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Atypical *Listeria monocytogenes* Serotype 4b Strains Harboring a Lineage II-Specific Gene Cassette

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***Listeria monocytogenes* is the etiological agent of listeriosis, a severe food-borne illness. The population of *L. monocytogenes* is divided into four lineages (I to IV), and serotype 4b in lineage I has been involved in numerous outbreaks. Several serotype 4b epidemic-associated clonal groups (ECI, -II, and -Ia) have been identified. In this study, we characterized a panel of strains of serotype 4b that produced atypical results with a serotype-specific multiplex PCR and possessed the *lmo0734* to *lmo0739* gene cassette that had been thought to be specific to lineage II. The cassette was harbored in a genomically syntenic locus in these isolates and in lineage II strains. Three distinct clonal groups (groups 1 to 3) were identified among these isolates based on single-nucleotide polymorphism-based multilocus genotyping (MLGT) and DNA hybridization data. Groups 1 and 2 had MLGT haplotypes previously encountered among clinical isolates and were composed of clinical isolates from multiple states in the United States. In contrast, group 3 consisted of clinical and environmental isolates solely from North Carolina and exhibited a novel haplotype. In addition, all group 3 isolates had DNA that was resistant to MboI, suggesting methylation of adenines at GATC sites. Sequence analysis of the *lmo0734* to *lmo0739* gene cassette from two strains (group 1 and group 3) revealed that the genes were highly conserved (>99% identity). The data suggest relatively recent horizontal gene transfer from lineage II *L. monocytogenes* into *L. monocytogenes* serotype 4b and subsequent dissemination among at least three distinct clonal groups of *L. monocytogenes* serotype 4b, one of which exhibits restrictions in regional distribution.**

Listeria monocytogenes is a Gram-positive bacterium that is ubiquitously present in the environment and can cause severe food-borne disease (listeriosis). At risk for listeriosis are pregnant women and their fetuses, the elderly, and immunocompromised individuals. Symptoms include meningitis, encephalitis, septicemia, stillbirths, and abortions. In the United States, *L. monocytogenes* is estimated to cause 1,600 cases and 250 deaths annually, suggesting that it is relatively infrequent but accompanied with a high mortality rate (16%) (29).

Even though 13 serotypes have been recognized, most cases of human disease involve strains of three serotypes, i.e., serotypes 1/2a, 1/2b, and 4b (12, 32). Studies on the population structure of *L. monocytogenes* have revealed serotype-associated lineages designated lineages I to IV (16, 28, 35). Lineage I consists of strains with serotype 4b, 1/2b, and 3b (8) and is characterized with pronounced clonality (5, 21, 25, 27, 37), while lineage II strains are of serotype 1/2a, 1/2c, 3a, and 3c (28, 37). Lineage III includes strains of serotype 4a and 4c, as well as certain strains of serotype 4b (28, 37). Recently, a subgroup of lineage III has been assigned into an independent lineage (lineage IV) (25, 35).

Analysis of differences in genomic content among strains of different serotypes and lineages has led to the development of a PCR-based method to determine serotype designations as an attempt to simplify the canonical serotyping method that employs different antibodies and can be done only in a small number of reference laboratories. In this method, inclusion of five primer pairs in the multiplex PCR divided *L. monocytogenes* into four serotype-associated groups: serotypes 1/2a or 3a, 1/2b or 3b, 4b complex (serotypes 4b [lineage I], 4d, and 4e), and 1/2c or 3c. Lineage III or IV isolates cannot be identified via this multiplex PCR, regardless of serotype (7).

During routine confirmation of serotype designations of serotype 4b *L. monocytogenes* by multiplex PCR, we noted certain isolates with atypical profiles. In addition to the amplicons expected for serotype 4b, the multiplex PCR with these isolates also yielded the amplicon expected for serotype 1/2a (or 3a) strains (Fig. 1). This novel atypical profile was first reported by Graves et al. at ISOPOL XVI (13). The PCR profiles were the same as those recently described for four serotype 4b isolates which were isolated from human listeriosis cases in Australia and for 22 serotype 4b isolates from human cases and food from France, Switzerland, Algeria, and Brazil; these isolates harbored the gene (*lmo0737*) from which the lineage II-specific primers were derived (15, 18). This gene is a member of a gene cassette (*lmo0734* to *lmo0739*) until now considered to be unique to lineage II strains and absent from those of lineage I (8, 22). It has been proposed that the designation "IVb-v1" is given to the multiplex PCR profile of serotype 4b isolates harboring *lmo0737* (18).

The objectives of the current study were to characterize the prevalence and genotypic characteristics of serotype 4b isolates from the United States that yield this atypical profile (IVb-v1) with the serotype-specific multiplex PCR. We wished to determine whether such isolates harbored the entire *lmo0734* to

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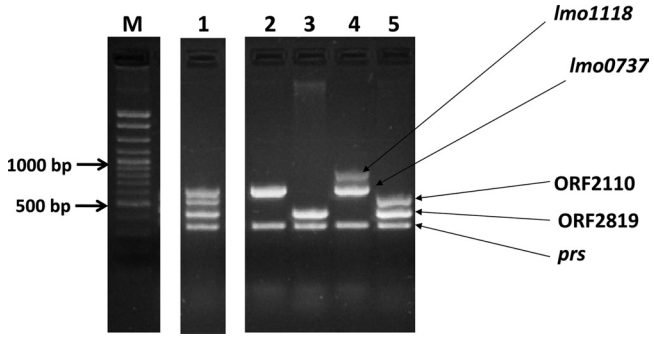

FIG 1 Exemplary multiplex PCR serotyping results and atypical PCR profile of serotype 4b strain 2007-618, which harbors the *lmo0737* to *lmo0739* cassette. Unlike the typical serotype 4b isolate, strain 2007-618 exhibited the extra band corresponding to *lmo0737*. Lanes: 1, 2007-618; 2, strain LW-A113 (serotype 1/2a); 3, strain G3986 (serotype 3b); 4, strain G3969 (serotype 1/2c); 5, strain G3992 (serotype 4b); M, exACTGene cloning DNA ladder (Fisher Scientific, Fair Lawn, NJ). From top to bottom, the bands correspond to *lmo1118* (906 bp), *lmo0737* (691 bp), ORF2110 (597 bp), ORF2819 (471 bp), and *prs* (370 bp).

lmo0739 gene cassette and whether the cassette was in a genomically syntenic location in such isolates and in lineage II. Lastly, we wished to characterize genotypic diversity among such isolates. In this study, we describe three distinct clonal clusters of serotype 4b *L. monocytogenes* isolates that harbor this lineage II-specific cassette and that were derived from clinical cases and the food processing plant environment in the United States.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The serotype 4b *L. monocytogenes* isolates that were investigated in this study and had atypical multiplex PCR results are listed in Table 1. Bacterial cells were routinely grown at 37°C overnight either in brain heart infusion (BHI; Becton, Dickinson and Co., Sparks, MD) broth or on BHI plates containing 1.2% agar (Becton, Dickinson and Co.). Bacterial stock cultures were cryopreserved in BHI with 20% glycerol at −80°C.

Genomic DNA extractions, enzyme digestions, PCR, multiplex PCR serotyping, and DNA-DNA hybridizations. Genomic DNA isolation, digestion with Sau3AI and MboI (New England BioLabs, Ipswich, MA), and PCR were done as described before (39). The primers used in this study were synthesized by Eurofins MWG operon (Huntsville, AL) and are listed in Table 2. Multiplex PCR for determination of serotype designations was done as described by Doumith et al. (7).

For hybridizations, DNA probes were obtained by PCR using primers listed in Table 2 and labeled as described previously (3). As a template of PCR, genomic DNA from *L. monocytogenes* serotype 1/2a EGDe was used for probe lmo0737; serotype 4b strain H7550 (ECII, 1998 multistate outbreak) for probes H18RP9/10, ECIIC-WAP, and Hsp001219; and serotype 4b strain F2365 (ECI, 1985 California Jalisco cheese outbreak [24]) for probes 4bSF18, non-ECIIC-WAP, and 85M. Spotting of DNA on nylon membranes and hybridizations were done as previously described (3).

Sequence analysis and phylogenetic assessments. For DNA sequencing, desired PCR fragments were purified with the QIAquick gel extraction kit (Qiagen, Valencia, CA), and sequencing was conducted by

TABLE 1 Bacterial strains used in this study

Clonal group	Strain	Isolation yr	Origin	State, country	Susceptibility[a]		MLGT[b]	Source or reference(s)[c]
					Sau3AI	MboI		
Group 1	2007-0904	2007	Clinical	NC, USA	+	+	1.17_4b	NCDHHS
	J2255	2003	Clinical	GA, USA	+	+	1.17_4b	3, 17, 31, 38
	J3139	2004	Clinical	WI, USA	+	+	1.17_4b	CDC
	J3913	2006	Clinical	IN, USA	+	+	1.17_4b	CDC
	J4016	2006	Clinical	SC, USA	+	+	1.17_4b	CDC
	J4500	2007	Clinical	NE, USA	+	+	1.17_4b	CDC
	J5000	2008	Clinical	VA, USA	+	+	1.17_4b	CDC
Group 2	2007-618	2007	Clinical	NC, USA	+	+	1.46_4b	NCDHHS
	J3026	2004	Clinical	CT, USA	+	+	1.46_4b	38
	J3053	2004	Clinical	MI, USA	+	+	1.46_4b	CDC
	J3195	2004	Clinical	OH, USA	+	+	1.46_4b	CDC
	J4458	2007	Clinical	MN, USA	+	+	1.46_4b	CDC
	J4490	2007	Clinical	SC, USA	+	+	1.46_4b	CDC
	J4953	2008	Clinical	OH, USA	+	+	1.46_4b	CDC
Group 3	2001-7R	2001	Clinical	NC, USA	+	−	1.60_4b	31
	2001-8R	2001	Clinical	NC, USA	+	−	1.60_4b	31
	2006-296	2006	Clinical	NC, USA	+	−	1.60_4b	31
	2007-454	2007	Clinical	NC, USA	+	−	1.60_4b	NCDHHS
	2008-894	2008	Clinical	NC, USA	+	−	1.60_4b	NCDHHS
	18-2a	2003	Processing plant (floor)	NC, USA	+	−	1.60_4b	This study
	128b-1[d]	2004	Processing plant (drain)	NC, USA	+	−	1.60_4b	23
	363b-1[d]	2005	Processing plant (drain)	NC, USA	+	−	1.60_4b	23
	491a-5[d]	2006	Processing plant (table)	NC, USA	+	−	1.60_4b	23

[a] Negative and positive signs represent resistance and susceptibility to the restriction endonuclease digestion, respectively.
[b] MLGT haplotypes for J2255 and J3026 were reported before (38).
[c] CDC, Centers for Disease Control and Prevention; NCSU, *Listeria* Strain collection at North Carolina State University; and NCDHHS, North Carolina Department of Health and Human Services.
[d] Isolates 128b-1, 363b-1, and 491a-5 were designated 377 (3), 1157, and 2616 (9), respectively, in the paper by Mullapudi et al. (23).

TABLE 2 List of the primers employed in this study

Probe	Primer	Sequence (5′ to 3′)	Target	Reference
	lmo0731F	TACTTGCTGCTCTTTCTGGC	*lmo0731*	This study
	lmo0740Rl	GTGGAAGGTTATGCTGCAGTTG	*lmo0740*	This study
lmo0737	lmo0737F	AGGGCTTCAAGGACTTACCC	*lmo0737*	7
	lmo0737R	ACGATTTCTGCTTGCCATTC	*lmo0737*	7
H18RP9/10	H18RP9/10F	CTGGATTTGCAGCTTATGAT	*LMOh7858_0487*	17
	H18RP9/10R	CCTATTCTTTCCATAAGTAAT	*LMOh7858_0487*	17
ECIIC-WAP	ECIIC-WAPF	GGGAACTTTCCATTAGCC	*LMOh7858_0479*	3
	ECIIC-WAPR	TTAAATGGGATATGATGT	*LMOh7858_0479*	3
Hsp001219	Hspec01219F	GAGGCTATCGAAATTGCTCG	*LMOh7858_1168*	3
	Hspec01219R	AGGATTCGGAATTTCATCCA	*LMOh7858_1168*	3
4bSF18	4bSF18_F	ACGGGCGTTTTATATTAAATGGG	*LMOf2365_0466*	10
	4bSF18_R	AATATCTCGAAAACTCCGAGT	*LMOf2365_0466*	10
Non-ECIIC-WAP	ECIC-WAPF	ATGGAAATTGGGCATGGC	*LMOf2365_0450*	3
	ECIC-WAPR	GTAGTTCCAGTGGACATG	*LMOf2365_0450*	3
85M	F2365_85MF	AATATATTTTCAATGTTTGATGGT	*LMOf2365_0327*	40
	F2365_85MR	GCTAATTCAATCCCTATTCT	*LMOf2365_0327*	40

GeneWiz (South Plainfield, NJ). Sequencing data were manually combined into a contiguous strand, and open reading frames (ORFs) were identified and annotated with ORF finder (www.ncbi.nlm.nih.gov/projects/gorf/), BLAST (1), and a conserved domain search (19) provided by the National Center for Biotechnology Information (NCBI). Sequences were compared with EMBOSS Align (http://www.ebi.ac.uk/Tools/emboss/align/), ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) (4), or the online Artemis comparison tool (WebACT; http://www.webact.org/WebACT/home) (2). Average homology between genomes of F2365 and EGDe was assessed by examining with ClustalW the concatenated sequences of seven housekeeping genes that were used for multilocus sequence typing (MLST) (27).

DNA sequences from the *inlA* and *inlB* genetic region (*inlAB* locus; ~4.5 kb) were used to assess phylogenetic relationships within the 4b complex of lineage I. PCR amplification and sequencing were performed as described previously (36). DNA sequences were aligned using the MUSCLE algorithm implemented in MEGA version 5.05 (http://www.megasoftware.net/). A neighbor-joining phylogeny was constructed using maximum composite likelihood distances (33). Support for individual nodes in the neighbor-joining tree was assessed by bootstrap analysis with 1,000 replications (11, 26).

PFGE and MLGT. Pulsed-field gel electrophoresis (PFGE) was conducted with AscI (New England BioLabs) and ApaI (Roche) as described previously (14), and BioNumerics (Applied Maths, Austin, TX) was employed for analysis of the PFGE profiles. For multilocus genotyping (MLGT), analyses of single nucleotide polymorphisms (SNPs) were conducted using a 49-probe version of the assay described by Ducey et al. (9). The reduced version of 49 probes was obtained by deleting 11 probes (ACC1, ACC6, AMI1, INLA2, INLA5, INLA9, INLA12, INLB2, INLB3, SIG1, and SIG3) that did not provide additional haplotype discrimination.

Nucleotide sequence accession numbers. The nucleotide sequences of the *lmo0731* to *lmo0740* region in *L. monocytogenes* strains 2007-454 and 2007-0904 have been submitted to GenBank (accession no. JN236006 and JN236007, respectively).

RESULTS

The atypical serotype 4b isolates (PCR profile IVb-v1) constitute three clonal groups. The serotype 4b isolates investigated in this study were first recognized due to their atypical multiplex PCR serotyping results (profile IVb-v1) that consisted of the bands expected for serotype 4b strains as well as an extra band of the same size as the band generated from strains of serotype 1/2a, 1/2c, 3a, and 3c with lineage II-specific *lmo0737* primers (Fig. 1 and data not shown). The presence of *lmo0737* was confirmed with hybridizations using an internal portion of this gene as the DNA probe (data not shown).

To obtain further information on the distribution of *lmo0737* in *L. monocytogenes* isolates of various serotypes, a total of 463 isolates were hybridized with the *lmo0737* DNA probe, including 210 of serotype 4b, 82 of serotype 1/2b (or 3b), 114 of serotype 1/2a (or 3a), 11 of serotype 1/2c (or 3c), and 46 of serotypes 4a and 4c or of indeterminate serotype (i.e., no serotype designation could be obtained with the multiplex PCR). The 210 serotype 4b isolates included 145 isolates from clinical cases (mostly sporadic) in different states in the United States. Of these 210 serotype 4b isolates, 23 (11%) hybridized with the *lmo0737* probe, including 19 from human cases of listeriosis and four from the processing plant environment (Table 1). All tested serotype 1/2b (or 3b) isolates were negative with the *lmo0737* probe. On the other hand, *lmo0737* was present in all tested lineage II strains (serotype 1/2a or 3a and 1/2c or 3c) except for one clinical isolate of serotype 1/2a (or 3a) (OLM 75; MLGT haplotype 2.12_1/2a).

Genomic DNAs of the 23 serotype 4b isolates that were positive with *lmo0737* did not hybridize with DNA probes specific for two major serotype 4b epidemic-associated clonal groups, ECI and ECII (ECI-specific 85M and ECII-specific Hsp001219) (data not shown). The isolates could be grouped into three clusters (groups 1, 2, and 3) based on their hybridizations with probes derived from a genomic region (region 18) adjacent to *inlAB*, which exhibits diversity in ECII strains, as well as with probes ECIIC-WAP and non-ECIIC-WAP, which correspond to the variable 3′ portion of *wap* adjacent to region 18 (3, 10) (Fig. 2). It was noted that isolates of groups 1 and 2 did not hybridize with either of the *wap*

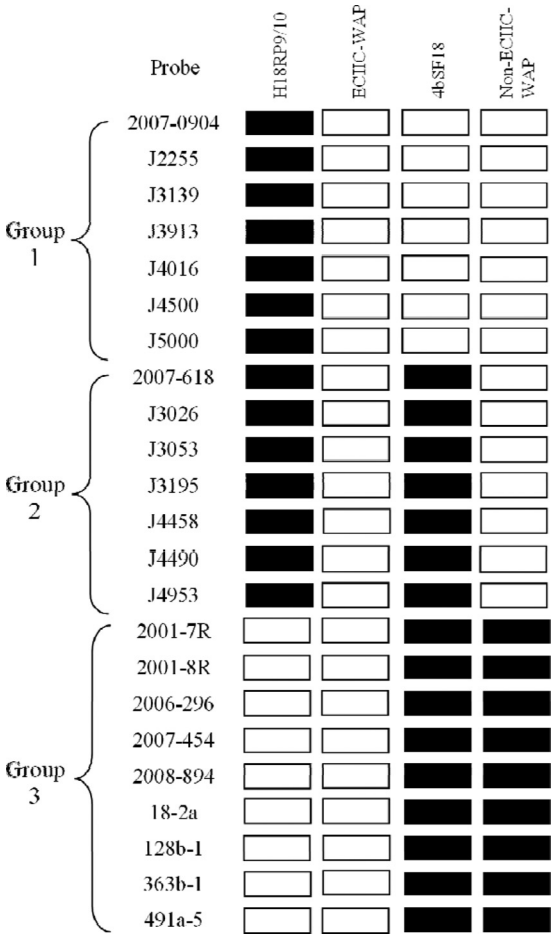

FIG 2 DNA-DNA hybridization analysis of serotype 4b isolates harboring the *lmo0737* to *lmo0739* cassette using DNA probes from two variable regions (region 18 [probes H18RP9/10 and 4bSF18] and the 3′ end of *wap* [probes ECIIC-WAP and non-ECIIC-WAP]). Positive and negative results are represented with black and white squares, respectively.

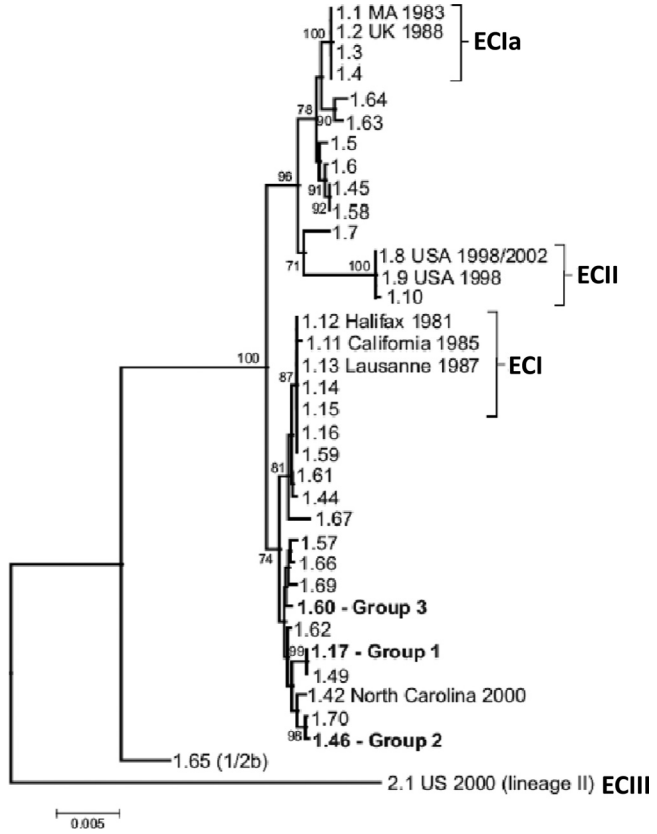

FIG 3 Neighbor-joining tree inferred from analysis of *inlAB* sequences representing unique MLGT haplotypes in the 4b complex of lineage I. Outbreak types and epidemic clonal groups are annotated following Ducey et al. (9). The MLGT haplotypes associated with the three groups of strains in Table 1 are shown in bold. The tree was rooted with sequences from a serotype 1/2b subtype and a lineage II subtype. The frequency (%) with which a given branch was recovered in 1,000 bootstrap replications is shown for branches recovered in more than 70% of bootstrap replicates. Similar results were obtained under different models of molecular evolution, when maximum parsimony was used as the optimality criterion, and after accounting for recombination using the program ClonalFrame version 1.1 (6).

probes, whereas group 3 hybridized with non-ECIIC-WAP. Group 3 isolates were also distinguished from the others by resistance of their genomic DNA to digestion by MboI, suggesting methylation of adenines at GATC sites (20) (Table 1). DNA of group 3 (and all other isolates) was susceptible to digestion by Sau3AI, which also recognizes GATC but is not affected by adenine methylation at GATC sites, being instead inhibited by methylation of cytosines at these sites (Table 1) (20). Another distinguishing attribute of group 3 isolates was their geographical origin. In contrast to group 1 and 2 isolates, which were from human cases of listeriosis from various states, all group 3 isolates were from a single state, North Carolina, and were derived from cases of listeriosis in different years between 2001 and 2007 as well as from the processing plant environment (Table 1).

The 23 isolates were also placed into groups 1, 2, and 3 by MLGT analysis of variation at SNP sites. Isolates in each group exhibited distinct MLGT haplotypes (Table 1). The seven group 1 isolates shared MLGT haplotype 1.17_4b, which was previously observed in two *L. monocytogenes* isolates (a rhesus monkey clinical isolate used to establish a nonhuman primate model for listeriosis [9, 30, 38] and the human isolate J2255 [38]); the latter isolate was also included in the current study. The seven group 2 isolates shared MLGT haplotype 1.46_4b, which was previously observed in clinical isolates (J1312 and J2630) from Michigan in 2002 and Connecticut in 2003, respectively, as well as in the clinical isolate J3026, which was also included in the current study (38). Multiplex PCR with these previously reported strains with MLGT haplotypes 1.17_4b or 1.46_4b indicated that these were also of type IVb-v1 (data not shown). The nine group 3 isolates shared MLGT haplotype 1.60_4b, which has not been reported before (Table 1).

A neighbor-joining analysis of the *inlAB* sequences was conducted to assess the phylogenetic positions of MLGT haplotypes 1.17_4b (group 1), 1.46_4b (group 2), and 1.60_4b (group 3) within the serotype 4b complex of lineage I (Fig. 3). These three MLGT haplotypes were part of a largely unresolved clade that included the MLGT pattern associated with a previously described listeriosis outbreak from North Carolina and which formed a sister group to the clade that included epidemic clone I subtypes (Fig. 3). While the relationships among the three groups of *lmo0737*-positive 4b isolates were not strongly supported, they clearly did

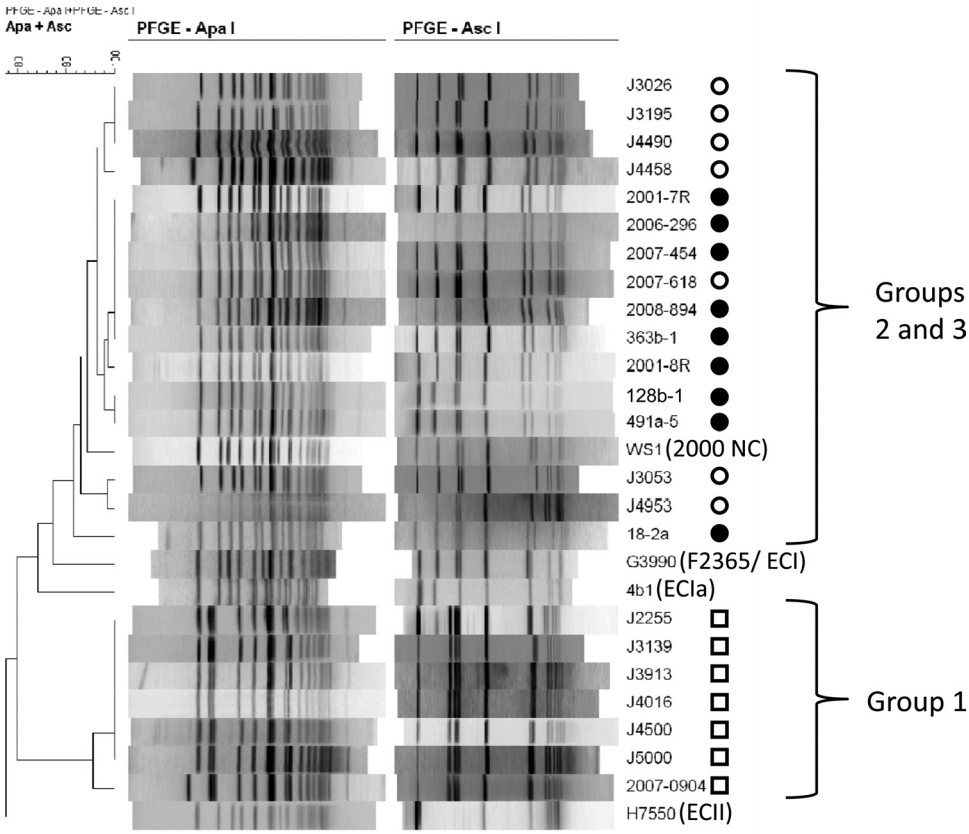

FIG 4 PFGE dendrogram of serotype 4b isolates harboring the *lmo0737* to *lmo0739* cassette. Isolates of groups 1, 2, and 3 are marked with open rectangles, open circles, and black circles, respectively. Outbreak-associated serotype 4b strains F2365 (ECI), H7550 (ECII), 4b1 (ECIa), and WS1 (2000 North Carolina outbreak strain) were included for reference.

not form a monophyletic group within the neighbor-joining phylogeny. The MLGT haplotypes associated with group 1 and group 2 isolates were strongly supported as being most closely related to haplotypes 1.49_4b and 1.70_4b, respectively; strains of these haplotypes (1.49_4b and 1.70_4b) had typical serotype 4b profiles with the multiplex PCR (data not shown).

Clustering of PFGE patterns with AscI and ApaI resulted in two clusters at 90% identity. In spite of their unique genotype based on hybridizations and MLGT, group 3 isolates could not be differentiated from those of group 2 by PFGE (though certain subclusters of isolates from either group 2 or group 3 could be identified). In contrast, group 1 isolates were a distinct group by PFGE (Fig. 4).

The *lmo0734* to *lmo0739* gene cassette is highly conserved and in a genomically syntenic region in *lmo0737*-positive serotype 4b isolates and lineage II strains. In the serotype strain 1/2a EGDe, *lmo0737* is part of the lineage II-specific gene cluster encompassing *lmo0734* to *lmo0739* and putatively involved in ribose metabolism (8) (Fig. 5). In the EGDe genome, the above-mentioned lineage II-specific genes are flanked by *lmo0733*, encoding a putative DNA binding protein, and *lmo0740*, encoding a hypothetical protein with a cAMP-binding protein domain. In the serotype 4b strain F2365, these flanking genes (*LMOf2365_0769* and *LMOf2365_0770*, respectively) are conserved and adjacent to each other, although part of *lmo0739* was also present in the intergenic region of this strain and other serotype 4b strains (Fig. 5 and data not shown). With the exception of lineage II strains, other *L. monocytogenes* genomes lacked the *lmo0734* to *lmo0739* cassette. However, sequences homologous to *lmo0734* to *lmo0736* were detected in the same genomic region of *Listeria welshimeri* SLCC5334 (Fig. 5).

In the proximity of the *lmo0734* to *lmo0739* lineage II-specific cluster, we identified a gene harbored by both F2365 (*LMOf2365_0768*) and EGDe (*lmo0732*) and encoding an internalin family protein with an LPXTG motif (Fig. 5). Interestingly, there was marked diversity between *lmo0732* and its counterpart in F2365 (77% and 75% identity at the nucleotide and deduced protein levels, respectively), especially in the internal portions. *In silico* analysis confirmed that this gene was divergent not only between EGDe and F2365 but also between sequenced lineage I (serotypes 4b and 1/2b) and lineage II (serotypes 1/2a and 1/2c) isolates (data not shown). To determine whether serotype 4b isolates harboring *lmo0737* also harbored the other genes in the lineage II-specific cassette, and whether the putative internalin gene was lineage II-like as well, we sequenced the DNA region between the *lmo0731* and *lmo0740* homologs of two strains, 2007-0904 (group 1) and 2007-454 (group 3).

Sequencing results showed that these strains harbored the entire cassette, in the same genomic region as lineage II strains (Fig. 5). The *lmo0734* to *lmo0739* gene cassette was highly conserved (99% identity at the nucleotide level) between 2007-0904 and 2007-454 and between these strains and EGDe. The homolog of *lmo0732* (putative internalin) in 2007-0904 and 2007-454 was lin-

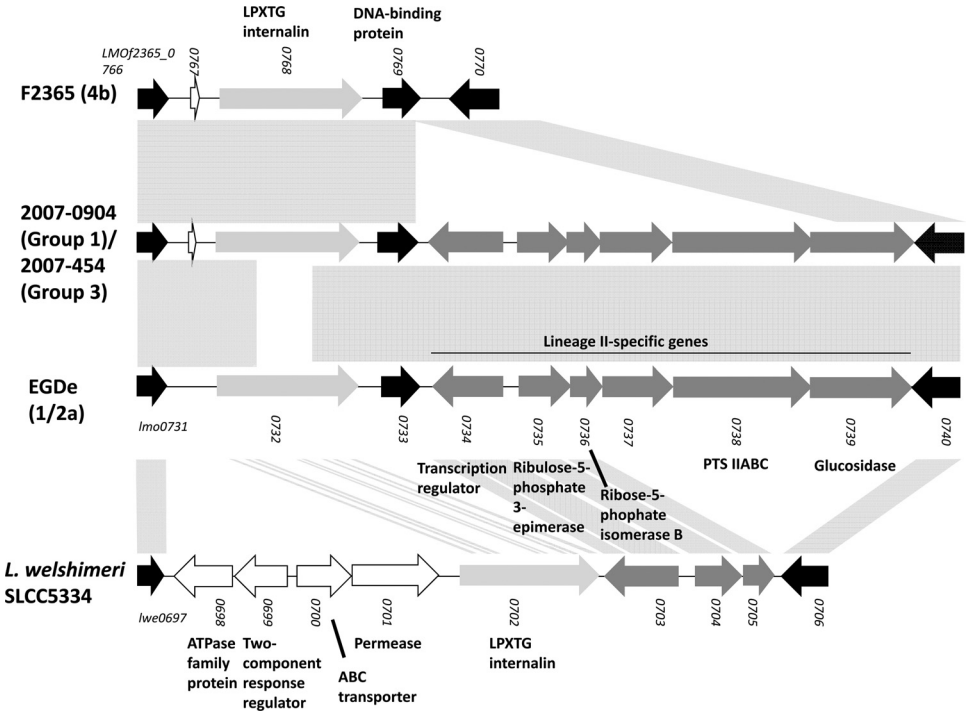

FIG 5 Comparative organization of the genomic region harboring the *lmo0737* to *lmo0739* cassette. Data on atypical serotype 4b strains (2007-454 and 2007-0904) were derived from our sequencing results, and others were retrieved for comparison from the genome sequences available on the NCBI database.

eage I-like, exhibiting >99% identity at the nucleotide sequence level with its homolog in F2365 (*LMOf2365_0768*). Sequences flanking this cassette in 2007-0904 and 2007-454 were also highly conserved between these strains and between EGDe and F2365.

Close scrutiny of the sequence alignment of the *lmo0734* to *lmo0739* cassette revealed two SNPs between 2007-454 and 2007-0904 in the *lmo0734* and *lmo0738* homologs, respectively. The SNP in the *lmo0734* homolog caused a change in amino acid. In contrast, a greater number of SNPs was detected between EGDe and 2007-454 ($n = 29$, including 9 nonsynonymous SNPs) as well as between EGDe and 2007-0904 ($n = 29$, including 8 nonsynonymous SNPs). Furthermore, *lmo0737* sequences of 2007-454 and 2007-0904 were compared with the previously published *lmo0737* sequence of the four Australian strains described before (15). The sequences were highly conserved with only two SNPs identified, neither of which resulted in changes in amino acid sequence. However, both SNPs differentiated between the Australian isolates and the isolates sequenced in this study.

For the remaining 21 *lmo0737*-positive serotype 4b isolates, the location of the *lmo0737* homolog was verified with PCR using primer sets annealing to *lmo0737* and the flanking gene. All isolates produced amplicons of the size expected for 2007-0904, suggesting that the lineage II-specific cassette was located in the same genomic region in these isolates (data not shown).

DISCUSSION

In this study, we showed that approximately 11% of the serotype 4b isolates that we characterized harbored a lineage II-specific gene cassette composed of highly conserved homologs of *lmo0734* to *lmo0739* harbored by *L. monocytogenes* EGDe (serotype 1/2a). These serotype 4b isolates with multiplex PCR profile IVb-v1 were all from the United States. Serotype 4b isolates with the IVb-v1 profile have been recently identified from other regions, including France, Algeria, Brazil, Switzerland, and Australia (15, 18).

Annotation of *lmo0734* to *lmo0739* implies that this gene cassette is associated with ribose metabolism. However, the biological consequences of the presence of this cassette in serotype 4b strains are currently unclear. Possible functions of these genes in lineage II strains have also not been determined. A deletion mutant of this cassette in EGDe was found to have normal growth *in vitro*, including under ribose-deficient conditions, and was not significantly impacted in invasion and intracellular growth (22). However, tiling microarray assessments in EGDe revealed that all genes in this cassette were upregulated in the stationary phase at 37°C. Furthermore, *lmo0735*, *-0736*, *-0738*, and *-0739* were upregulated in the intestines of infected mice while *lmo0739* was downregulated in the blood (34). Isogenic constructs of serotype 4b strains with and without this cassette would be useful in assessments of the functional impact of these genes for strains of this serotype.

In the genome of EGDe and other lineage II strains, the lineage II-specific cassette was located adjacent to a gene encoding a putative internalin protein with an LPXTG cell-wall-anchoring motif and exhibiting noticeable diversity between lineage I and II strains. However, sequence analysis revealed that in serotype 4b strains harboring the lineage II cassette, the sequence of the putative internalin protein gene was typical of those of other serotype 4b strains. Thus, the presence of the lineage II cassette in these serotype 4b strains does not appear to be accompanied by lineage II-like genetic content in the variable internalin gene in the vicinity of the cassette.

Hybridization with a panel of DNA probes from two variable regions (region 18 and the 3′ end of *wap*) and MLGT data revealed

that 4b isolates harboring the *lmo0734* to *lmo0739* lineage II-specific cassette could be divided into three groups. It is noteworthy that groups 1 and 2 did not generate a hybridization signal from either of the *wap*-based probes representing ECII and non-ECII strains, suggesting that the 3′ end of *wap* in these isolates is either absent or divergent from that in ECII or other serotype 4b strains (non-ECII), the genome of which has been sequenced. Group 3 and was unusual because it consisted of clinical and environmental isolates from a single state, North Carolina, and DNA from isolates of this group was resistant to MboI, suggesting methylation of adenine at GATC sites (20). Furthermore, the MLGT haplotype of group 3 isolates was novel, not having been detected previously among numerous other serotype 4b isolates (38). These unique features of group 3 suggest that it is a clonal group that emerged in North Carolina relatively recently and has not yet spread to geographically distant locations in the United States. Further studies will be needed to accurately monitor distribution of members of this group in North Carolina and other regions.

The origin of the *lmo0734* to *lmo0739* cassette in the serotype 4b strains described here is unknown. Phylogenetic analyses suggest that in the evolution of *L. monocytogenes*, a common ancestral lineage diversified into serotypes 1/2b and 1/2a, with serotypes 4b and 1/2c subsequently emerging from serotype 1/2b and 1/2a, respectively (8, 25, 27, 35). The conserved presence of the *lmo0734* to *lmo0739* cassette in lineage II and its absence from serotype 1/2b suggests that the cassette was lost in the evolution of serotype 1/2b strains (and thus was also lacking from typical serotype 4b strains that emerged subsequently). If this cassette were maintained by some members of serotype 1/2b and subsequently by certain 4b isolates, we would expect to detect it in serotype 1/2b isolates. However, none of the serotype 1/2b isolates screened by us and others (8) harbored this cassette.

The observed similarity in the *lmo0734* to *lmo0739* cassette between the atypical serotype 4b isolates and the serotype 1/2a EGDe (99% identity at the DNA sequence level) was higher than typically observed between homologous genes in serotype 1/2a and serotype 4b; for instance, 96% identity was observed between F2365 (serotype 4b) and EGDe (serotype 1/2a) in the seven housekeeping genes employed in MLST-based analysis of *L. monocytogenes* (27). This high conservation of the cassette between the serotype 4b strains that harbor it and EGDe argues against an ancestral origin of the cassette in these serotype 4b strains. The available data support horizontal gene transfer of this cassette from lineage II to certain serotype 4b clonal groups. Similar conclusions were reached by other investigators following analysis of the sequence of a 691-nucleotide fragment of *lmo0737* from serotype 4b strains with PCR profile IVb-v1 (18).

The available *lmo0737* sequences revealed only two sites with SNPs between the four Australian isolates (15), 2007-454 (group 1) and 2007-0904 (group 3). Interestingly, both SNPs differentiated between the Australian isolates and the ones investigated here. It is currently unknown whether the Australian isolates belong to any of the three clonal groups described in this study. Published PFGE profiles of IVb-v1 isolates from France, Brazil, and Algeria (18) were similar to those of group 2 and 3 isolates in the current study; however, our hybridization, MboI susceptibility assays, and MLGT data clearly showed major differences between groups 2 and 3. Characterization and comparison of isolates collected from different regions will shed further light onto the evolution and ecology of serotype 4b strains harboring the *lmo0734* to *lmo0739* cassette.

In conclusion, we have described three distinct clonal groups of serotype 4b *L. monocytogenes* that harbor a lineage II-specific cassette putatively involved in ribose metabolism. Notably, one clonal group (group 3) was unique in that its members were all from North Carolina. It had a novel MLGT haplotype and had DNA with methylated adenines at GATC sites. The genetic and epidemiological characteristics of these clonal groups of serotype 4b *L. monocytogenes* warrant further investigation to deepen our understanding of their evolution and ecology.

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REFERENCES

1. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
2. **Carver TJ, et al.** 2005. ACT: the Artemis comparison tool. Bioinformatics **21**:3422–3423.
3. **Cheng Y, Kim JW, Lee S, Siletzky RM, Kathariou S.** 2010. DNA probes for unambiguous identification of *Listeria monocytogenes* epidemic clone II strains. Appl. Environ. Microbiol. **76**:3061–3068.
4. **Chenna R, et al.** 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. **31**:3497–3500.
5. **den Bakker HC, Didelot X, Fortes ED, Nightingale KK, Wiedmann M.** 2008. Lineage specific recombination rates and microevolution in *Listeria monocytogenes*. BMC Evol. Biol. **8**:277.
6. **Didelot X, Falush D.** 2007. Inference of bacterial microevolution using multilocus sequence data. Genetics **175**:1251–1266.
7. **Doumith M, Buchrieser C, Glaser P, Jacquet C, Martin P.** 2004. Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. J. Clin. Microbiol. **42**:3819–3822.
8. **Doumith M, et al.** 2004. New aspects regarding evolution and virulence of *Listeria monocytogenes* revealed by comparative genomics and DNA arrays. Infect. Immun. **72**:1072–1083.
9. **Ducey TF, et al.** 2007. A single-nucleotide-polymorphism-based multilocus genotyping assay for subtyping lineage I isolates of *Listeria monocytogenes*. Appl. Environ. Microbiol. **73**:133–147.
10. **Evans MR, et al.** 2004. Genetic markers unique to *Listeria monocytogenes* serotype 4b differentiate epidemic clone II (hot dog outbreak strains) from other lineages. Appl. Environ. Microbiol. **70**:2383–2390.
11. **Felsenstein J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39**:783–791.
12. **Gasanov U, Hughes D, Hansbro PM.** 2005. Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: a review. FEMS Microbiol. Rev. **29**:851–875.
13. **Graves LM, Broeker R, Garette N, Swaminathan B.** 2007. Comparison of a multiplex PCR assay and conventional serotyping for sero-classification of *Listeria monocytogenes* isolates in the U.S.A., 2005–2006, poster communication P02. Proceedings of the 16th International Symposium on Problems of Listeriosis. ISOPOL XVI, Savannah, GA.
14. **Graves LM, Swaminathan B.** 2001. PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. Int. J. Food Microbiol. **65**:55–62.
15. **Huang B, et al.** 2011. Observation of a new pattern in serogroup-related PCR typing of *Listeria monocytogenes* 4b isolates. J. Clin. Microbiol. **49**:426–429.
16. **Kathariou S.** 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. J. Food Prot. **65**:1811–1829.
17. **Kathariou S, et al.** 2006. Involvement of closely related strains of a new clonal group of *Listeria monocytogenes* in the 1998-99 and 2002 multistate outbreaks of foodborne listeriosis in the United States. Foodborne Pathog. Dis. **3**:292–302.
18. **Leclercq A, et al.** 2011. Characterization of the novel *Listeria monocyto-*

genes PCR serogrouping profile IVb-v1. Int. J. Food Microbiol. **147**: 74–77.
19. **Marchler-Bauer A, et al.** 2011. CDD: a conserved domain database for the functional annotation of proteins. Nucleic Acids Res. **39**:D225–D229.
20. **McClelland M, Nelson M, Raschke E.** 1994. Effect of site-specific modification on restriction endonucleases and DNA modification methyltransferases. Nucleic Acids Res. **22**:3640–3659.
21. **Meinersmann RJ, Phillips RW, Wiedmann M, Berrang ME.** 2004. Multilocus sequence typing of *Listeria monocytogenes* by use of hypervariable genes reveals clonal and recombination histories of three lineages. Appl. Environ. Microbiol. **70**:2193–2203.
22. **Milillo SR, Badamo JM, Wiedmann M.** 2009. Contributions to selected phenotypic characteristics of large species- and lineage-specific genomic regions in *Listeria monocytogenes.* Food Microbiol. **26**:212–223.
23. **Mullapudi S, Siletzky RM, Kathariou S.** 2008. Heavy-metal and benzalkonium chloride resistance of *Listeria monocytogenes* isolates from the environment of turkey-processing plants. Appl. Environ. Microbiol. **74**: 1464–1468.
24. **Nelson KE, 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.
25. **Orsi RH, Sun Q, Wiedmann M.** 2008. Genome-wide analyses reveal lineage specific contributions of positive selection and recombination to the evolution of *Listeria monocytogenes.* BMC Evol. Biol. **8**:233.
26. **Penny D, Hendy MD.** 1985. The use of tree comparison metrics. Syst. Zool. **34**:75–82.
27. **Ragon M, et al.** 2008. A new perspective on *Listeria monocytogenes* evolution. PLoS Pathog. **4**:e1000146.
28. **Roberts A, et al.** 2006. Genetic and phenotypic characterization of *Listeria monocytogenes* lineage III. Microbiology **152**:685–693.
29. **Scallan E, et al.** 2011. Foodborne illness acquired in the United States-major pathogens. Emerg. Infect. Dis. **17**:7–15.
30. **Smith MA, et al.** 2003. Nonhuman primate model for *Listeria monocytogenes*-induced stillbirths. Infect. Immun. **71**:1574–1579.
31. **Sperry KE, Kathariou S, Edwards JS, Wolf LA.** 2008. Multiple-locus variable-number tandem-repeat analysis as a tool for subtyping *Listeria monocytogenes* strains. J. Clin. Microbiol. **46**:1435–1450.
32. **Swaminathan B, Gerner-Smidt P.** 2007. The epidemiology of human listeriosis. Microbes Infect. **9**:1236–1243.
33. **Tamura K, Nei M, Kumar S.** 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. U. S. A. **101**:11030–11035.
34. **Toledo-Arana A, et al.** 2009. The *Listeria* transcriptional landscape from saprophytism to virulence. Nature **459**:950–956.
35. **Ward TJ, Ducey TF, Usgaard T, Dunn KA, Bielawski JP.** 2008. Multilocus genotyping assays for single nucleotide polymorphism-based subtyping of *Listeria monocytogenes* isolates. Appl. Environ. Microbiol. **74**: 7629–7642.
36. **Ward TJ, et al.** 2010. Molecular and phenotypic characterization of *Listeria monocytogenes* from U.S. Department of Agriculture Food Safety and Inspection Service surveillance of ready-to-eat foods and processing facilities. J. Food Prot. **73**:861–869.
37. **Ward TJ, et al.** 2004. Intraspecific phylogeny and lineage group identification based on the *prfA* virulence gene cluster of *Listeria monocytogenes.* J. Bacteriol. **186**:4994–5002.
38. **Ward TJ, Usgaard T, Evans P.** 2010. A targeted multilocus genotyping assay for lineage, serogroup, and epidemic clone typing of *Listeria monocytogenes.* Appl. Environ. Microbiol. **76**:6680–6684.
39. **Yildirim S, et al.** 2010. Conservation of genomic localization and sequence content of Sau3AI-like restriction-modification gene cassettes among *Listeria monocytogenes* epidemic clone I and selected strains of serotype 1/2a. Appl. Environ. Microbiol. **76**:5577–5584.
40. **Yildirim S, et al.** 2004. Epidemic clone I-specific genetic markers in strains of *Listeria monocytogenes* serotype 4b from foods. Appl. Environ. Microbiol. **70**:4158–4164.