogenes* lineage III and *L. innocua* subgroup D fell between the three clusters (Fig. 1), indicating their special evolutionary status from a phylogenic perspective. *L. innocua* subgroup D was suggested as an evolutionary link in our previous report (Chen *et al*., 2010a). However, 16S rRNA is highly conserved, limiting its use for subtyping of strains within the same species or lineage (subgroup). Therefore, further partitioning of *L. monocytogenes* lineage III strains requires additional subtyping methods.

MLST revealed high diversity in *L. monocytogenes* lineage III

In order to construct a fully resolved phylogenetic tree with maximum support, a concatenation of a minimum of 20 genes was needed (Rokas *et al*., 2003). Virulence genes evolve more rapidly than housekeeping genes, and may provide a higher degree of nucleotide sequence polymorphisms with improved discriminatory power for subtyping (Zhang *et al*., 2004). Thus, in this study, the MLST was based on 21 genes including 4 virulence genes. The concatenated sequences had a total of 1006 polymorphic sites (7.65% on average ranging from 1.13% to 15.70% per gene) and recognized 12 sequence types with D.I. at 0.99. The average nucleotide diversity (π) was 0.03012, ranging from 0.00548 to 0.06909 (Table 2). Consistent with previous reports (Ward *et al*., 2008), lineage III strains showed a significantly higher level of nucleotide diversity than those of lineages I and II. When sequence data were analyzed after stratification by sublineages, the number of polymorphisms and genetic diversity within each subpopulation were reduced (Table 2), suggesting a barrier for genetic exchange between these lineage III subgroups. Roberts *et al*. (2006) proposed

that IIIA was the most genetically diverse subpopulation within lineage III based on the elevated measures of nucleotide diversity of *actA* and *sigB*. However, this study showed similar levels of nucleotide diversity within three sublineages (Table 2). Such discrepancy was possibly due to selection of target genes. To our knowledge, this study used the largest-ever gene set for MLST subtyping of foodborne pathogens.

Tajima's D test demonstrated that, apart from *sigB* ($p<0.05$) and *gap* ($0.05<p<0.10$), other genes in lineage III evolved under neutrality, as none of the Tajima's D test statistics were significantly different from the expected value of zero (Table 2). The large Tajima's D test statistics became smaller or negative upon stratification by sublineages (Table 2), supporting that lineage III represented a subdivided population.

The phylogenetic tree revealed four major branches of *L. monocytogenes*, corresponding to lineages I, II, IIIB, and IIIA/IIIC. IIIA could be further divided into 3 subbranches, one harboring strains 54006 and M7, one bearing strain F2-525 and the other containing strains F2-695, J1-168, and J2-071 (Fig. 2). Sublineage IIIB was phylogenetically distinct from IIIA and IIIC, and represented a separate unit at the lineage level. Ward *et al*. (2008) suggested that sublineage IIIB should be designated as lineage IV through analysis of sequences from three genomic regions (*prfA* island, *lmo0298-lmo0300*, *inlAB*) in order to make clear that this unique lineage was not part of, or even closely related to, lineage III. However, considering the small collection of lineage III strains and relatively limited knowledge on its phylogeny, we still name this lineage as IIIB in this study.

L. monocytogenes lineage III strains exhibited varied *in vitro* and *in vivo* pathogenic potential

As *L. monocytogenes* spreads from cell to cell, plaques are formed in the L929 cell monolayer due to its cytopathic effects (Liu *et al*., 2007). Apart from two IIIA strains 54006 and M7 that failed to form visible plaques, the other lineage III strains formed plaques with relative sizes from 87.2±3.3% to 98.9±3.3%, regardless of whether they possessed an intact *actA* gene or had a 105 nucleotide deletion that resulted in the removal of one of the four proline-rich repeats (PRRs) (Table 3).

Consistently, IIIA strains 54006 and M7 with impaired cell-to-cell motility exhibited reduced virulence in mice (LD_{50} $10^{8.21}$-$10^{8.35}$ CFU), while other lineage III strains showed comparable virulence (LD_{50} $10^{4.14}$-$10^{6.60}$ CFU) to those of lineages

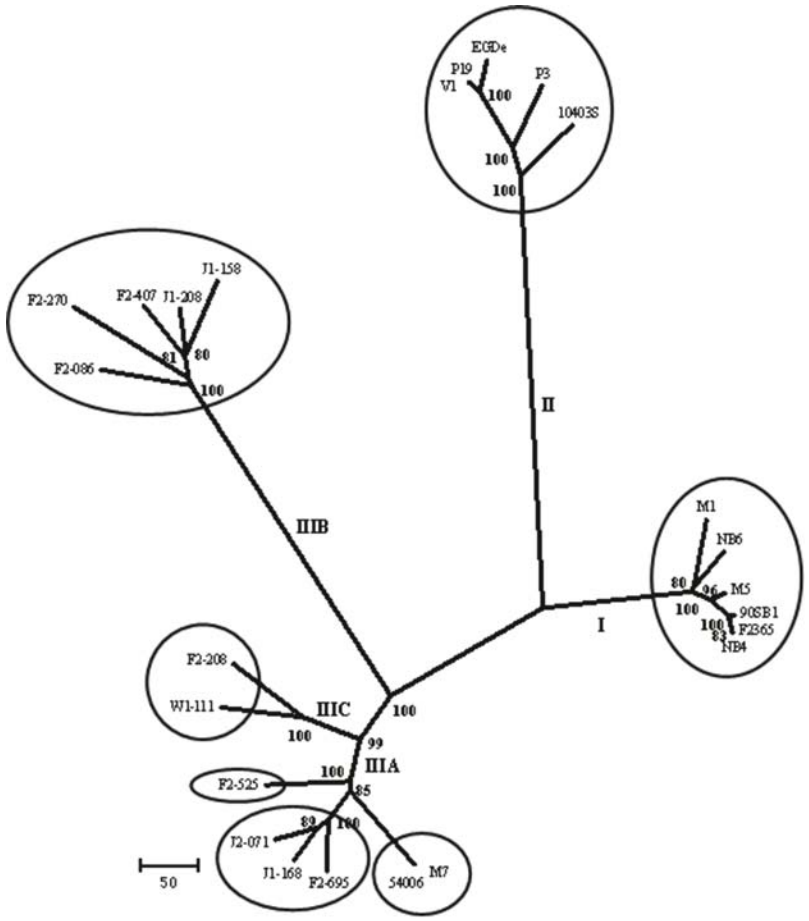

Fig. 2. An unrooted phylogenetic tree of 24 *L. monocytogenes* strains by the concatenated data set *gyrB-arcA-prs-prfA-actA-ldh-dapE-bglA-inlA-inlB-hisJ-sigB-ribC-lhkA-daaA-purM-betL-gap-tuf-abcZ-kat*. The numbers, I, II, IIIA, IIIB, and IIIC on the branches represent *L. monocytogenes* lineages I, II, IIIA, IIIB, and IIIC respectively. The values on the branches (expressed as percentages) indicate the robustness of the corresponding branches, as determined by a bootstrap analysis evaluated from 1,000 replications.

Table 3. *In vitro* and *in vivo* virulence of *L. monocytogenes* lineage III strains

Strain	Source	Lineage	Serovar	*actA* (bp)[a]	Relative size of plaque±SD (%)	log LD_{50}	
						Untreated mice	Immunocom-promised mice[a]
M5	Seafood	I	4b	432	100±0	3.86	0.90
10403S	Reference	II	1/2a	537	92.3±5.8	5.49	0.93
F2-525	Human	IIIA	4b	537	97.5±5.7	4.94	0.65
F2-695	Human	IIIA	4a	537	98.9±3.3	4.73	0.93
J1-168	Human	IIIA	4a	537	96.5±4.5	5.56	0.81
J2-071	Animal	IIIA	4c	537	98.2±3.9	5.33	0.80
54006	Unknown	IIIA	4a	432	0	8.35	7.68
M7	Milk	IIIA	4a	432	0	8.21	7.18
F2-407	Human	IIIB	4a	432	92.1±2.7	5.20	1.20
J1-208	Animal	IIIB	4a	432	94.0±5.1	4.14	0.64
J1-158	Animal	IIIB	4b	432	90.9±5.8	6.61	0.85
F2-270	Human	IIIB	4a	432	91.2±3.3	5.25	0.90
F2-086	Human	IIIB	4a	432	93.3±6.2	5.15	0.65
F2-208	Human	IIIC	4a	537	88.5±3.7	6.50	1.50
W1-111	Unknown	IIIC	4c	432	87.2±3.3	5.68	1.93

[a] The *actA* gene in some *L. monocytogenes* strains contained a 105 nucleotides deletion.

I and II reference strains (LD_{50} $10^{3.86}$-$10^{5.49}$ CFU) (Table 3). Because the differences of LD_{50} values in normal mice were relatively small between virulent and low-virulent strains, immunocompromised mice were used to examine their virulence. The LD_{50} of strains 54006 and M7 ($10^{7.18}$-$10^{7.68}$ CFU) were significantly higher than those of virulent strains ($10^{0.64}$-$10^{1.93}$ CFU), about a 6 to 7 log reduction ($p<0.01$) (Table 3).

These results suggest that (i) *L. monocytogenes* lineage III consists of low-virulent IIIA strains (corresponding to one of the three subbranches within IIIA) as well as virulent IIIA, IIIB, and IIIC strains; and (ii) three PRRs of ActA appear to be sufficient for lineage III strains to spread intracellularly and intercellularly, and cause listeriosis in the murine model, similar to the findings for lineages I and II strains in our previous study (Chen *et al*., 2009d).

Biochemical profiling demonstrated 8 biotypes of *L. monocytogenes* lineage III strains

Among 46 biochemical reactions, 20 were positive for all *L. monocytogenes* lineage III strains, 17 negative for all lineage III strains, and 9 displayed distinct patterns amongst these strains (Table 4). Overall, 8 biotypes (BTs) were identified. Specifically, low-virulent IIIA strains belonged to BT2, other virulent IIIA strains to BT1, IIIC strains to BT8, and each IIIB strain represented an individual BT from 3 to 7 (Table 4). The biochemical typing was consistent with sublineage structures, dividing lineage III strains into low-virulent IIIA, and virulent IIIA, IIIB, and IIIC. IIIB represented the sublineage having the most diverse biochemical characteristics. IIIA (including virulent and low-virulent strains) was specifically positive for L-rhamnose (lRHA), and low-virulent IIIA was exclusively L-lactate alkalinization (lLATk) positive (Table 4). These two reactions could serve as potential phenotypic markers for recognizing IIIA.

Internalin profiling identified 10 internalin types of *L. monocytogenes* lineage III strains

Upon examination of 19 *L. monocytogenes*-specific, 14 *L.*

Table 4. Biotypes (BTs) of *L. monocytogenes* lineage III strains based on biochemical reactions

Strain	Lineage	LAC	dMAL	SAC	lRHA	AGLU	APPA	AspA	AlaA	lLATk	Biotype
F2-525	IIIA	+	+	–	+	–	–	–	–	–	1
F2-695	IIIA	+	+	–	+	–	–	–	–	–	1
J1-168	IIIA	+	+	–	+	–	–	–	–	–	1
J2-071	IIIA	+	+	–	+	–	–	–	–	–	1
54006	IIIA	+	+	–	+	–	+	+	+	+	2
M7	IIIA	+	+	–	+	–	+	+	+	+	2
F2-407	IIIB	+	–	–	–	–	+	+	+	–	3
J1-208	IIIB	+	+	–	–	–	–	–	–	–	4
J1-158	IIIB	+	–	+	–	+	+	+	+	–	5
F2-270	IIIB	+	+	–	–	–	+	–	+	–	6
F2-086	IIIB	–	+	–	–	–	–	–	–	–	7
F2-208	IIIC	+	+	–	–	–	+	+	+	–	8
W1-111	IIIC	+	+	–	–	–	+	+	+	–	8

LAC, dMAL, SAC, lRHA, AGLU, APPA, AspA, AlaA, and lLATk represent Lactose, D-maltose, Sucrose, L-rhamnose, α-galactosidase, Ala-Phe-Pro arylamidase, L-aspartate arylamidase, Alanine arylamidase and L-lactate alkalinization

Table 5. Internalin profiling of *L. monocytogenes* and *L. innocua* strains based on 37 internalin genes

Strain	Lineage/ Subgroup	*L. monocytogenes* -specific	*L. innocua* -specific	*L. monocytogenes*-*L. innocua*-common	Subtotal	Internalin type (IT)	Internalin group
L. monocytogenes							
F2365	I	12	0	13	25	–	–
H7858	I	12	0	13	25	–	–
CLIP80459	I	12	0	13	25	–	–
EGD-e	II	15	0	10	25	–	–
F6854	II	16	0	11	27	–	–
F2-525	IIIA	11	0	11	22	1	1
F2-695	IIIA	10	0	11	21	2	1
J1-168	IIIA	10	0	11	21	3	1
J2-071	IIIA	9	0	11	20	4	1
54006	IIIA	4	0	11	15	5	2
M7	IIIA	4	0	11	15	5	2
F2-407	IIIB	3	0	6	9	6	3
J1-208	IIIB	4	0	6	10	7	3
J1-158	IIIB	4	0	7	11	8	3
F2-270	IIIB	4	0	6	10	7	3
F2-086	IIIB	3	0	6	9	6	3
F2-208	IIIC	6	0	11	17	9	4
W1-111	IIIC	6	0	10	16	10	4
L. innocua							
ATCC33090	A	0	4	14	18	–	–
NB2	B	0	4	13	17	–	–
0063	C	0	4	12	16	–	–
L43	D	1	4	13	18	–	–
Subtotal		19	4	14	37	–	–

monocytogenes-L. innocua-common, and 4 *L. innocua*-specific internalin genes, *L. monocytogenes* lineage III strains harbored fewer internalin genes (ranging from 9 to 22) than lineages I and II (ranging from 25 to 27). Specifically, 3 to 11 *L. monocytogenes*-specific and 6 to 11 *L. monocytogenes-L. innocua*-common internalin genes were identified in lineage III strains, and there were no *L. innocua*-specific internalin genes detected (Table 5).

Based on internalin profiling, lineage III strains were classified into 10 internalin types (ITs), with virulent IIIA strains belonging to ITs 1 to 4, low-virulent IIIA strains to IT5, IIIB strains to ITs 6 to 8, and IIIC strains to ITs 9 and 10. These ITs were clustered into 4 groups. Group 1 contained ITs 1 to 4 with 20 to 22 internalin genes. Group 2 correlated with IT5 bearing 15 internalins. Group 3 included ITs 6 to 8, harboring the fewest internalins ranging from 9 to 11. Group 4 covered ITs 9 and 10 with 16 to 17 internalins (Table 5). This grouping was also consistent with the observation that lineage III encompassed four subpopulations, corresponding to low-virulent IIIA, virulent IIIA, IIIB, and IIIC.

Table 6. Internalins specific for *L. monocytogenes* lineage III subpopulation

Internalin	Virulent IIIA	Low-virulent IIIA	IIIB	IIIC
inlD	+	–	–	–
inlE	+	–	–	–
inlG	+	–	–	–
inlI	+	–	–	–
inlJ	+	–	–	–
inlC2	+	–	–	+
lmo1290	–	+	+	+
lmo2445	+	+	–	+
F2365_0289	+	+	–	+
F2365_2416	+	+	–	+

inlD, *inlE*, *inlG*, *inlI*, and *inlJ* were exclusively identified in virulent IIIA (internalin group 1), *inlC2* in virulent IIIA and IIIC (internalin group 4), *lmo1290* in low-virulent IIIA (internalin group 2), IIIB (internalin group 3), and IIIC, and *lm02445*, *F2365_0289* and *F2365_2416* in virulent IIIA, low-virulent IIIA and IIIC (Table 6). These internalins could serve as potential genetic markers for lineage III subpopulations.

In conclusion, *L. monocytogenes* lineage III represents a rare but highly diverse lineage. Based on systematic phenotypic and genotypic characterization, lineage III could be partitioned into at least four subpopulations, i.e., virulent IIIA-1 and IIIC, low-virulent IIIA-2, and virulent lineage-like sublineage IIIB (Table 7). Two biochemical reactions and 10 internalins were identified as potential markers for lineage III subpopulations. This study provides new insights into the biodiversity and population structure of lineage III strains, which are important for understanding the evolution of the *L. monocytogenes-L. innocua* clade. There is no doubt that the evolutionary and phylogenetic links between *L. monocytogenes* lineages and the *L. monocytogenes-L. innocua* clade will be much clearer when a larger collection of lineage III strains and the whole genomic sequence data of representative strains become available.

Table 7. Summary of four distinct subtyping schemes

Strain	Virulence	Biotype	Internalin group-internalin type	Branch-sequence type	Subpopulation
F2-525	virulent	1	1-1	IIIA-1	IIIA-1
F2-695	virulent	1	1-1	IIIA-2	IIIA-1
J1-168	virulent	1	1-3	IIIA-3	IIIA-1
J2-071	virulent	1	1-4	IIIA-4	IIIA-1
54006	low-virulent	2	2-5	IIIA-5	IIIA-2
M7	low-virulent	2	2-5	IIIA-5	IIIA-2
F2-407	virulent	3	3-6	IIIB-6	IIIB
J1-208	virulent	4	3-7	IIIB-7	IIIB
J1-158	virulent	5	3-8	IIIB-8	IIIB
F2-270	virulent	6	3-7	IIIB-9	IIIB
F2-086	virulent	7	3-6	IIIB-10	IIIB
F2-208	virulent	8	4-9	IIIC-11	IIIC
W1-111	virulent	8	4-10	IIIC-12	IIIC

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