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Ribotypes and Virulence Gene Polymorphisms Suggest Three Distinct *Listeria monocytogenes* Lineages with Differences in Pathogenic Potential

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A total of 133 *Listeria monocytogenes* isolates were characterized by ribotyping and allelic analysis of the virulence genes *hly*, *actA*, and *inlA* to uncover linkages between independent phylogenetic and specific virulence markers. PCR-restriction fragment length polymorphisms revealed 8 *hly*, 11 *inlA*, and 2 *actA* alleles. The combination of these virulence gene alleles and ribotype patterns separated *L. monocytogenes* into three distinct lineages. While distinct *hly* and *inlA* alleles were generally found to cluster into these three lineages, *actA* alleles segregated independently. These three phylogenetic lineages were confirmed when 22 partial *actA* DNA sequences were analyzed. The clinical history of the *L. monocytogenes* strains showed evidence for differences in pathogenic potential among the three lineages. Lineage I contains all strains isolated during epidemic outbreaks of listeriosis, while no human isolates were found in lineage III. Animal isolates were found in all three lineages. We found evidence that isolates from lineages I and III have a higher plaquing efficiency than lineage II strains in a cell culture assay. Strains from lineage III also seem to form larger plaques than strains from lineage II. A distinctive ribotype fragment and unique 16S rRNA gene sequences furthermore suggest that lineage III might represent a *L. monocytogenes* subspecies. None of the 20 human isolates available but 11% of our animal isolates were grouped in this lineage, indicating that strains in this lineage might have reduced virulence for humans.

Strain differences in virulence and host range have been described for a variety of bacterial pathogens (24). For example, multiple clone families of *Streptococcus agalactiae* have been shown to be the predominant cause of invasive neonatal disease (25). *Listeria monocytogenes*, an intracellular pathogen of humans and animals, is another gram-positive bacterium for which there are indications of virulence differences among strains (11). Most clinical *L. monocytogenes* isolates from epidemics or human sporadic cases belong to only 3 (1/2a, 1/2b, and 4b) of the 13 serotypes known for this species (11, 30). Furthermore, the majority of *L. monocytogenes* strains found in conjunction with large outbreaks of human food-borne listeriosis (1, 11, 12, 17, 19–21, 29–31) appear to form a very homogeneous group. Most are serotype 4b and fall within two multilocus enzyme electrophoresis (MEE) types (15, 19, 23, 24) and two ribotypes. It seems plausible that these strains are clones particularly virulent for humans.

Two primary MEE phylogenetic divisions exist for *L. monocytogenes*, with serotypes 1/2b, 4a, and 4b in division I and serotypes 1/2a and 1/2c in division II (24). Isolates from sporadic human cases as well as animal isolates did not represent distinctive subsets of electrophoretic types (ETs). Nevertheless, as mentioned above, isolates from human food-borne epidemics belong to just two closely related ETs (ETs 1 and 7) of 45 known ETs. Pulsed-field fingerprinting (2) as well as ribotyping (15) studies also confirmed the existence of two distinct divisions. DNA sequence polymorphisms in virulence genes identified by either PCR-restriction fragment length polymorphism (RFLP) or DNA sequencing further verified the presence of two evolutionary lineages in *L. monocytogenes* (16, 26, 34). The two subgroups defined by these different methods appear to be equivalent in grouping common strains and different serotypes. One subgroup seems to primarily contain serotypes a and c, while the other one contains serotype b (16). Recently, Rasmussen et al. (27) defined three genetic lineages of *L. monocytogenes* based on sequence analysis of listeriolysin O (*hly*), invasion-associated protein (*iap*), and flagellin (*flaA*) genes. Serotype 4a appears to occupy a unique position, as it falls into the third lineage defined by Rasmussen et al. (27) and is the only tested 4a strain grouped with b serotypes by MEE (24).

To our knowledge, the role of different *L. monocytogenes* strains in animal disease has not been investigated. Listeriosis in animals can have four distinct clinical presentations, including encephalitis, abortion, septicemia, and uveitis. Within an outbreak, usually only one of these symptom complexes is observed in the infected animals. This could indicate that virulence differences exist for *L. monocytogenes* strains, with some preferentially causing distinctive clinical symptoms.

In this study we attempted to define genetic lineages based on ribotyping-generated pattern types and virulence gene alleles and to correlate them to pathogenic potential in *L. monocytogenes*. Ribotyping was chosen to initially type different *L. monocytogenes* strains because it is a reliable and reproducible typing method for which a large database exists (3). Further population genetic analysis of *L. monocytogenes* in conjunction with analysis of the clinical history of these strains might suggest why only certain clones are involved in human listeriosis outbreaks and might facilitate the identification of specific virulence markers. Comparisons between human and animal isolates could lead to greater insight about the transmission of

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TABLE 1. PCR primers used in this study

Primer	Sequence (5′→3′)
actA-F	TAG CGT ATC ACG AGG AGG
actA-R	TTT TGA ATT TCA TAT CAT TCA CC
actA-946F	TAA AAG TGC AGG GTT AT TG
actA-1834R	GGA TTA CTG GTA GGC TCG G
hly-F	TGC GTT TCA TCT TTA GAA GC
hly-R	AAG CCT GTT TCT ACA TTC TTC A
hly-β_n	GTC GAT GAT TTG AAC TTC ATC TTT
inlA-F	CAG GCA GCT ACA ATT ACA CA
inlA-R	ATA TAG TCC GAA AAC CAC ATC T
16S-P5SH	TGA AGA GTT TGA TCA TGG CTC AG
16S-DG74	AGG AGG TGA TCC AAC CGC A
16S-P3SH	CTA CGG TTA CCT TGT TAC GAC TT
16S-Lm2Ra	ATC CAT TGT AGC ACG TGT GTA GC

L. monocytogenes along the food chain and the role of animals as potential sources of human infection.

MATERIALS AND METHODS

Strains and isolates. A total of 74 *L. monocytogenes* animal isolates were collected by the Diagnostic Laboratory, College of Veterinary Medicine, Cornell University, as part of the routine diagnostic testing for listeriosis. Sixty-four of these were obtained from ruminants (cattle, sheep, and goats) and 10 were from nonruminants. An additional two clinical isolates from birds as well as two food isolates from chickens were obtained from the National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa. Twelve silage and two bulk-milk isolates were also included in this study. Furthermore, strains of ribotype dd 0647 ($n = 22$) isolated from foods, humans, and animals as well the type strains for ribotypes dd 0648 (ATCC 19114), dd 0652, dd 8842, dd 3823, dd 6821, and dd 6824 were tested (3). Three strains with attenuated virulence as described by Chakraborty et al. (5), four commonly used laboratory strains (10403S, ScottA, 2289, and EGD), and six isolates from human listeriosis cases and outbreaks (7) were also included.

Virulence gene polymorphisms. The *L. monocytogenes* virulence genes encoding listeriolysin O (*hly*), ActA (*actA*), and internalin A (*inlA*) were PCR amplified and screened for allelic polymorphisms by PCR-RFLP. PCR primers used are detailed in Table 1. PCR products were digested separately by using *Hha*I and *Hpa*II for *hly*, *Sac*I for *actA*, and *Apo*I, *Hin*fI, and *Hha*I for *inlA*.

Partial DNA sequencing and phylogenetic analysis of *actA* and the 16S rRNA gene. PCR amplification was performed by using primers actA-F and actA-R for *actA* and 16S-P5SH and 16S-DG74 for the 16S rRNA gene (Table 1). PCR products were purified with the QIAquick PCR purification kit (Qiagen Inc., Chatsworth, Calif.). Sequencing was performed with primer actA-946F (*actA*) and primers 16S-P3SH, 16S-P5SH, and 16S-Lm2Ra (16S rRNA gene) (Table 1) by using the Perkin-Elmer cycle sequencing kit and an Applied Biosystems model 373A automated sequencer. Alignment of the DNA sequences was performed by using MEGALIGN (DNASTAR Inc., Madison, Wis.). Phylogenetic analysis of the *actA* alignment was performed with PAUP 3.1.1 (33).

Ribotyping. Ribotyping with normalized data was performed as previously described (3, 18). This typing method involves *Eco*RI digestion of chromosomal DNA followed by Southern hybridization probing with an *Escherichia coli rrnB* rRNA operon probe (3). Images were acquired with a charge-coupled device camera and processed by using custom software (3). This software normalizes fragment pattern data for band intensity and relative band position compared to the molecular weight marker.

Cell culture plaque assay. The cytopathogenicity of 23 selected *L. monocytogenes* animal isolates, 2 *L. monocytogenes* strains with attenuated virulence (L19 and L99), and strains 10403S, EGD, and 2289 was determined by a plaque assay with mouse L cells as described by Smith et al. (32). *L. monocytogenes* was grown overnight to stationary phase at 30°C and inoculated onto the cell monolayer at approximately 3×10^4 CFU per well. Serial dilutions of the inoculum were plated on Oxford agar (Unipath Ltd., Basingstoke, United Kingdom) to determine the number of CFU used for inoculation. Results were reported as average plaque diameter and as plaquing efficiency (CFU/PFU). Plaque sizes were measured for all plaques formed in at least two wells as described by Smith et al. (32). Plaque sizes were expressed as percent relative to strain 10403S, which was included as a control in each experiment and assigned a value of 100%. Average plaque size and standard deviations (SDs) for a given strain were calculated by pooling individual plaque sizes from different experiments by using BMDP 7.0 (BMDP Statistical Software, Inc., Los Angeles, Calif.). Strains were grouped according to their average plaques sizes by using the Student-Newman-Keuls multiple-range test at a 95% confidence interval.

Nucleotide sequence accession numbers. The 16S rRNA gene DNA sequences for strains DD 3823, DD 6821, and ATCC 19114 can be found in the GenBank database under accession numbers U84148 to U84150, respectively.

RESULTS

Ribotype pattern types. A total of 23 different ribotype pattern types were observed for the 133 strains tested (Fig. 1). Five of these pattern types (dd 11900, dd 11903, dd 11696, dd 11698, and dd 12388) had not previously been described for any of 1,346 *L. monocytogenes* strains classified by ribotyping (3).

A total of 15 ribotypes (including the new ribotype dd 11698) were found among the 64 ruminant isolates. All ribotypes which had a frequency of >0.05 in a previous survey of 1,346 *L. monocytogenes* strains were also represented among these ruminant isolates, with the exception of ribotype dd 1284 (3).

Virulence gene polymorphisms. PCR-RFLP analysis revealed 8 *hly* and 11 *inlA* alleles. Schematic representations of the different restriction patterns are shown in Fig. 2. For strains with *hly* alleles *4a* and *4b*, no amplification with primers hly-F and hly-R was observed but amplification with primers hly-F and hly-β_n gave a PCR product of the expected size (1,193 bp). Primers hly-F and hly-R are located outside the *hly* open reading frame (ORF), while hly-β_n is located within the 3′ end of the ORF. For alleles *4a* and *4b* the restriction sites present in the amplified fragment were identical to those found in alleles *1*, *1b*, *2*, and *1c*, respectively.

For *actA*, two alleles were differentiated by PCR-RFLP. Subsequent DNA sequence analysis of representative strains (see below) showed that these two alleles differ solely by the presence or absence of a 105-nucleotide (nt) direct repeat which encodes a proline-rich repeat (PRR) structure. The *actA* PCR product obtained with primers actA-F and actA-R also showed a size difference for strains carrying the two different alleles consistent with the absence or presence of a PRR. The precise location of the deletion was further confirmed by PCR amplification with two internal primers (actA-946F and actA-1834R) flanking the PRR region. The two *actA* alleles were designated *3* and *4* (Table 2), for three and four PRRs, respectively. These results as well as a distribution of the virulence gene alleles to different ribotypes are summarized in Table 2.

The DNA sequence of nt 775 to 1313 (numbering refers to the first base of the coding sequence for *actA* [10]) of *actA* was determined for 21 selected strains. An alignment of the deduced ActA sequences, including the published sequence for strain EGD (10), is shown in Fig. 3. A phylogram representative of the four minimal trees determined by a branch-and-bound search of the 22 *actA* sequences is displayed in Fig. 4A. The four most parsimonious trees differed only in position and resolution of the branches containing strains 662205 (dd 0653, 4 PRRs) and 782779 (dd 0566, 4 PRRs). A consensus tree (cladogram) constructed by the majority-rule consensus is displayed in Fig. 4B. This phylogenetic analysis indicates that strains of lineages I and III represent two subclusters. Strains within lineage II are placed on branches distinctive from the other two lineages but show far more sequence divergence than strains within lineages I and III.

The deletion of a 105-nt repeat coding for a PRR was confirmed for the 14 three-repeat strains scored by PCR-RFLP. Deletion of a PRR-encoding region in *actA* did not cause a frameshift in the *actA* ORF in any of these strains. A total of 53 polymorphic nucleotides were found within the 539 nt sequenced leading to 22 amino acid changes within a total of 179 amino acids (aa). All nucleotide differences were base substitutions; no single base insertions or deletions were observed. All four ribotype dd 0647 strains showed a unique signature amino acid sequence, FPLMP (aa 380 to 384), in the third PRR, in contrast to FPP(I/M)P in the other strains (Fig. 3).

Correlation between virulence gene alleles and ribotypes. Based on ribotypes and virulence gene alleles, we were able to

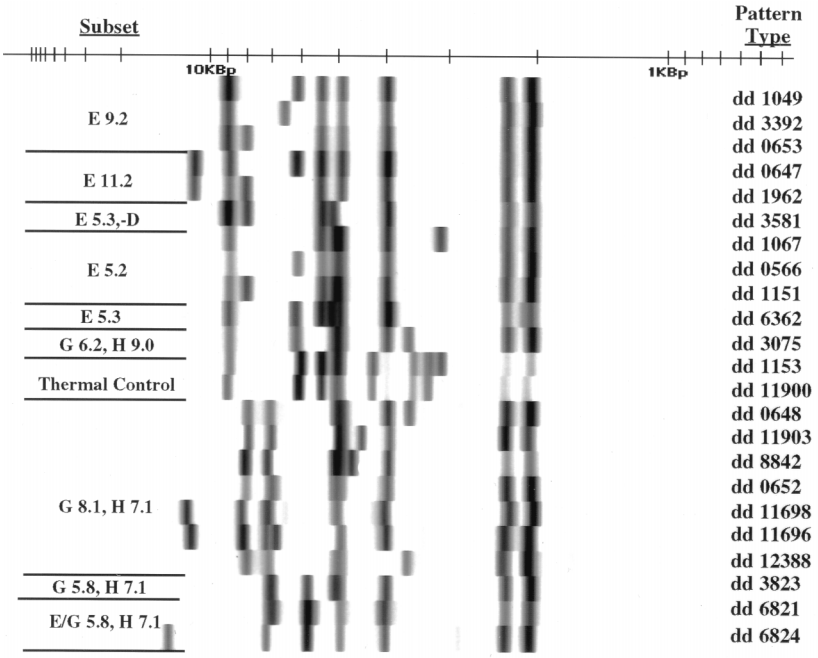

FIG. 1. *Eco*RI pattern types for the *L. monocytogenes* isolates used in this study. Ribotype pattern types are indicated on the right, and the *Eco*RI subsets defining a group of ribotypes which have identical *Eco*RI rRNA operon restriction fragments (3) are indicated on the left. For example, subset E 9.2 contains all ribotypes which have a 9.2-kbp fragment; details regarding the subset designations can be found in reference 3. Fragment sizes are indicated at the top. Images were acquired with a charge-coupled device camera (Star One camera; Photometrics, Tucson, Ariz.) and processed by using custom software as previously described (3). Additional methodological details are given in references 3 and 18.

divide the species *L. monocytogenes* into three distinct genotypic lineages. Commonly used laboratory or type strains representative of these lineages include 2289 and ScottA (lineage I), EGD and 10403S (lineage II), and ATCC 19114 (lineage III). An overview of the three different lineages, their characteristics, and their frequency among human and animal isolates as well as in a survey of mainly environmental and food isolates (3) is presented in Table 3.

16S rRNA gene sequencing results. The 16S rRNA gene DNA sequence was determined for three strains of lineage III chosen to cover all three pattern type subsets classified in this lineage (Table 3). The complete 16S rRNA gene DNA sequence was determined for strains DD 3823 (G 5.8, H 7.1 subset) and DD 6821 (E/G 5.8, H 7.1 subset). For strain ATCC 19114 (G 8.1, H7.1, −F subset) only the sequence for about 500 bp of the 5′ end, including the V2 region, was determined. Some differences with the DNA sequences previously determined for *L. monocytogenes* ScottA (8) and NCTC 10357 (6) were observed, although the sequences determined here are generally consistent with the sequence reported for at least one of these strains. Only the V2 region showed consistent differences with previously reported 16S rRNA sequences from different *L. monocytogenes* strains (8). Strains within lineage III showed a distinctive signature sequence, AAA (DD 3823) or AGA (DD 6821, ATCC 19114, and SLCC 53) (nt 194 to 196) and CT (nt 214 and 215), in this region, in contrast to GAA and TT, GAG and TC, or AAG and TT in strains from other lineages (8). Sequence data for SLCC 53 (ribotype dd 0648) have previously been reported (8).

Virulence-attenuated *L. monocytogenes* isolates. Three virulence-attenuated isolates (5) were shown to have a unique combination of only three PRRs and a glutamic acid residue at position 117 in their ActA proteins (Fig. 3). Isolates L83 (dd 12388) and L99 (dd 0648) belong to lineage III, while L19 (dd 1151) belongs to lineage I. In the plaque assay, L19 and L99 formed relatively small plaques (Table 4) although strain EGD, a fully virulent isolate, did not significantly differ in plaque size from strain L19 (Table 4). Strains L19 and L99 nevertheless had a lower plaquing efficiency than fully virulent strains. The plaquing efficiencies were 4.7×10^3 (L19), 5.8×10^3 (L99), and $>1.7 \times 10^6$ (L83) CFU/PFU, in contrast to averages of 756 to 1,217 CFU/PFU for fully virulent strains in the three lineages (Table 3).

In vitro cytopathogenicity. The sizes of plaques formed by selected *L. monocytogenes* isolates in mouse L cells are shown in Table 4. The average plaque size as well as the average plaquing efficiency for the selected strains within each of the three genotypic lineages is included in Table 3. The average plaque size and plaquing efficiency for all tested strains, except the two virulence-attenuated strains, are 115.5% (SD = 17.5) and 940 CFU/PFU (SD = 360). Although there is no clear correlation between plaque size and genotypic lineage in this assay, there is some evidence that lineage III strains seem to form larger plaques than strains in lineage II if the two virulence-attenuated strains (L19 and L99) are excluded ($P < 0.1$, Tukey-Cramer method for unequal group sizes). All strains in lineage III, with the exception of the virulence-attenuated isolate L99, have plaque sizes of >116% (Table 4). Furthermore, strains in lineage II appear to have a lower plaquing efficiency than strains in lineage I ($P < 0.01$) and lineage III ($P < 0.05$, Tukey-Cramer method for unequal group sizes) (Table 3).

DISCUSSION

Genetic structure of *L. monocytogenes*. Our study shows that there is a clear correlation between ribotype pattern types and alleles for *actA*, *hly*, and *inlA*. Allelic analysis of the three virulence genes and pattern types allowed us to separate *L. monocytogenes* into three lineages. There is no evidence for

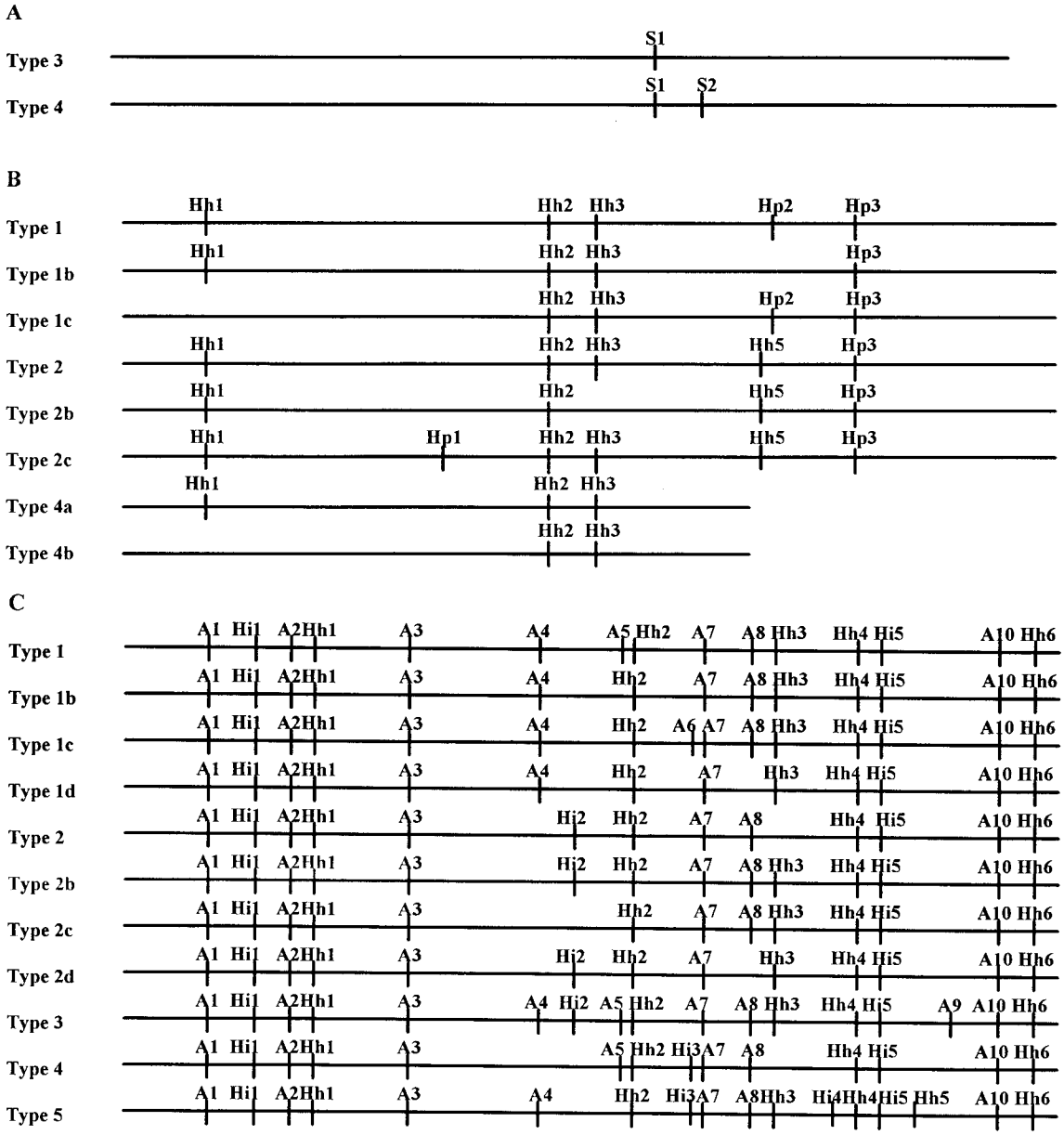

FIG. 2. Schematic representation of RFLP patterns obtained for *actA* (1,993 bp) (A), *hly* (1,812 or 1,193 bp) (B), and *inlA* (2,341 bp) (C) PCR products. Recognition sites for restriction enzymes are designated as follows: S, *Sac*I; Hh, *Hha*I; Hp, *Hpa*II; A, *Apo*I; and Hi, *Hin*fI. Recognition sites are numerically designated in a linear fashion from left to right.

horizontal transfer of virulence genes as manifested by a strong linkage disequilibrium of the alleles investigated. This indicates that the population sampled is predominantly clonal. These findings are in agreement with MEE data as well as with sequencing data for *flaA*, *iap*, and *hly* (27).

Previously, different groups (2, 15, 24, 26, 34) have described two lineages of *L. monocytogenes*. Recently, Rasmussen et al. (27) extended their analysis of *flaA*, *iap*, and *hly* sequence types in *L. monocytogenes*, defining a third type containing the only two serotype 4a strains tested. This type seems to be equivalent to lineage III defined in this paper, as the reference strain, *L. monocytogenes* ATCC 19114, falls into both of these groups. Our results support the conclusion reached by Rasmussen et al. (27) that *L. monocytogenes* can be divided into three sequence types for which there is a correlation between flagellar antigens, MEE types, randomly amplified polymorphic DNA types, chromosomal pulsed-field gel electrophoresis types, PCR-RFLP types of *hly* and *inlA*, and DNA sequence types of *flaA*, *actA*, *hly*, and *iap*. A comparison with previously published data indicates that our lineages I and II are equivalent to the subgroups containing b serotypes or a and c serotypes, respectively (16).

Sequence analysis of *actA*. An unexpected finding of our study was that about 38% of the animal isolates obtained in New York and adjacent states between 1986 and 1996 showed a deletion of one PRR-encoding region in *actA*. The loss of a PRR was found in strains from all three genotypic lineages of *L. monocytogenes*. While this could be taken as an indication

TABLE 2. Relationship of *actA*, *hly*, and *inlA* alleles and pattern types[a]

Pattern type subset	Pattern type	Allele(s) for:		
		hly	*inlA*	*actA*
E 9.2 (22)	dd 1049 (6)	*1* (6)	*1* (4), *1b* (2)	*3* (4), *4* (2)
	dd 3392 (1)	*1* (1)	*1b* (1)	*4* (1)
	dd 0653 (15)	*1* (15)	*1* (13), *3* (2)	*3* (6), *4* (9)
E 11.2 (43)	dd 0647 (37)	*1* (37)	*1* (37)	*3* (37)
	dd 1962 (6)	*1* (6)	*1* (6)	*3* (6)
E 5.3, −D (5)	dd 3581 (5)	*1* (5)	*1* (5)	*3* (5)
E 5.2 (40)	dd 1067 (8)	*2* (8)	*2* (5), *2b* (1), *2d* (1), *1d* (1)	*3* (1), *4* (7)
	dd 0566 (27)	*2* (25), *2b* (1), *2c* (1)	*2* (19), *2b* (8)	*3* (3), *4* (24)
	dd 1151 (5)	*2* (4), *2b* (1)	*2* (1), *2b* (3), *2d* (1)	*3* (1), *4* (4)
E 5.3 (2)	dd 6362 (1)	*2b* (1)	*2* (1)	*4* (1)
G 6.2, H 9.0 (1)	dd 3075 (1)	*2* (1)	*2b* (1)	*4* (1)
Thermal control (5)	dd 1153 (4)	*2* (4)	*2* (4)	*4* (4)
	dd 11900 (1)	*2* (1)	*2b* (1)	*4* (1)
G 8.1, H 7.1 (13)	dd 0648 (6)	*1b* (3), *4a* (2), *4b* (1)	*1b* (3), *1c* (1), *2c* (2)	*3* (2), *4* (4)
	dd 11903 (1)	*4a* (1)	*1b* (1)	*4* (1)
	dd 8842 (1)	*4b* (1)	*1b* (1)	*3* (1)
	dd 0652 (2)	*4b* (2)	*1b* (2)	*4* (2)
	dd 11696 (1)	*1c* (1)	*1b* (1)	*4* (1)
	dd 11698 (1)	*4a* (1)	*1b* (1)	*4* (1)
	dd 12388 (1)	*4a* (1)	*2c* (1)	*3* (1)
G 5.8, H 7.1 (1)	dd 3823 (1)	*4a* (1)	*5* (1)	*3* (1)
E/G 5.8, H 7.1 (2)	dd 6821 (1)	*4b* (1)	*4* (1)	*3* (1)
	dd 6824 (1)	*4b* (1)	*4* (1)	*3* (1)
Total (133)		*1* (70), *1b* (3), *1c* (1), *2* (43), *2b* (3), *2c* (1), *4a* (6), *4b* (6)	*1* (65), *1b* (12), *1c* (1), *1d* (1), *2* (30), *2b* (14), *2c* (3), *2d* (2), *3* (2), *4* (2), *5* (1)	*3* (70), *4* (63)

[a] The number of isolates falling into each respective category is indicated in parentheses.

that horizontal transfer of *actA* between the different lineages occurred, phylogenetic trees constructed by maximum parsimony by using the branch-and-bound alogarithm revealed that the phylogeny of partial ActA proteins including the four (or three) PRRs is related to the three genetic lineages. Three *actA* subclusters which concur with the three genotypically distinct lineages of *L. monocytogenes* can be defined. It is important to note that the trees constructed are based on only a few nucleotide differences (Fig. 4A), so the relationship within some subclusters cannot be resolved reliably. Signature sequence analysis (data not shown) revealed a unique nucleotide for all strains of lineage I, although there was no unique signature sequence present in all strains of either lineage II or III. All lineage I strains had a T at position 1184 (numbering according to that for strain EGD [10], with the first base pair of the coding sequence designated 1), in contrast to a G for lineage II and III strains. Each of the three lineages contains strains with either three or four repeats. This indicates that a gain or loss of one of those repeats occurred multiple times during the evolution of this species. A loss of a PRR-encoding region could have occurred by intragenic recombination in a single *cis* crossover event. The frequency of strains with three or four PRRs significantly differs between lineages and pattern type subsets. Only 5 of the 47 strains in lineage II have three PRRs, in contrast to 58 of the 70 strains in lineage I (including E 11.2). All 42 pattern type subset E 11.2 strains (which include ribotype dd 0647 strains) have only three PRRs. Ribotype dd 0647 strains furthermore showed a unique signature sequence of FPLMP in the third ActA PRR, in contrast to FPP(I/M) P. The unique *actA* sequence found in ribotype dd 0647 isolates links for the first time a distinct virulence gene allele to this bacterial clone responsible for the majority of the human listeriosis outbreaks. This allele can be used to rapidly detect this clone. For example, preliminary experiments in our laboratory have shown that an allele-specific PCR assay based on this sequence is able to specifically detect dd 0647 isolates.

Current working models of ActA function propose that the internal fragment of ActA including the PRR region promotes or controls host actin polymerization (13). ActA of a dd 0647 strain has been shown to be nonphosphorylated in infected cells (24a), while ActA of other strains is phosphorylated (4). This is particularly interesting because a *Shigella flexneri* strain carrying a mutation of the ActA homolog IcsA, that is not phosphorylated, shows increased cell-to-cell spread (9). Therefore, we hypothesize that the ActA sequences of dd 0647 strains might lead to increased cell-to-cell spread. Plaque size in tissue culture cells is likely to be a complex phenotype which might depend on multiple phenotypes. Experiments in an isogenic background will be necessary to specifically evaluate the

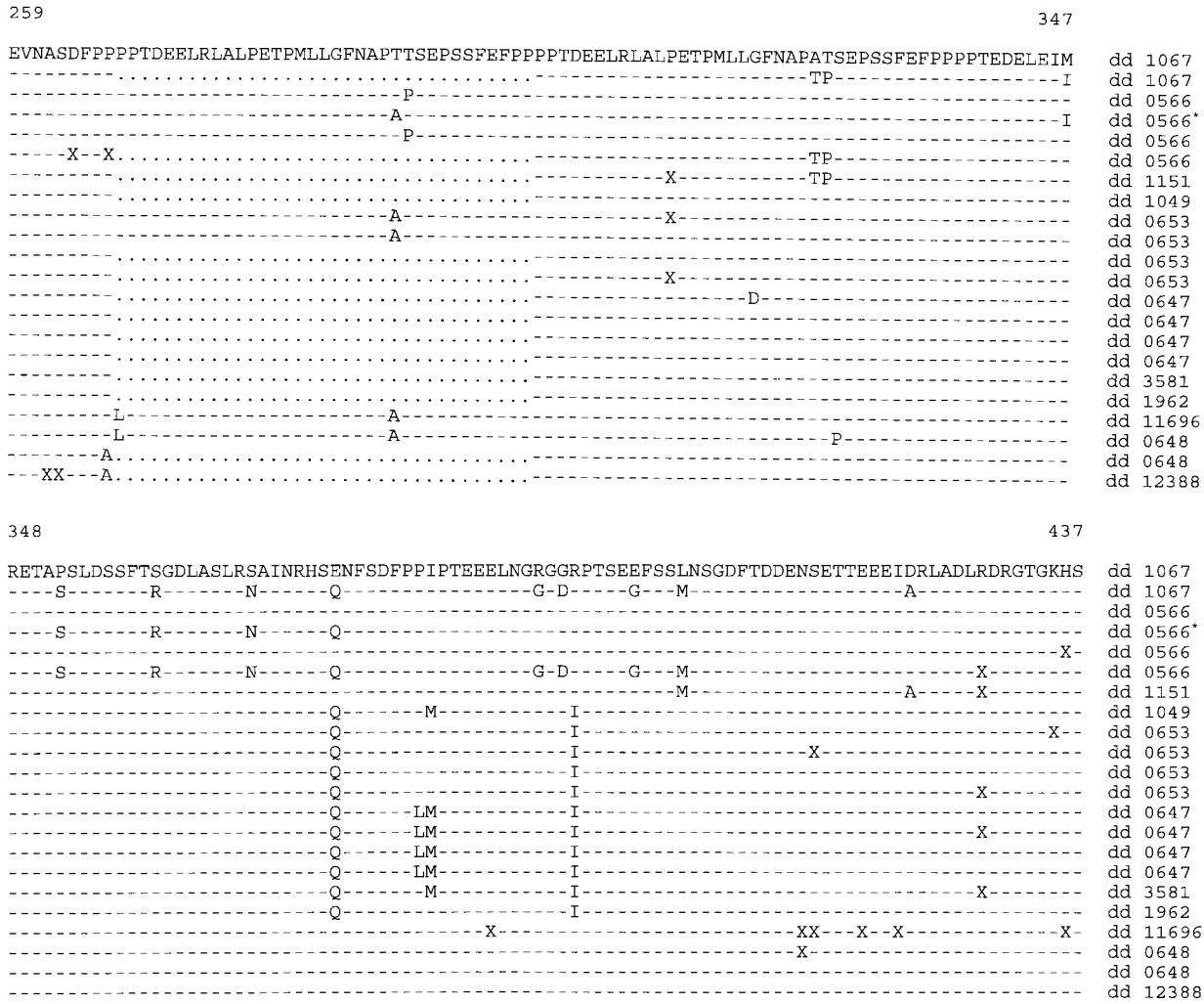

FIG. 3. Alignment of deduced partial ActA sequences for 22 *L. monocytogenes* strains. The amino acid sequence for the first strain is given. For all other strains dashes indicate amino acids identical to those in the first strain, while dots indicate amino acid deletions. X, ambiguous amino acid. Numbers at the top indicate the positions according to the numbering for strain EGD (designated with an asterisk), which has four PRRs. The four PRRs are located from aa 263 to 297, aa 298 to 332, aa 334 to 345, and aa 378 to 389 (10).

phenotypes associated with the different naturally occurring *actA* alleles.

The significance of a variable number of PRRs is difficult to determine, as we have no indications if the number of PRRs confers a selective advantage. It is not clear whether duplication or deletion of a PRR is responsible for the presence of strains with three and four repeats. Yang and Gabriel (37) recently showed that after intragenic recombination among homologous repeats *Xanthomonas* spp. can lose pathogenic or avirulence phenotypes as well as gain new pathogenic phenotypes. We currently favor the hypothesis that the loss of a PRR occurred similarly and independently in different lineages of *L. monocytogenes*. The relatively high frequency of strains with three PRRs in the surveyed *L. monocytogenes* population and the fact this trait is fixed in the E 11.2 pattern type subset (Table 2) could indicate a selective advantage of three PRRs.

Rodrigues et al. (28) have hypothesized that the most common characteristics of a clonal group give clues to the ancestral condition. For example, the loss of a PRR might therefore have occurred in lineage II more recently than in lineage I, particularly in subset E 11.2.

The finding of a variable repeat number in *L. monocytogenes* ActA could reflect the importance of direct repeats in the evolution of virulence genes (38). Two other *L. monocytogenes* virulence genes (*iap* and *inlA*) also contain direct-repeat structures; for *iap* between 11 and 20 repeats have been described for different strains (27). Similarly, for *iactA*, the *Listeria ivanovii* homolog of *actA*, between 5 and 8 PRR structures have recently been reported (14).

Twenty-two of the 53 nucleotide changes in the 539-bp *actA* fragment are nonsynonymous, while a 150-bp fragment of *hly* from 75 strains contained only 12 polymorphic nucleotides (26, 27). Only 2 of the nucleotide changes in *hly* were nonsynonymous and found in one strain each, while 20 of the nonsynonymous changes in *actA* were found in more than one strain. These data suggest differences in the selection pressure on these two genes, which are located in the same virulence cluster. These data indicate a functional constraint in *hly* but not in *actA*. It seems feasible that positive selection favors certain *actA* alleles. For example, the presence of an *actA* sequence in ribotype dd 0647 strains, in contrast to the rest of the lineage I strains, all of which carry *hly* allele *1*, might suggest that this

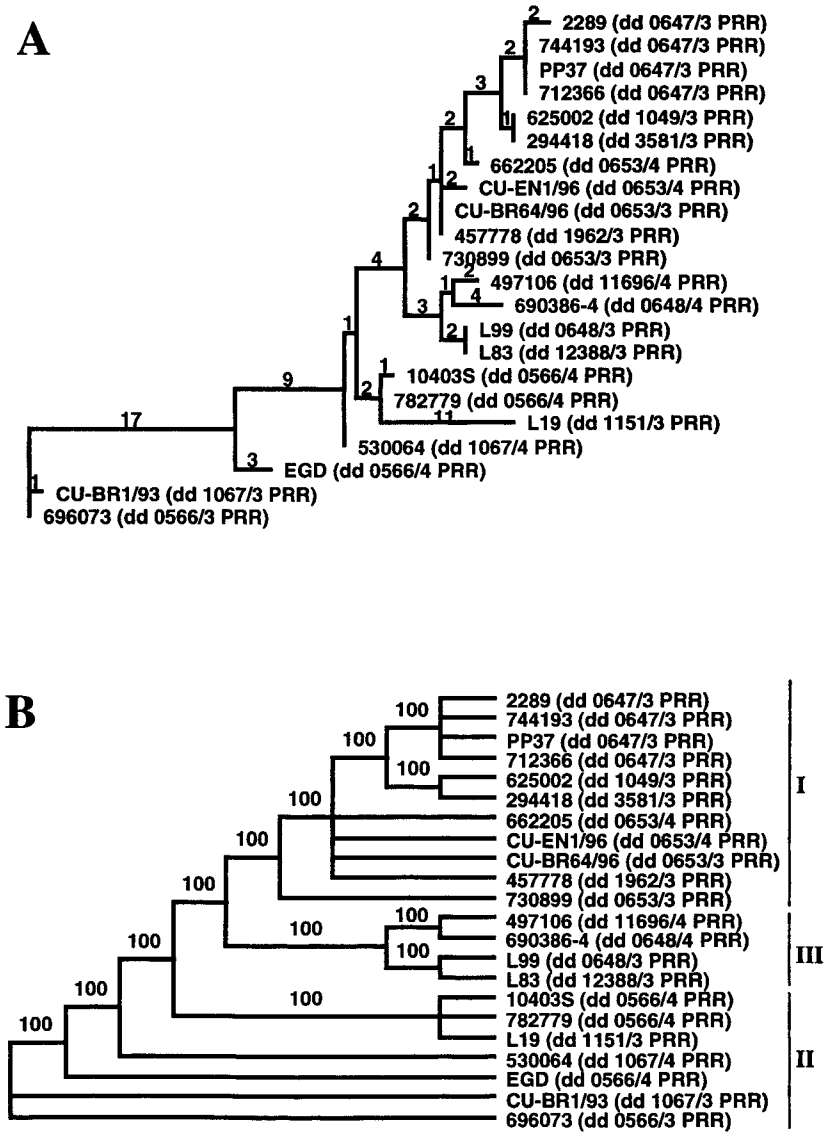

FIG. 4. Phylogram and cladogram for partial *actA* sequences from 22 *L. monocytogenes* strains. Maximum-parsimony analysis of 22 partial *actA* sequences by using the branch-and-bound alogarithm gave four minimal trees. (A) Representative phylogram. The values above the branches indicate the numbers of nucleotide differences between the nodes. (B) Cladogram constructed by using the majority-rule consensus. Strain designations (ribotype, number of PRRs) are on the right. Strains of the same lineage are grouped together, and the respective lineages are indicated on the rightmost side. The numbers on the branches are the percentages of all trees in which this branch was found.

actA sequence type is linked to increased virulence. This hypothesis is currently under investigation, using isogenic *L. monocytogenes* strains carrying different naturally occurring *actA* alleles.

Pathogenic potential among strains of the genotypically distinct lineages. Comparison of the clinical histories of the strains in this study reveals evidence for associations among the three lineages, specific virulence genes, and pathogenic potential. Lineage I contains all food-borne-epidemic isolates as well as isolates from sporadic cases in humans and animals. Lineage II contains both human and animal isolates but apparently no isolates from food-borne epidemics. Most interestingly, so far only strains isolated from animals and none from humans have been found in lineage III. Strains of this lineage represent only 1% of the predominant food and environmental isolates previously surveyed (3), in contrast to 8% of our ruminant and 25% of our other animal isolates. We therefore hypothesize that strains of lineages I and III may be particularly virulent for humans and animals, respectively.

Rasmussen et al. (26) found that while isolates of both of their sequence types can cause invasive disease in humans and mice, all human clinical isolates from epidemic outbreaks belong to their sequence type 1 (corresponding to our lineage I). Furthermore, Vines et al. (34) reported that human perinatal listeriosis seems to be caused more frequently by strains equivalent to our lineage I than by strains of lineage II. McLauchlin (22) found some indications for associations between virulence differences and serotypes 1/2b, 1/2c, and 4b. For example, serotype 4b was found in 64% of all human cases and occurred more often in pregnancy-associated then in non-pregnancy-associated cases of listeriosis. Serotypes 1/2b and 4b make up 74% of all human isolates serotyped by McLauchlin (22).

TABLE 3. Genetic divisions of *L. monocytogenes* and their correlation to sources

Lineage[a]	Pattern type subset[b]	Allele(s) for:			Mean plaque size[c] (SD)	Mean no. of CFU/PFU[d] (SD)	No. (%) of human isolates (n = 20)[e]	No. (%) of ruminant isolates (n = 64)	No. (%) of other animal isolates (n = 12)	No. (%) in survey by Bruce et al.[f] (n = 1,346)
		hly	*actA*	*inlA*						
I	E 9.2	*1*	*3*, *4*	*1*	121.3 (12.5)	756 (239)	13 (65)	26 (40.5)	6 (50)	740 (55)
	E 11.2			*1b*						
	E 5.3, −D			*3*						
II	E 5.2	*2*	*3*, *4*	*1d*	106.7 (22.0)	1,217 (361)	7 (35)	33 (51.5)	3 (25)	578 (43)
	E 5.3	*2b*		*2*						
	G 6.2, H 9.0	*2c*		*2b*						
	Thermal control			*2d*						
III	G 8.1, H 7.1	*1b*	*3*, *4*	*1b*	126.2 (6.8)	799 (246)	0 (0)	5 (8)	3 (25)	11 (1)
	G 5.8, H 7.1	*1c*		*1c*						
	E/G 5.8, H 7.1	*4a*, *4b*		*2c*, *4*, *5*						

[a] Lineages are designated as by Rasmussen et al. (27), e.g., "type 1" in reference 27 equals "lineage I."
[b] Designated as by Bruce et al. (3).
[c] Plaque size was determined as described in Materials and Methods, and the average of all tested strains within one lineage was determined; plaque sizes for strains with attenuated virulence (L19 and L99) were not included. For lineage I, n = 11; for lineage II, n = 10; and for lineage III, n = 5. As determined by analysis of variance, the lineages have different means for plaque sizes (P = 0.06), the Tukey-Cramer method for unequal group sizes shows a difference between the means for lineages II and III ($P < 0.1$), and Scheffe's method shows no differences between the means ($P > 0.1$).
[d] The number of CFU necessary to form 1 PFU was determined as described in Materials and Methods, and the average of all tested strains within one lineage was determined; data for strains with attenuated virulence (L19 and L99) were not included. The number of strains of each lineage is the same as in footnote *c*. As determined by analysis of variance, the lineages have different means for CFU/PFU (P = 0.004), the Tukey-Cramer method for unequal group sizes shows a difference between the means for lineages I and II ($P < 0.01$) and lineages II and III ($P < 0.05$), and Scheffe's method shows a difference between the means for lineages I and II ($P < 0.01$) and lineages II and III ($P < 0.1$).
[e] The frequency of the different lineages among human isolates is based on unpublished data (3a).
[f] Numbers are from reference 3.

These serotypes are most likely included in our lineage I, since the serotype 4b strains from epidemic outbreaks are classified as ribotypes dd 0647 and dd 0653 (lineage I). Further studies will be necessary though to clarify the correlation between serotypes and ribotypes. Nevertheless, these data further support our hypothesis that strains in lineage I might show an increased pathogenic potential for humans compared to that of strains in the other two lineages.

Ribotype dd 0647 represents a clone which has been implicated in at least four human food-borne listeriosis outbreaks. All dd 0647 isolates involved in epidemics are serotype 4b, although this serotype is also found in other ribotypes (data not shown). It is possible that the epidemic clone actually represents a subgroup of dd 0647 strains which also carry the serotype 4b antigen, as preliminary data indicate that serovars other than 4b are found in this ribotype (36). ET 1 as described by Nørrung and Skovgaard (23) as well as Piffaretti et al. (24) seems to be related to dd 0647 based on ribotyping of strains with known ETs. dd 0647 isolates have been found to occur frequently in human and animal listeriosis but were isolated only sporadically from foods and food factories. Piffaretti et al. (24) also report that ETs 1 and 7 account for two-thirds of the human and animal disease cases in their study. ET 7 is most likely related to ribotype dd 0653, which also falls into our lineage I. Ribotype dd 0647 strains account for <10% of the ruminant isolates in our study, indicating that they do not play a predominant role in animal disease. This might further indicate a genetic basis of virulence for humans different from that for ruminants.

To our knowledge, this is the first study investigating linkages between genotypes and the clinical presentation of disease in animals. dd 0647 seems to be the only ribotype that specifically causes listerial encephalitis in ruminants, as all six ruminant dd 0647 isolates were from animals with encephalitis. Besides this, there appears to be no association between genotypic lineages and pathogenic potential for ruminants, par-

TABLE 4. Plaque sizes of selected *L. monocytogenes* isolates in mouse L cells

Strain[a]	Lineage	Pattern type	Virulence allele for:			Mean plaque size (SD)
			actA	*inlA*	*hly*	
L99[A]	III	dd 0648	*3*	*2c*	*4a*	53.5 (7.9)
EGD[B]	II	dd 0566	*4*	*2*	*2*	61.9 (8.8)
L19[B]	II	dd 1151	*3*	*2b*	*2*	63.4 (14.3)
CU-BR27/93[C]	II	dd 0566	*4*	*2*	*2*	81.3 (8.7)
782779[D]	II	dd 0566	*4*	*2b*	*2*	98.4 (10.4)
10403S[D]	II	dd 0566	*4*	*2*	*2*	100.8 (11.4)
CU-BR9/93[D]	I	dd 3581	*3*	*1*	*1*	100.9 (13.9)
294418[E]	I	dd 3581	*3*	*1*	*1*	106.3 (13.5)
457778-1[E]	I	dd 1962	*3*	*1*	*1*	108.4 (11.5)
696073-1[E,F]	II	dd 0566	*3*	*2*	*2*	109.4 (9.4)
530064[F,G]	II	dd 1067	*4*	*2*	*2*	116.0 (21.3)
497106[F,G]	III	dd 11696	*4*	*1b*	*1c*	116.5 (10.9)
773014[E,F,G]	I	dd 0647	*3*	*1*	*1*	116.9 (18.1)
740359[E,F,G,H]	II	dd 1153	*4*	*2*	*2*	119.0 (11.0)
529871[F,G,H]	II	dd 0566	*4*	*2*	*2*	119.3 (13.6)
744193[F,G,H]	I	dd 0647	*3*	*1*	*1*	121.5 (12.3)
689426-1[G,H]	III	dd 11698	*4a*	*1b*	*4a*	122.0 (19.2)
625002[G,H]	I	dd 1049	*3*	*1*	*1*	123.1 (16.9)
662205[G,H]	I	dd 0653	*4*	*1*	*1*	123.8 (15.7)
689201[G,H,I]	II	dd 1067	*4*	*2b*	*2*	125.4 (13.3)
712366[H,I]	I	dd 0647	*3*	*1*	*1*	126.7 (12.3)
717468[H,I]	I	dd 0647	*3*	*1*	*1*	128.0 (11.5)
637135[I]	III	dd 0648	*4b*	*1c*	*4b*	129.0 (17.4)
690386[I]	III	dd 0648	*4*	*1b*	*1b*	130.5 (21.1)
758453[I]	III	dd 0652	*4b*	*1b*	*4b*	132.9 (19.0)
CU-BR1/93[K]	II	dd 1067	*3*	*2*	*2*	135.9 (16.2)
730899[K]	I	dd 0653	*3*	*1*	*1*	139.3 (20.1)
2289[K]	I	dd 0647	*3*	*1*	*1*	139.4 (18.4)

[a] Identical superscript letters after strain names indicate that the respective strains belong to the same group as determined by the Student-Newman-Keuls multiple-range test at a 95% confidence interval.

ticularly with regard to distinctive clinical symptoms (data not shown).

Reevaluation of *L. monocytogenes* taxonomy. The data presented here argue for a reevaluation of the *L. monocytogenes* taxonomy. We propose that the *L. monocytogenes* strains grouped in lineage III represent at least one subspecies. All strains in this lineage have an H 7.1 pattern type fragment which is not present in *L. monocytogenes* strains of lineages I and II or in *L. innocua* (3). 16S rRNA gene DNA sequence analysis confirmed the distinct taxonomic position of lineage III with a unique V2 sequence which differs by at least two nucleotides from that of other *Listeria* strains. As determined by DNA-DNA homology, one representative of the pattern type subset G 8.1, H 7.1 (ATCC 19114, dd 0648) is only 72% related to the type strain of *L. monocytogenes* and 54% related to the type strain of *L. innocua* (3). This low level of DNA homology with the *L. monocytogenes* type strain further supports the possibility that a new subspecies has been identified (35).

The taxonomy within lineage III may warrant further evaluation, as strains in the pattern type subsets G 8.1, H 7.1, G 5.8, H 7.1, and E/G 5.8, H 7.1 actually may represent either two new subspecies or a new species (G 5.8, H 7.1 and E/G 5.8, H 7.1) and a new subspecies (G 8.1, H 7.1). Strains in the subsets carrying the G 5.8 fragment are rhamnose negative, while *L. monocytogenes* is characteristically rhamnose positive and *L. innocua* shows variable fermentation of rhamnose (11). Octal codes obtained with the Micro-ID listeria system (Organon Teknika-Cappel) for this subset are consistent with *L. innocua* (3). We confirmed that these strains contain the *L. monocytogenes* virulence genes *actA*, *hly*, and *inlA*. As these genes are not present in *L. innocua*, subsets G 5.8, H 7.1 and E/G 5.8, H 7.1 should not be classified as *L. innocua*, even though they share the G 5.8 fragment. DNA-DNA homology analysis may further clarify the taxonomic status of this group.

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