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Identification and Characterization of Nucleotide Sequence Differences in Three Virulence-Associated Genes of *Listeria monocytogenes* Strains Representing Clinically Important Serotypes

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Abstract. *Listeria monocytogenes* is a Gram-positive, facultative intracellular bacterium that causes invasive, often fatal, disease in susceptible hosts. As a foodborne pathogen, the bacterium has emerged as a significant public health problem and has caused several epidemics in the United States and Europe. Three serotypes (1/2a, 1/2b, 4b) of *L. monocytogenes* are responsible for nearly 95% of all reported cases of human listeriosis. *L. monocytogenes* serotype 4b has caused all well-characterized foodborne epidemic outbreaks in North America and Europe between 1981 and 1993. However, most of the genetic studies to characterize virulence factors of *L. monocytogenes* have been done by using serotypes 1/2a and 1/2c. In this investigation, we examined three virulence-associated genes (*hly* encoding listeriolysin, *plcA* encoding phosphotidylinositol-specific phospholipase C, and *inlA* encoding internalin) of two serotype 4b and two serotype 1/2b strains. We chose these virulence-associated genes on the basis of published sequence differences among strains from *Listeria* subgroups containing serotypes 1/2a and 1/2c versus 4b, respectively. They correspond to sequence homologies that include very highly conserved (*hly*A), highly conserved (*plc*A) and mostly conserved (*inl*A). We found by using nucleotide sequence analysis of the *hly*, *plc*A, and *inl*A genes, the two *L. monocytogenes* strains (including a strain associated with a foodborne disease outbreak in California in 1985) in this study, two serotype 1/2b strains from a study that we recently reported, and other similar published data for serotypes 1/2a, 1/2c, and 4b, had a high degree of sequence conservation at the gene and protein levels for all three genes. However, the sequences for the *hly* gene of *L. monocytogenes* strains of serotypes 1/2b and 4b were more closely related to each other and showed significant divergence from serotypes 1/2a and 1/2c. A unique nonsynonymous mutation was found in the *hly* gene of *L. monocytogenes* isolates that were associated with the 1985 California outbreak and were the epidemic phage type. When 158 *L. monocytogenes* isolates from the collection at the Centers for Disease Control and Prevention were screened, the mutation was found only in one other strain that had been isolated in California 3 years before the epidemic. Although the California epidemic clone was lactose negative, other *L. monocytogenes* serotype 4b isolates that were lactose negative did not possess the unique mutation observed in that epidemic clone.

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Listeria monocytogenes is a Gram-positive, facultative intracellular bacterium that is ubiquitously distributed in the environment. It is capable of causing severe invasive diseases such as septicemia, meningitis, or meningoencephalitis in susceptible hosts, most of whom are immunocompromised with underlying conditions such as acquired immunodeficiency syndrome (AIDS), cancer, renal

Table 1. Primers for amplification of virulence-associated genes of *L. monocytogenes*

Gene	Primer set	Nucleotide sequences[a] (5′-3′)	References
hly	1-LLO	gagaggaggggctaaacagtat	[30]
	2-LLO	tcgtgtgtgtgttaagcggt	
plcA	pipinfront	accaggtacacatgaatacga	[25, 32]
	pipoutback	ccgaggttgctcggagatat	
inlA	inlafp2	ttgtaaaacgacggccagtggatatagacccgctt	[13]
	inlarp1	aaacagctatgaccatgttctatttactactagcac	

[a] M13 sequences underlined.

failure, or organ transplants [14]. *L. monocytogenes* also causes abortions and stillbirths in otherwise normal pregnancies. Over the past decade, the organism has caused several epidemics in the United States and Europe. Epidemiologic studies have implicated contaminated foods such as poultry, dairy products, and fresh vegetables in the transmission of *L. monocytogenes* [43]. Thus, the organism has become increasingly important in the clinical setting and a substantial problem for the food industry.

At least 13 serotypes of *L. monocytogenes* have been identified; however, only 3 (1/2a, 1/2b, and 4b) cause 95% of human listeriosis [14]. Despite a lower overall frequency of isolation from *Listeria*-contaminated foods [35], *L. monocytogenes* serotype 4b appears to be more associated with human disease than serotypes 1/2a and 1/2b. In fact, all six major outbreaks of foodborne listeriosis in North America and Europe since 1981 were caused by serotype 4b [4, 12, 27, 29, 39, 42]. A specific clone (identified by bacteriophage typing and multilocus enzyme electrophoresis [MEE]) of serotype 4b was associated with epidemic disease in outbreaks in California and Switzerland [3, 7, 33]. A very closely related strain (by serotyping, phage typing, and DNA restriction patterns) was implicated in a French listeriosis outbreak attributed to contaminated pork tongue in jelly [26, 39]. Also, *L. monocytogenes* serotype 4b is the most frequent cause of sporadic foodborne listeriosis. Investigations of foodborne listeriosis indicate foods that contain serotype 4b isolates are more likely to contain the patient-matching strain [35]. Because available evidence does not indicate an overwhelming presence of serotype 4b in the environment to account for its increased association with human disease, serotype-specific differences that may enhance virulence must be explored.

MEE divides *L. monocytogenes* into two distinct clusters (ETGA and ETGB), separable from each other at a genetic distance of approximately 0.45[3, 33]. ETGA is composed of *L. monocytogenes* serotypes 1/2a and 1/2c, and ETGB is composed of serotypes 1/2b and 4b. These data suggest an evolutionary divergence within the species *L. monocytogenes*. Whether or not these differences are related to virulence differences among serotypes of *L. monocytogenes* is not known.

At least seven different virulence-associated genes of *L. monocytogenes* have been characterized [36]. In this investigation, we sequenced three virulence-associated genes (*hly* gene encoding listeriolysin [LLO], *plc*A gene encoding phosphotidylinositol-specific phospholipase C, and *inl*A gene encoding internalin) for four ETGB strains of *L. monocytogenes* that included the 1985 California epidemic strain. The nucleotide sequences were compared with published sequences for the same genes for ETGA strains (serotype 1/2a and 1/2c) and analyzed to determine the extent of evolutionary diversity among serotypes and the presence of unique mutations in the epidemic-associated strain.

Materials and Methods

Bacterial strains. The *Listeria* strains used in this study came from human and environmental sources and were obtained from the collections at the Centers for Disease Control and Prevention (CDC). Bacterial cultures were stored frozen in sheep blood at −70°C. The bacteria were cultured by plating on tryptic soy agar containing 5% sheep blood at 35°C for 16–18 h.

Four *L. monocytogenes* strains were used for sequence analysis and included an epidemic serotype 4b strain (F2365) associated with the 1985 California epidemic [3] and three nonepidemic strains that were chosen based on the frequency of isolation of the selected enzyme types (ET): F5782 (serotype 4b); F6798 (serotype 1/2b); and F4233 (serotype 1/2b).

A battery of 158 *L. monocytogenes* isolates from the collections at CDC were used in a DNA probe assay to screen them for a specific mutation found in the *hly* gene of an epidemic strain. The following serotypes were represented in the strain set (numbers in parentheses indicate the number of isolates in a specific serotype): 1/2a (17), 1/2b (20), 1/2c (2), 3b (1), 4b (113), 4 (1), 4bX (3), and undetermined (1). Isolates from the outbreaks in Nova Scotia (1), Massachusetts (8), California (11), Costa Rica (1), England and Wales (19), Switzerland (3), and France (3) were included. The 158 isolates comprised 100 clinical and 58 food isolates. One *L. innocua* isolate from an environmental source was used as the negative control for species identification in DNA hybridization experiments (see below).

DNA isolation. Chromosomal DNA was extracted from *Listeria* spp. by a method developed in our laboratory [16, 17]. For some polymerase chain reaction (PCR) amplication experiments, the target DNA from *Listeria* strains was prepared by a rapid cell lysis protocol [20].

Amplification and sequencing of virulence genes. The *hly*, *plc*A, and *inl*A genes were amplified by PCR by using a Perkin Elmer thermocycler and GeneAmp DNA amplification reagent kit (Perkin Elmer Cetus, Norwalk, CT). The PCR reaction mixture contained 15 pmoles each of two gene-specific primers (Table 1), 100 ng of *Listeria* target DNA or 10

μl of cell lysate, PCR reaction buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 200 μM each of dATP, dGTP, TTP, dCTP, and 2.5 units *Taq* polymerase in a reaction volume of 100 μl. The reaction mix was overlaid with 100 μl of mineral oil. Amplifications were carried out for 25–30 cycles (96°C/30 s denaturing, 45°–58°C/15–30 s for annealing, and 60°–72°C/1–2 min extension, depending on the thermal denaturing characteristics of the primer pair), with a final extension for 10 min at 60°C. Generation of amplicons (PCR-amplified products) was verified by agarose gel electrophoresis. Additional primers (20-mers) for sequencing of the virulence-associated genes were made as needed by designing them from published data [9, 13, 25, 30].

Excess nucleotides and enzyme were removed from the PCR mixture with a Qiagen PCR purification spin kit (Qiagen, Chatsworth, CA). The double-stranded templates were sequenced by using the dideoxy chain termination method [41] and *Taq* Dye-Primer Cycle Sequencing kit or *Taq* Dye-Terminator Cycle Sequencing kit on a model 373A DNA sequencer (Applied Biosystems, Foster City, CA). Nucleotide and protein sequences were analyzed by DNASTAR (DNASTAR, Madison, WI) or DNASIS (Hitachi Software Engineering America, Brisbane, CA). A BioImage DNA sequence analysis system (BioImage, Ann Arbor, MI) equipped with sequence assembly manager software was used for the assembly of nucleotide sequences.

The GenBank (GenBank, Los Alamos, CA) accession numbers for *hly* (F2365, 4b); (F5782, 4b); (F4233, 1/2b); (F6798, 1/2b) are as follows: U25443, U25446, U25449, and U25452, respectively. The accession numbers for *plc*A (F2365, 4b); (F5782, 4b); (F4233, 1/2b); (F6798, 1/2b) are: U25444, U25447, U25450, and U25453, respectively. Accession numbers for partial sequences for the *inl*A gene for these same strains are U25445, U25448, U25451, and U25454, respectively.

Phylogenetic analysis of the genes coding for LLO and other cytolysins. The nucleotide sequences of the cytolysins of *L. monocytogenes*, *L. ivanovii* subsp. *ivanovii*, *L. seeligeri*, *Streptococcus pneumoniae*, *Bacillus alvei*, and *Clostridium perfringens* [9, 15, 19, 24, 30, 31, 38, 45, 48] obtained from GenBank data base were aligned along with the four *hly* nucleotide sequences from *L. monocytogenes* determined in this investigation by using the multisequence alignment program PILEUP [8; Genetic Computer Group (GCG), Madison, WI] running on a VAX computer. Sequence alignments were edited to remove nucleotide sequence gaps at the 5′ and 3′ ends, leaving 1427 positions for calculation of evolutionary distance. The phylogenetic relationship among the cytolysins was determined by using version 3.5 of the PHYLIP software package [11]. The bootstrap method for placing confidence intervals on inferred phylogenies [10] was utilized with 100 samplings of the nucleotide sequences. A similarity matrix was calculated by using the method of Jukes and Cantor [22]. The neighbor-joining method of Saitou and Nei [40] was employed to convert the similarity values to an (unrooted) phylogenetic tree. To generate an alternative (unrooted) phylogenetic tree, maximum parsimony method was done with DNAPENNY from the same PHYLIP software package.

Dot blot hybridization. *Listeria* chromosomal DNA was denatured and immobilized on nylon membranes as previously described by Kafatos et al. [23]. For each probe, experiments were repeated at least three times.

The probes (20-mers) for hybridization with *Listeria* DNA were synthesized on Applied Biosystems model 380A or 380B DNA synthesizers with standard phosphoramidite chemistry [2, 21]. The 20-mers were purified to a single homogeneous band on a Waters W600 high-performance liquid chromatographic system. Buffers and gradient conditions used are described elsewhere [44]. The 20-mers were 5′-end labeled with ^{32}P, according to the methods described by Maniatis et al. [28], except that after labeling, unlabeled and labeled probes were not separated.

For labeling, each oligonucleotide probe was diluted with deionized water to a final concentration of 200 ng/μl, and 1 μl was mixed with 6 μl of 10× elution buffer (1.0 mM Tris-HCl [pH 7.4], 0.01% SDS, 5.0 mM NaCl), 10 μl of γ - [^{32}P]ATP (10 mCi/ml, New England Nuclear Research Products, Boston, MA), and 41 μl of deionized water. Three units of T4 polynucleotide kinase (United States Biochemical [USB], Cleveland, OH) were added. The reaction mix was incubated at 37°C for 45 min and used directly in hybridization experiments.

Prehybridizations and hybridizations were done according to the procedure of Wood et al. [51]. Briefly, prehybridization was done in 30 ml of prehybridization solution containing Denhart's solution (0.02% Ficoll, 0.02% bovine serum albumin, and 0.02% polyvinylpyrrolidone) and 0.1 mg/ml of fish sperm DNA (USB). The hybridization was done in 3 ml of prehybridization solution containing the labeled probe at 37°C overnight. Washings were done in tetramethylammonium chloride (TMAC) solution (3 M TMAC, 50 mM Tris-HCl [pH 8], 2 mM EDTA, 0.1% SDS) at 37°C, 45°C, 58°C, and 65°C. After each wash, filters were exposed to X-ray film for 16–72 h at −70°C by using Kodak X-Omatic film cassette (Kodachrome X-ray film; Kodak) with intensifying screens.

DNA probe assay to screen *L. monocytogenes* for a specific mutation found in the epidemic isolates. Dot blot hybridization was performed with all *Listeria* strains listed above by using two probes derived from *hly* gene sequence. One probe, LLO-900M: 5′AATTTCATCCATAGCACCAC3′, contained the sequence from the 1985 California epidemic strain and the other, LLO900:5′AATTTCATCCATGGCACCAC3′, contained the sequence from a serotype 4b strain previously sequenced by Rasmussen et al. [38]. In addition, the 900 designation in the name of these two oligonucleotides corresponded to the position in the *hly* gene of a *L. monocytogenes* serotype 4b strain [38], where the mismatch between 4b (wild-type) and the epidemic-associated strain (F2365) occurred. Probe LLO-100: 5′ATGAAAAAAATAATGCTAGT3′ derived from a conserved area of the *hly* gene specifically reacted with *L. monocytogenes* strains and was used as a positive control for hybridization.

Lactose fermentation reaction. Lactose reactions were determined for all *L. monocytogenes* strains by inoculating 10 μl of bacterial suspensions into 5 ml of lactose fermentation media (Enteric Fermentation Media base [pH 7.2], supplemented with 1% lactose; Difco Laboratories, Detroit, MI). The tubes were incubated at 35°C for 7 days. A positive reaction was indicated by a color change to red.

Results

Comparative sequence analysis of virulence-associated genes. Nucleotide sequence analyses of the *hly*, *plc*A, and *inl*A genes from the four ETGB *L. monocytogenes* strains (two of serotype 1/2b and two of 4b) and two previously published sequences for ETGA strains (one of serotype 1/2a and one of 1/2c) showed significant conservation of nucleotide and amino acid sequences (>97%) among these strains (Table 2).

Although virulence-associated gene products from different *L. monocytogenes* strains may be identical, the primary nucleotide sequences that encoded them may not be because of silent or synonymous mutations. We therefore analyzed the nucleotide sequences in the three virulence-associated genes for the four *Listeria* strains. Most of the heterogeneity (including synonymous and

Table 2. Nucleotide and amino acid sequence homologies between the virulence-associated genes of *Listeria monocytogenes* used in this study

Strain no. (serotype) *hly* gene	Nucleotides (total no. 1590)		Amino acids (total no. 529)	
	No. of differences from reference strain[a]	% homology	No. of differences from reference strain	% homology
F2365[b] (4b)	42	97.4	4	99.2
F4233 (1/2b)	43	97.3	3	99.4
F5782 (4b)	42	97.4	3	99.4
F6798 (1/2b)	43	97.3	3	99.4
12067[c] (4b)	42	97.4	3	99.4
	Nucleotides (total no. 954)		Amino acids (total no. 317)	
plcA gene	No. of differences from reference strain[d]	% homology	No. of differences from reference strain	% homology
F2365[b] (4b)	24	97.5	7	97.8
F4233 (1/2b)	23	97.6	7	97.8
F5782 (4b)	24	97.5	7	97.8
F6798 (1/2b)	23	97.6	7	97.8
	Nucleotides[e] (total no. 1027)		Amino acids (total no. 344)	
inlA gene	No. of differences from reference strain[f]	% homology	No. of differences from reference strain	% homology
F2365[b] (4b)	19	98.8	4	99.2
F4233 (1/2b)	22	97.6	4	99.2
F5782 (4b)	23	98.6	5	98.1
F6798 (1/2b)	22	97.6	4	99.2

[a] Domann and Chakraborty, [9] (reference strain-serotype 1/2a).
[b] *L. monocytogenes* strain from 1985 California epidemic outbreak.
[c] Rasmussen et al. [38] (serotype 4b).
[d] Mengaud et al. [32] (reference strain-serotype 1/2c).
[e] *inlA* gene is 2232 bp long and encodes a protein of 744 amino acids.
[f] Gaillard et al. [13].

nonsynonymous mutations) in the three virulence-associated genes was found between the two previously identified subgroups for *L. monocytogenes* strains: ETGA (serotypes 1/2a and 1/2c) and ETGB (serotypes 1/2b and 4b) [3, 33]. The *hly* genes from the four ETGB strains showed one to five differences in their nucleotide sequences. The most nucleotide differences in the *hly* gene from ETGB were found between F2365 (an epidemic-associated strain) and F4233 (a high-frequency ET serotype 1/2b), which had five nucleotide differences (Table 3). A single nucleotide difference was found between the following ETGB strain-pairs for this gene: F6798 and F5782, F5782 and F2365, and 12067 and F5782 (Table 3).

In the *plc*A gene, the two ETGB strains of serotype 4b (F2365 and F5782) had identical nucleotide sequences; however, no more than three nucleotide differences were present among all ETGB strains (Table 3).

Partial sequence analysis of the *inl*A gene in the four *L. monocytogenes* strains from ETGB indicated greater divergence in the *inl*A gene than *hly* or *plc*A genes from *L. monocytogenes*. As shown in Table 3, more nucleotide changes were found in the *inl*A gene than the *hly* gene and *plc*A gene among the four ETGB strains. The location of specific deduced amino acid changes in the *hly*, *plc*A, and *inl*A genes is shown in Table 4. Three amino acid changes were observed between the ETGA and ETGB strains in the LLO sequence at positions 35, 438, and 523, respectively, from the NH_2-terminal. Except for California epidemic strain F2365, all ETGA and ETGB strains had methionine at position 39 of LLO; F2365 had an isoleucine at this location.

Three of the four ETGB strains (F2365, F4233, F5782) had identical deduced amino acid sequences encoded by the *plc*A gene. In one strain (F6798) a different amino acid in the *plc*A gene product was predicted at position 211 (Table 4). The deduced amino acid at position 211 in that strain was asparagine, a neutral amino acid. The weakly basic (positively charged) histidine was present at that position in the other ETGB strains (Table 4).

There was no specific pattern to the nonsynonymous mutations in the *inl*A gene. In contrast, three of four deduced amino acid sequences for *hly* or six or seven for *plc*A were identical in the four ETGB strains (Table 4).

Comparative phylogenetic analysis of thiol-activated cytolysins. The phylogenetic relationships of various thiol-activated cytolysins are shown in Fig. 1. Because both neighbor-joining and maximum-parsimony analyses generated essentially the same phylogenetic trees, for simplicity, Fig. 1 shows only the neighbor-joining tree. The *hly* genes encoding LLO in *L. monocytogenes* serotypes 1/2b and 4b formed a cluster that was separated from *hly* of *L. monocytogenes* serotypes 1/2a and 1/2c. The genes encoding seeligerolysin (*L. seeligeri*) and ivanolysin (*L. ivanovii* subsp. *ivanovii*) were more closely related to *hly* of serotypes 1/2a and 1/2c, which were identical. The other genes for cytolysins, including streptolysin O, alveolysin, perfringolysin O, and pneumolysin were found to all share a common line of descent with the *Listeria* cytolysin genes. Alveolysin was the most distantly related.

Table 3. Nucleotide sequence differences between pairs of *hly, plcA,* and *inlA* gene sequences among *Listeria monocytogenes* serotypes

Strain no. (serotype)	Gene	No. of nucleotide differences from serotype					
		LO28/EGD 1/2a, 1/2c	F2365 4b	F4233 1/2b	F5782 4b	F6798 1/2b	12067 4b
	hly						
LO28, EGD (1/2a, ½c)[a]		—					
F2365 (4b)		42	—				
F4233 (1/2b)		43	5	—			
F5782 (4b)		42	1	4	—		
F6798 (1/2b)		43	2	3	1	—	
12067 (4b)[b]		42	2	3	1	2	—
	*plc*A						
EGD[c] (1/2c)		—					
F2365 (4b)		24	—				
F4233 (1/2b)		23	1	—			
F5782 (4b)		24	0	1	—		
F6798 (1/2b)		23	3	1	2	—	
	*inl*A						
LO28[d] (1/2a)		—					
F2365 (4b)		19	—				
F4233 (1/2b)		22	11	—			
F5782 (4b)		23	4	7	—		
F6798 (1/2b)		22	11	2	7	—	

[a] Domann and Chakraborty [9]; Mengaud et al. [30].
[b] Rasmussen et al. [38].
[c] Mengaud et al. [32].
[d] Gaillard et al. [13].

Screening of *L. monocytogenes* isolates for the mutation found in the *hly* gene of the California epidemic strain. Using the oligonucleotide probe LLO-900M in a dot blot hybridization format, we screened the 158 isolates of *L. monocytogenes* for the specific mutation found at position 117 of the *hly* gene. Eleven strains of serotype 4b reacted with the probe indicating the presence of the mutation (Table 5). Of these, 10 were associated with the 1985 California epidemic and were the epidemic phage type. The eleventh strain (F7188) was also from California but had been isolated from a case in 1982.

Of the 158 strains of *L. monocytogenes* tested for lactose utilization that included the epidemic prototype strain (F2365), 17 were lactose negative (Table 5). They included all 11 strains that reacted with the LLO-900M oligonucleotide probe.

Discussion

Differences at the molecular level among serotypes of *L. monocytogenes* have been reported by several investigators. MEE, which samples approximately 1% of the *Listeria* genome for nonsynonymous mutations that result in changes in net charge of metabolic enzymes, divides *L. monocytogenes* isolates into two groups, one consisting of serotypes 1/2a, 1/2c, and 3a, and the other consisting of serotypes 1/2b, 3b, and 4b [3, 33]. Similar findings are also obtained by ribosomal DNA fingerprinting [18]. These observations suggest an evolutionary divergence within the species *L. monocytogenes*, resulting in two lineages, one consisting of serotypes 1/2a, 3a, and 1/2c, and the other consisting of serotypes 1/2b, 3b, and 4b. Whether such a divergence is evident in virulence-associated genes among serotypes of *L. monocytogenes* was not known.

The clustering of epidemic *L. monocytogenes* strains exclusively within ETGB prompted us to investigate virulence-associated genes from that cluster. The four *L. monocytogenes* strains selected for sequence analysis from ETGB included the 1985 California epidemic strain (F2365) and three nonepidemic strains (F4233, F5782, F6798). The nonepidemic strains were either serotype 1/2b or 4b and were selected on the basis of the frequency of isolation of their ET from clinical specimens and foods. F5782 (serotype 4b) is a low-frequency ET (6 of 1414 isolates from foods and cases characterized by MEE at CDC are this ET); F6798 (serotype 1/2b) is also a low-frequency ET (3/1414). F4233 (serotype 1/2b) is a high-frequency ET (78/1414) (M.W. Reeves, CDC, unpublished data).

We recently reported for the first time data comparing the nucleotide sequences for the *hly* and *plc*A genes

Table 4. Differences in the deduced amino acid sequences of three virulence-associated genes of *Listeria monocytogenes* strains

Gene	Amino acid position	Amino acid in[a] Strain no.: Serotype:	F2365 4b	F4233 1/2b	F5782 4b	F6798 1/2b	12067[b,c] 4b	LO28[d] 1/2a	EGD[e] 1/2c
hly									
	35		L	L	L	L	L	S	S
	39		I	M	M	M	M	M	M
	438		I	I	I	I	I	V	V
	523[g]		S	S	S	S	S	K	K
plcA									
	13		V	V	V	V	V	L	L
	19		C	C	C	C	C	Y	Y
	57		S	S	S	S	S	N	N
	76		M	M	M	M	M	I	I
	112		K	K	K	K	K	N	N
	211		H	H	H	N		R	R
	220		S	S	S	S		P	P
inlA[f]									
	68		S	T	S	T			T
	113		S	N	S	N			N
	119		F	L	L	L			F
	179		L	W	L	W			L
	218		R	P	P	R			S
	342		E	E	E	E			A

[a] Amino acid symbols: Alanine (A), Cysteine (C), Glutamic acid (E), Phenylalanine (F), Arginine (R), Serine (S), Lysine (K), Leucine (L), Methionine (M), Asparagine (N), Proline (P), Histidine (H), Isoleucine (I), Threonine (T), Valine (V), and Tryptophan (W).
[b] Rasmussen et al. [38] (*hly* and *plc*A, serotype 4b).
[c] Partial amino acid sequence information available for *plc*A.
[d] Domann and Chakraborty [9] (*hly*A, serotype 1/2a) and Leimeister-Watchter et al. [25] (*plc*A).
[e] Mengaud et al. [30] (*hly*); Mengaud et al. [32] (*plc*A); and Gaillard et al. [13] (*inl*A).
[f] Partial sequence analysis of the *inl*A gene (i.e., only a third of one strand from the 5′-end was sequenced).
[g] Position 523 (not 532 as reported [47]) is correct.

for two serotype 1/2b strains of *L. monocytogenes* [47]. In this report, we extend these studies to investigate observed differences between epidemic (4b) and nonepidemic *L. monocytogenes* (1/2b and 4b) strains. In addition, we furnish nucleotide sequences for the *inl*A (partial sequence only) gene.

We found a high level of nucleotide sequence homology (>97%) in the three virulence-associated genes for the four *L. monocytogenes* strains. The deduced amino acid sequences for LLO from three of the four ETGB strains (F4233, F5782, and F6798) were identical. One nonsynonymous mutation was found in the epidemic-associated ETGB strain, F2365. It predicted a change in the deduced amino acid sequence for LLO at position 39 from methionine to isoleucine. Comparison of the deduced amino acid sequences for LLOs and ivanolysin, a related thiol-activated cytolysin from *L. ivanovii* subsp. *ivanovii*, showed that methionine is also conserved at this location in ivanolysin [19]. The nonsynonymous mutation in the *hly* gene of the California epidemic strain is immediately downstream of the signal peptide in LLO and may affect processing of the mature LLO molecule in the epidemic-associated strain. However, isoleucine and methionine are both nonpolar and hydrophobic; therefore, this represents a neutral amino acid substitution. Thus, the functional significance of this mutation is not clear.

Three of the four ETGB *L. monocytogenes* strains had identical deduced amino acid sequences for phosphotidylinositol-specific phospholipase C encoded by *plc*A. One (F6798; serotype 1/2b) strain had a nonsynonymous mutation that changed the predicted amino acid at position 211 from histidine to asparagine. Because asparagine is uncharged (polar) and histidine is positively charged, this substitution in phosphotidylinositol-specific phospholipase C sequence results in a change in net charge.

When *plc*A sequences of ETGA and ETGB were compared, seven differences were observed in the amino acid composition. Five of these had been previously identified by Rasmussen et al. [38].

Four or five amino acid differences were found in the partial sequence of the *inl*A gene product in the four ETGB strains when compared with an ETGA strain.

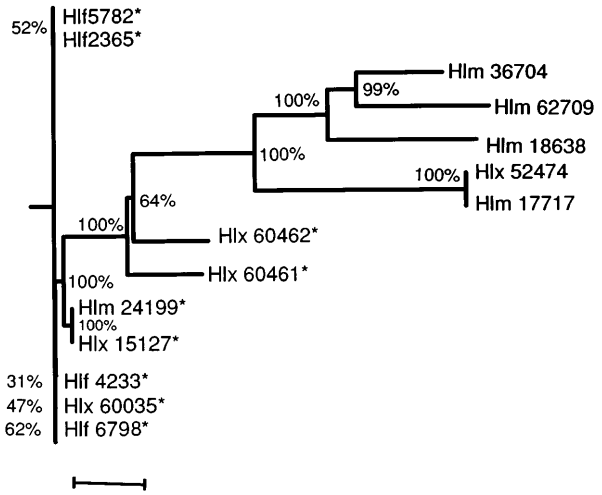

Fig. 1. Phylogenetic tree showing the position of *Listeria* cytolysins among related thiol-activated cytolysins. The tree was inferred from cytolysin-gene similarity data by the neighbor-joining method [40] with bootstrap analysis [10]. The percentage of bootstrap replications that yielded each group is indicated. Horizontal length represents genetic distance, and the bar represents 5% nucleotide difference. Vertical lengths are not meaningful. The *Listeria* cytolysin genes are indicated with an asterisk. Branch designations: hlx60035, *hly* serotype 4b [38]; hlf4233, *hly* (this study, serotype 1/2b); hlf5782, *hly* (this study, serotype 4b); hlf6798, *hly* (this study, serotype 1/2b); hlf2365, *hly* (this study, serotype 4b); hlm18638, streptolysin O gene [24]; hlm62709, alveolysin gene [15]; hlm36704, perfringolysin O gene [45]; hlm17717 and hlx52474, pneumolysin gene [48]; hlx60462, seeligerolysin gene[19]; hlx60461, ivanolysin gene [19]; hlm24199, *hly* (serotype 1/2c,[30]); and hlx15127, *hly* (serotype 1/2a [9]).

These amino acid differences were not always the same from strain to strain. Therefore, *inl*A gene appears to be more divergent than the other two virulence-associated genes. Poyart et al. [37] have recently identified DNA polymorphism in an intragenic region of *inl*A, which spans a region not investigated in this study (Table 3). These mutations may contribute to the variability in pathogenicity observed among virulent *L. monocytogenes* strains [34]. However, the Poyart et al. [37] study could not determine a correlation between the genetic polymorphism of *inl*A and epidemic or sporadic behavior of the *L. monocytogenes* strains. Gaillard et al. [13] suggested that the *inl*A gene product (internalin) may exhibit functional and antigenic variability. The functional variability may explain why certain clones are apparently more virulent than others [3, 5, 33]. The antigenic variability may aid *L. monocytogenes* in evading the host's immune response mechanisms.

Phylogenetic analysis of the gene sequences encoding cytolysins revealed interesting information about the relatedness of *Listeria* cytolysins to each other and to other thiol-activated cytolysins. The genes encoding alveolysin were found to be most distantly related to the genes encoding for *Listeria* cytolysins. The genes encoding LLO in *L. monocytogenes* separated into two clusters. Cluster I consisted of serotypes 1/2a and 1/2c, and Cluster II consisted of serotypes 1/2b and 4b. The genes encoding seeligerolysin and ivanolysin were more closely related to Cluster I. Cluster II, which included the epidemic strain, appears to have diverged the farthest from the other *Listeria* cytolysins (i.e., Cluster I LLO, seeligerolysin, and ivanolysin).

Table 5. Correlation between lactose-negative *Listeria monocytogenes* strains serotype 4b and their reactivity with LLO-900M probe[a]

Strain	Lactose reaction[b]	Probe LLO-900M reactivity	Comments[c]
F2365	−	+	California epidemic (1985); epi-phage type
F2380	−	+	California epidemic (1985); epi-phage type
F2383	−	+	California epidemic (1985); epi-phage type
F2379	−	+	California epidemic (1985); epi-phage type
F6823	−	+	California epidemic (1985); epi-phage type
F7004	−	+	California epidemic (1985); epi-phage type
F7394	−	+	California epidemic (1985); epi-phage type
F6902	−	+	California epidemic (1985); epi-phage type
F7188	−	+	California sporadic (1982); not phage typed
F6955	−	+	California epidemic (1985); epi-phage type
F7008	−	+	California epidemic (1985); epi-phage type
F6803	−	−	California sporadic (1989); epi-phage type
G2120	−	−	California sporadic (1989); epi-phage type
F7209	−	−	California epidemic (1985); phage type different from epi-phage type
G2113	−	−	California sporadic isolate (1989); not phage typed
G2111	−	−	California sporadic isolate (1989); not phage typed
G2116	−	−	California sporadic isolate (1989); not phage typed

[a] LLO-900M: 5′AATTTCATCCATAGCACCAC3′.
[b] 7 days at 35°C.
[c] Phage typing data obtained from Prof. Dr. A. Audurier, Laboratoire de Bactériologie, Hôpital Trousseau, Paris, France.

There was no pattern to the amino acid differences found in the *inl*A gene from ETGA and ETGB strains. In contrast, sequence differences in the *hly* and *plc*A genes varied according to serotype (based on somatic and flagellar antigens) or ET (based on variations in electrophoretic mobility of constitutive metabolic enzymes). Thus, in *L. monocytogenes*, *hly* and *plc*A genes appear to be evolving at approximately the same rate as the changes in the genes coding for essential cytoplasmic enzyme and the immunogenic surface antigens that are utilized in serotyping. In contrast, *inl*A gene (belonging to *inl*AB operon) appears to be evolving at a faster rate. The evolution of the *inl*A gene in *L. monocytogenes* may be similar to the evolution of genetic diversity in uropathogenic *Escherichia coli*. In this organism, the pyelonephritis-associated, pilus-related gene clusters that are associated with virulence appear to be evolving at a faster rate than ribosomal operons or the genes coding for constitutive enzymes [1].

In this investigation, epidemic and nonepidemic *L. monocytogenes* strains were screened for the unique mutation identified in *hly* gene of epidemic strain F2365 to determine whether the mutation was a marker for epidemic strains. The mutation was specific only for the California epidemic strain. Although MEE subtyping and bacteriophage typing suggested that a single clone was responsible for the California and Switzerland outbreaks [3, 7, 33], none of the Swiss outbreak strains tested had the specific mutation in the *hly* gene. Also, the strain responsible for the recent French outbreak, which is very closely related to the California and Switzerland epidemic strains by serotyping, phage typing, and DNA restriction patterns[26, 39], did not have the mutation. The only other isolate that had the unique mutation found in the California epidemic strain had been isolated in California 3 years before the California epidemic. The isolate was obtained from a 66-year-old man who had lung cancer and a dual infection with *L. monocytogenes* and *Salmonella* Group D (serotype Dublin). This isolate was indistinguishable from the Mexican-style cheese isolate of *L. monocytogenes* when they were characterized by DNA macrorestriction analysis by pulsed-field gel electrophoresis with two restriction enzymes (*Apa*I and *Asc*I) according to the method of Brosch et al. [6] (data not shown). The patient had a history of drinking certified raw milk. Because contaminated, Mexican-style cheese responsible for the California epidemic was presumably made from contaminated raw milk, it is possible that these cases may have resulted from dietary exposure to the same clone from raw milk. It is not clear why this clone resurfaced after 3 years to cause the outbreak and why it disappeared after 1985. None of the recent California isolates tested had the unique mutation in the *hly* gene.

All of the *L. monocytogenes* strains that had the mutation were also lactose negative. However, no definite correlation was found between the inability of *L. monocytogenes* strains to utilize lactose (*lac*$^-$) and the occurrence of the *hly* mutation. All strains that had the mutation were *lac*$^-$; however, not all *lac*$^-$ strains had the mutation. The inability of *L. monocytogenes* strains to utilize lactose coupled with the unique mutation found in the *hly*A gene, which correlated with the 1985 California epidemic strain, may indicate phage involvement. However, both F6803 and G2120 strains with the epi-phage type (Table 5) did not utilize lactose nor have the mutation. It is probably more likely that the correlation between epi-phage type and *lac*$^-$ phenotype in the California epidemic strain is just coincidental. Whether some other underlying genetic defect, unrelated to lactose utilization, may have occurred in strains not phage typed is not known (Table 5).

Because *L. monocytogenes* isolates belonging to ETGB have been responsible for several epidemics in North America and Europe, this group may contain strains that have enhanced virulence [3, 5, 33]. Because mutations unique to the epidemic-associated strains were not found in the three virulence-associated genes investigated in this study, further studies should be directed to the characterization of other known virulence-associated genes (e.g., *actA*, *prfA*, *plcB*, *inlB*) or new virulence-associated genes. Subtractive hybridization [46] is a powerful tool for exploring the epidemic-associated strains for new virulence factors. Using this approach, investigators may find unique sequences in the epidemic strain that include genes encoding other virulence factors or genes coding for factors that regulate the synthesis of virulence factors. In addition, subtractive hybridization of a strain grown in vivo from the same strain grown on laboratory culture media may provide information on new and unique gene products expressed by the clone under in vivo growth conditions.

Finally, serotype-specific and strain-specific mutations in virulence-associated genes of *L. monocytogenes* have been identified in this study. Methods such as PCR [40], ligase chain reaction [50], and in situ hybridization [49] could be developed by using oligonucleotides made from regions that include specific mutations. These methods could be used to rapidly screen a large number of isolates to identify a specific virulent clone such as the one that caused the epidemic in California in 1985. This would be beneficial in investigations of foodborne epidemic outbreaks and for screening of foods.

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