Genetic and phenotypic characterization of *Listeria monocytogenes* lineage III

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Listeria monocytogenes has been previously grouped into three evolutionary groups, termed lineages I, II and III. While lineages I and II are commonly isolated from various sources, lineage III isolates are rare and have several atypical and unique phenotypic characteristics. Relative to their prevalence in other sources, lineage III strains are overrepresented among isolates from food-production animals, and underrepresented among isolates from human clinical cases and foods. This work describes an extensive genotypic and phenotypic characterization of 46 lineage III isolates. Phylogenetic analyses of partial *sigB* and *actA* sequences showed that lineage III represents three distinct subgroups, which were termed IIIA, IIIB and IIIC. Each of these lineage III subgroups is characterized by differentiating genotypic and phenotypic characteristics. Unlike typical *L. monocytogenes*, all subgroup IIIB and IIIC isolates lack the ability to ferment rhamnose. While all IIIC and most IIIB isolates carry the putative virulence gene *lmaA*, the majority of subgroup IIIA isolates lack this gene. All three lineage III subgroups contain isolates from human clinical cases as well as isolates that are cytopathogenic in a cell culture plaque assay, indicating that lineage III isolates have the potential to cause human disease. The identification of specific genotypic and phenotypic characteristics among the three lineage III subgroups suggests that these subgroups may occupy different ecological niches and, therefore, may be transmitted by different pathways.

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INTRODUCTION

Listeria monocytogenes is a Gram-positive foodborne pathogen of humans and animals. It is the aetiological agent of the disease listeriosis, a rare but severe foodborne disease, which causes approximately 2500 human cases and 500 deaths each year in the United States (Mead *et al.*, 1999). Listeriosis symptoms in humans can include encephalitis, meningitis, septicaemia and abortion (Low & Donachie, 1997). Epidemiological, population genetics and evolutionary studies of *L. monocytogenes* using various subtyping methods have been critical to improving our understanding of how *L. monocytogenes* is transmitted from animals or the environment through foods to humans. Commonly used subtyping methods for *L. monocytogenes* include serotyping, multi-locus enzyme electrophoresis, and DNA-based methods such as pulsed-field gel electrophoresis (PFGE), ribotyping, PCR-restriction length polymorphism (PCR-RFLP) analysis and multilocus sequence typing (Wiedmann, 2002a, b). Results from subtyping studies utilizing many of these methods have established that *L. monocytogenes* can be

divided into at least three distinct evolutionary lineages. Two phylogenetic lineages were identified initially by multi-locus enzyme electrophoresis (Piffaretti *et al.*, 1989), and the existence of these lineages was subsequently confirmed by partial DNA sequencing of a virulence gene (Rasmussen *et al.*, 1991), PFGE (Brosch *et al.*, 1994) and ribotyping (Graves *et al.*, 1994). Rasmussen *et al.* (1995) provided the first evidence of a third *L. monocytogenes* phylogenetic lineage, lineage III, based on analyses of partial DNA sequences for *flaA*, *iap* and *hly*. Ribotyping and PCR-RFLP analyses of *L. monocytogenes* virulence genes, as well as comparative genomics and DNA sequencing studies, further confirmed the existence of at least three phylogenetic lineages in *L. monocytogenes* (Doumith *et al.*, 2004; Ward *et al.*, 2004; Wiedmann *et al.*, 1997). Interestingly, these three *L. monocytogenes* lineages appear to correlate well with serotypes: lineage I isolates represent serotypes 1/2b, 3b, 3c and 4b, while lineage II isolates represent serotypes 1/2a, 1/2c and 3a (Nadon *et al.*, 2001). While lineage III isolates initially were reported to represent serotypes 4a and 4c (Nadon *et al.*, 2001), recent characterization of a larger number of lineage III isolates indicated that a number of isolates in this lineage are serotype 4b (Nightingale *et al.*, 2005; Ward *et al.*, 2004).

L. monocytogenes isolates belonging to lineage III are rare and have several unique genetic and phenotypic characteristics.

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Of the more than 1800 *L. monocytogenes* isolates in our strain collection that have been subtyped, for example, only 52, or less than 3 %, belong to lineage III (<http://www.pathogentracker.net>). Lineage III isolates often have unique ribotypes (e.g. DUP-1061, DUP-1059) (Jeffers *et al.*, 2001), *hly* PCR-RFLP types and serotypes (e.g. 4a, 4c) (Nadon *et al.*, 2001; Wiedmann *et al.*, 1997). Interestingly, lineage III strains are more prevalent among isolates from animals with clinical listeriosis than among human clinical isolates. For example, 10.5 % of the 76 animal isolates characterized by Jeffers *et al.* (2001) were lineage III, while human lineage III prevalence has been consistently less than 2.5 % (Jeffers, 1998; Jeffers *et al.*, 2001). While these findings led some to hypothesize that lineage III isolates may have reduced human pathogenic potential (Jeffers, 1998; Jeffers *et al.*, 2001; Wiedmann *et al.*, 1997), more recent data reporting an exceptionally low prevalence (<1.0 %) of lineage III among food isolates (Gray *et al.*, 2004; Ward *et al.*, 2004) suggest that the uncommon occurrence of human listeriosis cases due to lineage III strains may be explained by the rarity of foodborne exposure to these strains.

The goal of this study was to gain a more comprehensive knowledge of the phenotypic and genotypic characteristics of *L. monocytogenes* lineage III strains to facilitate a better understanding of the ecology and transmission of these strains. To achieve this goal, we assembled the largest set of lineage III isolates characterized to date (46 isolates) to (i) determine the phylogenetic relationship among lineage III isolates, (ii) compare *actA*, *hly* and *lmaA* genotypes and rhamnose fermentation phenotypes of lineage III isolates, and (iii) characterize the pathogenic potential of lineage III isolates using a cell culture plaque assay.

METHODS

Bacterial isolates. Fifty-two *L. monocytogenes* isolates in our strain collection (www.pathogentracker.net) were identified as belonging to lineage III based on their *EcoRI* ribotypes and *hly* PCR-RFLP types (Bruce *et al.*, 1995). Forty-six of those isolates were chosen for inclusion in this study (Table 1); six isolates were eliminated because their sources of isolation (e.g. isolation from two animals on the same farm), ribotypes, *actA* types and *hly* types suggested that they were clones of other isolates already included in our isolate set. Our final lineage III isolate set characterized here included isolates from human clinical cases ($n=20$), animals with and without signs of listeriosis ($n=13$), foods ($n=6$), as well as seven isolates of unknown origin. All stock cultures were stored at -80°C in 15 % (v/v) glycerol and grown and, unless otherwise noted, maintained in brain heart infusion (BHI) agar or broth (Difco/Becton Dickinson).

Ribotyping. Automated ribotyping was performed as previously described (Bruce *et al.*, 1995). The Riboprinter generated DuPont IDs (e.g. DUP-1061) for the majority of the isolates analysed. If the Riboprinter was unable to assign a DuPont ID (i.e. for a new pattern with <0.85 similarity to existing patterns in the DuPont database), we assigned a unique type designation based on the 'Ribogroup' that had been assigned by the instrument (e.g. ribogroup 116-110-S-2). All DuPont IDs were confirmed by visual inspection. If an assigned DuPont ID included more than one distinct ribotype pattern (e.g. patterns differing by a single weak band) then each pattern was

designated with an additional letter (e.g. DUP-1059A and DUP-1059B). Ribotype patterns for isolates in this study are available for comparison through Pathogen Tracker (www.pathogentracker.net).

Virulence gene allele characterization. Virulence gene alleles for *actA* and *hly* were determined by PCR-RFLP as described previously (Wiedmann *et al.*, 1997). Detection of the putative virulence gene *lmaA* was carried out by PCR using primers *lmaA*-F (5'-TTC TGC TGG TGC TAC AGG TG-3' and *lmaA*-R (5'-CCA ACA AGG TCT AAC TGT AAA CCG-3'), which amplify an approximately 420 bp fragment of the *lmaA* ORF (Schäferkordt & Chakraborty, 1997). Each reaction contained 1 unit *Taq* polymerase (Promega), 1.5 mM MgCl_2 , $1 \times$ PCR buffer, 50 μM of each dNTP and 0.5 μM of each primer. Thermocycling conditions for the *lmaA* PCR were 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s.

***actA* and *sigB* sequencing.** *actA* and *sigB* PCR amplification and sequencing was performed using standard methods as described by Nightingale *et al.* (2005). For *actA*, an approximately 600 bp region at the 3' end of this gene (nt 1275 to 1920), previously identified as being the most discriminatory region within *actA* (Cai *et al.*, 2002), was amplified and sequenced. For *sigB*, an 840 bp DNA fragment (nt -30 to 810) containing the complete *sigB* ORF was amplified and sequenced. DNA sequencing was performed at Cornell University's Bioresource Center or by Macrogen (Seoul, Korea). All DNA sequences are available online through Pathogen Tracker (www.pathogentracker.net).

Descriptive analysis of sequence data. Descriptive analyses were performed as described by Nightingale *et al.* (2005). Analysis was based on a 561 nt *actA* alignment and a 573 nt *sigB* alignment (representing 29.2 % and 73.5 % of the respective ORFs). *actA* and *sigB* sequences for lineage I and II isolates reported by Nightingale *et al.* (2005) were used for comparison purposes. Analysis was performed for alignments containing all isolates studied here ($n=163$) as well as separately for alignments that were stratified by lineage (I, II and III) and subgroups within lineage III (i.e. IIIA, IIIB and IIIC). Briefly, nucleotide diversity (π , mean pairwise nucleotide difference per site; and k , mean pairwise nucleotide difference per sequence), number of polymorphic sites, number of mutations, number of alleles, Tajima's D test for neutrality, number of synonymous mutations (S), number of nonsynonymous mutations (N), and d_N/d_S ratio averaged over all isolates were calculated for each gene using DnaSP version 3.99 (Rozas *et al.*, 2003). Alleles, defined by a unique combination of polymorphisms within an individual gene, and sequence types, defined by a unique combination of alleles, were also assigned using DnaSP.

Phylogenetic analysis. Phylogenetic analyses were performed essentially as described by Nightingale *et al.* (2005). Briefly, MODELTEST (Posada & Crandall, 1998) was used to optimize parameters to infer maximum-likelihood phylogenetic trees in PAUP* (Swofford, 1997). Due to the large size of our dataset ($n=163$, lineages I, II and III), a single isolate was selected to represent each unique sequence type ($n=67$) to infer phylogenetic trees. Maximum-likelihood trees were generated for each individual gene in PAUP*. Heuristic searches were performed using equal weights for all sites and the tree-bisection-reconnection branch-swapping algorithm was employed.

Plaque assay. We used a plaque assay previously described by Sun *et al.* (1990) to determine the ability of selected lineage III isolates to spread from cell to cell as represented by plaque size. Briefly, duplicate wells of mouse fibroblast L2 cells (a gift from D. A. Portnoy, Department of Molecular and Cell Biology, University of California, Berkeley) were infected with 1×10^5 c.f.u. and 3×10^4 c.f.u. of *L. monocytogenes*. After a 1 h incubation at 37°C , the monolayers were washed and overlaid with Dulbecco's Modified Eagle's Medium (DMEM) containing 10 μg gentamicin ml^{-1} and 1.4 % agar.

Table 1. Phenotypic and genetic characteristics of *L. monocytogenes* lineage III isolates

Lineage III subset	Strain	Source	Ribotype	<i>actA</i> allele	<i>sigB</i> allele	Sequence type (ST)	<i>actA</i> type	<i>hly</i> type	<i>lmaA</i>	Rhamnose utilization
Subset IIIA	E1-011*	Animal	DUP-1061A	2	2	2	4	4b	—	+
	E1-016*	Animal	DUP-1061A	3	3	3	4	4b	—	+
	F2-016	Human	DUP-1059A	4	4	4	4	4b	—	+
	F2-318*	Animal	DUP-1059A	8	7	8	3	4b	+	+
	F2-458*	Human	DUP-1061A	10	8	10	4	1	+	+
	F2-501*	Human	DUP-18606	11	9	12	4	1	+	+
	F2-524*	Human	DUP-1061A	12	10	13	4	1d	+	+
	F2-525*	Human	DUP-1061A	13	11	14	4	1d	+	+
	F2-650*	Human	DUP-1061A	14	12	15	4	1b	+	+
	F2-655*	Human	DUP-18606	15	3	16	4	4a	+	+
	F2-695*	Human	DUP-1061A	16	3	17	4	1	—	+
	J1-031	Human	DUP-1059A	4	4	4	4	4b	—	+
	J1-168*	Human	DUP-18606	17	3	18	4	3	—	+
	J2-067*	Animal	DUP-1059C	11	7	11	4	1b	—	+
	J2-068	Animal	DUP-14003B	11	7	11	4	1b	—	+
	J2-069	Animal	DUP-1059A	4	4	4	4	1b	—	+
	J2-070*	Animal	DUP-1059A	4	4	4	4	4b	—	+
	J2-071	Animal	DUP-1061A	3	3	3	4	2	—	+
	J2-074*	Animal	DUP-10146	20	7	22	4	4a	+	+
	J2-076*	Food	DUP-10145	21	13	23	4	4a	—	+
	N1-211	Human	DUP-18606	11	9	12	4	1	+	+
	R2-128*	Food	DUP-1061A	24	16	26	4	1c	+	+
	R2-381*	Unknown	DUP-1059B	26	17	28	3	1	—	+
	R2-652	Human	DUP-18606	15	3	16	4	1d	+	+
	X1-002	Food	DUP-1059B	26	17	28	3	4a	—	+
	X1-004	Food	116-1001-S-5 (dd 12388)†	26	17	28	3	4a	—	+
	X1-006	Unknown	DUP-1059B	26	17	28	3	4a	—	+
	X1-007	Unknown	DUP-1061A	3	3	3	4	4b	—	+
	X1-011*	Food	DUP-1036A	30	10	32	3	4b	+	+
Subset IIIB	F2-086*	Human	DUP-10142	5	5	5	3	4b	—	—
	F2-407*	Human	DUP-18036	9	5	9	4	1c	+	—
	J1-158	Animal	DUP-10142	18	5	19	4	4b	+	—
	J1-208*	Animal	DUP-10142	19	5	21	4	4b	+	—
	M1-001*	Animal	DUP-10142	18	5	19	4	4b	+	—
	M1-002*	Animal	DUP-10142	18	14	20	3	4b	+	—
	M2-030*	Unknown	DUP-10142	23	15	25	4	1d	+	—
	R2-142*	Food	DUP-18036	25	5	27	4	4b	+	—
	W1-111*	Unknown	DUP-18036	28	5	30	3	4b	+	—
	W1-112*	Unknown	DUP-1033A	29	5	31	3	4b	—	—
Subset IIIC	C1-418*	Human	DUP-10148	1	1	1	3	4b	+	—
	F2-208*	Human	DUP-10148	6	6	6	4	4b	+	—
	F2-270*	Human	DUP-18007A	7	1	7	3	4b	+	—
	F2-569	Human	DUP-18007A	7	1	7	3	4b	+	—
	F2-595	Human	DUP-18007A	7	1	7	3	1	+	—
	M1-003*	Animal	DUP-10148	22	1	24	4	4a	+	—
	W1-110*	Unknown	DUP-1055A	27	18	29	3	4a	+	—

*Indicates an isolate included in the phylogenetic tree.

†The first ribotype designation represents the *EcoRI* ribotype patterns generated using the RiboPrinter; the second designation (dd 12388) represents the manual ribotype designation as previously reported (Wiedmann *et al.*, 1997).

Following 3 days of incubation, a second overlay of DMEM containing 6% Neutral Red solution and 1.4% agar was added. Plaques were counted and their sizes determined using the SigmaScan Pro 5.0 software (Statistical Solutions) to calculate plaque area. Plaque size and number were normalized by expressing them as a percentage of the internal control strain, 10403S.

Rhamnose fermentation. We tested the ability of our isolates to ferment rhamnose by inoculating Oxidative Fermentative (OF) Test Medium (prepared as described by Atlas, 1993) containing 0.55 mM rhamnose with a stab of cells taken from individual colonies on agar plates. Two tubes per isolate were stabbed; one was overlaid with mineral oil, and both were incubated at 37 °C for 48 h. Observations were taken at 24 and 48 h, and a reaction was scored as positive if the media turned yellow in both tubes, indicating the fermentative formation of acid from rhamnose.

Statistical analysis. The chi-squared test was used to test for significant associations between lineage III subgroup and isolate source, *lmaA* genotype, *hly* PCR-RFLP type, *actA* type and rhamnose fermentation phenotype. A one-way analysis of variance (ANOVA) was used to compare mean plaque sizes of isolates classified to different lineage III subgroups (i.e., IIIA, IIIB and IIIC). A two-sided one-sample *t*-test was used to determine if the mean plaque size for all lineage III isolates was significantly different from the plaque size for the internal control strain, 10403S. Statistical significance was declared at $P \leq 0.05$ and all analyses were performed in Statistix 7 (Analytical Software).

RESULTS

Sequencing, phylogenetic analysis and identification of subgroups IIIA, IIIB and IIIC

Partial *sigB* and *actA* ORF DNA sequence data for 117 previously characterized lineage I and II isolates (Nightingale *et al.*, 2005) and 46 lineage III isolates were used to infer the molecular phylogeny of isolates previously classified as lineage III based on *EcoRI* ribotype and *hly* PCR-RFLP analyses. *sigB* and *actA* were chosen for sequencing because they are located on different regions of the *L. monocytogenes* chromosome (721.2 kb apart), and because they have different functions: *actA* is a virulence gene while *sigB* is a general stress-response gene. Moreover, previous work showed that *actA* and *sigB* are highly polymorphic and discriminatory, but show limited indication of horizontal gene transfer, making them suitable for reconstructing the evolutionary history of a group of *L. monocytogenes* isolates (Cai *et al.*, 2002). A total of 57 unique *actA* alleles and 30 unique *sigB* alleles, resulting in 67 unique sequence types (STs) were differentiated among all 163 isolates (I, II and III). Among the 46 lineage III isolates, a total of 30 *actA* and 18 *sigB* alleles, resulting in 32 STs, were differentiated (Table 2). One lineage III food isolate (FSL J2-076) contained a point mutation in *sigB* leading to a premature stop

Table 2. Descriptive analysis of nucleotide sequence data for all *L. monocytogenes* genetic lineages

Dataset	Gene (% ORF)*	Polymorphic sites†	Mutations‡	Alleles§	π	k ¶	Tajima's D	S#	N**	d_N/d_S ††
All isolates	<i>actA</i> (29.2)	148	168	57	0.0631	35.40	0.62	61	79	0.2896
Lineage I	<i>actA</i>	15	15	14	0.0061	3.39	0.25	6	9	0.9679
Lineage II	<i>actA</i>	16	16	13	0.0064	3.56	−0.04	9	7	0.4724
Lineage III	<i>actA</i>	102	108	30	0.0449	25.19	0.09	41	57	0.2503
IIIA	<i>actA</i>	38	39	17	0.0144	8.06	−0.70	15	24	0.3626
IIIB	<i>actA</i>	13	13	8	0.0084	4.73	0.14	4	9	0.6495
IIIC	<i>actA</i>	19	19	5	0.0122	6.85	−0.65	6	13	0.5957
All isolates	<i>sigB</i> (73.5)	68	76	30	0.0289	16.51	0.72	70	3	0.0169
Lineage I	<i>sigB</i>	9	9	8	0.0036	2.40	0.74	9	0	NA
Lineage II	<i>sigB</i>	27	27	4	0.0036	2.42	−2.0‡‡	26	1	0.0088
Lineage III	<i>sigB</i>	54	55	18	0.0268	15.34	0.79	53	2	0.0072
IIIA	<i>sigB</i>	14	14	12	0.0047	2.67	−0.85	12	2	0.0156
IIIB	<i>sigB</i>	2	2	3	0.0007	0.40	−1.40	2	0	NA
IIIC	<i>sigB</i>	4	4	3	0.0027	1.52	−0.32	4	0	NA

*% ORF indicates that fraction of the full ORF for the respective gene that was used for descriptive analyses.
†Number of segregating sites in nucleotide alignment.
‡Number of nucleotide changes in the alignment.
§Unique combination of polymorphisms within a given locus.
||Average number of nucleotide differences per site between two sequences.
¶Average number of nucleotide differences per sequence between two sequences.
#Number of synonymous (silent) substitutions.
**Number of nonsynonymous (amino acid replacement) substitutions.
††(no. nonsynonymous substitutions/no. nonsynonymous sites)/(no. synonymous substitutions/no. synonymous sites); NA indicates that this ratio could not be calculated since the number of nonsynonymous substitutions was zero.
‡‡ $P < 0.05$.

codon at codon 51; at the first nucleotide position in this codon (nt 151) a 'T' substitution, which resulted in a TAA stop codon instead of the CAA codon found in all other isolates, was identified.

Partial *sigB* and *actA* maximum-likelihood phylogenetic trees, created using unique lineage I, II and III sequence types, both clearly show consistent grouping of lineage III isolates into three distinct subgroups that were designated IIIA, IIIB and IIIC to be consistent with previous classification of these isolates into lineage III (Fig. 1). Subgroup IIIA includes 29 isolates, representing 18 STs and 10 *EcoRI* ribotypes. Subgroup IIIB includes 10 isolates, 9 STs and 3 ribotypes, while subgroup IIIC includes 7 isolates, 5 STs and

3 ribotypes. *EcoRI* ribotypes, STs, and *actA* and *sigB* alleles were unique for each lineage III subgroup; i.e. no *EcoRI* ribotype, ST or allelic type was represented in more than one lineage III subgroup, further supporting isolate classification into these subgroups.

Descriptive analysis of sequence data using alignments containing all isolates studied here ($n=163$) showed that *L. monocytogenes* is a diverse bacterial species: partial *actA* and *sigB* sequences contained 148 and 68 polymorphic sites, respectively (Table 2). *actA* showed a considerably higher level of nonsynonymous mutations as compared to *sigB* sequences (Table 2). Most of the genetic diversity within *L. monocytogenes* appears to be attributable to the presence of

Fig. 1. Maximum-likelihood cladograms for *actA* (a) and *sigB* (b). Cladograms were inferred based on a 561 nt *actA* and a 573 nt *sigB* alignment (representing 29.2% and 73.5% of the respective ORFs) using a single isolate representing each unique sequence type ($n=67$), which was defined based on both *actA* and *sigB* alleles. A homologous *sigB* sequence from *Bacillus subtilis* was used as an outgroup in the *sigB* phylogeny. No outgroup was used for the *actA* phylogeny because no homologous *actA* sequences from closely related genera are available. Confidence measures for maximum-likelihood tree branch points were generated by performing 100 bootstrap replicates. Bootstrap values from the 50 per cent bootstrap consensus tree were imposed as node-labels on maximum-likelihood trees. Taxa belonging to *L. monocytogenes* lineage III are labelled with their sequence type (ST, see Table 1). Phylogenetic lineages (i.e. I, II, IIIA, IIIB and IIIC) are indicated by arcs and are labelled accordingly.

divergent evolutionary lineages within this bacterial species. When sequence data were analysed after stratification by lineage (i.e. lineage I, II, IIIA, IIIB and IIIC), the genetic diversity observed within each of these subpopulations was considerably reduced (Table 2). While lineage III isolates showed the greatest level of genetic diversity, as these isolates contained the majority of *actA* and *sigB* polymorphisms, the number of polymorphisms observed within lineage IIIA, IIIB and IIIC subgroups for both *actA* and *sigB* was much lower, suggesting a barrier for genetic exchange between these lineage III subgroups (Table 2). Lineage IIIA appears to be the most genetically diverse subpopulation within lineage III, as determined by the elevated measures of nucleotide diversity (i.e. π and k values; Table 2).

Tajima's D test not only tests the hypothesis that sequences have evolved according to the neutral theory but also encompasses information about a given population's demographics and thus can be used to make inferences about an organism's population structure (Nielsen, 2001; Simonsen *et al.*, 1995). Our data indicate that *actA* and *sigB* have evolved under neutrality, as none of the Tajima's D test statistic values, based on all isolates, were significantly different from the expected value of zero. In a previous study, we showed that large positive Tajima's D test statistics, indicating a subdivided population structure for *L. monocytogenes*, became much smaller or negative when analyses were performed for lineage I and II isolates separately (Nightingale *et al.*, 2005). Similarly, the large positive Tajima's D values observed for both *actA* and *sigB* based on alignments containing all isolates studied here (i.e. lineages I, II and III), which were reduced upon stratification of the full dataset by lineage, supports the concept that *L. monocytogenes* has a subdivided population structure. Similarly, for the most part, Tajima's D values observed for all lineage III isolates became smaller or negative when lineage III isolates were divided into the subgroups that were resolved in *actA* and *sigB* phylogenetic trees (IIIA, IIIB and IIIC), providing additional evidence that lineage III also represents a subdivided population.

Genetic and phenotypic characteristics of lineage III subgroups

Based on preliminary evidence that at least some lineage III strains lack the ability to ferment rhamnose (Wiedmann *et al.*, 1997), we assayed all 46 lineage III isolates for rhamnose fermentation. There was an exclusive and significant association ($P < 0.001$) between lineage III subgroup and ability to ferment rhamnose. Specifically, all lineage IIIA isolates fermented rhamnose, while all lineage IIIB and IIIC isolates were rhamnose fermentation negative (Table 1).

Since preliminary evidence also indicated that lineage III isolates often had unique genotypic characteristics (Jeffers *et al.*, 2001; Wiedmann *et al.*, 1997), we further characterized all lineage III isolates for *actA* type (type 3 or 4, indicating absence or presence of a proline-rich repeat, respectively), *hly* PCR-RFLP type, and the presence or absence of the

virulence-associated gene *lmaA* (Table 1). There was an overall significant association ($P = 0.034$) between lineage III subgroup and *actA* type. Specifically, *actA* type 4 was significantly overrepresented among lineage IIIA isolates (79 % of IIIA isolates carried *actA* type 4), while *actA* types 3 and 4 appeared to be equally prevalent among lineage IIIB and IIIC isolates. Overall, lineage III isolates represented eight different *hly* PCR-RFLP types; however, there was no significant association between lineage III subgroup and *hly* type. In general, lineage IIIA had the most *hly* type diversity, with all eight *hly* PCR-RFLP types represented, while lineages IIIB and IIIC contained only three types each (1c, 1d, 4b and 1, 4a, 4b, respectively). Subgroups IIIA, IIIB and IIIC contained predominantly *hly* PCR-RFLP type 4b (28 %, 80 % and 57 %, respectively). Interestingly, 19 lineage III isolates did not yield a PCR product for *lmaA*, indicating the absence of this putative virulence gene (Schaferkordt & Chakraborty, 1997) in some lineage III isolates. While the majority (88 %) of IIIB and IIIC isolates carried *lmaA*, only 58 % of lineage IIIA isolates were confirmed to carry this gene, and this difference was statistically significant ($P = 0.002$). Serotypes were available for 31 of the 46 lineage III isolates characterized and included serotypes 4a ($n = 16$), 4b ($n = 7$) and 4c ($n = 8$). There were no associations between lineage III subgroup and serotype, and all three subgroups contained at least one isolate representing each serotype.

Distribution of lineage III isolates among different sources

Of the 46 lineage III isolates characterized, 20 were obtained from human clinical cases, 13 from animals both with and without clinical listeriosis, 6 from foods, and 7 from unknown sources. Human clinical isolates were found in all lineage III subgroups and there were no significant associations between lineage III subgroup and source of isolation. There were, furthermore, no significant associations between isolate source (human, animal or food) and *actA* type, *hly* type, *lmaA* type or rhamnose fermentation phenotype.

Cell culture characterization of lineage III isolates

Since isolates in all three lineage III subgroups were obtained from human listeriosis cases, selected isolates from these three subgroups were characterized using a cell culture plaque assay to assess their ability to spread from cell to cell. Plaque size for the 14 isolates characterized ranged from 53 % to 114 %, with an overall mean plaque size of 88 ± 21 % (mean $\pm$ SD) which represents a marginally significantly smaller size than the lineage II internal control strain, 10403S ($P = 0.05$). There was no significant difference in the mean plaque size of isolates representing the three lineage III subgroups; specifically, lineage IIIA isolates had a mean plaque size of 100 ± 23 % ($n = 4$), lineage IIIB isolates had a mean plaque size of 84 ± 22 % ($n = 7$), while lineage IIIC isolates had a mean plaque size of 80 ± 8 % ($n = 3$). In contrast, Wiedmann *et al.* (1997) reported a mean plaque size for lineage III isolates ($n = 5$) that was significantly

larger than the 10403S control ($126.2 \pm 6.8\%$). In that study, however, one small plaque-forming isolate (L99, plaque size $53.5 \pm 7.9\%$) was excluded from analysis based on its previously reported virulence-attenuated phenotype (Chakraborty *et al.*, 1994). When isolate L99 is included, however, the mean lineage III plaque size is $114 \pm 30\%$, which is not significantly different from the mean plaque size for the lineage III isolates characterized here.

DISCUSSION

A few previous studies have identified and characterized *L. monocytogenes* strains that are distinct from the two common *L. monocytogenes* phylogenetic lineages (lineages I and II), which represent most human clinical and food isolates. Limited information on the genetic and phenotypic characteristics of these strains (which have been generally referred to as lineage III), is available though due to the rarity of lineage III isolates. We have thus assembled and characterized the largest set of lineage III *L. monocytogenes* isolates described to date. Our data indicate (i) *L. monocytogenes* isolates previously designated lineage III represent a subdivided population that contains at least three distinct phylogenetic lineages (termed subgroups IIIA, IIIB and IIIC), (ii) while lineage III isolates are most prevalent among animal clinical cases, they are also isolated from human cases and appear to have the ability to cause human disease, even though at low frequency, which is possibly caused by a reduced ability to survive and multiply under stress conditions typically found in foods, (iii) some lineage III isolates have unusual phenotypic characteristics which may interfere with their identification.

***L. monocytogenes* isolates previously designated lineage III represent a subdivided population that can be partitioned into at least three genetically and phylogenetically distinct subgroups**

Using phylogenetic analysis of *sigB* and *actA* sequences for 46 isolates, we found that *L. monocytogenes* isolates identified as lineage III based on *EcoRI* ribotyping and *hly* PCR-RFLP analysis represent three distinct subgroups, which we have termed IIIA, IIIB and IIIC. The relevance of these three lineage III subgroups is further supported by the fact that *EcoRI* ribotypes are unique to lineage III subgroups; i.e. a given *EcoRI* ribotype does not occur in more than one subgroup, similar to the observation that *EcoRI* ribotypes as well as ribotype subset are unique to lineages I and II (Jeffers, 1998; Jeffers *et al.*, 2001; Wiedmann *et al.*, 1997). Additionally, we showed that *actA* and *sigB* alleles and thus sequence types are exclusive to these lineage III subgroups. The absence of shared alleles has been proposed as a criterion to resolve subpopulations within a set of intraspecific data (Lan & Reeves, 2001) and our data strongly suggest that the three subgroups identified within lineage III do, in fact, represent genetically distinct populations. Interestingly, classification of lineage III isolates into multiple distinct subgroups is also

consistent with previous studies, which used phylogenetic analysis of DNA sequences for one or more *L. monocytogenes* gene(s) to classify and characterize *L. monocytogenes* isolates; most of these studies showed that lineage III isolates were highly diverse and often formed multiple distinct clusters in phylogenetic trees (Meinersmann *et al.*, 2004; Moorhead *et al.*, 2003; Ward *et al.*, 2004). For example, the *sigB* phylogeny for 8 lineage III (including 6 isolates used here) and 15 other *L. monocytogenes* isolates reported by Moorhead *et al.* (2003) also showed separation of lineage III isolates into subgroups, including completely separate clustering of the IIIC isolate FSL W1-110. Similarly, lineage III isolates also grouped in multiple separate clusters in the *addB* and *truB* phylogenetic trees for 5 lineage III and 30 other *L. monocytogenes* isolates reported by Meinersmann *et al.* (2004), even though the IIIC isolate FSL W1-110 generally clustered with the two IIIA isolates. However, these previous studies were limited by the small number of lineage III isolates characterized, and the larger lineage III isolate collection used in the current study allowed the lineage III subgroups, particularly subgroup IIIC, to be defined. We conclude that there is abundant and convincing evidence that isolates previously grouped into *L. monocytogenes* lineage III represent at least three distinct populations. While we refer to the lineage III subgroups as IIIA, IIIB and IIIC to be consistent with previous designation of these isolates as lineage III and reflecting that serotypes 4a, 4b and 4c are found among all three subgroups, phylogenetic analyses indicate that these three subgroups may represent separate evolutionary lineages.

While lineage III isolates are most prevalent among animal isolates, they are occasionally isolated from human cases and appear to have the ability to cause human disease

Interestingly, we found that all three lineage III subgroups identified here included isolates from human clinical cases, indicating that lineage III strains have the ability to cause human disease. Our finding that all lineage III isolates tested were able to form plaques in a cell culture cytopathogenicity assay indicates that lineage III strains possess the virulence genes necessary to spread from cell to cell, demonstrating the pathogenic potential of this *L. monocytogenes* population. These data indicate that lineage III may not show attenuated human virulence as previously proposed (Jeffers *et al.*, 2001; Wiedmann *et al.*, 1997). While over 40 % of the lineage III isolates characterized here came from human clinical cases of listeriosis, it is important to point out that human clinical isolates are overrepresented in our strain collection used to assemble the lineage III isolates set for this study. In general, lineage III isolates are more common among animal listeriosis cases (representing about 10 % of animal clinical isolates: Jeffers *et al.*, 2001) than among human clinical isolates or food isolates, representing 2.2 % and 0.4 % of isolates in these two categories, respectively (Gray *et al.*, 2004). Consistent with the results reported by Ward *et al.* (2004), these data thus suggest that rarity of human exposure to

lineage III isolates through consumption of contaminated foods, rather than reduced virulence, may account for the fact that lineage III isolates cause few human listeriosis cases and have never been linked to a human listeriosis outbreak. We hypothesize that the genetic and phenotypic characteristics that are unique to lineage III, both those characteristics that have been already identified and those that remain to be discovered, make lineage III strains, on average, less well suited to survive the stressful conditions associated with food and food-production environments, explaining the rare human foodborne exposure to lineage III strains. Supporting this hypothesis, a study on thermal inactivation *D*-values and growth characteristics of lineage I, II and III isolates (De Jesus & Whiting, 2003) previously found that lineage III isolates had the longest mean lag duration time at 7 °C (a temperature typical of refrigerated food storage) and the lowest mean thermal inactivation *D*-value, suggesting that lineage III isolates are, on average, less resistant to heat and cold stress than isolates belonging to lineages I and II. There were no differences in lag duration times between the lineages at 37 °C, which supports our assertion that lineage III strains have the potential to grow normally and be fully virulent once consumed by a mammalian host. Djordjevic *et al.* (2002) also found that lineage III, along with lineage II, has a decreased ability to form biofilms on PVC microtitre plates. Since biofilm formation is likely to contribute to the ability of *L. monocytogenes* to persist in different environments and to subsequently be introduced into the human food chain, a reduced ability to form biofilms may also contribute to a lower prevalence of lineage III isolates in foods. Interestingly, we also found here that one subgroup IIIA isolate carried a premature stop codon in *sigB*, which encodes the stress-responsive alternative sigma factor σ^B (Becker *et al.*, 1998). While this mutation was only found in a single isolate sequenced, occurrence of this mutation may indicate that at least some lineage III strains experience limited selection to maintain functional stress-response systems in their natural reservoirs, consistent with their potentially reduced stress resistance. While the observations outlined above provide initial evidence that lineage III strains are characterized by reduced environmental survival capability while maintaining the ability to cause mammalian disease, future research on genetic and phenotypic characteristics of the different lineage III subgroups and their ecology and epidemiology is needed to better understand the evolution and transmission of these *L. monocytogenes* strains.

Lineage III isolates have unusual phenotypic characteristics that may complicate their identification

Our data confirm and extend previous initial evidence (Wiedmann *et al.*, 1997) that lineage III isolates have unique phenotypic and genetic characteristics that may complicate their detection using various standard assays for the identification of *L. monocytogenes*. While previous studies noted that some lineage III isolates are unable to ferment

rhamnose, rhamnose fermentation is frequently included as a differential biochemical test in rapid identification kits such as API *Listeria* (BioMérieux) and Micro-ID (Organon Technika) used to identify pathogenic *L. monocytogenes* (Bille *et al.*, 1992; Sado *et al.*, 1998). Since all subgroup IIIB and IIIC isolates are rhamnose-negative, identification of these *L. monocytogenes* isolates based on biochemical patterns could be ambiguous. In addition, the lack of *lmaA* PCR amplification in some lineage III isolates combined with the absence of a number of other *L. monocytogenes*-specific genes, including several confirmed and putative internalin genes (e.g. as determined by DNA array analyses; Doumith *et al.*, 2004; Y. Jia and others, unpublished data) indicates that PCR assays targeting certain *L. monocytogenes*-specific genes may also fail to detect some or all *L. monocytogenes* lineage III strains. Our results indicate the importance of including *L. monocytogenes* isolates representing all three lineage III subgroups when validating assays for the specific detection of *L. monocytogenes*, particularly considering that all three lineage III subgroups have been found to cause human listeriosis cases. To this end, the lineage III isolates characterized here are available to other researchers and commercial entities.

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