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Prevalence and Distribution of *Listeria monocytogenes inlA* Alleles Prone to Phase Variation and *inlA* Alleles with Premature Stop Codon Mutations among Human, Food, Animal, and Environmental Isolates

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In *Listeria monocytogenes*, 18 mutations leading to premature stop codons (PMSCs) in the virulence gene *inlA* have been identified to date. While most of these mutations represent nucleotide substitutions, a frameshift deletion in a 5′ seven-adenine homopolymeric tract (HT) in *inlA* has also been reported. This HT may play a role in phase variation and was first identified among *L. monocytogenes* lineage II ribotype DUP-1039C isolates. In order to better understand the distribution of different *inlA* mutations in this ribotype, a newly developed multiplex real-time PCR assay was used to screen 368 DUP-1039C isolates from human, animal, and food-associated sources for three known 5′ *inlA* HT alleles: (i) wild-type (WT) (A_7), (ii) frameshift (FS) (A_6), and (iii) guanine interruption (A_2GA_4) alleles. Additionally, 228 DUP-1039C isolates were screened for all *inlA* PMSCs; data on the presence of all *inlA* PMSCs for the other 140 isolates were obtained from previous studies. The statistical analysis based on 191 epidemiologically unrelated strains showed that strains with *inlA* PMSC mutations ($n = 41$) were overrepresented among food-associated isolates, while strains encoding full-length InlA ($n = 150$) were overrepresented among isolates from farm animals and their environments. Furthermore, the A_6 allele was overrepresented and the A_7 allele was underrepresented among food isolates, while the A_6 allele was underrepresented among farm and animal isolates. Our results indicate that genetic variation in *inlA* contributes to niche adaptation within the lineage II subtype DUP-1039C.

Listeria monocytogenes is a food-borne pathogen and the etiological agent of listeriosis, a severe invasive disease that can affect both humans and animals (1). More than 99% of human listeriosis cases are estimated to be transmitted through food (2). Despite its presence in a wide range of environments and foods, the majority of human listeriosis infections appear to be linked to consumption of contaminated ready-to-eat (RTE) foods (3) that support *L. monocytogenes* growth. Numerous studies have indicated that not all *L. monocytogenes* strains are equally associated with invasive disease. For example, McLauchlin reported that three (1/2a, 1/2b, and 4b) of the 13 serotypes of *L. monocytogenes* were responsible for 90% of 1,363 listeriosis cases from the United Kingdom (4). Additionally, multiple studies using both DNA band-based and sequence-based subtyping methods have shown that *L. monocytogenes* forms a structured population composed of at least four divergent lineages (I, II, III, and IV), which in a number of studies have been suggested to differ in their associations with different sources and in their pathogenic potentials (5–9). The majority of *L. monocytogenes* isolates belong to lineages I and II, which contain the serotypes most commonly associated with human clinical cases; serotypes 1/2b and 4b group into lineage I, while serotypes1/2a and 1/2c group into lineage II. Lineage III and IV strains are rare and usually isolated from ruminants (8, 10), where they have been found to be responsible for disease cases and outbreaks. Studies in a number of countries indicate that lineage I strains are overrepresented among human cases compared to lineage II strains, even though lineage II strains still frequently cause disease in humans (9, 11). Nevertheless, overrepresentation of lineage I strains among human cases has not been observed in some countries, such as Finland (12) and Sweden (13). In many studies, lineage II seems to be the most common lineage among isolates from foods and food-related environments, such as food processing plants (14), posing the question of why these lineage II isolates are not more frequently found among human clinical cases.

Interestingly, a number of studies suggest that the frequent occurrence of virulence-attenuating premature stop codon (PMSC) mutations in *inlA* may at least be partially responsible for the underrepresentation of lineage II strains among human clinical cases (9, 15–18). Recent studies specifically indicate that a considerable proportion (approximately 45%) of *L. monocytogenes* isolates from RTE foods in the United States carry a mutation leading to a PMSC in *inlA* (16, 17, 19). The virulence factor internalin A (InlA; encoded by *inlA*) facilitates the crossing of the host intestinal barrier, which enables subsequent establishment of a systemic infection by *L. monocytogenes* (20). *L. monocytogenes* isolates carrying a PMSC in *inlA* produce a truncated form of InlA that is secreted rather than anchored to the bacterial cell wall; these strains have been shown to be virulence attenuated in a guinea pig

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model (17, 21–23). A number of studies specifically indicate that lineage II strains carry an *inlA* PMSC more frequently than lineage I strains (18, 23, 24). Among the 18 PMSCs found in *inlA*, one (PMSC type 4) is due to the deletion of an adenine residue from a 5′ homopolymeric tract (HT) of seven adenines (wild type [WT]) (A_7), leading to an allele with 6 adenines (frameshift [FS]) (A_6).

HTs are hypermutable regions frequently associated with insertion and deletion mutations due to slipped-strand mispairing (25). The hypermutability of HTs has led them to be implicated as a mechanism for phase variation (the rapid switch between two different states of protein expression) in a number of bacteria (26, 27). Previous research by our group specifically identified three alleles in the HT region in the 5′ end of *inlA* (starting at *inlA* nucleotide [nt] 6); in addition to isolates carrying either an AAA AAAA allele (A_7; *inlA* with the correct frame) or an AAAAAA allele (A_6; with a frameshift mutation leading to a premature stop codon that is the same as PMSC type 4), some isolates carry an AAG AAAA allele (A_2GA_4; *inlA* with the correct frame). The strains carrying the A_2GA_4 allele were significantly less likely to undergo a frameshift mutation in *inlA* than the isogenic strains carrying either the A_7 or the A_6 allele (28). While all lineage I and lineage III isolates characterized to date carry the A_2GA_4 allele (28, 29), only a few lineage II strains carry the A_2GA_4 allele; the majority of lineage II strains carry the A_7 or A_6 allele. Collectively, the observations from these studies suggest that (i) disease-associated (i.e., lineage I and III) *L. monocytogenes* strains have evolved to carry an A_2GA_4 allele that facilitates stable expression of the key virulence factor InlA, (ii) generalist strains (i.e., lineage II strains) have evolved a phase variation mechanism for *inlA*, allowing for selective silencing of *inlA* in environments where it may not be needed (e.g., food processing environments or foods), and (iii) some lineage II strains have acquired an A_2GA_4 allele by point mutation or by horizontal gene transfer, suggesting that a subpopulation of lineage II strains have adapted to a disease-associated lifestyle, consistent with reports of lineage II isolates being identified from human and animal disease cases (30).

The A_6 allele has been identified in *L. monocytogenes* lineage II ribotype DUP-1039C isolates and in other lineage II ribotypes (i.e., DUP-1038B, DUP-1041A, DUP-1043A, DUP-1045B, DUP-1052A, and DUP-1056A) (15, 16). Ribotype DUP-1039C has been reported to be one of the most common ribotypes among isolates obtained from foods and food-associated environments in the United States (9, 15, 31); in one study, this ribotype was reported to represent 7.0% of 502 food isolates (9). We chose to use *L. monocytogenes* DUP-1039C isolates as a model to further investigate the ecology and diversity of *inlA* PMSCs and the polymorphic 5′ *inlA* HT in *L. monocytogenes* isolates from different sources. *L. monocytogenes* ribotype DUP-1039C isolates represent a common and diverse subset of lineage II strains and are commonly isolated from a wide variety of sources, including food, food environments (e.g., processing plants), nonfarm environments (e.g., wilderness and urban areas), and, in some instances, human and animal clinical cases and asymptomatic animals (9, 15, 32–34). Additionally, DUP-1039C isolates frequently contain three unique mutations leading to a PMSC in *inlA*, including PMSC type 4 (the previously mentioned A_6 allele in the 5′ *inlA* HT, leading to a PMSC at codon 8), PMSC type 7 (a C-to-T substitution leading to a PMSC at codon 562), and PMSC type 12 (a G-to-A substitution leading to a PMSC at codon 684) (16, 24, 35). We thus assembled and analyzed a large and diverse collection ($n = 368$) of *L. monocytogenes* DUP-1039C isolates, which were screened using (i) a newly developed multiplex real-time PCR (RT-PCR) genotyping assay to determine the frequency and distribution of the three known alleles of the polymorphic 5′ *inlA* HT and (ii) a previously described single nucleotide polymorphism (SNP) genotyping assay (15) to determine the frequency and distribution of 18 known *inlA* PMSC mutations. These data were used to probe for associations between the *inlA* genotypes and the isolate source, which may suggest the genetic mechanisms for niche adaptation in *L. monocytogenes* lineages and specifically among ribotype DUP-1039C isolates.

TABLE 1 Source information for *L. monocytogenes* isolates used in this study

Isolate source	Total no. of isolates[a]	Total no. of epidemiogically unrelated strains[b]	Reference for original source of isolates
Animal clinical case	22	21	5
Fecal samples from asymptomatic animals	60	23	5
Farm environment	51	30	32
Food environment	77	10	38
Urban or natural environment	10	10	33
Food	100	57	9
Human clinical case	48	40	9
Total	368	191	

[a] All isolates were screened for detection of unique HT allelic types using the real-time PCR assay developed in this study.
[b] Isolates were grouped into epidemiologically unrelated strains to reduce the effect of oversampling the same isolates multiple times.

MATERIALS AND METHODS

Bacterial isolates for genotyping experiments. We queried the publicly available database Food Microbe Tracker (www.foodmicrobetracker.org) (36) on 5 April 2005 for all available DUP-1039C *L. monocytogenes* isolates. The query returned 368 total isolates from the various sources (e.g., human and animal clinical cases, fecal samples from an asymptomatic animal, farm environments, food, food environments, and urban or natural environments) (Table 1). All human isolates were associated with invasive cases of listeriosis (as opposed to the cases of flu-like symptoms that can occur among healthy low-risk individuals). Additional information on the isolates used in this study can be found in Data Set S1 in the supplemental material and online using the Food Microbe Tracker database search function.

The isolates were grouped into epidemiologically unrelated strains to reduce the effect of oversampling the same isolates multiple times. In order for two or more isolates to be considered epidemiologically related, they had to (i) have been isolated within the same month, (ii) have been isolated from the same source type (i.e., an animal clinical case, fecal samples from asymptomatic animals, a farm environment, a food environment, a nonfarm environment, food, or a human clinical case), and (iii) share the same genotype (e.g., share the same PMSC and the same genotype at the 5′ *inlA* HT) as identified by the assays described below. Additionally, if the isolates were isolated from a farm environment, they had to be isolated from the same farm, or if the isolates were isolated from a food environment or food, they had to come from the same source (e.g., same food or same food plant) in order to be considered epidemiologically related. Isolates from animals or humans also had to be isolated in the same state to be considered epidemiologically related. With these criteria, 213 epidemiologically unrelated isolates were identified. Among these 213

TABLE 2 List of primers and probes used in this study

Primer or probe	Sequence (5′ to 3′)[a]	Purpose[b]	Reference or source
RHO1inlATqMnF	CGG ATG CAG GAG AAA ATC CTA TAC	Amplification of a 134-bp fragment of the 5′ region of *inlA*	This study
RHO2inlATqMnR	CGC TGC CAA ATA CTA ATA TTG CTA CTA G	Amplification of a 134-bp fragment of the 5′ region of *inlA*	This study
inlA proF	TTT TAA AAG GTG GAA TGA CA	Sequencing primer for entire *inlA* ORF	24
inlA proR	GAA GCG TTG TAA CTT GGT CTA	Sequencing primer for entire *inlA* ORF	24
inlA F1	CAG GCA GCT ACA ATT ACA CA	Sequencing primer for entire *inlA* ORF	24
inlA S1R	GGA CTG ATG TTA CTT ATT TGG T	Sequencing primer for entire *inlA* ORF	24
inlA F2	AAG ATA TAG GCA CAT TGG CGA GTT	Sequencing primer for entire *inlA* ORF	24
inlA S2R	CGT ACT GAA ATY CCA KTT AGT TCC	Sequencing primer for entire *inlA* ORF	24
inlA seq	GTG GAC GGC AAA GAA ACA AC	Sequencing primer for entire *inlA* ORF	24
F inlA R	ATA TAG TCC GAA AAC CAC ATC T	Sequencing primer for entire *inlA* ORF	24
RHO8-inlA-TqMn-WT	TAGTGAGAAAAAAACGATATG (reporter, FAM; quencher, MGB)	TaqMan probe for detection of A_7 HT	This study
RHO9-inlA-TqMn-FS	AGTGAGAAAAAACGATATG (reporter, VIC; quencher, MGB)	TaqMan probe for detection of A_6 HT	This study
CSM1-inlA-TqMn-Gtrans	ATAGTGAGAAGAAAACGATATG (reporter, NED; quencher, MGB)	TaqMan probe for detection of A_2GA_4 HT	This study

[a] MGB, minor groove binder; FAM, 6-carboxyfluorescein. VIC and NED are commercially available fluorescent dyes.
[b] ORF, open reading frame.

isolates, 24 isolates (16 from a food environment and 8 from food) presented the same rare PMSC type 7 mutation, the A_2GA_4 allele, and were isolated from the same plant (food environmental isolates) or from foods originating from that food processing plant (food isolates) between 1998 and 2002 (37, 38). It has been well documented that *L. monocytogenes* strains can persist in food processing plants and recontaminate foods originating from those plants for long periods of time, while accumulating a few point mutations (39). Given the identical genotypes and the fact that these isolates originated from the same place, which strongly suggest that the same strain has persisted in this plant for a long period of time and had been sampled multiple times over that period, we decided, *ad hoc*, to select only one representative of these food environmental isolates and one representative of these food isolates to be used for statistical analyses. Exclusion of the other 22 isolates associated with this food processing plant reduced the total number of epidemiologically unrelated isolates to 191. Data for the other isolates were also reviewed to identify additional epidemiologically related isolates that may have passed our formal inclusion criteria detailed above; this approach did not identify any further isolates that might be identified as epidemiologically related. Although we used multiple approaches to reduce the risk of oversampling epidemiologically related isolates, we cannot formally exclude the possibility that our final isolate set includes some remaining epidemiologically related isolates, which might be identified with additional information, such as subtyping data or detailed metadata, which were not available for all isolates.

Detection of *inlA* PMSC mutations using a multiplex SNP genotyping assay. We used a previously described multiplex SNP genotyping assay to screen lineage II DUP-1039C *L. monocytogenes* isolates for all 18 known *inlA* PMSC mutations (15). The SNP genotyping assay detects known *inlA* PMSC mutations by the extension of unlabeled primers with fluorescently labeled dideoxynucleoside triphosphates (15). In the current study, this genotyping assay was used to screen 228 DUP-1039C *L. monocytogenes* isolates; the *inlA* PMSC data for the other 140 isolates were obtained from previous studies (15, 16).

Screening of the 5′ *inlA* HT genotype using a newly developed real-time PCR genotyping assay. We developed a real-time PCR assay using competitive hybridization of fluorescently labeled TaqMan (Applied Biosystems, Carlsbad, CA) probes to determine the allelic type of the polymorphic 5′ *inlA* HT in all 368 isolates (for primers and probes, see Table 2; see also Fig. S1 in the supplemental material). Oligonucleotide probes with MGB quencher dyes were designed to be specific for the wild-type HT (i.e., A_7; probe RHO8-inlA-TqMn-WT), the frameshift HT (i.e., A_6; probe RHO9-inlA-TqMn-FS), and the guanine-interrupted HT (i.e., A_2GA_4; probe CSM1-inlA-TqMn-Gtrans) (Table 2). A 25-μl reaction mix contained 12.5 μl of the TaqMan Universal PCR master mix reagent (Applied Biosystems), 4.5 μl of each primer (5 μM), 0.25 μl of each probe (25 μM), and 2.75 μl of distilled water (dH_2O). Thermal cycling was performed according to the TaqMan Universal PCR master mix kit manufacturer's instructions. Isolates with each allelic type, confirmed by DNA sequencing as reported by Orsi et al. (24), were used as positive controls. The genotype of each isolate was assigned, depending on which probe had the highest final signal above the 1.0 ΔRn threshold after 40 cycles. If results were inconclusive (i.e., no probe signal or multiple probe signals), samples were rerun up to three times. If no run produced unambiguous results (likely due to additional polymorphisms in the hybridization sequence and/or the primer-anchoring sequence), the 5′ *inlA* sequence of the isolate was sequenced using *inlA* sequencing primers (Table 2). The DNA sequencing was performed at the Colorado State University Proteomics and Metabolomics facility. The sequences were assembled, proofread, and aligned using DNAStar Lasergene software (DNAStar 8; DNAStar, Madison, WI).

Statistical analysis. All statistical analyses were performed using R (version 2.13.0); P values of <0.05 were considered to be statistically significant in all cases. Fisher's exact tests were used to assess the association between (i) the presence of *inlA* PMSC mutations and the isolate source (e.g., human clinical cases or foods) and (ii) the three polymorphic 5′ *inlA* HT alleles and the isolate source. When appropriate, Monte Carlo simulation was used to estimate the P values based on 200,000 replications. Bonferroni corrections were applied to correct for multiple testing. The sensitivities of the multiplex SNP genotyping assay and the real-time PCR (RT-PCR) genotyping assay for the detection of PMSC 4 (A_6 allele) were calculated as the total number of isolates determined to harbor PMSC 4 with a given method divided by the total number of isolates determined to harbor PMSC 4 with both methods (SNP assay and RT-PCR) or sequencing (considered here as being true positives). The specificities of the multiplex SNP genotyping assay and the real-time PCR genotyping assay for the detection of PMSC 4 (A_6 allele) were calculated as the total number of isolates determined not to harbor PMSC 4 with a given method divided by the total number of isolates determined not to harbor PMSC 4 with both methods and/or sequencing (considered here as being true negatives).

TABLE 3 Distribution of *inlA* genotypes across strain source

Isolate source	No. (%) of strains with[a]:				
	Truncated *inlA* with:				
	PMSC 4	PMSC 7	PMSC 12	Full-length *inlA*	Bonferroni-corrected *P* value[b]
Animal clinical case	0	0	0	21 (100)	0.0612
Fecal samples from asymptomatic animals	0	0	0	23 (100)	0.0351
Farm environment	0	0	0	30 (100)	0.0034
Food environment	3 (30)	1 (10)	0	6 (60)	1.00
Urban or natural environment	1 (10)	0	0	9 (90)	1.00
Food	26 (45)	1 (2)	1 (2)	29 (51)	<0.0001
Human clinical case	7 (18)	0	1 (2)	32 (80)	1.00
Total	37 (19)	2 (1)	2 (1)	150 (79)	

[a] Of the total of 368 isolates, 228 isolates were screened here for *inlA* PMSCs by a multiplex SNP genotyping assay; *inlA* PMSC data for the remaining 140 isolates were obtained from previous publications (15, 16). This table shows the data for 191 isolates that were classified as epidemiologically unrelated strains (as described in Materials and Methods). Isolates were classified into those carrying (i) truncated *inlA* (indicating the presence of an *inlA* PMSC) or (ii) full-length *inlA*.

[b] *P* value for Fisher's exact tests determining whether the *inlA* PMSC genotype (full length or truncated) was associated with a given isolate source. Fisher's exact test of an overall 2 by 7 table (*inlA* genotype by isolate source) indicated that the *inlA* PMSC genotypes differed between sources ($P < 0.001$).

RESULTS AND DISCUSSION

In this study, we screened a collection of *L. monocytogenes* lineage II ribotype DUP-1039C isolates using both a newly developed TaqMan real-time PCR (RT-PCR) genotyping assay and a previously described SNP genotyping assay to gain a better understanding of both the frequency and the distribution of *inlA* PMSC mutations and the nature of a polymorphic 5′ *inlA* HT. The resulting data were used to probe the role of *inlA* polymorphisms in the niche adaptation and virulence attenuation of *L. monocytogenes* lineage II and ribotype DUP-1039C. Based on the *L. monocytogenes* isolates included in the Cornell University Food Microbe Tracker database (www.foodmicrobetracker.org) as of 23 May 2014, isolates with ribotype DUP-1039C represent a considerable proportion of food isolates and human and animal clinical isolates (28.3%, 36.2%, and 30.4% of lineage II isolates with ribotype data, respectively) (see Table S1 in the supplemental material). We specifically focused this study on isolates representing the lineage II ribotype DUP-1039C because (i) isolates with this ribotype are common and have been obtained from diverse sources, (ii) at least three PMSC mutations have been found in isolates belonging to this ribotype, and (iii) this ribotype has been specifically associated with a frameshift mutation in a 5′ HT, which is thought to be involved in phase variation.

Multiplex SNP genotyping of *L. monocytogenes* DUP-1039C isolates supports the hypothesis that strains encoding a full-length InlA are better adapted for survival in mammalian hosts than for survival in food and food environments. While data on the presence of *inlA* PMSCs were available from published studies (29, 31) for 140 DUP-1039C isolates, a previously described SNP genotyping assay (15, 16) was used to screen the other 228 *L. monocytogenes* DUP-1039C isolates for 18 *inlA* PMSCs (16). Among the overall set of 368 *L. monocytogenes* DUP-1039C isolates, three different PMSC types were found, including PMSC types 7, 12, and 4.

We performed categorical data analyses to describe the association of *inlA* PMSC mutations for 191 epidemiologically unrelated strains from different sources (i.e., human clinical cases, food, food environments, urban and natural environments, farm environments, animal clinical cases, and fecal samples from asymptomatic animals). Among these 191 strains, 41 contained *inlA* PMSC mutations, including 2, 2, and 37 strains with PMSC types 7, 12, and 4, respectively (Table 3). Among the epidemiologically unrelated strains, there was an overall association between the presence of an *inlA* PMSC mutation and the isolation source ($P < 0.0001$, Fisher's exact test with Monte Carlo simulation). Specifically, Bonferroni-corrected Fisher's exact tests showed that strains containing any *inlA* PMSC mutation were overrepresented among strains from foods ($P < 0.0001$), whereas strains without an *inlA* PMSC mutation were overrepresented among isolates from farm environments ($P = 0.0034$), animal clinical cases ($P = 0.06124$), and fecal samples from asymptomatic animals ($P = 0.0351$) (Table 3). These results are consistent with those of previous studies, suggesting that *inlA* PMSC mutations are frequently encountered among *L. monocytogenes* lineage II isolates sourced from foods (31). Isolates carrying PMSC mutations in *inlA* have been shown to be virulence attenuated (17), which has been suggested to explain the apparent underrepresentation of lineage II *L. monocytogenes* isolates among isolates linked to human and animal disease (15–17, 23, 40) since isolates belonging to lineage II are more likely to carry a PMSC in *inlA* than isolates from other lineages (18, 41).

A real-time PCR assay allows reliable detection of different 5′ *inlA* HT alleles. Using a newly developed RT-PCR assay, we also investigated the frequency and distribution of three different 5′ *inlA* HT allelic types (i.e., A_7, A_6, and A_2GA_4) among the 368 *L. monocytogenes* DUP-1039C isolates. Since both the RT-PCR assay and the previously described SNP genotyping assay are capable of detecting *inlA* PMSC type 4 (which represents the A_6 allele), the data were analyzed to ensure that both assays were in agreement. Of the 368 isolates screened, only 2 isolates displayed inconsistent results; targeted *inlA* sequencing was used to resolve these inconsistencies. Briefly, the SNP genotyping assay failed to detect PMSC 4 in one isolate, whereas the RT-PCR assay failed to detect *inlA* PMSC type 4 in a different isolate. Hence, with regard to PMSC 4, both the RT-PCR assay and the previously described SNP genotyping assay showed the same sensitivity (97.3%) and the same specificity (100.0%). In contrast to the SNP genotyping assay, the RT-PCR assay can differentiate between the two (A_7 and A_2GA_4) 5′ *inlA* HT alleles expressing full-length InlA, which can provide important characterization data. The A_2GA_4 allele in *inlA* is note-

TABLE 4 Association between isolate sources and the different alleles observed at the 5′ homopolymeric tract of *inlA*

Source	No. (%) of isolates with[b]:			Odds ratio (95% confidence interval) for presence of the allele in given source[a]		
	A_7	A_6	A_2GA_4	A_7	A_6	A_2GA_4
Animal clinical case	13 (62)	0	8 (38)	1.14 (0.41–3.34)	0 (0.00–0.74)$^+$	2.54 (0.84–7.27)
Fecal samples from asymptomatic animals	15 (65)	0	8 (35)	1.34 (0.50–3.85)	0 (0.00–0.66)$^+$	2.17 (0.73–6.02)
Farm environment	23 (77)	0	7 (23)	2.58 (1.00–7.54)$^+$	0 (0.00–0.47)*	1.14 (0.38–3.04)
Food environment	4 (40)	3 (30)	3 (30)	0.44 (0.09–1.94)	1.85 (0.29–8.62)	1.61 (0.26–7.47)
Urban or natural environment	7 (70)	1 (10)	2 (20)	1.65 (0.36–10.19)	0.45 (0.01–3.43)	0.91 (0.09–4.83)
Food	22 (39)	26 (46)	9 (15)	0.30 (0.15–0.59)*	9.24 (3.92 −23.14)*	0.60 (0.23–1.41)
Human clinical case	29 (73)	7 (17)	4 (10)	2.10 (0.93–5.01)	0.86 (0.29–2.22)	0.34 (0.08–1.06)
Totals	113 (60)	37 (19)	41 (21)			

[a] With significance values, * indicates a Fisher's exact test *P* value that is significant after Bonferroni correction and $^+$ indicates a Fisher's exact test *P* value that is significant before, but not after, Bonferroni correction.

[b] The A_7 allele encodes full-length InlA. The A_6 allele leads to a frameshift in *inlA* and encodes InlA with a premature stop codon at amino acid 8. The A_2GA_4 allele is an *inlA* gene with an interruption in the 5′ homopolymeric tract, which has been shown to decrease the rate of deletion, leading to a premature stop codon in this region.

worthy since all *L. monocytogenes* lineage I and III (lineages often associated with human and animal clinical cases) isolates analyzed to date carry this allele (28, 29). Moreover, some lineage II 5′-end *inlA* sequences from isolates carrying the A_2GA_4 allele were previously reported to cluster with lineage I 5′-end *inlA* sequences, which has led to the hypothesis that the presence of the A_2GA_4 allele among some lineage II isolates is likely the result of multiple horizontal gene transfer events originating from lineage I strains (28). Acquisition of the A_2GA_4 allele by lineage II isolates may also allow for adaptation of these isolates to a disease-associated lifestyle due to stable expression of a full-length InlA (28). On the other hand, the A_6 allele represents a mutation hot spot that can revert to the A_7 allele at high frequency (i.e., at a frequency at least a 1,000-fold different from that for the A_2GA_4 allele); the A_7/A_6 alleles have thus been suggested to be involved in phase variations of InlA (28).

Different 5′ *inlA* HT alleles are associated with specific isolate sources. Among the overall 368 isolates, 182, 55, and 131 isolates carried the A_7, A_6, and A_2GA_4 alleles in the 5′ *inlA* HT, respectively. Among the 191 epidemiologically unrelated strains, 113, 37, and 41 strains carried the A_7, A_6, and A_2GA_4 alleles, respectively. Among the 74 epidemiologically unrelated strains from farm animals (44 isolates, 21 and 23 from animal clinical cases and asymptomatic animals, respectively) and farm environments ($n = 30$), none carried the A_6 allele, while 51 and 23 strains carried the A_7 and the A_2GA_4 alleles, respectively (Table 4). Although these numbers clearly show that isolates associated with animals tend to express a full-length InlA, neither the clinical nor the asymptomatic animal isolates showed a significant underrepresentation of the A_6 allele after Bonferroni correction (nominal $P < 0.02$ for both clinical and asymptomatic animal cases). We also did not find a significant association between the A_2GA_4 and A_7 alleles and animal strains (Table 4). The A_6 allele was significantly underrepresented among epidemiologically unrelated strains from farms though ($P = 0.0118$). Overall, these data indicate that strains with PMSC and specifically carrying PMSC 4 (A_6 allele) are unlikely to colonize and infect animal hosts, probably due to their low abundance in the farm environment and/or a requirement for expression of a full-length InlA for colonization and infection of ruminant farm animals.

Among the 10 epidemiologically unrelated strains from natural or urban environments, 1, 7, and 2 isolates carried the A_6, A_7, and A_2GA_4 alleles, respectively. None of these three allelic types were found to be significantly over- or underrepresented among isolates from natural or urban environments (Table 4).

Among the 57 epidemiologically unrelated strains from foods, 26 were found to carry the A_6 allele, while 22 and 9 isolates carried the A_7 and A_2GA_4 alleles, respectively. The A_6 allele was significantly ($P < 0.0001$) overrepresented among food isolates, which is consistent with our previous study (31); conversely, the A_7 allele was significantly ($P = 0.0015$) underrepresented among these strains. Interestingly, a previous study also showed that the average *L. monocytogenes* dose per positive serving was 3×10^6 CFU/serving for DUP-1039C with the 5′ *inlA* frameshift mutation (i.e., the A_6 allele), which was >1,000-fold higher than those for other subtypes, including ribotype DUP-1039C, expressing full-length InlA (2.0×10^3 CFU/serving), and ribotype DUP-1042B, representing an epidemic clone (9.0×10^2 CFU/serving) (21). This indicates that isolates with the A_6 allele are not only more frequent among RTE food isolates but also typically found at higher levels in RTE foods than other subtypes, including subtypes commonly implicated in sporadic and epidemic listeriosis. Among the 10 epidemiologically unrelated strains from food environments, 3 strains carried the A_2GA_4 allele, while 3 and 4 strains carried the A_6 and the A_7 alleles, respectively (Table 4). No association between isolates from food environments and *inlA* HT alleles was observed.

Among the 40 epidemiologically unrelated strains from human clinical cases, the majority ($n = 29$) were found to carry the A_7 allele, while 7 and 4 isolates carried the A_6 and A_2GA_4 alleles, respectively. No association between *inlA* HT alleles and humans was observed. This is noteworthy, as isolates that carried any PMSC in InlA were significantly underrepresented among farm isolates, clinical animal isolates, and asymptomatic animal isolates, which may suggest a greater importance of stable expression of full-length InlA for *L. monocytogenes* transmission in farm animal populations than in humans. Moreover, our hypothesis that the A_2GA_4 allele is associated with a more virulent lifestyle of certain isolates that acquired this allele through lateral gene transfer from other lineages was not supported. Rather, our data show that a considerable number of clinical human and animal isolates carry the A_7 allele that allows for phase variation.

Our results indicate that the hypermutable 5′ poly(A) HT in *inlA* shows considerable genetic variability and some association with the isolate source. These findings are consistent with those of previous studies that identified the A_6 allele (which leads to a truncated InlA protein) in *L. monocytogenes* isolates from multiple sample types and sources and various locations (2, 20). Our results reported here are also consistent with previous observations suggesting that the hypermutable 5′ poly(A) HT in *inlA* allows for phase variation among some lineage II *L. monocytogenes* isolates (19); frameshift mutations in the 5′ *inlA* HT have been shown to have an at least a 1,000-times-higher mutation rate than point mutations in the same region (28). This mechanism appears to allow *L. monocytogenes* isolates to rapidly shift from expression of a full-length and functional InlA protein to the truncated and secreted form of the protein. This variation may be advantageous in certain settings and environments and may, for example, facilitate successful transmission between foods and humans, while stable expression of full-length InlA (facilitated by the A_2GA_4 allele) may allow adaptation to other niches (e.g., infection of animals and perhaps transmission in animal-associated environments). Future phenotypic experiments will be needed to confirm the selective advantages of InlA phase variation and stable expression of full-length InlA and to specifically define the selective advantage of expressing a truncated InlA protein in certain environments.

In conclusion, overall, our results support a growing body of evidence that *L. monocytogenes* isolates from food and food environments frequently contain PMSC mutations in *inlA*, leading to a truncated InlA protein, particularly compared to those of isolates from human and animal clinical cases. It is worth pointing out that although *inlA* PMSC mutations are associated with food isolates, about 45% of the epidemiologically unrelated strains from the foods analyzed in this study express a full-length, functional InlA protein, and therefore a substantial fraction of food isolates do not have virulence-attenuating mutations in *inlA*. The newly developed real-time PCR genotyping assay described here represents a high-throughput approach for interrogating a 5′ *inlA* HT of *L. monocytogenes* for one of three known allelic types. Given the high prevalence of *inlA* PMSC mutations in food isolates, the increasing body of evidence showing that isolates with these mutations are virulence attenuated, and the apparent importance of *inlA* phase variation in transmission, the methods described here might be useful for assessing the pathogenic potential of *L. monocytogenes* isolates from various sources.

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