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Virulence Phenotyping and Molecular Characterization of a Low-pathogenicity Isolate of *Listeria monocytogenes* from Cow's Milk

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Abstract A low-pathogenicity isolate of *Listeria monocytogenes* from cow's milk, as screened in mouse and chicken embryonated egg models, was examined for virulence-related phenotypic traits. Corresponding virulence genes (*iap*, *prfA*, *plcA*, *hly*, *mpl*, *actA*, *plcB*, *InlA* and *InlB*) were compared with *L. monocytogenes* reference strains 10403S and EGD to elucidate the possible molecular mechanisms of low virulence. Although *L. monocytogenes* H4 exhibited similar patterns to strain 10403S in terms of hemolytic activity, *in vitro* growth and invasiveness and even had higher adhesiveness, faster intracellular growth and higher phospholipase activity *in vitro*, it was substantially less virulent than the strain 10403S in mouse and chicken embryo models (50% lethal dose: $10^{8.14}$ vs. $10^{5.49}$ and $10^{6.73}$ vs. $10^{1.9}$, respectively). The genes *prfA*, *plcA* and *mpl* were homologous among *L. monocytogenes* strains H4, 10403S and EGD (>98%). Genes *iap*, *hly*, *plcB*, *InlA* and *InlB* of *L. monocytogenes* 10403S had higher homology to those of strain EGD (>98%) than isolate H4. The homology of the gene *hly* between strain 10403S and isolate H4 was 96.9% at the nucleotide level, but 98.7% at the amino acid level. The *actA* gene of isolate H4 had deletions of 105 nucleotides corresponding to 35 amino acid deletions falling within the proline-rich region. Taken together, this study presents some clues as to reduced virulence to mice and chicken embryos of the isolate H4 probably as a result of deletion mutations of *actA*.

Key words *Listeria monocytogenes*; virulence factor; virulence-associated gene

Listeria monocytogenes is a gram-positive facultative intracellular pathogen of humans and several animal species. It is capable of causing serious foodborne illness in pregnant and immunocompromised individuals [1]. *L. monocytogenes* encompasses a diversity of strains or genotypes with varying pathogenic potential. Many *L. monocytogenes* strains are potentially highly pathogenic, but others are less virulent or even avirulent and produce little harm in the host [2,3].

At least seven different virulence-associated genes have been characterized from *L. monocytogenes* [4]. The gene *iap* (invasion associated protein) encodes the protein p60, a murein hydrolase enzyme that is essential during the final stage of cell division of *L. monocytogenes* [5]. The *hly* gene encodes listeriolysin O (LLO), a pore-forming exotoxin essential for invasion into the host cells and lysis of the phagosomes [6]. The actin nucleator protein ActA encoded by *actA* is required for its intracellular or intercellular spreading by initiating actin filament assembly within host cell cytosol. The gene *plcA* encodes a phosphatidylinositol-specific phospholipase C which might contribute to lysis of the phagosomal membrane [7], whereas *plcB* encodes a phospholipase or lecithinase [8], which has been demonstrated to be important for secondary vacuolar escape [9], neurovirulence [10], and NF-κB-mediated inflammatory responses in mice [11]. The gene *mpl* encodes a metalloprotease Mpl, which participates in the activation of phosphatidylcholine phospholipase C (PC-PLC) and thus appears to play an indirect role in its pathogenicity [9,12]. The above virulence-associated genes, present as a cluster in a small region of the *L. monocytogenes* chromosome, are all regulated by the transcription factor PrfA [4]. The genes *InlA* and *InlB* encode *Listeria*

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surface proteins InlA and InlB, respectively, which are involved in the uptake of *L. monocytogenes* into mammalian cells [13].

As part of our food safety research project, we have so far identified over 20 *L. monocytogenes* isolates from dairy and seafood-processing plants. Our preliminary examination indicated that one isolate from cow's milk was of substantially lower pathogenicity than the others. In the present study, we attempted to compare this *L. monocytogenes* isolate with the reference strain 10403S in terms of virulence to mice and embryonated chickens, hemolytic titers, *in vitro* growth and invasiveness. Major virulence-associated genes (*iap, hly, prfA, plcA, actA, plcB, mpl, InlA and InlB*) of both strains were sequenced for functional genomic analyses. The results thus obtained might provide some insights into the molecular basis of its low pathogenicity.

Materials and Methods

Bacterial strains, plasmid, growth media and primers

The reference strain of *L. monocytogenes* 10403S (serotype 1/2a) was kindly provided by Dr. M. WIEDMANN (Cornell University, Ithaca, USA). *L. monocytogenes* H4 was originally isolated from cow's milk in a dairy company (Hangzhou, China) and identified by an API system BioMerieux, Marcy l'Etoile, France) and a polymerase chain reaction procedure targeting *iap* and *hly* fragments specific for the bacterium. *Escherichia coli* Topo-10 was used as the host strain for plasmid pMD18-T (TaKaRa Biotech, Dalian, China). *L. monocytogenes* was grown in trypticase soya broth with yeast extract (TSB-Y) (Oxoid, UK) or BHI (Luqiao, Beijing, China) for routine subculturing. The primers used in this study were synthesized by Huanuo Biotechnology (Shanghai, China), and are listed in **Table 1**.

Virulence to mice and chicken embryos

The 50% lethal dose (LD_{50}) assay of *L. monocytogenes* strains H4 and 10403S to mice and chicken embryos was performed as described previously [14]. Briefly, female ICR mice weighing approximately 20–22 g (Zhejiang College of Traditional Chinese Medicine, Hangzhou, China) were divided into five groups, six mice per group, and acclimatized for 2 d in a standard class II laboratory animal facility. Mice of each group were intraperitoneally inoculated with 0.1 ml of an appropriate dilution of *L. monocytogenes* strain H4 or 10403S in phosphate-buffered saline (PBS, 0.01 M, pH 7.2) from the 12 h cultures grown in TSB-Y at 37 ºC. The LD_{50} was estimated based on the death rate within a 10 d observation period using the trimmed Spearman-Karber method. For virulence assay in chicken embryos, 0.1 ml of appropriate 10-fold dilutions of the two strains was inoculated through the chorioallantoic membrane into 14-day old specific pathogen-free embryonated eggs (Shandong SAIS Poultry Science & Technology, Jinan, China). Six eggs were used for each inoculum size of either strain tested. Eggs receiving PBS were included as the control. Inoculated eggs were incubated at 37.5 ºC, and embryo death was monitored daily

Table 1 Primer pairs used to amplify virulence-associated genes *iap, hly, prfA, plcA, actA, plcB, mpl, InlA* and *InlB*

Primer	Sequence (5′→3′)	Product length (bp)
iap-a	CGCGAAGAGAGGAGTTTTATGAAT	1503
iap-b	GCGGCTCCACAGGTTACTTTA	
hly-a	AAGAATTCTGTAGAAGGAGAGTGAAAC	1646
hly-b	GGCGGATCCTTTTTATTACTTTTACAAT	
prfAplcA-a	AAGGATCCATTGAGGAATACTGTTTTTG	2001
prfAplcA-b	TAAAGCTTATACTAATCAAAGGAGGGG	
actAplcB-a	CAGGTACCTATAAGTGGGATTAAACAGAT	2883
actAplcB-b	CGAAGCTTCGCTAACGAGTGGATAA	
mpl-a	GCGAATTCAATTAAAAAGGAGCGGTG	1597
mpl-b	GCGGGATCCAATTAATTTTTCTCTAACAT	
InlA-a	ACGTATTGTTTCTAACTATTGA	2457
InlA-b	GCTCTTTACACTACTTCTATTTA	
InlB-a	GCGTAAATAGAAGTAGTGTAAAGAGC	2036
InlB-b	TAAAAGAAAAGAAGGCGAAAAT	

for 7 d by transillumination for LD_{50} calculation. The inoculum sizes for both mice and embryos were confirmed by enumeration on TSB-Y agar plates of the viable counts in appropriate dilutions of the bacteria resuspended in PBS.

Titration of hemolytic activity and hemolysis on blood plates

Hemolytic activity assay was performed as previously described [14]. *L. monocytogenes* strains H4 and 10403S, grown in TSB-Y at pH 5.0 and 7.2 with shaking at 37 ºC for 12 h, were centrifuged at 10,000 *g* for 10 min. Supernatant samples were saved for hemolytic assay. The culture supernatants (75 μl) were serially diluted two-fold in a 96-well V-bottom microplate with saline (8.5 g/L NaCl), and 75 μl of sheep red blood cells in sterile saline was added to each well. Plates were then incubated at 37 ºC for 1 h. Hemolytic activity is expressed as the reciprocal of the dilution of the culture supernatant required to lyse 50% of the erythrocytes. Experiments were carried out in duplicate and repeated twice for each strain. The two strains were also subcultured on TSB-Y agar plates containing 7% sheep blood to visualize the hemolytic zone.

Growth in broth medium

Growth kinetics of *L. monocytogenes* strains H4 and 10403S in BHI at pH 5.0 vs. 7.2 or at pH 7.2 with different NaCl concentrations (0.5%, 2%, 4%, 6% and 8%; *W*/*V*) were performed in a 96-well U-bottom microplate. The two strains were grown in BHI overnight at 37 ºC with shaking, centrifuged at 10,000 *g* for 10 min and adjusted the absorbance at 600 nm (A_{600}) to 0.1–0.12 (approximately 10^8 CFU/ml) after resuspension in PBS. Volumes of 20 μl bacterial suspensions were added to 180 μl TSB-Y with or without NaCl in the wells. The microplate was then mounted onto the controllable thermo-plate in the SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, USA) setting at 37 ºC. Bacterial growth was measured every 30 min for 12 h or 18 h with shaking before every measurement. Growth curves were analysed for lag phase and growth rate (slope) using the software SoftMax Pro (Version 4.7.1; Molecular Devices).

Plaque-forming assay in L929 cells

Plaque-forming assay was performed on L929 cell monolayers in six-well tissue culture plates (Corning, USA) according to the method described previously [15,16]. Briefly, cell monolayers at approximately 80% confluence were inoculated with 5 μl suspensions of *L. monocytogenes* strains H4 and 10403S (1×10^8 CFU/ml). After 1 h at 37 °C, the monolayers were washed three times with PBS then overlaid with 3 ml medium containing 7 g/L agarose and 20 μg/ml gentamicin. After 2–3 d, plaques were visualized by the addition of 2 ml medium containing 7 g/L agarose and 0.1 g/L filter-sterilized neutral red (Sigma, St. Louis, USA).

Invasion and intracellular growth assay

The gentamicin-based invasion assay was performed on HeLa cell monolayers in 12-well plates (Corning, USA) according to the method previously described [17]. Briefly, HeLa monolayers at approximately 80% confluence were inoculated with 300 μl of suspensions *L. monocytogenes* strains H4 and 10403S ($1–2\times10^7$ CFU/ml) to obtain a multiplicity of infection of 10:1 and incubated for 1 h at 37 ºC in 5% CO_2. Half of the monolayers wells were then washed four times with PBS, pH 7.2 and treated with 0.1% Triton X-100 (Sigma) for enumeration of total bacteria (adhered and invaded), and the remaining half subjected to gentamicin inactivation of extracellular bacteria using 100 μg/ml gentamicin in Dulbecco's modified Eagle's medium (DMEM) for 1 h at 37 ºC in 5% CO_2. The cell monolayers were washed four times with PBS and lysed with 0.1% Triton X-100 for enumeration of invaded bacteria. Invasion index was expressed using the formula:

$$\text{Invasion index}=(CFU_{Inv}/CFU_{Total})\times100\%$$

where CFU_{Inv} represents bacterial numbers that invaded the cells, and CFU_{Total} represents the total number of bacteria adhered to and invaded into the cells. The experiment was repeated three times, in triplicate wells for each strain.

For intracellular growth assay, the cell monolayers after 1 h invasion were maintained for an additional 6 h in DMEM containing 10 μg/ml of gentamicin before cell lysis. Ten-fold dilutions of the cell lysates were made and plated on TSB-Y agar plates for bacterial counting. Intracellular growth was calculated as the log increase of bacterial numbers over additional incubation for 6 h against those right after gentamicin inactivation of extracellular bacteria.

Phospholipase activity assay

Egg yolk assay was performed essentially as described previously [16,18]. Activated charcoal powder (Hangzhou Charcoal, Hangzhou, China) was added to the base agar medium TSB-Y before autoclaving. A 5% (*V*/*V*) sample of an egg yolk suspension, prepared by diluting fresh egg yolk by 50% with sterile saline, was added aseptically to the melted base agar plate medium tempered at 55 ºC. The two *L. monocytogenes* strains 10403S and H4 were streaked onto the TSB-Y agar and incubated at 37 ºC for

48 h. A positive reaction was seen as a definitive zone of opacity surrounding the streak. *Listeria ivanovii* Li01 provided by Dr. M. JAKOBSEN (The Royal Veterinary and Agricultural University, Danmark) was used as the positive control.

Cloning of the main virulence genes of *L. monocytogenes* into pMD18-T vector

The genes *iap*, *hly*, *mpl*, *InlA*, *InlB* and *prfA* in-frame with *plcA* as well as *actA* in-frame with *plcB* were amplified from the genomic DNA of *L. monocytogenes* strains H4 and 10403S using the primer pairs listed in **Table 1**. The target fragments were purified and inserted into the pMD18-T vector using the T-A cloning strategy. The resulting plasmids were confirmed by restriction digestion with different enzymes and polymerase chain reaction amplification of the target fragments. The resulting plasmids were named pMD18-T-iap, pMD18-T-hly, pMD18-T-mpl, pMD18-T-InlA, pMD18-T-InlB, pMD18-T-prfA-plcA and pMD18-T-actA-plcB.

DNA sequencing and sequence data analysis

The recombinant plasmids pMD18-T-iap, pMD18-T-hly, pMD18-T-mpl, pMD18-T-InlA, pMD18-T-InlB, pMD18-T-prfA-plcA and pMD18-T-actA-plcB of the two strains were sent to Huanuo Biotechnology (Shanghai, China) for sequencing of the target genes using the dideoxy method. The nucleotide sequences of the genes and their deduced amino acid sequences were analyzed using the MegAlign version 5.0 (DNAStar, USA).

Statistical analysis

One-way ANOVA was used for analysis of statistical differences of intracellular invasion and growth in HeLa cells.

Nucleotide sequence accession numbers

GenBank accession numbers of *iap*, *hly*, *mpl*, *prfA*, *plcA*, *actA* and *plcB* genes of the two strains are from DQ054582 to DQ054595. *InlA* and *InlB* genes accepted by GenBank are under accession numbers DQ132794 to DQ132797.

Results

Virulence to mice and embryonated eggs

In our preliminary testing of the virulence of *L. monocytogenes* isolates from dairy and seafood-processing plants using single inoculum sizes, the isolate H4 was of the least virulence to mice and embryonated eggs. This strain was chosen for further examination. **Table 2** illustrates that *L. monocytogenes* isolate H4 showed reduced virulence to mice, having about 2.6 logs less than the strain 10403S in LD_{50}. The chicken embryo model also showed its decreased virulence by over 4.8 logs in LD_{50} in comparison with *L. monocytogenes* 10403S.

Table 2 Comparison of pathogenicity and hemolytic titers between *Listeria monocytogenes* strains H4 and 10403S in terms of 50% lethal dose (LD_{50}) in mouse and chicken embryo models

Strain	LD_{50}		Hemolytic titer
	Mouse	Embryonated egg	
10403S	$10^{5.49}$	$10^{1.90}$	2^4
H4	$10^{8.14}$	$10^{6.73}$	2^5

Phenotypic traits relevant to virulence

To look into the bases of reduced virulence of *L. monocytogenes* H4 as compared to *L. monocytogenes* 10403S, attempts were made to examine several phenotypic characteristics related to its virulence, such as growth potential in broth medium, hemolytic titers of the culture supernatants, phospholipase activity on egg yolk agar plates, invasiveness and intracellular growth, and plaque forming ability. The strains showed a similar growth pattern in BHI at pH 7.2 with the strain H4 showing a longer lag phase, whereas at pH 5.0, *L. monocytogenes* H4 grew faster than the strain 10403S as shown by a shorter lag phase and higher maximum absorbance (**Fig. 1**). The two strains also had similar

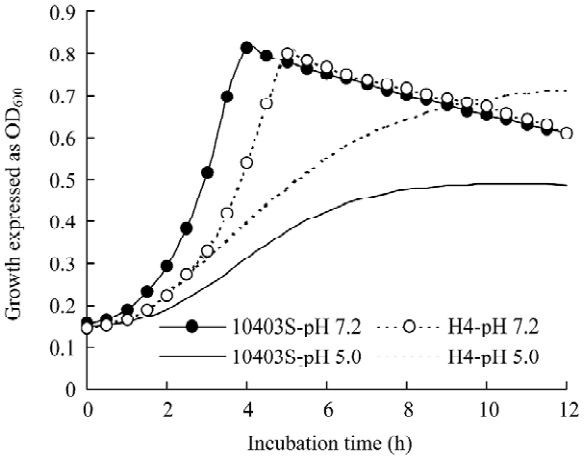

Fig. 1 Growth of *Listeria monocytogenes* strains H4 and 10403S in brain heart infusion at pH 5.0 and pH 7.2

growth profiles in the presence of NaCl (**Fig. 2**) with a longer lag phase and slower growth rate with increasing concentrations of NaCl, although isolate H4 did show a longer lag phase than strain 10403S.

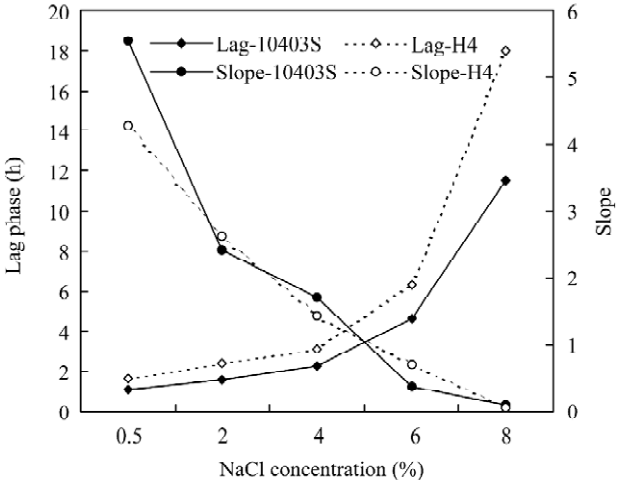

Fig. 2 Growth of *Listeria monocytogenes* strains H4 and 10403S in brain heart infusion at pH 7.2 with different NaCl concentrations (0.5, 2, 4, 6, 8 and 10%) expressed as lag phase and growth rate (slope)

L. monocytogenes H4 did not have significant difference in hemolytic titer compared to the strain 10403S in their respective culture supernatants (**Table 2**), and both strains showed similar hemolytic patterns on TSB-Y agar plates with 7% sheep blood. However, *L. monocytogenes* H4 exhibited apparent phospholipase activity with a definite zone of opacity surrounding the streak similar to that of positive control *L. ivanovii* Li01, whereas the strain 10403S lacked this activity even with the addition of activated charcoal (**Fig. 3**). *L. monocytogenes* isolate H4 was more adhesive to HeLa cells than strain 10403S with statistical difference at $P<0.01$ [**Fig. 4(A)**] and also grew faster than the latter ($P<0.05$) [**Fig. 4(C)**]. However, both strains had similar invasiveness [**Fig. 4(B)**]. *L. monocytogenes* 10403S formed clear plaques in L929 cell monolayers, but there

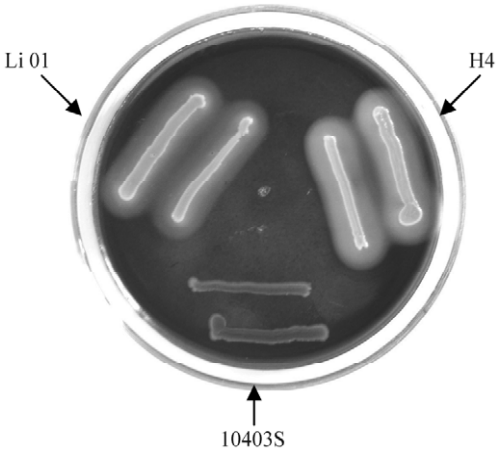

Fig. 3 Phospholipase activity of *Listeria monocytogenes* strains H4 and 10403S grown on TSB-Y agar plate plus 5% egg yolk and 0.5% activated charcoal at 37 ºC for 48 h

Listeria ivanovii Li01 was used as the positive control.

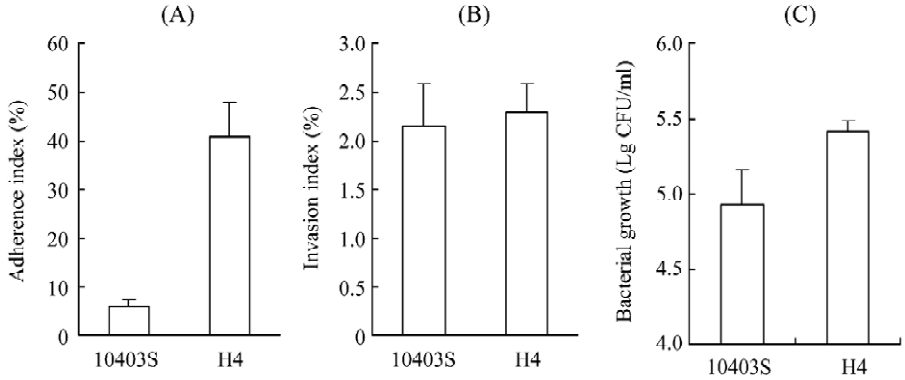

Fig. 4 Adherence index, invasion index and bacterial growth of *Listeria monocytogenes* H4 and reference strain 10403S

(A) *Listeria monocytogenes* H4 was more adhesive than strain 10403S as tested by gentamicin-killing invasion assay in cultured HeLa cell model ($P<0.05$), each data point representing the average invasion index±SD of three experiments, each in triplicate wells. (B) Both strains showed similar invasiveness in cultured HeLa cell monolayers ($P>0.05$), each data point expressed as the average of triplicate wells. (C) *L. monocytogenes* H4 showed faster intracellular growth than strain 10403S in cultured HeLa cell monolayers ($P<0.05$), each data point representing the average in triplicate wells.

was no visible plaque by the isolate H4 (**Fig. 5**), indicating its defect of cell-to-cell spread in the cell monolayers.

Fig. 5 Formation of plaques in infected L929 mouse fibroblasts

L929 cells were infected with *Listeria monocytogenes* strains 10403S (A) and H4 (B). Cell monolayers were stained with neutral red after 2–3 d, highlighting clear areas of dead cells resulting from *L. monocytogenes* 10403S intracellular growth and cell-to-cell spread. There was no visible plaque in *L. monocytogenes* H4, indicating its defect of cell-to-cell spread in the cell monolayers.

Sequence analyses of virulence-associated genes

In view of the apparent differences of several virulence-related traits between *L. monocytogenes* strains H4 and 10403S, major virulence genes of the species were cloned and sequenced for comparison of their differences at the nucleotide and deduced amino acid levels. The sequence information of *L. monocytogenes* EGD (GenBank accession No. NC003210) was used as reference.

The genes *prfA, plcA and mpl* were homologous among *L. monocytogenes* strains H4, 10403S and EGD either at the nucleotide or at the amino acid level (>98%) (**Table 3**). Genes *iap*, *hly*, *plcB*, *InlA* and *InlB* of *L. monocytogenes* 10403S had higher homology to those of strain EGD than isolate H4 (>98%). For the gene *hly* between strain 10403S and isolate H4, there were differences of 49 nucleotides (96.9% homology), but there were differences of only six deduced amino acids (98.7% homology), indicating silent mutations in the isolate H4 (**Table 4**). *L. monocytogenes* H4 had the same PEST sequence as strain EGD. The PEST sequence of the above strains was almost identical except one mutation in the strain 10403S from M to V at position 39.

There were 67 nucleotide differences of *plcB* (95.4%)

Table 3 Nucleotide and amino acid sequence homologies of the virulence-associated genes of *Listeria monocytogenes* strains H4 and 10403S with those of strain EGD

Gene	Homology to strain EGD (%)			
	10403S		H4	
	Nucleotide	Amino acid	Nucleotide	Amino acid
iap	99.1	99.2	94.0	92.4
hly	99.7	99.4	97.2	99.1
prfA	99.9	99.6	99.4	98.3
plcA	98.8	98.5	99.4	98.5
actA	96.7	95.4	94.7	91.5
plcB	99.4	98.3	95.5	95.5
mpl	99.6	99.4	99.5	99.2
InlA	98.9	98.8	96.9	97.4
InlB	99.2	98.9	91.8	91.9

Table 4 Nucleotide and amino acid sequence homologies between the virulence-associated genes of *Listeria monocytogenes* strains H4 and 10403S

Gene	Nucleotide sequence		Amino acid sequence	
	Different nucleotides (*n*)	Homology (%)	Different amino acids (*m*)	Homology (%)
iap	123 (24†)	93.5	38	93.4
hly	49	96.9	6	98.7
prfA	5	99.3	3	98.3
plcA	15	98.1	6	97.4
actA	214 (105†)	93.0	85	91.2
plcB	67 (27†)	95.4	23	94.8
mpl	2	99.9	1	99.6
InlA	73	97.0	19	97.5
InlB	161	91.5	52	91.6

† nucleotide deletion or additional nucleotide.

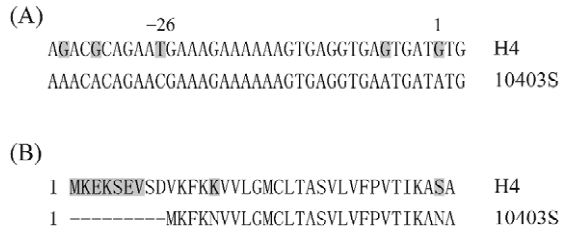

Fig. 6 Fragment of the intergenic sequence between actA and plcB as well as plcB's partial nucleotide sequence (A) and deduced amino acid sequence (B) from *Listeria monocytogenes* strains H4 and 10403S showing major differences around the start codon

between strains 10403S and H4. The first 27 nucleotides were additional due to two mutations from A to G at position 1 of the *plcB* open reading frame (ORF) and C to T at position −26 [**Fig. 6(A)**]. Thus, the ORF of *plcB* in isolate H4 started from position −27 relative to that in strain 10403S or EGD with nine extra amino acids [**Fig. 6(B)**].

When *iap* was analyzed, both strains of 10403S and H4 had deletions of six consecutive deduced amino acids compared to strain EGD (**Fig. 7**). These amino acids were within the Thr-Asn repeat (TN repeat) units. Within this same region, *L. monocytogenes* H4 had additional deletions of 10 deduced amino acids in close proximity (mostly Thr and Asn) generating six and nine less TN repeats than strains 10403S and EGD, respectively.

The *actA* gene of both *L. monocytogenes* strains H4 and 10403S were more divergent than the virulence genes described above, as compared with the EGD strain (**Table 3**). More typically, the H4 strain had deletions of 35 deduced amino acids in succession from position 286 (**Fig. 8**), which fell into the central region of proline-rich repeats (265−396 aa) required for binding to vasodilator-stimulated phosphoprotein (VASP).

When *InlB* was analyzed, there were discrete mutations throughout the gene with differences of 161 nucleotides and of 52 deduced amino acids between strains 10403S and H4 (91.5% and 91.6%, respectively) (**Table 4**). The homology between strains 10403S and H4 was higher at both the nucleotide and amino acid levels (≥97%) for *InlA*.

Discussion

The pathogenicity of *L. monocytogenes* is dependent on expression of a set of genes such as *hly*, *plcA*, *plcB*, *mpl* and *actA*, which are organized in a regulon known to be controlled by the transcriptional activator *PrfA* [4]. Although *L. monocytogenes* H4 exhibited similar patterns to strain 10403S in terms of hemolytic activity, *in vitro* growth and invasiveness [**Table 2**; **Figs. 1, 2** and **4(B)**] and even had higher adhesiveness, faster intracellular growth and higher phospholipase activity *in vitro* [**Figs. 3** and **4(A, C)**], it was substantially less pathogenic than the strain 10403S in the mouse and chicken embryo models (**Table 2**). Efforts were made to examine the virulence-associated genes between the strains to see if there would be any molecular bases of such low pathogenicity with the isolate H4.

Among the target genes sequenced, *mpl*, *prfA* and *plcA* were more conserved in all three strains than the others (>98%) (**Table 3**). *L. monocytogenes* 10403S generally

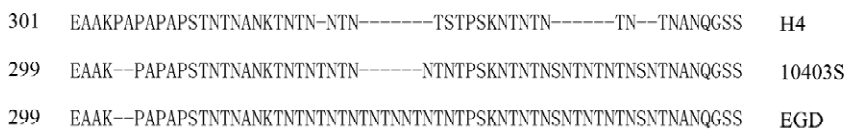

Fig. 7 Amino acid sequence analysis of the partial iap gene fragment from *Listeria monocytogenes* strains H4, 10403S and EGD showing deletions of the Thr-Asn repeats

The shaded amino acid letters indicate the mutation. –, the deletion mutations at the central repeat region of Thr-Asn units.

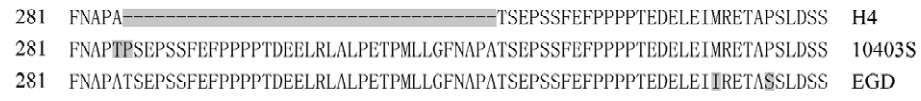

Fig. 8 Amino acid sequence analysis of the partial actA gene fragment from *Listeria monocytogenes* strains H4, 10403S and EGD indicating partial deletion of the Mena/vasodilator-stimulated phosphoprotein binding region

The shaded amino acid letters indicate the mutation. –, the deletion mutations beginning at position 286.

had higher homology to strain EGD than isolate H4 at the nucleotide and amino acid levels. These genes and their products did not appear to be responsible for the reduced virulence of isolate H4, nor did the listeriolysin O seem to contribute, because its *hly* gene was of high homology to that of strains EGD and 10403S (**Tables 3** and **4**). There was also no difference among these strains of the PEST sequence of LLO, which supposed to act as a eukaryotic protein degradation signal for eventual inactivation in the host cell cytosol, thus restricting its activity within the host cell vacuole for its intracellular persistence [19].

In contrast, genes *iap*, *plcB* and *actA* between strains H4 and 10403S were more divergent than those above with apparent insertions or deletions (**Table 4**; **Figs. 6**–**8**). The *ActA* gene of isolate H4 had deletions of 105 nucleotides corresponding to 35 amino acids deletions from position 286. This deletion region fell within the proline-rich repeats (265–396 aa) required for binding of the focal contact proteins VASP and Mena to stimulate actin-based motility [20,21]. *L. monocytognes* mutants lacking this region are still able to recruit actin filaments to the bacterial surface and to move intracellularly, albeit at significantly lower speeds [21–23]. In the plaque assay, no visible plaques were found in L929 cell monolayers infected with isolate H4 [**Fig. 5(B)**]. Thus, we postulate that its reduced virulence to mice might be related to deletion mutation of the proline-rich region of *actA*. This hypothesis is also supported by the findings of Smith *et al*. [23] and Sokolovic *et al*. [24] who described *L. monocytogenes* strains with deletion or absence of one or two proline-rich repeats were less virulent than the wild type or reference strain.

The broad-range lecithinase PC-PLC of *L. monocytogenes* is secreted as an inactive precursor [8], which is activated *in vitro* by proteolytic cleavage at its N terminus by a bacterial metalloprotease (Mpl) [12]. PC-PLC is not only involved in lysis of the primary and secondary vacuoles but also in efficient cell-to-cell spread [25,26]. The *plcB* gene encoding PC-PLC is part of the lecithinase operon, consisting of *mpl*, *actA* and *plcB*. Apparently, this operon in isolate H4 operated more effectively than strain 10403S for its higher lecithinase activity than the latter (**Fig. 3**). The major difference between these two strains was that isolate H4 had two mutations from A to G at position 1 of the *plcB* ORF and C to T at position –26 [**Fig. 6(A)**]. Thus, there are two possibilities. One is that the ORF of *plcB* in isolate H4 started from position –27 relative to that in strain 10403S or EGD with nine extra amino acids [**Fig. 6(B)**]. The other could be that *plcB* still had the same ORF but with GTG as the start codon, as with the genes *inlA* and *inlB* (GenBank accession No. NC003210). In the former case, the ribosome binding site would be downstream of the start codon. Because both membrane damaging genes *plcB* and *hly* were highly expressed in isolate H4, they could be the molecular bases of its lower virulence if synergistic cytotoxic activity would exit *in vivo* exposing the bacteria to the host immune systems, as is the case with LLO with deletions of the PEST sequence [19].

Previous data indicated that protein p60 encoded by *iap* is involved in the invasion of nonprofessional phagocytic mammalian cells by *L. monocytogenes* [27] and its deletion leads to abnormal division of bacterial cells and loss of actin-based motility [28]. In the isolate H4, there were partial deletions of TN repeats (**Fig. 7**). Neither the deletions in the *iap* gene nor the discrete mutations in the *InlB* gene appeared to affect the bacterial invasiveness [**Fig. 4(B)**].

This study presents some clues as to the reduced pathogenicity to mice and chicken embryos of the isolate H4, probably as a result of deletion mutations of the proline-rich region of *actA* which is responsible for binding to VASP as well as for efficiency of the cell-to-cell spread [20, 21]. We speculate this might not be the single factor of decreased pathogenicity. Further studies are required to examine how *plcB* in the H4 isolate is expressed as a result of mutations at the initiation code and its upstream region, and if its highly expressed phospholipase PC-PLC interacts with LLO in enhanced cytotoxicity, rendering the host immune systems accessible to the bacteria with the result of reduced virulence, as seen with the highly cytotoxic *L. monocytogenes* mutant lacking the PEST sequence of LLO [19]. Research is also needed to test if partial deletion of the TN repeats plays any role in ActA-based bacterial motility [28].

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