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Intraspecific Phylogeny and Lineage Group Identification Based on the *prfA* Virulence Gene Cluster of *Listeria monocytogenes*†

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Listeria monocytogenes **is a serious food-borne pathogen that can cause invasive disease in humans and other animals and has been the leading cause of food recalls due to microbiological concerns in recent years. In order to test hypotheses regarding *L. monocytogenes* lineage composition, evolution, ecology, and taxonomy, a robust intraspecific phylogeny was developed based on *prfA* virulence gene cluster sequences from 113 *L. monocytogenes* isolates. The results of the multigene phylogenetic analyses confirm that *L. monocytogenes* comprises at least three evolutionary lineages, demonstrate that lineages most frequently (lineage 1) and least frequently (lineage 3) associated with human listeriosis are sister-groups, and reveal for the first time that the human epidemic associated serotype 4b is prevalent among strains from lineage 1 and lineage 3. In addition, a PCR-based test for lineage identification was developed and used in a survey of food products demonstrating that the low frequency of association between lineage 3 isolates and human listeriosis cases likely reflects rarity of exposure and not reduced virulence for humans as has been previously suggested. However, prevalence data do suggest lineage 3 isolates may be better adapted to the animal production environment than the food-processing environment. Finally, analyses of haplotype diversity indicate that lineage 1 has experienced a purge of genetic variation that was not observed in the other lineages, suggesting that the three *L. monocytogenes* lineages may represent distinct species within the framework of the cohesion species concept.**

Listeria monocytogenes is a ubiquitous gram-positive bacterium that can cause serious invasive disease (listeriosis) in humans and other animals, resulting in severe clinical features, including meningitis, septicemia, and abortion. Contaminated food is believed to be the primary source of human exposure to *L. monocytogenes* and has been repeatedly linked to sporadic cases and large outbreaks of listeriosis. *L. monocytogenes* has the highest hospitalization rate (92%) and second-highest case-fatality rate (20%) of any food-borne pathogen and is responsible for more than one-quarter of food-borne disease-related deaths linked to known pathogens (17). The ability of this bacterium to persist in the food-processing environment, its ability to grow at refrigeration temperatures, and its pathogenic potential make *L. monocytogenes* a unique and significant regulatory problem, which is reflected by the fact that *L. monocytogenes* contamination has been the leading cause of food recalls due to microbiological concerns in recent years (21, 32).

Two primary evolutionary divisions, or lineages, have been identified within *L. monocytogenes* on the basis of multilocus enzyme electrophoresis, pulsed-field gel electrophoresis, ribotyping, and amplified fragment length polymorphism studies (3, 11, 24, 26). However, ribotype and virulence gene polymorphism data were used to describe the existence of a third lineage (25, 33), with some researchers suggesting that this lineage may represent a distinct taxonomic unit requiring recognition as a new species or subspecies (33, 34). Lineage-specific associations with serotypes commonly found in connection with human listeriosis (4b, 1/2b, and 1/2a) and genetic characterization of isolates from human and animal listeriosis cases have led to the suggestion that *L. monocytogenes* lineages differ in their pathogenic potential and host specificity (13, 33–35). However, disagreement persists about the number and composition of the major phylogenetic divisions within *L. monocytogenes* (2, 18, 33), the evolutionary history of lineage divergence within *L. monocytogenes* remains unclear, and perceived differences in virulence or host specificity need to be evaluated with respect to relative frequencies of exposure.

A solid evolutionary framework is essential for understanding the ecology and population dynamics of *L. monocytogenes* and for evaluating proposals regarding taxonomic revision of this important food-borne pathogen. Therefore, *prfA* virulence gene cluster (*p*VGC) sequences from 113 *L. monocytogenes* isolates, *Listeria seeligeri*, and *Listeria ivanovii* were used to develop a robust intraspecific phylogeny for *L. monocytogenes*. The *p*VGC is stably integrated in the same chromosomal location in these three *Listeria* species, and the *p*VGC of each species contains homologs of six virulence genes: a transcrip-

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tional regulator (*prfA*), two phospholipases (*plcA* and *plcB*) and a hemolysin (*hly*) required for lysis of host phagosomes, a metalloprotease (*mpl*) involved in extracellular activation of *plcB*, and a surface protein (*actA*) responsible for actin-based motility and cell-to-cell spread (31).

The primary objectives of the present study were to (i) determine the number of major phylogenetic divisions within *L. monocytogenes*, the genetic diversity within each of these lineages, and the distribution of serotypes across lineages; (ii) develop and use an accurate PCR-based approach for lineage identification to evaluate hypotheses of lineage-specific differences in virulence and host specificity with respect to the prevalence of individual lineages in food products; and (iii) combine analyses of phylogeny and historical demography to reconstruct the evolutionary history of lineage divergence within *L. monocytogenes* and to evaluate the taxonomic status of *L. monocytogenes* lineages within an appropriate evolutionary framework.

MATERIALS AND METHODS

Isolates and serotype determination. The *L. monocytogenes* isolates sequenced in the present study are listed in Table 1. All *Listeria* isolates were maintained in the Agricultural Research Service Culture Collection (NCAUR, Peoria, Ill.) in liquid nitrogen vapor at −175°C and were cultured at 37°C in brain heart infusion broth or tryptic soy agar containing 0.6% (wt/vol) yeast extract (Difco, Sparks, Md.). Serotype determinations were made by using the 96-well enzyme-linked immunosorbent assay procedure described by Palumbo et al. (22).

DNA sequencing. DNA isolation was performed as described by Fliss et al. (9). Primers were designed to amplify and sequence overlapping segments of the *p*VGC from 112 *L. monocytogenes* isolates (Table 1), *L. seeligeri* isolate NRRL 33019 (LMG11386; Belgian Coordinated Collections of Microorganisms, Ghent, Belgium), *L. ivanovii* subsp. *ivanovii* isolate NRRL 33017 (LMG11388; Belgian Coordinated Collections of Microorganisms), and *L. ivanovii* subsp. *londoniensis* isolate NRRL 33021 (DSM12491; Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ], Braunschweig, Germany). Amplifications were performed with Platinum *Taq* DNA Polymerase High-Fidelity (Invitrogen Life Technologies, Carlsbad, Calif.), and amplification products were purified by using Montage PCR cleanup filter plates (Millipore, Billerica, Mass.). Sequencing reactions were performed by using ABI BigDye version 3.0 sequencing chemistry (Applied Biosystems, Foster City, Calif.). Reaction products were purified via ethanol precipitation and run on an ABI3100 or an ABI3730 genetic analyzer (Applied Biosystems). Primer sequences and PCR protocols are presented in the supplemental material.

Phylogenetic analyses and genetic distance estimation. DNA sequences were edited and aligned by using Sequencher (version 4.1.2; Gene Codes, Ann Arbor, Mich.). In addition to the sequences generated in the present study, the *p*VGC sequence from *L. monocytogenes* strain EGD-e (GenBank accession no. AL591824) was included in the phylogenetic analyses. Prior to phylogenetic analyses, ambiguously aligned characters and nonunique *p*VGC haplotypes identified by using Collapse (version 1.1 [http://inbio.byu.edu/Faculty/kac/crandall_lab/programs.htm]) were removed from the data set.

Phylogenetic reconstructions were performed under both distance and maximum-parsimony frameworks. Distance analyses were performed by using the neighbor-joining algorithm and the Kimura two-parameter model of molecular evolution (15) as implemented in MEGA version 2.1 (http://www.megasoftware.net). Maximum-parsimony analyses were conducted by using the tree-bisection and reconnection method of branch swapping and the heuristic search algorithm of PAUP* version 4.0b (Sinauer Associates, Sunderland, Mass.). Relative support for individual nodes was assessed by nonparametric bootstrapping (8, 23) with 1,000 pseudoreplications of the data. For the combined *p*VGC data, bootstrap analyses were performed under both maximum-parsimony and distance frameworks. However, due to computational constraints, bootstrap analyses for the individual *p*VGC genes were performed only with the neighbor-joining algorithm. Genetic distance estimates were obtained as described for phylogenetic analyses with MEGA version 2.1, with standard errors estimated by using the bootstrap method and 1,000 pseudoreplications of the data. The significance of differences in genetic distance estimates was assessed by using one-tailed *t* tests and infinite degrees of freedom.

Development of an ASO-PCR multiplex for lineage identification. Three sets of primers were designed from *p*VGC sequences for the specific identification of isolates from each of the three *L. monocytogenes* lineage groups via an allele-specific oligonucleotide PCR (ASO-PCR) multiplex (Table 2). Amplifications were performed in 10-μl volumes with 0.5 μM concentrations of each primer, 2 mM $MgCl_2$, 0.2 mM concentrations of each deoxynucleoside triphosphate, 0.5 U of Ampli*Taq* Polymerase (Applied Biosystems), and 100 ng of genomic DNA. Amplifications consisted of 25 cycles of 15 s at 94°C, 10 s at 56°C, and 10 s at 72°C. Amplification products were resolved on 1.5% (wt/vol) agarose gels, and scored relative to a 100-bp DNA size ladder (Invitrogen Life Technologies, Carlsbad, Calif.).

Nucleotide sequence accession numbers. DNA sequences have been deposited in the GenBank database under accession numbers AY510072 to AY510074 and AY512391 to AY512502.

RESULTS

Intraspecific phylogeny and *L. monocytogenes* lineage evolution. The sequenced region consists of 8,750 aligned nucleotides, includes the entire *p*VGC with the exception of the last 12 bp of the *prfA* gene, and corresponds to nucleotides 203652 to 212294 in the complete genome sequence for *L. monocytogenes* strain EGD-e (GenBank accession no. AL591824). After ambiguously aligned characters were excluded, 61 unique *p*VGC haplotypes were identified among the sequenced *L. monocytogenes* isolates.

Phylogenetic analyses of the combined *p*VGC data resolved three distinct *L. monocytogenes* lineages, with each of the lineages recovered as monophyletic groups in 100% of bootstrap replicates from both neighbor-joining and maximum-parsimony analyses (Fig. 1). Lineage designations were assigned according to the convention of Rasmussen et al. (25), by including partial *hly* sequences from this previous study into phylogenetic analyses of the *p*VGC haplotypes reported here (not shown). Based on these phylogenetic reconstructions 21, 23, and 17 unique haplotypes were identified within the *p*VGC data for *L. monocytogenes* lineages 1, 2, and 3, respectively (Fig. 1). Neighbor-joining and maximum-parsimony analyses both resolved *L. monocytogenes* lineages 1 and 3 as sister groups that formed a larger monophyletic group referred to here as the L1/L3 clade (Fig. 1). These lineage relationships were supported by 99 and 86% of bootstrap replicates from neighbor-joining and maximum parsimony analyses, respectively. Topological constraints that forced lineages 2 and 3 to form a single monophyletic group required nine additional steps in maximum-parsimony analyses, and constraints that forced lineages 1 and 2 into a sister-group relationship provided the worst fit to the observed data with respect to lineage relationships, requiring 15 additional steps in maximum-parsimony analyses.

With the exception of a single lineage 3 isolate (NRRL 33227), which was recovered as the nearest relative of a monophyletic lineage 1 in the *plcA* neighbor-joining tree, the three *L. monocytogenes* lineages identified in analyses of the combined data also were resolved as monophyletic clades within individual gene trees derived from each of the six genes within the *p*VGC (Fig. 2). Clustering of NRRL 33227 with lineage 1 was not supported by bootstrap analyses, and examination of individual character differences revealed that no character states were uniquely shared between NRRL 33227 and lineage 1 isolates. In addition, all three lineages were recovered as monophyletic groups in maximum-parsimony analyses, sug-

TABLE 1. *L. monocytogenes* isolates used in analyses of intraspecific phylogeny and serotype evolution

NRRL no.[a]	Equivalent no.	Source[b]	Origin	Serotype	Lineage	NNRL no.[a]	Equivalent no.	Source[b]	Origin	Serotype	Lineage
33001	RM2205	WRRC	Human	4b	1	33116	2847	NADC	NA	4d	1
33002	RM2212	WRRC	Food	1/2a	2	33120	2848	NADC	NA	4b	1
33004	RM2215	WRRC	Food	4b	1	33123	2110	NADC	Environmental	1/2b	1
33005	RM2216	WRRC	Food	1/2b	1	33124	2111	NADC	Food	1/2b or 3b	1
33007	RM2218	WRRC	Food	4b	1	33125	3869	NADC	Animal	4b	1
33008	RM2387	WRRC	Food	4b	1	33126	7034	NADC	Animal	1/2b	1
33009	RM2388	WRRC	Food	1/2a	2	33127	2063	NADC	Animal	1/2a	2
33010	G3990	CFSAN	NA	4e or 4b	1	33128	2153	NADC	Food	1/2a	2
33011	G3982	CFSAN	Human	4e or 4d	1	33130	2071	NADC	Food	1/2b	1
33012	H7550	CFSAN	Human	4e or 4b	1	33140	2617	NADC	Animal	4b	1
33013	Scott A	CFSAN	Human	4b	1	33141	2218	NADC	Human	4b	1
33014	12443	CFSAN	Animal	1/2a	2	33143	2149	NADC	Human	4b	1
33015	12375	CFSAN	Animal	4b	1	33144	2112	NADC	Food	4b	1
33022	DSM20600	DSMZ	Animal	1/2a	2	33145	2401	NADC	Human	4b	1
33027	OB001075	FSIS	Food	1/2a	2	33148	5713	NADC	Environmental	1/2b	1
33028	OB001102	FSIS	Food	1/2b	1	33152	2072	NADC	Food	1/2a	2
33029	OB001124	FSIS	Food	1/2c	2	33154	2364	NADC	Food	1/2b	1
33030	OB001171	FSIS	Food	1/2b	1	33157	2355	NADC	Environmental	4b	1
33031	OB001183	FSIS	Food	1/2a	2	33160	3682	NADC	Food	1/2b	1
33032	OB001186	FSIS	Food	1/2b	1	33164	5712	NADC	Food	1/2b	1
33033	OB001206	FSIS	Food	1/2b	1	33166	2196	NADC	Human	4b	1
33034	OB001241	FSIS	Food	1/2a	2	33167	2362	NADC	Environmental	1/2a	2
33035	OB001270	FSIS	Food	1/2a	2	33169	SE 106	CFSAN	NA	1/2a	2
33036	OB001325	FSIS	Food	1/2b or 3b	1	33171	H 6900	CFSAN	Human	1/2a	2
33037	OB001350	FSIS	Food	1/2b	1	33176	20240-954	LDDC	Animal	1/2b	1
33038	OB001385	FSIS	Food	1/2b	1	33177	28838-95	LDDC	Animal	4c	3
33039	OB001410	FSIS	Food	1/2c	2	33178	32736-96	LDDC	Animal	1/2b	1
33040	OB001411	FSIS	Food	1/2a	2	33179	25734-97	LDDC	Animal	4b	1
33041	OB001412	FSIS	Food	1/2a	2	33180	41966-97	LDDC	Animal	1/2a	2
33042	OB000208F	FSIS	Food	1/2b	1	33181	1709-98	LDDC	Animal	4b	3
33043	OB000217B	FSIS	Food	1/2a	2	33182	7259-98	LDDC	Animal	4c	3
33044	OB000220(IA)	FSIS	Food	1/2a	2	33183	20842-98	LDDC	Animal	4b	3
33045	OB000223C	FSIS	Food	1/2b or 3b	1	33184	11466-01	LDDC	Animal	4c	3
33046	OB000255J	FSIS	Food	1/2b	1	33185	12459-01	LDDC	Animal	4b	3
33047	2202	NADC	Human	4b	1	33186	20674-01	LDDC	Animal	1/2b	1
33049	2395	NADC	Human	4b	1	33187	22409-01	LDDC	Animal	4b	3
33056	2220	NADC	Human	4b	1	33188	23594-01	LDDC	Animal	4c	3
33064	2064	NADC	Animal	1/2a	2	33189	32285-01	LDDC	Animal	1/2a	2
33068	8058	NADC	Animal	1/2b	1	33190	36087-01	LDDC	Animal	4b	3
33069	2070	NADC	Food	1/2a	2	33191	50301-01	LDDC	Animal	4b	3
33073	3883	NADC	Animal	1/2b	1	33215	LMB0027	ADRU	Food	1/2a[c]	2
33074	8054	NADC	Animal	1/2b	1	33216	LMB0033	ADRU	Food	1/2a[c]	2
33077	7035	NADC	Animal	4b	3	33218	LMB0338	ADRU	Environmental	1/2b[c]	1
33078	7680	NADC	Animal	4b	1	33219	LMB0340	ADRU	Environmental	1/2a[c]	2
33080	7679	NADC	Animal	1/2b	1	33220	LMB0345	ADRU	Human	1/2b[c]	1
33083	2632	NADC	Food	4b	1	33221	LMB0347	ADRU	Human	4b[c]	1
33090	7675	NADC	Animal	1/2b	1	33223	LMB0366	ADRU	Human	1/2c[c]	2
33092	7678	NADC	Animal	4b	3	33225	LMB0455	ADRU	NA	3a[c]	2
33094	3889	NADC	Animal	4b	1	33226	LMB0456	ADRU	NA	3c[c]	2
33095	7037	NADC	Animal	4b	1	33227	LMB0459	ADRU	NA	4c[c]	3
33098	2427	NADC	Food	4b	1	33229	LMB0487	ADRU	Human	4c[c]	3
33100	2612	NADC	Animal	1/2a	2	33230	LMB0291	ADRU	Food	4b[c]	3
33105	7676	NADC	Animal	4b	3	33231	MFS 108	ERRC	Food	4c[d]	3
33106	2420	NADC	Food	1/2a	2	33232	MFS 53	ERRC	Food	4b[d]	1
33114	2613	NADC	Animal	1/2b	1	33233	MFS 96	ERRC	Food	4b[d]	1
33115	3890	NADC	Animal	4c	3	33234	MFS 110	ERRC	Food	1/2a[d]	2

[a] NRRL, U.S. Department of Agriculture, Agricultural Research Service Culture Collection, Peoria, Ill.

[b] WRRC, U.S. Department of Agriculture, Western Regional Research Center, Albany, Calif.; CFSAN, U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Washington, D.C.; NADC, U.S. Department of Agriculture, National Animal Disease Center, Ames, Iowa; LDDC, Livestock Disease Diagnostic Center, University of Kentucky, Lexington, Ky.; ADRU, U.S. Department of Agriculture, Animal Disease Research Unit, Pullman, Wash.; ERRC, U.S. Department of Agriculture, Eastern Regional Research Center, Wyndmoor, Pa.

[c] Serotype data reported by M. Borucki and D. R. Call, unpublished data.

[d] Serotype information provided with strain histories.

gesting that the paraphyletic distribution of lineage 3 isolates in the *plcA* neighbor-joining tree is an artifact resulting from shared-ancestral character states and relatively long terminal branches within lineage 3.

Neighbor-joining and maximum-parsimony trees derived from each of the *p*VGC genes except *hly* were congruent with the results of the combined data analyses in supporting a monophyletic L1/L3 clade exclusive of lineage 2 (Fig. 2). However, due to the inability to adequately assess positional homology between *L. monocytogenes* and the outgroup species *L. seeligeri* and *L. ivanovii*, the *actA* gene tree was rooted by the midpoint method (along the longest branch in the phylogeny). Although midpoint rooting indicated that lineages 1 and 3 are more closely related to each other than either is to lineage 2, bootstrap support for lineage relationships in the *actA* gene tree could not be assessed without the ability to root the tree

TABLE 2. ASO-PCR primer sequences and predicted product sizes.

Lineage	Primer	Primer sequences[a] (5′-3′)	PCR product size (bp)
1	*actA*1-f	AATAACAACAGTGAACAAAGC	373
	*actA*1-r	TATCACGTACCCATTTACC	
2	*plcB*2-f	TTGTGATGAATACTTACAAAC	564
	*plcB*2-r	TTTGCTACCATGTCTTCC	
3	*actA*3-f	CGGCGAACCATACAACAT	277
	*plcB*3-r	TGTGGTAATTTGCTGTCG	

[a] Underlined nucleotides are specific to the *L. monocytogenes* lineage listed in the first column.

with an outgroup sequence. In contrast to the results of the combined data analyses and gene trees recovered from the other *p*VGC genes, lineages 1 and 2 formed a clade exclusive of lineage 3 in neighbor-joining and maximum parsimony trees from *hly*. These lineage relationships were supported by 84% of neighbor-joining bootstrap replicates and likely reflect recombination between the ancestors of extant lineage 1 and lineage 2 haplotypes.

Branching patterns observed in the combined *p*VGC phylogeny (Fig. 1) suggest that the sampled lineage 1 haplotypes shared a single common ancestor more recently than haplotypes in the other two *L. monocytogenes* lineages. In order to test this hypothesis, the average genetic distance between haplotypes within each of the three lineages was determined. The

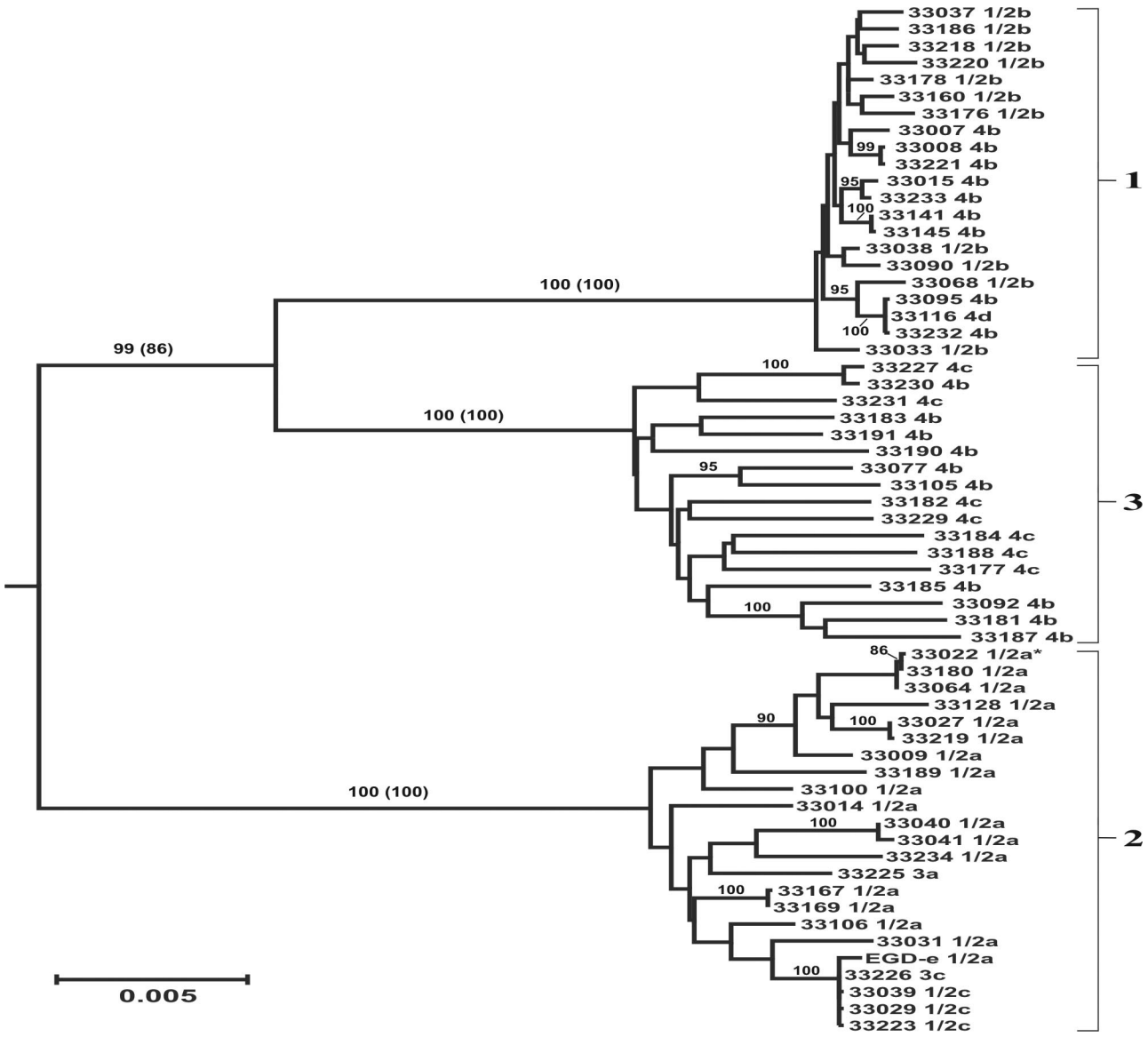

FIG. 1. Neighbor-joining phylogram inferred from analysis of the combined *p*VGC sequence data. Strains are identified by their NRRL numbers and serotype designations. Lineages are demarcated with numbered brackets, and the *L. monocytogenes* type strain is marked with an asterisk. The tree was rooted with *L. ivanovii* (NRRL 33017 and NRRL 33021) and *L. seeligeri* (NRRL 33019) sequences (not shown). The frequency (percent) with which a given branch was recovered in 1,000 neighbor-joining bootstrap replications is shown above branches recovered in more than 70% of bootstrap replicates, with bootstrap values from maximum-parsimony analysis given in parentheses.

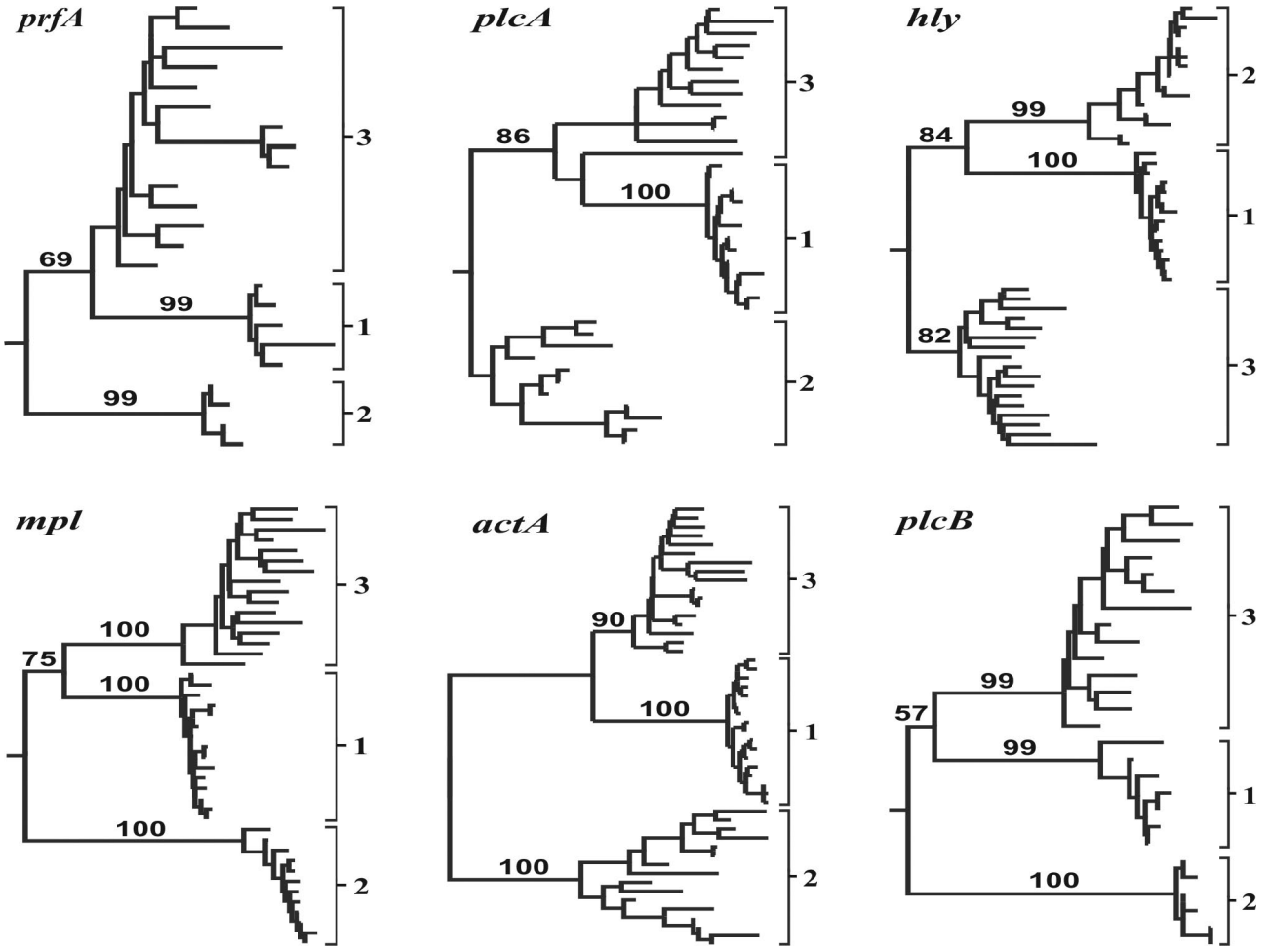

FIG. 2. Neighbor-joining phylogram inferred from analysis of individual *p*VGC genes, with lineages demarcated by numbered brackets. With the exception of the *actA* gene tree, which was midpoint rooted, individual gene trees were rooted with *L. ivanovii* (NRRL 33017 and NRRL 33021) and *L. seeligeri* (NRRL 33019) sequences (data not shown). The frequency (percent) with which a given branch was recovered in 1,000 neighbor-joining bootstrap replications is shown above branches recovered in >50% of the bootstrap replicates.

average genetic distance between sampled haplotypes was significantly ($P < 0.001$) less for lineage 1 (0.29% ± 0.04%) than for either lineage 2 (0.73% ± 0.05%) or lineage 3 (1.17% ± 0.08%). Although this could result from biased sampling of highly related lineage 1 isolates or from differences in population substructure, these explanations are unlikely as the maximum genetic distance between lineage 2 (1.29%) or lineage 3 haplotypes (1.58%) was >2.5-fold the maximum genetic distance between lineage 1 haplotypes (0.49%). In addition, the average genetic distance between lineage 1 isolates after exclusion of the lower quartile of values (0.33% ± 0.05%) also was significantly ($P < 0.001$) less than the average values for the other two lineages.

Serotype distributions. Unambiguous serotype determinations were made for 93 of the 96 *L. monocytogenes* isolates tested, with three isolates ambiguously typed as 1/2b or 3b (Table 1). However, due to conflicts with strain history data and previously reported problems distinguishing among serotypes 4b, 4e, and 4d (22), four serotype 4e isolates were retested. Upon retesting, one isolate was confirmed as serotype 4e, two isolates were identified as serotype 4b (in agreement with strain histories), and a fourth isolate was identified as serotype 4d, confirming the previously reported difficulties in distinguishing among the 4b, 4e, and 4d serotypes. In addition to the serotype data collected here, *L. monocytogenes* strain EGD-e has been reported as serotype 1/2a (10), and serotype information was previously published (1) or provided with strain histories for 16 isolates from which *p*VGC sequence data were collected (Table 1). Serotypes were almost exclusively associated with one of the three *L. monocytogenes* lineages. Serotypes 4b, 1/2b, 4e and 4d were identified among lineage 1 isolates. Serotypes 1/2a, 1/2c, 3a and 3c were identified among lineage 2 isolates. However, in addition to the 4a and 4c serotypes identified among lineage 3 isolates, 10 (59%) of the 17 unique *p*VGC haplotypes identified within lineage 3 were from serotype 4b isolates (Fig. 1).

Lineage identification by using an ASO-PCR multiplex. An ASO-PCR multiplex was used to determine the lineage of individual *L. monocytogenes* isolates (Fig. 3). The accuracy of this test was evaluated by comparing the ASO-PCR multiplex results with lineage identifications based on *p*VGC sequence data for the 112 *L. monocytogenes* isolates used in the phylo-

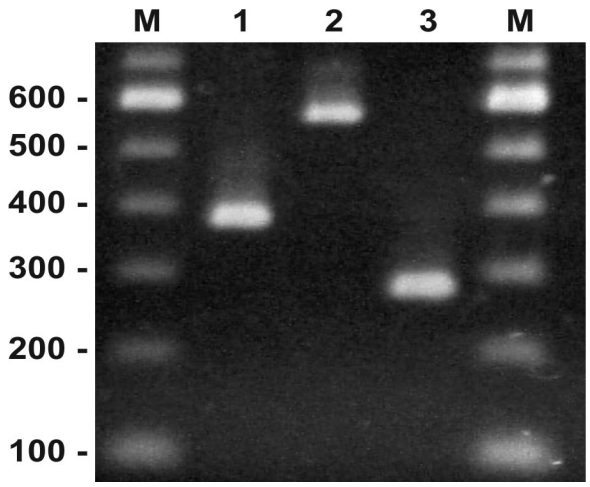

FIG. 3. ASO-PCR multiplex amplification results for representative *L. monocytogenes* strains from lineage 1, NRRL 33176 (lane 1); lineage 2, NRRL 33180 (lane 2); and lineage 3, NRRL 33185 (lane 3). Amplification products were scored relative to a 100-bp DNA size ladder (lane M).

genetic analyses. The ASO-PCR multiplex produced a single amplicon of the correct size for each of these isolates. In addition, no target amplicons were produced with isolates from any of the other *Listeria* species when the test was applied to four *L. innocua*, four *L. ivanovii*, two *L. grayi*, two *L. welshimeri*, and one *L. seeligeri* strain. In order to evaluate the utility of the test with a panel of isolates for which lineage identity was unknown and to estimate the frequency of the three *L. monocytogenes* lineages in food products, the ASO-PCR multiplex was also applied to 99 *L. monocytogenes* isolates from food products surveyed by the U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS) (Table 3). A single target amplicon was produced for each of the 99 food isolates, with lineages 1 (47%) and 2 (51%) present at nearly equal frequencies and lineage 3 (2%) nearly absent.

DISCUSSION

***L. monocytogenes* lineage composition.** The results of the multigene phylogenetic analyses presented here clearly demonstrate that *L. monocytogenes* comprises at least three primary evolutionary divisions (Fig. 1 and 2), corresponding to lineages proposed by Rasmussen et al. (25) and Wiedmann et al. (33). In contrast, Mereghetti et al. (18) concluded on the basis of ribotyping and random amplification of polymorphic DNA (RAPD) data that *L. monocytogenes* is composed of only two lineages, with lineage 3 interpreted as a branch of the lineage 1 group. Similarly, only two primary divisions were recognized by Borucki et al. (2) based on microarray analyses. However, the results presented here demonstrate that the average genetic distance between *p*VGC haplotypes was significantly ($P < 0.001$) greater for lineage 3 than for lineage 1 and that lineage 1 haplotypes share a common ancestor more recently than haplotypes from the other two lineages. These results demonstrate that lineage 3 cannot be considered a branch of the lineage 1 group and that there are at least three primary evolutionary divisions within *L. monocytogenes*.

Salcedo et al. (28) have suggested that the three primary divisions of *L. monocytogenes* are evident only from analyses of specific genes associated with virulence, and that housekeeping genes or random genetic markers are unable to distinguish more than two lineages. However, they did not include lineage 3 isolates in their analyses. In addition, all three lineages were monophyletic in analyses of mixed genome microarray data (2) and data from ribotyping and RAPD typing (18). Recognition of only two major divisions by the authors of these studies was likely due to an underestimation of diversity within lineage 3, since both included only two lineage 3 isolates, representing only one of the three known serotypes from this lineage, and no more than 2 of the 17 unique lineage 3 haplotypes reported here (Fig. 1). Therefore, the identification of three primary divisions within *L. monocytogenes* is not restricted to analyses of virulence associated genes but may have been hampered in some studies by inadequate sampling of variation within lineage 3.

Direct correlations between the three *L. monocytogenes* lineages and the most common serotypes have previously been reported, with lineage 1 containing serotypes 4b, 1/2b, 3b, and 3c; lineage 2 containing serotypes 1/2a, 1/2c, and 3a; and lineage 3 containing serotypes 4a and 4c (20). Such correlations are of interest because serotypes represent the traditional common language of *L. monocytogenes* subtyping, and because strains with serotypes 4b, 1/2b, and 1/2a are responsible for the vast majority of human listeriosis cases (7, 29). Similar correlations were observed in the present study, particularly with respect to serotypes 1/2a, 1/2c, and 1/2b. However, comparisons between serotype and lineage for 106 *L. monocytogenes* isolates for which both were unambiguously determined (Table 1 and Fig. 1) revealed for the first time that serotype 4b, which is responsible for the majority of human listeriosis cases (16) and virtually all major outbreaks of listeriosis in humans (13), is prevalent (59% of unique haplotypes) among strains from lineage 3, which is rarely associated with human listeriosis (13). These results demonstrate that serotype 4b isolates do not represent a distinct evolutionary group within *L. monocytogenes* and that serotype 4b cannot be used as a proxy for lineage identification.

Lineage identification, host specificity, and virulence differences. *L. monocytogenes* lineage-specific variation identified during the analyses of *p*VGC sequences was used to develop an ASO-PCR multiplex test for the specific identification of evolutionary lineage for individual *L. monocytogenes* isolates. This test proved to be 100% sensitive and specific in accurately assessing the lineage for 112 *L. monocytogenes* isolates for which lineage identity had been confirmed phylogenetically. Previously, Jinneman and Hill (14) developed a PCR-based assay for *L. monocytogenes* lineage identification based on sequences from the *hly* gene but reported that one of the lineage 3 isolates produced target amplicons indicative of both lineage 2 and lineage 3. Analyses of the *hly* sequences reported here indicated that by using the Jinneman and Hill test, multiple target amplicons would be produced with nine of the 17 unique lineage 3 haplotypes and that only the amplicon specific to lineage 2 isolates would be produced for one of the lineage 3 haplotypes. These predictions were confirmed by performing the Jinneman and Hill test on the isolates in Table 1. In addition to problems with specificity caused by undersampling

TABLE 3. ASO-PCR lineage identification for *L. monocytogenes* food isolates

NRRL no.[a]	FSIS equivalent no.	Origin	Lineage	NRRL no.[a]	FSIS equivalent no.	Origin	Lineage
33235	OB1441	Beef and pork franks	2	33308	OB10335	Seasoned chicken or beef	1
33236	OB1520	Beef and pork weiners	2	33309	OB10341	Wieners	1
33237	OB1547	Beef and pork franks	1	33310	OB10347	Ham	2
33238	OB1548	Beef jerky	2	33311	OB10348	Quesadilla with chicken	2
33239	OB1549	Beef and pork franks	1	33312	OB10349	Cooked ham	1
33240	OB1550	Beef and pork franks	1	33313	OB10350	Smoked ham	1
33241	OB1566	Cooked apple sausage	2	33315	OB10388	Semiboneless ham	1
33242	OB1597	Roast beef	1	33316	OB10390	Roast beef	2
33243	OB1608	Cooked beef	2	33317	OB10391	Deli turkey	2
33246	OB1648	White chicken salad	2	33318	OB10392	Deli turkey cheese	2
33247	OB1649	Roast beef	2	33319	OB10393	Beef franks	2
33248	OB1650	Barbeque chicken	1	33320	OB20002	Franks	1
33250	OB1720	Boneless smoked ham-steak	1	33321	OB20004	Roast duckling	2
33252	OB1777	Embotido	1	33322	OB20009	Pork barbeque	1
33253	OB1778	Cooked ham	2	33323	OB20012	Pork barbeque	1
33254	OB1779	Roast beef	1	33324	OB20017	Pork spring rolls	2
33255	OB1780	Chinese sausage	2	33325	OB20061	Barbeque sauce w/pork	1
33256	OB1781	Chinese sausage	2	33326	OB20062	Hungarian paprika salami	2
33257	OB10002	Roast beef	2	33327	OB20065	Smoked turkey drumsticks	1
33258	OB10003	Smoked boneless ham	1	33329	OB20091	Corn beef brisket	1
33259	OB10008	Cooked chicken meat strips	2	33330	OB20097	Liquid unpast whole egg	3
33260	OB10016	Beef sausage links	2	33331	OB20114	Chicken burrito	1
33261	OB10017	Beef jerky	3	33332	OB10004	Beef or pork smoked sausage	2
33262	OB10022	Boneless cooked country ham	1	33334	OB10216	Pork	1
33264	OB10065	Sliced cooked beef	2	33335	OB020094	Smoked pork chops	2
33265	OB10068	Boneless cooked country ham	1	33336	OB020122	Boneless pork chops	2
33276	OB10106	Mechanically separated chicken	2	33337	OB020132	Chicken burrito	1
33281	OB10112	Dried sausage	2	33338	OB020428	Pork links	2
33282	OB10113	Duck breast	2	33339	OB020429	Spicy cashew chicken egg roll	2
33283	OB10114	Chicken base	2	33340	OB020552	Kayseri soujouk	2
33284	OB10115	Boneless cooked country ham	1	33341	OB020632	Ham bologna	1
33285	OB10118	Smoked boneless turkey breast	2	33342	OB020663B	Turkey pastrami	2
33286	OB10119	Cooked sausage	2	33343	OB020709	Pork hash dumpling	1
33287	OB10120	Cooked roast beef brisket	1	33344	OB020735	Polish sausage	2
33288	OB10123	Sliced cooked beef	2	33345	OB020760	Buffet style ham	1
33289	OB10142	Sweet sopressata	1	33346	OB030003	Boneless deli ham	1
33290	OB10145	Quesadilla with beef	2	33347	OB030094	Sliced beef in barbeque sauce	1
33291	OB10146	Portuguese sausage with egg wrap	1	33348	OB030115	Sweet bologna	2
33292	OB10147	Smoked boneless turkey breast	2	33349	OB030116	Smoked pork chops	2
33293	OB10149	Cooked pork meat	1	33350	OB030145	Cooked hot Italian sausage	2
33294	OB10151	Cooked charbroil beef patty	1	33351	OB030159	Chicken in chipotle sauce burrito	1
33295	OB10153	Chorizo	2	33352	OB030205	Sliced roast beef	2
33296	OB10154	Boneless cooked country ham	1	33353	OB030305	Boneless ham	1
33297	OB10158	Dried sausage	2	33354	OB030306	Cooked beef brisket	2
33298	OB10167	Cooked sausage	2	33355	OB030469	Sliced sausage for pizza	1
33299	OB10169	Cooked sausage	2	33356	OB030631	Cooked pork pattie	1
33304	OB10205	Cooked pork sausage	1	33357	OB030758	Cajun chicken salad	1
33305	OB10206	Chicken bacon	1	33358	OB030759	Cooked sweet Italian sausage	1
33306	OB10219	Chicken chow mein	1	33359	OB030774	Pizza pocket	1
33307	OB10334	Chicken breast tenders	2				

[a] NRRL, U.S. Department of Agriculture, Agricultural Research Service Culture Collection, Peoria, Ill.

variation in lineage 3, this test also requires at least three separate PCRs. Similar problems exist with a lineage identification test developed by Moorehead et al. (19) that is based on only 23 isolates and also requires at least three separate PCRs.

Accurate, inexpensive, and high-throughput methods for *L. monocytogenes* lineage identification have the potential to inform studies of the population genetics, ecology, and epidemiology of this important food-borne pathogen and can also aid in understanding the biological and regulatory significance of the evolutionary lineages that have been identified within this species. For instance, the fact that lineage 3 isolates are rarely associated with human listeriosis but are common among animal isolates led Wiedmann et al. (33), and later Jeffers et al. (13), to suggest that lineage 3 isolates show a host specificity for nonprimate mammals and limited virulence in humans. However, application of the ASO-PCR multiplex to 99 *L. monocytogenes* isolates surveyed by FSIS provided the first direct estimate of the prevalence of individual lineages from a broad array of food products, and indicated that lineage 3 accounts for only 2% of *L. monocytogenes* isolates from food (Table 3). If we assume that contaminated food is the primary cause of listeriosis in humans, the frequency of lineage 3 isolates among human sporadic cases (1%) reported by Jeffers et al. (13) is entirely consistent with the relative frequency of lineage 3 isolates in food products (Table 3). Therefore, the low frequency of association between lineage 3 isolates and human listeriosis cases likely reflects rarity of exposure and not reduced virulence for humans or specificity for nonhuman hosts. In addition, the prevalence of lineage 3 among animal isolates (37% of the animal isolates in Table 1) and the near

absence among food isolates suggests that lineage 3 may be better adapted to the animal production environment than the food processing environment. Systematic comparisons of lineage-specific fitness in different environments are needed to fully evaluate this hypothesis. However, De Jesús and Whiting (6) have found that strains from lineage 3 are less likely to survive thermal inactivation than strains from the other two lineages of *L. monocytogenes*, indicating that lineages 1 and 2 may be better adapted to the food-processing environment than are lineage 3 isolates.

Comparison of the relative frequencies of the three *L. monocytogenes* lineages in food products (Table 3) and human listeriosis cases (13) suggests that lineage 1 is overrepresented and lineage 2 is underrepresented among isolates from human listeriosis cases. However, it is unclear if this reflects enhanced virulence for humans or unique ecological adaptations such as enhanced psychrotolerance and growth at refrigeration temperatures. Prevalence studies alone are insufficient to clearly demonstrate lineage-specific differences in virulence or ecological adaptations. However, the availability of complete genome sequences for *L. monocytogenes* lineage 1 (http://www.tigr.org) and lineage 2 (10) isolates will facilitate functional genomic studies and additional analyses of genomic variation within and between lineages that will complement comparative evaluations of virulence and comprehensive surveys of lineage prevalence in different environments. A combination of such studies will be required to fully test hypotheses regarding lineage-specific differences in virulence, host range, or ecology and to understand the genetic and evolutionary basis of such differences.

Lineage relationships and taxonomy. Previous analyses of relationships within *L. monocytogenes* based on shotgun DNA microarray data suggested that the single lineage 3 isolate examined in that study was distinct from a more derived group consisting of lineages 1 and 2 (36). However, these data were highly homoplasious (homoplasy index = 0.6491) in that they contained a high proportion of character state similarities that were not due to inheritance from a common ancestor, with over half of the polymorphisms distributed among polyphyletic groups (36). As noted by Zhang et al. (36), several comparative studies of *Listeria* genomes suggest a bias toward cell surface-related differences in genome content (4, 10, 12), indicating that different combinations of genes encoding cell surface characteristics may be favored by selection (36). This suggests that polymorphism data from such genome content studies may be inherently less reliable for use in phylogenetic reconstruction because these studies can be biased toward nonessential genes that may be lost independently in multiple evolutionary lineages or classes of genes that may be frequently involved in lateral gene transfer events due to selection.

Accurate reconstruction of the evolutionary relationships between the three *L. monocytogenes* lineages is essential to understanding the evolution of virulence traits and ecological adaptations within this species and is also critical in evaluating proposals to reassess the taxonomic rank of individual lineages. Both neighbor-joining and maximum-parsimony analyses of the combined *p*VGC data strongly support a sister-group relationship between lineages 1 and 3 (Fig. 1). The L1/L3 clade was also recovered in five of the six gene trees constructed from individual *p*VGC genes (Fig. 3). Lineages 1 and 2 were most closely related in the *hly* gene tree, which appears to reflect historical recombination between ancestors of present-day lineage 1 and lineage 2 haplotypes. However, despite the discordant *hly* gene tree, a sister-group relationship between lineages 1 and 2 provided the worst fit to the combined *p*VGC data, and the combined analyses of *p*VGC sequences strongly support the conclusion that lineages 1 and 3 share a common ancestor exclusive of lineage 2 (Fig. 1). This conclusion is congruent with the midpoint-rooted phylogenetic tree derived from combined analyses of ribotyping and RAPD typing data (18) and phylogenetic analyses based on mixed genome microarray data (2). In addition, the single serotype 4a isolate included in the multilocus enzyme electrophoresis study conducted by Piffaretti et al. (24) clustered with lineage 1 isolates. Although lineage association was not directly determined for this isolate, the 4a serotype appears to be specific to lineage 3 (Table 1) (20) and the other serotypes that have been identified within lineage 3 were either absent (serotype 4c) from the Piffaretti et al. (24) study or clustered with the lineage 1 group (serotype 4b), a finding consistent with the conclusion that lineages 1 and 3 are sister-groups.

Wiedmann et al. (33, 34) have suggested that lineage 3 represents a distinct taxonomic unit separate from lineages 1 and 2 and that lineage 3 should be recognized as a new species or subspecies because the small number of lineage 3 isolates examined had a distinctive ribotype fragment, a unique 16S rRNA sequence, and 70 to 76% DNA-DNA homology with the *L. monocytogenes* type strain from lineage 2 (27) and were predominantly serotype 4a or 4c. However, these differences were not evaluated relative to the phylogenetic history of lineage divergence within *L. monocytogenes*. The results of the phylogenetic analyses presented here strongly support a monophyletic L1/L3 clade exclusive of lineage 2 (Fig. 1). Therefore, recognizing lineage 3 as a new species or subspecies without equivalent recognition for lineage 1 would make *L. monocytogenes* paraphyletic, which is inconsistent with the modern systematic principles that taxonomy should reflect evolutionary history and taxonomic groups should comprise individuals that uniquely share a most recent common ancestor. In addition, the use of genetic or phenotypic features to circumscribe new species in the absence of an evolutionary framework for interpreting species boundaries is arbitrary and is not supported by population genetic or evolutionary theory.

The cohesion species concept proposed by Templeton (30) provides an evolutionary framework for understanding species as groups of organisms whose divergence is constrained by microevolutionary forces that maintain species as genetically and phenotypically cohesive groups. For species such as *L. monocytogenes*, which has a largely clonal population structure (4, 24, 25), evolutionary theory indicates that genetic drift and natural selection are the primary forces influencing species cohesion (5, 30). The results presented here, indicating a more recent coalescence for lineage 1 haplotypes than haplotypes from the other two lineages (Fig. 1), are interesting because they suggest that lineage 1 was exposed to a purge of genetic variation not observed in the other two lineages. This indicates a limitation on the extent to which these lineages are bound together by natural selection and suggests that they may represent distinct species within the framework of the cohesion species concept. Evidence from the survey of lineage preva-

lence in food products (Table 3) and from previous studies (6, 13), suggesting lineage-specific differences in ecological niche adaptations further support this interpretation. Given the phylogenetic relationships supported by the combined *p*VGC data and the fact that the *L. monocytogenes* type strain belongs to lineage 2 (Fig. 1), species recognition for lineage 1 would require reclassification of lineage 1 and lineage 3 isolates into two new species. However, the results of the present study should be viewed as hypothesis-generating with respect to taxonomic revision of *L. monocytogenes*, which will require a greater understanding of the ecology and demographic exchangeability of *L. monocytogenes* lineages and evaluations of the demographic history of these lineages based on genetic variation sampled from additional regions of the *L. monocytogenes* chromosome.

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